THE SYNTHESIS AND SECRETION OF STAPHYLOCOCCAL PENICILLINASE *in vivo* AND *in vitro*

A thesis submitted for the degree of Doctor of Philosophy, in the University of Adelaide, by

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STATEMENT

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

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

PETER R. GUNN

CONTENTS

CHAPTER SIX - INHIBITION OF LIPID SYNTHESIS AND ITS EFFECT ON PENICILLINASE SYNTHESIS

SUMMARY

This thesis describes studies on the synthesis and secretion of the staphylococcal extracellular enzyme penicillinase (= β-lactamase).

The penicillinase of *S.aureus* was chosen as a representative extracellular enzyme because of its well documented genetics, its relation to similar enzymes of other bacterial species, the ease of isolation of its genetic material (plasmid DNA), and the possible role played by extracellular proteins in the pathogenicity of the clinically important *S.aureus*, not the least of which is its resistance to β-lactam antibiotics.

Chapters 3 and 4 of this thesis describe initial studies on the synthesis penicillinase in a cell-free DNA-directed protein synthesizing system. It is shown that an *E.coli*-based system is capable of producing an enzymatically active protein in response to staphylococcal penicillinase DNA. The protein-synthesizing system is less responsive to staphylococcal DNA than to *E.coli* DNA, and is, for reasons explained in Chapter 3, not sensitive to the genetic tontrol mechanisms which operate on the operon *in vivo*.

Chapter 4 shows that the protein synthesizing system normally produces a penicillinase indistinguishable from the extracellular protein found *in vivo*, in contrast to parallel studies by other researchers. Modifications of the *in vitro* system show that synthesis of the extracellular enzyme penicillinase produces a significantly different polysome-profile than does the synthesis of intracellular proteins. While this does not greatly affect the nature of the final protein product *in vitro* it does indicate that the synthesis of an extracellular protein is markedly different from that of an intracellular protein.

Chapters 5 and 6 describe the effects of disruption of the normal physiological state of the cell, on the synthesis of staphylococcal extracellular proteins. It has been shown by other workers that the synthesis of secreted proteins is especially sensitive to the disruption of lipid synthesis by the antibiotic cerulenin, which has led them to suggest that the two processes are intimately linked. The results of Chapter 5 suggest that this is not the case, but rather that extracellular penicillinase synthesis is disrupted by cerulenin because the antibiotic interferes with induction of the enzyme: constitutive penicillinase synthesis is insensitive to the antibiotic, whereas inducible penicillinase is

sensitive to the antibiotic. A theory is presented which could explain these results, and those of other workers.

Chapter 6 reinforces this idea, by utilizing a glycerol auxotroph of *E.coli* harboring the staphylococcal penicillinase operon. Disruption of lipid synthesis in such cells does not interfere with penicillinase production. The results of this chapter do not remove the possibility that some high-level turnover of lipids occurs in lipid-synthesis mutants (or cerulenin-treated cells), but do suggest that lipid synthesis *per se* is <u>not</u> a pre-requisite of extracellular protein synthesis.

This thesis therefore examines the production of staphylococcal penicillinase from two approaches: firstly, by its synthesis in an *in vitro* system, which is controlled primarily by the added DNA, and secondly by the modification of the environment of cells carrying those genes. The amalgamation of the two approaches is essential for a complete understanding of the nature of the synthesis and secretion of extracellular proteins.

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ABBREVIATIONS

A_x:	absorbance of a solution at x nanometers (nm)
DNA:	deoxyribonucleic acid
NNG:	N-methyl-N^1-nitro-N-nitrosoguanidine
OD_x:	optical density (turbidity) of a culture or solution at x nm
PEG:	polyethylene glycol 6000
RNA:	ribonucleic acid
mRNA:	messenger RNA
tRNA:	transfer RNA
SDS:	sodium dodecyl sulphate
TCA:	trichloracetic acid
tris:	tris (hydroxymethyl) aminomethane

CHAPTER ONE

GENERAL INTRODUCTION

1.1 WHY STUDY STAPHYLOCOCCAL PENICILLINASE?

The work described in this thesis is aimed at gaining a greater understanding of the mechanism of secretion of extracellular proteins through membranes. The secretion of penicillinase by *Staphylococcus aureus* was chosen for study for several reasons:

1. The staphylococcal penicillinase operon is almost always carried on a plasmid (Novick, 1965), unlike those, for example of *Bacillus* species. This enables one to readily isolate the genes for *in vitro* analysis.

2. Unlike other staphylococcal operons, which have only recently been studied in detail (e.g. Pattee, 1976; Thompson and Pattee, 1977), the genetics of the penicillinase operon have been studied intensively (Imsande, 1978). In particular, investigation of the genetics of other extracellular enzymes of *S.aureus* has been hampered by the difficulty in isolating specific mutants; attempts to find mutants defective in the production of a particular extracellular protein almost always yield a variety of pleiotropic mutants (e.g. Forsgren *et al.*, 1971; Ryden, Lindberg and Philipson, 1973). While this may

indicate that production is co-ordinately controlled at some level (e.g. induction or secretion), which is in itself an interesting and worthwhile area of study, it does complicate attempts to examine a particular protein. In contrast, a number of structural and control mutants of penicillinase have been isolated and studied (e.g.Novick, 1965).

3. Penicillinase activity is readily assayable, by many methods, and so its production is easily monitored.

4. Different staphylococcal penicillinases have differing degrees of extracellularity. For example, the antigenically and enzymatically identical enzymes produced by the plasmids pI524 and pI258, are found approximately 40% and 25% in the culture supernatant, when grown under specified conditions, while the related enzyme of pII147 is predominantly cell associated (less than 10% being found in the supernatant) (Novick and Richmond, 1965).

So, despite the evolutionary similarities between the proteins (Richmond, 1965) and their host plasmids (Shalita, Murphy and Novick, 1980), there are obviously differences

in the proteins, or their operons, which affect their extracellularity. Investigation of these differences could be informative in understanding the nature of protein secretion.

Thus the penicillinases represent a well-understood, related group of proteins, ideal for investigation of protein secretion because their DNA is readily isolatable, the protein can be easily identified and because they show some variations in extracellularity they offer the chance to examine differential effects in the secretory process.

5. *S.aureus* is a gram-positive organism, so that it produces truly extracellular proteins, as distinct from gram-negative bacteria, where secretory proteins are located primarily in the periplasm.

6. *S.aureus* produces a large number of extracellular proteins, which can be classified broadly into two groups. Firstly, the degradative enzymes whose principal role is the breakdown of macromolecules in the environment into components that can be taken up and utilized by the organism. These include lipases, nucleases, proteases and phosphatases

(see Abrahamson, 1972). Secondly, proteins classed as "toxins", which may contribute to the pathogenicity of *S.aureus*. The most important of these are the so-called hemolysins (alpha, beta, gamma and delta), exfoliative toxin, leucocidin (Rogolsky, 1979) and the enterotoxins (Bergdoll, 1972). Thus *S.aureus* is an organism well endowed with the capacity to produce extracellular proteins, and a study of their production may have important implications in understanding and controlling the virulence of this clinically important organism.

The remainder of this chapter will review the current state of knowledge on the synthesis of secretory proteins, both in eukaryotes and prokaryotes, much of which has been gained since the beginning of this work; the staphylococcal penicillinases; and an outline of the work in this thesis.

1.2 PROTEIN SECRETION IN EUKARYOTIC CELLS

Most eukaryotic cells synthesise proteins destined for export from the cell, but some, such as those of the liver, and the acinus cells of the pancreas, are especially adapted for the secretion of large amounts of protein. The prominent feature of such cells in the subdivision of the cytoplasm into membrane-enclosed cisternal compartments, the most extensive of which are the endoplasmic reticulum (E.R.), consisting of an elaborate interconnecting network of sac-like cisternae, and the Golgi complex-a collection of flattened membrane vesicles, cisternae and vacuoles.

The activities of these separate cytoplasmic compartments, and the interrelationships between them, have been elucidated in the classical studies of Palade and his co-workers, extending over more than twenty years (reviewed by Palade 1975). Their studies on the pancreatic acinar cells have shown that, together, the E.R., the Golgi complex and intracellular secretory granules constitute a membrane-enclosed "secretory pathway" which prepares, concentrates and stores the secretory proteins. Proteins destined for export are synthesized by polysomes associated with the E.R. (the "rough" endoplasmic reticulum) and secreted

across that membrane into the cisternal space, from whence they are transported via the smooth E.R. to the Golgi complex. Here the proteins are further processed and packaged into the secretory vesicles which then fuse with the plasma membrane, releasing their contents by reverse pinocytosis (for review see Palade 1975).

Redman and Sabatini (1966) were the first to propose that the synthesis of proteins on membrane ribosomes was coupled to their secretion through the E.R. membrane. They studied synthesis of guinea pig liver secretory protein using isolated rough microsomes, and showed that completed proteins and nascent peptide chains released from the ribosome by treatment with puromycin remained associated with the microsomes, and could only be released by solubilization of the microsomal membranes with detergent. Subsequent studies by Redman (1967) and Sabatini and Blobel (1970) confirmed their results and on the basis of these studies were formulated the concepts of vectorial extrusion and cotranslational secretion whereby the proteins are unidirectionally incorporated into and through the membrane during their synthesis on the membrane polysomes. Blobel and Dobberstein (1975 a,b) isolated mRNA specific for immunoglobulin light chains and translated it in *in vitro* cell free

systems in the presence of ribosome-free microsomes. Newly translated protein was found inside vesicles, whereas when globin (an intracellular protein) mRNA was substituted, synthesized protein remained outside the microsomes. These results indicate firstly, that membrane ribosomes are derived from the pool of free ribosomes, and secondly, that the particular message or the peptide produced from it, can "direct" the ribosome to the membrane, if the protein is destined for export.

Blobel and his co-workers have extended this concept to provide what is now known as the "signal hyposhesis" (Blobel and Dobberstein, 1975 a,b; Blobel 1977 a,b). All mRNAs for secretory proteins, they hypothesize, code for an amino terminal signal sequence of between 15 - 30 amino acids longer than the mature protein. Initially as the 5' end of the message is translated on a free ribosome this peptide signal is shielded within the ribosome (Malkin and Rich, 1967; Blobel and Sabatini, 1970), presumably in a tunnel or groove in the large subunit of the ribosome (Blobel and Dobberstein, 1975; Blobel, 1977 a,b) or elsewhere (Unwin, 1977). As translation continues, the signal portion of the growing peptide chain becomes exposed on the surface of the ribosome, and at this point there occurs an interaction

between the ribosome-peptide and certain receptors on the membrane surface, resulting in an association between the polysome and the membrane. Blobel and Dobberstein (1975 a,b) then propose the formation of a pore in the membrane, through which growing peptide can pass. The proteins comprising the pore must have an affinity for the signal peptide, whereas the remainder of the peptide passes through the pore, resulting in the formation of a hairpin loop in the vicinity of the pore (Inouye and Halegova, 1980). It is then proposed that a specific membrane-associated endopeptidase cleaves off the signal peptide allowing the polypeptide (now with the mature amino-terminus) to pass vectorially through the pore. After translation is complete, the ribosome-mRNA complex dissociates, and the pore is eliminated.

A large body of evidence exists in support of the original hypothesis. The list of pre-secretory proteins which have been shown to be synthesized in cell-free systems devoid of membranes is now substantial and their signal sequences have in many cases been determined (for example see Davis and Tai, 1980). The signal may vary from 15 to 29 amino-acid residues and is characterized in general by a stretch of non-polar residues at either end. The signal

hypothesis implies that the leader peptide is responsible (in concert with the ribosomes) for attachment to the membrane, but although there is some sequence homology for some signals (e.g. for several pancreatic secretory protein precursors (Devillers-Thiery *et al.*, 1975) there are in general considerable differences between signals in their primary structure. Thus it is not yet apparent exactly how the signal sequences interact with either the ribosome or the membrane. There appears to be little species specificity involved - thus Dobberstein and Blobel (1975) were able to synthesize and process mouse immunoglobin light chains in an *vitro* system containing dog panreatic smooth microsomes and in a wheat germ cell-free extract.

So far there is little information on the mechanism of assembly and disassembly of the ribosome-membrane complex, although recently Walter *et al.* (1979) have begun studying the translocation activity of smooth microsomal vesicles. Using trypsin, they reported that the translocation activity can be separated into two fractions, one soluble and one insoluble. The trypsinized membrane fraction had lost its translocation activity, but recombination of the two fractions regenerates the active complex. The

work of Warren and Dobberstein (1978) however is not in complete agreement, suggesting that peripheral, rather than integral, membrane-proteins have the translocation activity. So the nature of the membrane proteins involved in the ribosome-membrane complex has yet to be resolved.

Signal peptidase activity has been reported in detergent-solubilized rough endoplasmic reticulum (Jackson and Blobel, 1977) and its insensitivity to proleolytic enzymes in intact vesicles (Walter *et al.*, 1979) is consistent with the notion that the activity is located on the cisternal side of the RER. However its precise nature has yet to be determined.

Blobel and Dobberstein (1975 b) also suggested that the signal hypothesis could apply to the synthesis of membrane proteins, as well as secretory proteins, in that a leader peptide could "direct" the polysome to the membrane. Evidence that this is the case comes primarily from the *in vitro* translocation experiments with the membrane-protein glycoprotein G of vesicular stomatitis virus (Katz *et al.*, 1977; Rothman and Lodish, 1977; Lingappa *et al.*, 1978 b). In the absence of microsomes translocation of mRNA glycoprotein G *in vitro* results in the synthesis of a precursor molecule that contains a 16 amino acid N-terminal

extension, and lacks the sugar moiety found in the mature protein (Lingappa *et al.*, 1978 b). Translocation of the mRNA in the presence of microsomes resulted in the appearance of the mature protein (Katz *et al.*, 1977; Rothman and Lodish, 1977; Lingappa *et al.*, 1978 b), inserted in the membrane in the correct orientation (Katz *et al.*, 1977). The fact that glycoprotein G mRNA and prolactin (a secretory protein) mRNA competed for membrane receptors (Lingappa *et al.*, 1978 a) is further evidence that the signal hypothesis can apply to both secretory and membrane-proteins. Similarly, Lingappa *et al.* (1979) synthesized a peripheral membrane-protein, β-microglobin, in the absence and presence of a microsomal fraction, and demonstrated that its processing and sequencing are directly comparable to those of secretory and integral membrane-proteins.

Although the data presented provides strong evidence in favour of the signal hypothesis, there have been a number of studies which are inconsistent with it. Ovalbumin, which is synthesized as a secreted protein in the oviduct, has been synthesized *in vitro* in a rabbit reticulocyte lysate primed with Ovalbumin mRNA. Even in the absence of membranes, this protein is synthesized in a form identical (by amino acid sequencing and gel

electrophoresis) to the extracellular form obtained *in vivo*, thus there was no evidence of a "signal peptide" (Palmiter *et al.*, 1978).
Dobberstein, Blobel and Chua (1977) found evidence which also conflicts with the idea of co-translational secretion of proteins, when they studied the synthesis of the small subunit of the chloroplast protein riboulose-1,5-biphosphate carboxylase of the alga *Chlamydomonas*. The protein is synthesized on free ribosomes as a precursor, and then processed post-translationally to the mature form. The leader sequence of 44 amino-acids (Highfield and Ellis, 1978) is far more acidic than the leaders of other secretory proteins.

Furthermore, *in vitro* synthesis and translocation of yeast mitochondrial ATPase submits shows that they too are processed after synthesis as larger precursors (Maccecchini *et al.*, 1979), and the peroxisomal proteins catalase and uricase are also synthesised exclusively by free ribosomes and translocated post-translationally (Goldman and Blobel, 1978). Thus there is some evidence that the signal hypothesis as originally proposed by Blobel and Dobberstein (1975 a, b), incorporating the concept of co-translational secretion, does not cover all possible mechanisms of synthesis of functional secretory proteins.

1.3 PROTEIN SECRETION IN PROKARYOTES

By analogy with eukaryote systems, secreted proteins in prokaryote cells might also be synthesized on membrane-bound ribosomes, and secreted through the membrane in a similar manner. There is now strong evidence that this similarity does exist, in that the three key factors in the signal hypothesis: membrane-associated polysomes, the existence of precursors of secreted proteins and the nature of the peptide's passage through the membrane, have all been demonstrated in prokaryotes, primarily in *E.coli*.

1.3.1 Membrane-associated polysomes

Cancedda and Schlessinger (1974) examined the synthesis of the *E.coli* periplasmic enzyme alkaline phosphatase by isolated membrane-bound and free polysomes, and found that the enzyme was preferentially synthesized on membrane-bound polysomes, while the majority of mRNA for the cytoplasmic enzymes of the *lac* and *trp* operons was associated with free polysomes. Their results can be criticized as they used lysozyme in their isolation of polysomes, which has been shown to atrifactually bind polysomes to the cell membrane (Patterson *et al.*, 1970). More

recently, however, their results have been confirmed and extended by Randall and Hardy (1977). They isolated membrane-bound and free polysomes from sonicated cell suspensions and analysed the translation products of these polysomes in an *in vitro* system. They showed that the membrane-associated fraction synthesized several proteins which are exported from the cell, including some outer membrane proteins and the periplasmic maltose binding protein, whereas free polysomes primarily produced cytoplasmic proteins such as the elongation factor Tu. This result has also been confirmed by Smith *et al.* (1977) and Varenne *et al.* (1978), thus the analogy exists between the RER of eukaryotic cells and the membrane-bound ribosomes of prokaryotes.

A key point in the signal hypothesis in eukaryotes is the anchoring of the ribosome to the membrane by binding to specific membrane proteins. Such an attachment has been reported in eukaryotes, since the membrane polysomes were found to remain associated with the membrane even after release of the nascent peptide chain by puromycin, and required high salt concentrations to remove them (Adelman, Sabatini and Blobel, 1973; Rosbash and Penman, 1971). However, in *E.coli*, evidence has recently been published which suggests that there is no specific attachment of the ribosome

to the membrane other than by way of the nascent peptide chain (Smith, Tai and Davis, 1978). When isolated polysomes were treated with puromycin, 75% of the polysomes were released from the membrane, without the need for salt treatment. These results imply that the only attachment between the membrane and the ribosome is via the growing peptide chain. However, these results can be criticized on the basis that about 25% of puromycin-treated ribosomes remained attached to the membrane, even after salt treatment. These ribosomes did not react with extremely high concentrations of puromycin (500 μg/ml), nor could they be released with Mg^{++} or KCl, as are similarly treated eukaryotic membrane-bound ribosomes. The authors did not characterize the proteins produced by these polysomes, so the reason for their different behaviour cannot be determined. They may be artifactual, or could be indicative of a specific membrane ribosome attachment.

The suggestion that membrane-ribosomes are only attached to the membrane by the growing peptide raises the question: where does the energy for translocation arise? The suggestion (Blobel and Dobberstein, 1975 b) that the energy for movement of the growing chain through the membrane comes from the elongation of the peptide

itself, may be valid for eukaryotic systems, where the evidence suggests a specific membrane protein-ribosome attachment, but would not be so for prokaryotes, if there is no such attachment. One possibility (Inouye and Halegoua, 1980) is that the stabilization of the protein, by folding of the peptide as it crosses the membrane, may provide that energy. Recent theoretical analysis by von Heijne (1980) has suggested that the free-energy involved in the conversion of leader-sequence peptides from random coil to alpha-helical structures could provide the energy necessary for "pulling" the protein into, and through, the membrane.

Another interesting problem has been raised by Glenn (1976). If exoproteins are synthesized on membrane-associated ribosomes, then either the mRNA must move from the DNA, in the cytoplasm, to the translation sites at or near the membrane, or the polysome itself must travel through the cytoplasm (under what force?) to the membrane. Work on the extracellular enzymes of *Bacillus amyloliquefaciens* by Elliott and his co-workers has been interpreted by Glenn (1976) as supporting the postulated existence of a primitive mRNA transport system. Late log phase cells of *B.amyloliquefaciens* are capable of sustaining rapid *de novo* synthesis of extracellular protease, amylase and ribonuclease for up to 60 minutes in the absence of mRNA synthesis (Both *et al.*, 1972). Similar results by other workers (Kadowaki *et al.*, 1965;

Yudkin *et al.*, 1965) were interpreted as implying that mRNAs for extracellular enzymes are very stable, but Both *et al.* (1972) and Glenn *et al.*(1973) showed that the *Bacillus* protease message is unstable. Both *et al* (1972) proposed that the exo-enzyme mRNA molecules migrate from the gene to translation sites in the membrane, and that the "pool" of message implied by their protein synthesis data represents message in transit. This pool would have to be of such a size that, even after many half-life decay periods, a quantity sufficient to saturate the postulated membrane translation sites remains intact. However, hybridization studies (Brown and Coleman, 1975 a,b) have failed to demonstrate a large pool of preformed mRNA in *B.subtilis* or *B.amyloliquefaciens*.

Thus there is no conclusive evidence in favour of the existence of a pool of exo-protein mRNA. A more likely possibility is that secretory proteins are first translated on cytoplasmic ribosomes, which are somehow "directed" to the membrane by the signal sequence. Exactly how this occurs is still unknown, although it is worth mentioning in this context the well documented association between DNA and the membrane (see for example, Ryter and Chang, 1975; Helland and Nygaard, 1975; and Dworsky, 1976), and the

close coupling of transcription and translation in prokaryotes.

This still leaves unanswered the observation (Both *et al.*, 1972) that translation of *B.amyloliquefaciens* exo-enzyme mRNAs can occur in rifamycin-treated cells, i.e. in the absence of mRNA synthesis. In relation to this, it is interesting to note that a number of antibiotics have a differential effect on the synthesis of secretory proteins. Hirashima *et al.* (1973) examined the effects of various antibiotic inhibitors of protein synthesis on the biosynthesis of *E.coli* cytoplasmic and envelope proteins. They established that there are major differences between the synthesis of the two types of protein. In general, the synthesis of membrane proteins was much more resistant to kasugamycin and puromycin; but more sensitive to tetracycline and sparsomycin. There were also differences in the sensitivity of synthesis of various envelope proteins. For example *tolG* protein was more resistant to kasugamycin, sparsomycin and chloramphenical than other envelope proteins, while lipoprotein was more sensitive to chloramphenicol. Likewise, even at 600 µg/ml, puromycin did not greatly inhibit synthesis of lipoprotein (compare this to the effect of puromycin on membrane ribosomes, reported by Smith, Tai and

Davis, 1978, and discussed earlier), where almost all membrane and cytoplasmic proteins were completely inhibited by this concentration of antibiotic. Further experiments (Halegoua *et al.*,1976) led the authors to suggest that puromycin resistance was an intrinsic property of the lipoprotein biosynthetic machinery. Therefore, the differential sensitivity of membrane-associated polysomes to various antibiotics may be a function of that membrane binding. Similar results have been reported for other extracellular proteins; for example, staphylococcal coagulase, alpha-hemolysin and lipase synthesis are especially sensitive to inhibition by lincomycin (Gemmell and Shibl, 1975). These results will also be discussed later in this Chapter. It is possible especially if there is a close coupling of transcription and translation of extracellular proteins, that their transcription is also peculiarly resistant to inhibition (e.g. by rifamycin), which may help to explain the results of Both *et al.*(1972).

1.3.2 Precursors of membrane proteins and secretory processes

The most complete studies of the synthetic pathway of a prokaryote membrane protein have been on the outer membrane lipoprotein of *E.coli* (see DiRenzio *et al.*, 1978 for review). Initial efforts to detect a precursor for this, and other outer membrane proteins by pulse-label experiments were unsuccessful (Lee and Inouye, 1974; and Leij *et al.*, 1978), suggesting that if a precursor existed, it was processed very rapidly. An alternative approach, used by Halegoua *et al.* (1976 a; 1977) involved perturbing the membrane structure of the cells with toluene, which breaks down the cellular permeability barrier, with partial dissolution of the cytoplasmic membrane. Under precise conditions these cells can continue to synthesize protein. When the membrane proteins of treated cells were reacted with anti-lipoprotein serum, and analysed by SDS-polyacrylamide gel electrophoresis, two bands were found to immuno-precipitate - one corresponding to the *in vitro* lipoprotein, and the other migrating more slowly. The new form of the protein had the same carboxy-terminus as the *in vivo* protein, but an amino-terminal peptide extension of 18 - 19 amino acids (Halegoua *et al.*, 1976, 1977). Thus toluene treatment

appeared to have blocked the processing of the precursor ("prolipoprotein") to lipoprotein. This conclusion was verified when lipoprotein mRNA was purified and translated in cell-free systems, yielding a product identical to the prolipoprotein found in toluene treated cells (Hirashima *et al.*, 1974; Pirtle, *et al.*, 1978; Wang, *et al.*, 1976). Similarly, toluene treatment has allowed identification of precursors of other outer membrane proteins, *tol*G and the matrix protein (Sekizawa *et al.*, 1977), both of which contain an amino-terminal extension of about 20 amino acids. Perturbation of membranes by phenethyl alcohol also results in the accumulation of precursors to outer membrane proteins.

H.Inouye *et al.* (1977) primed a cell-free protein synthesizing system with DNA from the transducing phage ϕ80*pho*A which contains a functional gene for the periplasmic protein alkaline phosphatase, and were able to demonstrate the synthesis of active enzyme, which initially appeared indistinguishable from the *in vivo* enzyme. However, in later experiments (Inouye and Beckwith, 1977) they concluded that the protein was synthesized as a higher molecular weight precursor, which could be converted to the mature protein by inclusion of either a crude membrane preparation, or purified outer membrane fragments. The precursor appears (by its binding to decyl-agarose) to be more hydrophobic than the

mature protein, which is consistent with the idea that the signal sequence is responsible for the attraction of the precursor for the membrane. The major inconsistency in their work, however, is that the (presumably proteolytic) activity which converts the precursor to the mature form of the protein, resides in the outer membrane of the cell, rather than the cytoplasmic membrane. This implies either

(a) the precursor is processed by the outer membrane post-translationally, or
(b) their results are not a true reflection of *in vivo* events.

Recently, Chang, C.N., *et al.* (1980) have shown that an inner membrane preparation of *E.coli* provides co-translational cleavage of alkaline phosphatase, and f1 coat protein, although the outer membrane has some (possibly non-specific) proteolytic activity.

There is evidence for the existence of signal sequences in other prokaryotic secretory systems. Particularly important among these is the penicillinase of *Bacillus licheniformis*, as studied by Lampen and his colleagues, which will be reviewed more extensively later in

this chapter. They have shown that the membrane-bound penicillinase can be the precursor of the extracellular enzyme (see Lampen, 1978). The membrane-enzyme is highly hydrophobic (Crane and Lampen, 1974), and has been shown (Yamamoto and Lampen, 1976 a,b) to have a 26 amino-acid amino-terminal extension, but is otherwise similar to the exo-enzyme. As there is only one structural gene for penicillinase (Sherratt and Collins, 1973), it seemed highly likely that the membrane-enzyme is converted to the extracellular enzyme during or after synthesis. This view was strengthened when it was shown that limited trypsin treatment of purified membrane-enzyme yielded a product almost identical to the extracellular penicillinase (Yamamoto and Lampen, 1976 a), and that a protease isolated from *Bacillus* could specifically cleave the membrane-enzyme to yield the mature enzyme (Aiyappa and Lampen, 1977; Aiyappa, *et al.*, 1977).

There are, however, several disturbing features about their work; primarily their assertion that the amino-terminal residue of the membrane penicillinase is a phosphatidylserine (Yamamoto and Lampen, 1975) which confers hydrophobicity

on the membrane enzyme. This is inconsistent with amino acid analyses of other signal sequences (see Inouye and Halegoua, 1980) which all have methionine as the terminal residue (as expected, if this represents the initiation codon). Also, the signal sequence that Yamamoto and Lampen propose (1976 b) is not particularly hydrophobic, unlike other known signals (Inouye and Halegoua, 1980), and the existence of the phosphatidyserine residue was not confirmed by Simons *et al.* (1978). Finally, very little of the membrane-penicillinase is converted to the extracellular form (Crane, *et al.*, 1973) which is inconsistent with the essence of the signal hypothesis - i.e. that processing occurs co-translationally. It is possible that processing of *B.licheniformis* penicillinase is not as simple as predicted by the signal hypothesis. Simons *et al.* (1978) have tentatively identified four forms of the enzyme and Kelly and Brammar (1973) suggested, on the basis of genetic studies, that there may be a carboxy-terminal sequence of 4 - 6 amino acids that is removed by processing. Other exported proteins have been shown to appear first as higher molecular weight precursors. Randall, Hardy and Josefsson (1978) used the same approach

as Randall and Hardy (1977) to monitor the synthesis of arabinose binding protein, maltose binding protein and receptor protein. All these periplasmic proteins first appeared in a membrane associated, higher molecular weight form which immunoprecipitated with antisera to the respective periplasmic protein. The arabinose binding protein was later shown, by pulse-chase experiments, to contain a peptide extension at or near the amino-terminus (Hardy and Randall, 1978).

The existence of a precursor for *E.coli* β-lactamase, a periplasmic enzyme, has been deduced from the nucleotide sequence of its plasmid gene (Sutcliffe, 1978). It was found that the first 23 amino acids predicted by the DNA sequence did not appear in the mature, secreted protein, thus implying that the enzyme is first synthesized as a precursor.

Similarly, mRNA for the major coat protein of bacteriophage fd (a cytoplasmic membrane protein) contains a sequence coding for 23 amino-terminal acids (Sugimoto *et al.*, 1977) and the coat proteins of bacteriophage M13 (Wickner, *et al.*, 1978) and F1 (Chang, Model and Blobel, 1979) are synthesized *in vitro*, as higher molecular weight precursors,

processed into inverted membrane vesicles in a manner indistinguishable from the *in vitro* protein.

Thus there is strong evidence that the membrane and secretory proteins of prokaryotes are synthesized as precursors, in a way analogous to that in eukaryotes. Whether this is the only way prokaryotes produce such proteins cannot be answered yet. However it is worth noting, firstly, that *E.coli* can effectively synthesize and process chicken ovalbumin, from cloned genes (Fraser and Bruce, 1978), although pre-ovalbumin does not contain a classical amino-terminal leader sequence (Palmiter,Gragnon and Walsh, 1978) and secondly, as mentioned previously, the circumstantial evidence that *Bacillus* penicillinase is not processed as simply as predicted by the signal hypothesis.

1.3.3 The nature and role of the precursor signal sequence

Inouye and Halegoua (1980) have compared the signal sequences of several periplasmic and membrane precursors, and have pointed out several common features which may play important roles in the translocation of the proteins. Firstly, the amino-terminal section of the signal is basic, being rich in histidine, arginine and lysine residues. There is then a relatively hydrophobic section of about 50Å long, between the basic region and the site of cleavage of the precursor, with a proline or glycine residue at least 15 - 20Å from the cleavage site, and an amino acid with a short side chain (glycine, alanine, serine or crysteine) at the cleavage site. Based on this information, they propose a mechanism whereby the signal sequence can function. Their model (Inouye and Halegoua, 1980) is somewhat different from the linear translocation model of Blobel and Dobberstein (1975 a,b) in that it involves the formation of a loop of peptide through the membrane, prior to cleavage, rather than linear extrusion of the peptide. They suggest that the basic, amino-terminal region of the signal allows the initial attachment of the precursor to the membrane (the inner surface of which, in *E,coli,* is usually negatively charged). The hydrophobic section of the signal is then extruded into the membrane, in a loop until it spans the lipid bilayer. Eventually the cleavage site

is exposed to the outer surface of the membrane, while the positively-charged amino-terminus remains on the inside surface of the membrane. It is then proposed that the signal peptidase (presumably located on the outer surface of the membrane) cleaves the peptide, allowing the nature protein to be extruded linearly through the membrane. This model has the advantage that it explains more precisely the role of the signal sequence, but as yet there is no evidence to favour one or other of the models. The necessity of the signal sequence for the export of secretory proteins has recently been demonstrated. Bassford and Beckwith (1979) isolated transducing phage carrying fusions of *lac*, *lac* Z, *mal* E (the maltose binding protein) genes and *mal* control genes. When induced with maltose, bacteria carrying the phage accumulated a hybrid protein with β-galactosidase activity at or near the cytoplasmic membrane, implying that the signal sequence of the maltose binding protein portion of the hybrid was directing the protein to the membrane. Accumulation of this protein is lethal to the cell, so that the cell is sensitive to the presence of maltose. The authors isolated $lac^{+}mal^{R}$ mutants (i.e. cells that still produced β-galactosidase activity on maltose induction, but were not killed by such induction), and then re-fused their *mal* operon fragment to wild-type *mal* segments to produce an intact *mal* operon. These hybrids failed to secrete the maltose binding protein, implying that the secretion defect was not in the protein synthetic machinery, but in the coding material itself; DNA sequencing of

the mutants (Bedouelle *et al.*, 1980) showed that the mutations were, in most cases, due to change of single hydrophobic or uncharged amino acid to a charged amino acid.

Similarly, isolation of mutants defective in export of λ receptor protein, and DNA sequence analysis of the mutant gene, has shown that substitution of a charged amino acid into the hydrophobic region of the signal peptide prevents export of this protein Emr *et al*, 1980).

These studies support the central idea of the signal hypothesis - that the leader sequence is the "signal" which dictates the destiny of proteins carrying it. Although there is considerable variation in the amino acid sequence of the leaders of various proteins (Inouye and Halegoua, 1980), their overall structure is obviously an important feature of the secretory process.

As yet, little is known about the protease which is believed necessary for removal of the signal peptide. Aiyappa, Traficante and Lampen (1977) isolated an extracellular protease from the supernatant of cultures of *B.licheniformis*, which cleaved membrane penicillinase, to yield the mature protein. Its specificity (Aiyappa and Lampen, 1977) suggests that it may be

the endopeptidase which processes penicillinase *in vivo*, but the doubts previously expressed about the true nature of the *B.licheniformis* penicillinase precursor, do not yet enable the true role of this protease to be properly evaluated.

Chang, Blobel and Model (1978) have detected in cytoplasmic membrane fragments of *E.coli*, a peptidase which specifically cleaves bacteriophage f1 pre-coat protein, yielding mature protein and a discrete peptide fragment corresponding to the signal sequence. Further investigation of the enzyme will be invaluable in studying the events in protein secretion.

1.3.4 Involvement of the lipids in the secretion process

The question of how lipids are involved in the secretion of extracellular proteins is raised primarily by the extensive work of Lampen and his colleagues on the penicillinase of *Bacillus licheniformis*. They have identified two forms of penicillinase, although there is only a single structural penicillinase gene (Sherratt and Collins, 1973). The extracellular enzyme is a highly hydrophilic protein of molecular weight about 29,000 daltons, while the cell-associated form is hydrophobic and has a molecular weight of 33,000 daltons, and is found exclusively in the cell membrane. Under some growth conditions the membrane-bound form is converted, *in vitro*, to the extracellular form (Crane, *et al.*, 1973).

The membrane-bound enzyme has been purified (Yamamoto and Lampen, 1976 b) and extensively characterized. It is twenty-six amino acids longer than the extracellular protein, these being at the amino-terminus, and, on the basis of the Rudd reaction (Rudd, 1962) appears to contain phospholipid in a 1:1 molar ratio to the protein (Yamamoto and Lampen, 1976 a,b). The phospholipid could not be removed from the peptide by very stringent procedures such as boiling in 2% SDS and 4M urea (Yamamoto and Lampen, 1976 a,b) which led Lampen to suggest that the phospholipid

is covalently attached to the peptide.

Cleavage of the purified membrane-bound enzyme with trypsin yields two fragments, one almost identical in size and hydrophilicity to the extracellular enzyme and one of the 25 amino acids longer, containing the phospholipid moiety (Yamamoto and Lampen, 1975). Analysis of this fragment revealed that the peptide itself was not hydrophobic, but that the amino-terminal residue was phosphatidylserine. Thus their work suggests that the hydrophobicity of *B.licheniformis* penicillinase is due entirely to the phospholipid moiety at the amino-terminus of the protein, and they suggest that it is this moiety which "directs" or "binds" the penicillinase to the cell membrane, prior to its conversion to the mature, extracellular form. The discovery of tRNA-phosphatidylserine in RNA extracts of *B.licheniformis* (Dancer and Lampen, 1977) suggests that the phospholipid could be covalently attached to the peptide chain during its elongation - thus conferring on the peptide the hydrophobidity necessary for membrane attachment.

The concept of a covalently bound lipid assisting a hydrophilic protein through the cell membrane is attractive, and fits in well with the concepts of vectorial extrusion and co-translational processing of extracellular proteins, however the work of Lampen *et al.*

has been seriously challenged by several recent papers. In particular, Simons *et al.*, (1978) repeated much of Lampen's work on membrane penicillinase, using the same strain of *Bacillus*, and preparing the enzyme with a similar procedure. Their studies could not confirm the existence of a covalently bound phospholipid in membrane penicillinase. After chloroform-methanol extraction less than 0.03 moles of phosphorous per mole of protein could be found associated with the polypeptide. If the membrane penillinase was lyophilized before extraction, higher values were obtained, suggesting that their presence in Lampen's samples may be artifactual. Similarly, Sawai *et al*. (1973) reported that treatment of membrane penillinase with phospholipase D removed the hydrophobic moiety of the molecule, whereas Simons' protein retained its hydrophobic properties after phospholipase treatment. Further, sequence analysis of the amino-terminus of the membrane protein by Simons yielded a totally different amino acid composition than that obtained by Yamamoto and Lampen (1976 c). Simons' sequence indicated that the signal sequence was slightly hydrophobic in contrast to that of Lampen. So, while Simons *et al*. agree with Lampen that the membrane penillinase contains a hydrophobic leader, their results do cast considerable doubt on the significance of Lampen's results.

Similarly, when Dancer and Lampen (1975) used a mRNA-directed, *in vitro* protein synthesizing system to synthesize penillinase, they found that all enzyme produced was of the higher molecular weight, hydrophobic form. This is consistent with the *de novo* synthesis of the larger, membrane-bound (or "precursor") penicillinase. After phospholipase C treatment, the protein electrophoresed identically to the extracellular protein in SDS-acrylamide gels. This should NOT be the case - one would expect that, if synthesized as the 33,000 dalton protein, phospholipase treatment should not greatly affect its behaviour in SDS-acylamide gels, i.e. the peptide should still be 26 amino acids longer than the exo-enzyme. Thus the behaviour of their precursor is questionable.

Paton *et al.* (1979) isolated alpha-amylase and protease precursors from the cell membranes *B.amyloliquefaciens* grown in the presence of ^{32}P-orthophosphate, on SDS-acrylamide gels. No radioactivity was associated with bands corresponding to these proteins, implying that no phospholipid was covalently attached to the peptides.

Thus the role of phospholipids in the hydrophobicity of proteins destined for export remains unclear. However, there must be some interaction between such proteins and the cell membrane, either between polysomes and the membrane (involving the ribosome-membrane

binding site postulated by Blobel and Dobberstein (1975 a,b)) or between the polypeptide and the membrane, involving the incorporation of the protein into the membrane prior to its export. Consequently studies on the role of lipids in extracellular protein synthesis and transport have become popular.

Recently there have been several such studies utilizing the antibiotic cerulenin. This antibiotic is produced by the fungus *Cephalosporium caerulens* and is an unsaturated fatty amide which specifically inhibits fatty acid synthesis by binding to β-ketoacyl carrier protein synthetase (D'Angolo *et al.*, 1973; Omura, 1976).

Fishman *et al.* (1978) have shown that when cultures of *B.licheniformis* 749 are treated with sub-inhibitory levels of cerulenin (10 μg/ml) there is a 80% reduction in the appearance of extracellular penicillinase. Concommittantly, there is a reduced rate of fatty acid synthesis, although cell growth and total protein synthesis remain almost unaffected by this concentration of cerulenin. The inhibition could, under some circumstances, be partially reversed by the addition of exogenous fatty acids, thus circumventing the effect of the antibiotic.

Altenbern (1977) also found that addition of saturated fatty acids to cerulenin treated cultures

could partially reverse the inhibitory effect of the antibiotic, but only over a very narrow range of concentrations, and fatty acids also stimulated enterotoxin production in control cultures. The general conclusion of these authors is that inhibition of lipid synthesis jointly and preferentially suppresses the appearance of extracellular proteins, implying that lipid synthesis is necessary for their production. Fishman *et al.*, (1978) found no accumulation of cell-associated penicillinase in cerulenin treated cells, suggesting that its effect is not on the release of the protein from the membrane, but at an earlier stage. They suggest than penicillinase continues to be synthesized at a normal rate, but, lacking the necessary phospholipid attachment, is conformationally unstable and thus rapidly degraded inside the cell. Alternatively, attachment of ribosomes to the membrane could be impaired in the absence of lipid synthesis.

In contrast to these results, there is some evidence which suggests that lipid synthesis is not necessary for the production of membrane associated or extracellular proteins. For example lactose permease can be induced in the absence of lipid synthesis, both in *E.coli* (Hsu and Fox, 1970; Weisberg *et al.*, 1975) and in *S.aureus* (Mindich, 1971). Similarly, Beacham *et al.* (1976) observed synthesis

and secretion of the periplasmic enzymes alkaline phosphatase and acid hexose phosphatase in the absence of phospholipid synthesis. They used a *gpsA*$^-$ mutant of *E.coli*, which needs an exogenous source of glycerol for lipid synthesis. Removal of glycerol from the growth media resulted in an immediate cessation in phospholipid synthesis (measured by incorporation of ^{32}P into chloroform-methanol extracts), but synthesis of the phosphatases continued for about one generation before being affected. Although they could not discount the possibility of a pool of phospholipids, or their immediate precursors, being present there was no need for *de novo* phospholipid synthesis for extracellular enzyme production, in direct contrast to the implications of the cerulenin studies, where a hypothetical phospholipid pool would also be present.

Rouslin (1979) studied the production of the membrane-associated respiratory enzymes of *Saccharomyces cerevisiae* mitochondria in the presence of cerulenin and under lipid-limiting conditions, and he concluded that they were no more sensitive to cerulenin than to chloramphenicol. He suggested that cerulenin leads to a general slowing of synthesis of all cell proteins. Similarly, Chopra (1975) found that cerulenin did not prevent induction of tetracycline resistance in *S.aureus*. Tetracycline resistance in his strain is mediated by a membrane associated protein.

Thus there is the possibility that the action of cerulenin on extracellular protein production is not specific. Cerulenin has a structure similar to that of a fatty acid; a 12-carbon aliphatic chain with a terminal amide group, and may possible interfere directly with the cell membrane thus disrupting membrane-associated protein synthesis. Recent work by Fishman *et al.*, (1980) shows that a number of membrane-modifying agents such as phenethyl alcohol, benzyl alcohol, procaine, and a number of aliphatic alcohols, selectively suppress production of active extracellular enzymes by various bacteria - presumably by directly upsetting membrane structure. Conceivably cerulenin may be acting in a similar manner.

Additionally, sub-inhibitory concentrations of tetracycline and spectinomycin have been observed by Wainwright and Beacham (1977) to preferentially suppress appearance of periplasmic alkaline phosphatase in *E.coli*, raising the possibility that extracellular protein synthesis is, in general more sensitive to disruption of normal cellular metabolism than intra-cellular protein synthesis. Gemmell and Shibl (1975) have shown that sub-inhibitory levels of lincomycin (0.09 µg/ml) completely suppressed the formation of extra-cellular lipase, coagulase, DNAase and esterase and partly suppressed formation of alpha and delta toxins in various strains of *S.aureus*. Fusidin, also

an inhibitor of protein synthesis, only inhibited DNAase formation, while chloramphenicol had no effect on any of the extracellular proteins examined. Interestingly, polymyxin, an antibiotic which affects the permeability of the cell membrane, could reverse the effect of lincomycin; completely in the case of DNAase and esterase, and partially for alpha and delta toxins, and lipase. The authors suggest that this is due to a release of pre-formed protein in the presence of polymyxin, implying that lincomycin can

a) selectively reduce synthesis of some extracellular proteins;
b) interfere with release of membrane-associated proteins.

Similarly, Nordstrom and Lindberg (1978) have shown that streptomycin and novobiocin can inhibit production of staphylococcal alpha and beta hemolysins, while stimulating production of protein A, in mutants resistant to the antibiotics. In these mutants propagation of phage was also inhibited, implying that the antibiotics interfere with some general function of the cell, despite its overall resistance to the antibodies.

Thus is it not feasible, on the basis of the data so far accumulated, to attribute the effect of cerulenin

on extracellular protein synthesis to its supposedly specific inhibition of lipid synthesis, as other, unrelated, antibiotics can have similar selective effects.

1.4 STAPHYLOCOCCAL PENICILLINASE

The β-lactamases (= penicillinases) of *S.aureus* are similar to those of other gram-positive bacteria - they are inducible, unlike the comparable enzymes of *E.coli* (Richmond, 1975 b) are predominantly extracellular (Citri and Pollock, 1966), and have essentially the same substrate specificities, their major activity being against benzylpenicillin, ampicillin, and related semisynthetic penicillins, while being relatively inactive against methicillin and the cephalosporins (see Richmond, 1975 a). Unlike the related enzymes of *Bacillus* species, however, staphylococcal penicillinases are almost always coded for by plasmid-borne genetic determinants (Novick, 1967 b).

There are four minor variants of staphylococcal penicillinase. Three were described by Richmond (1965): types A and B are antigenically related, but have different specific activities and substrate specificities, whereas type C is antigenically distinct from types A and B, but is enzymatically identical to type A. A fourth group, type D, has been described by Rosdahl (1973); although antigenically similar to type A enzyme, it is produced constitutively, and was judged by Rosdahl (1973) to be a distinct enzyme.

The amino acid composition of types A, B and C enzymes are similar (Richmond, 1965), suggesting that they are closely related proteins, and their overall similarity to the *Bacillus* penicillinases (Richmond, 1975a; Ambler, 1975), both physically and enzymatically also suggest a common evolutionary origin. This view has been reinforced strongly in recent studies on the physical nature of a number of staphylococcal plasmids, by restriction enzyme and DNA heteroduplex analysis (Shalita, Murphy and Novick, 1980). Their comprehensive work shows that plasmids from diverse sources share some very similar restriction fragments, and have much homologous DNA, despite many variations in their size and genetic makeup. The authors argue for a common evolutionary origin, such as an "ancestor" plasmid, being modified in different hosts by the rearrangement and addition of genetic material, for example by the movement of transposons.

The genetics of the penicillinase operon (designated bla for β-lactamase) have been reviewed recently by Imsande (1978). The operon contains two genes, the penicillinase structural gene, bla Z (or pen P) and an adjacent repressor gene (bla or pen I), with the associated operator-promoter region. On the basis of the work of several authors (Cohen and Sweeney, 1968; Cohen, Vernon and Sweeney,1970; Imsande, 1973;

Imsande and Lilleholme, 1976), Imsande (1978) has postulated that ultimate control of penicillinase induction rests with the product of another, unlinked regulating gene, which reacts directly with the inducing agent (e.g. penicillin) to form an active "antipressor" molecule, which then interacts with, and inactivates the bla I repressor protein, allowing transcription of the bla Z gene. As the antirepressor gene is probably chromosomal, and so not directly associated with the bla operon, it is possible that the bla operon "uses" a resident penicillin-binding protein, such as an enzyme involved in cell-wall synthesis, as its antirepressor. However, there is as yet little information on the nature or identity of either the repressor or antirepressor proteins.

There have been few studies on the synthesis and secretion of staphylococcal penicillinase. However the similarities to the *Bacillus* enzymes, as reviewed earlier, suggest that the staphylococcal enzymes are probably produced by a similar mechanism. Certainly the enzyme exists in both membrane-associated and extracellular forms (Coles and Gross, 1967 a,b; 1969; 1973), as do other staphylococcal secretory proteins such as α-toxin (Coulter, 1966) and nuclease (Okabayashi and Mizuno, 1974 a,b; Davis et al., 1977). In particular, nuclease B has been

shown to exist in several forms, some of which contain amino-terminal peptide extensions (Davis *et al.*, 1977) analogous to the *B.licheniformis* penicillinase (see section 1.3.4).

1.5 OUTLINE AND APPROACHES OF THIS WORK

The work described in this thesis is divided into two parts:

A. Chapters 3 and 4 describe the synthesis of a staphylococcal penicillinase in an *in vitro* protein synthesizing system based on that of Zubay (1973). Most of these studies were performed with the plasmid pI258 *bla i* 433, which carries a mutation in the repressor gene of the penicillinase operon, allowing the enzyme to be produced constitutively (Novick, 1965). This plasmid was considered an ideal *in vitro* template as it allows the synthesis of the enzyme to be studied independently of the induction process.

The initial aim of this work was to determine whether the synthesis of an extracellular protein is dependent on the presence of membrane-bound ribosomes, and to examine the co-ordinated synthesis-peptide processing-secretion mechanism. It was decided that an *in vitro* protein synthesizing system was most suitable for such investigations - it allows examination of the synthesis of a particular protein in relative isolation from other proteins, and in isolation from other cellular activities while accurately reflecting the protein synthesis process of the whole cell.

Similarly, the use of a plasmid as template means that both transcription and translation of the protein can be studied, circumventing difficulties in isolation of purified mRNA, and allowing transcription and translation to occur simultaneously - a more normal mode of protein synthesis.

As reviewed earlier in this chapter, a number of other studies on extracellular proteins have now increased knowledge of the process beyond the original aims of this project, in particular, the development of recombinant DNA technology has allowed synthesis of a number of extracellular proteins *in vitro*, while DNA sequence analysis has provided much information on the nature of the leader sequence.

B. Chapters 5 and 6 describe studies on the role of lipid synthesis in the secretory process. As reviewed earlier in this chapter, extracellular protein synthesis appears to be especially sensitive to disruption of the lipid state of the cell, indicating that the synthesis and secretion of proteins is not dependent solely on the information provided by the protein's genes, but also to some extent on the state of the cell itself, e.g. the disproportionate sensitivity of secretory proteins to interference by antibiotics, and to disruption of lipid synthesis.

In these chapters, the role of lipid synthesis was examined using the antibiotic cerulenin which specifically (or so it is believed) inhibits fatty acid synthesis, and a glycerol dependent auxotroph of *E.coli*.

Thus the work in this thesis takes two approaches to the secretion phenomenon: firstly, by examining the synthesis of the protein itself, in an isolated system, and secondly by looking at the synthesis of the enzyme, as it is affected by modifying the state of the cell.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains

The strains of *Staphylococcus aureus* and *Escherichia coli* used in this study are described in Tables 2.1 and 2.2 respectively.

2.1.2 Plasmids

Tables 2.3 lists the *S.aureus* and *E.coli* plasmids used in this work, their relevant genotype, and their source.

2.1.3 Bacteriophage strains

The hybrid phage λ*plac*5cI857 *Sam* was used as a source of the *lac Z* gene, for *in vitro* protein synthesis. The phage contains the *lac Z* gene and the operator-promoter region of the *lac* operon. It was isolated and described by Shapiro *et al.* (1969) and Ippen, Shapiro and Beckwith (1971).

The temperate staphylococcal phage ϕ11, (Novick 1967) was used for transduction of plasmids between strains of *S.aureus*. Low titre stocks were prepared by UV-induction of strain S80, as described later.

TABLE 2.1

Strains of *S.aureus* used in this study

Lab. no.	Other designations	Relevant phenotype	Source
S2	8325-4, RN450	NCTC8325, cured of prophages	R.Novick
S80	8325-4(ϕ11)	-	this laboratory
S111	8325-4(pI258*bla*i443)	constitutive penicillinase	this laboratory
S112	8325-4(pI258)	-	this laboratory
S138	FRI 184	enterotoxin negative	M.Bergdoll
S150	RN1465 8325-4(pII147)	-	R.Novick
S151	RN1466 8325-4(pII147*bla*i220)	constitutive penicillinase	R.Novick
S152	8325-4(pI258Δ*mad→bla*)	deletion to Δ*mad→bla*	R.Novick

TABLE 2.2

Strains of *E.coli* used in this study

Lab. no.	Other designations	Relevant phenotype	Source
E233	C600 (λp*lac*5)	LAC$^-$ host, lysogenic for λp*lac*5	R. Mailhamer
E234	LG4	ΔLAC	R.Mailhamer
E392	ED8654	R_k^- M_k^+	N.Murray
E234GLY$^-$	-	glycerol requirement	this work
E541	R163	Su$^+$3	D. Hogness

TABLE 2.3

Plasmids used in this study

A. *S.aureus* plasmids were kindly provided by R.Novick, and where necessary, transduced into S2, or S138, with the phage ϕ11.

B. The recombinant plasmid pMB9 was used for transfer of staphylococcal penicillinase DNA into *E.coli* (Chapter 6).

References:

a. Novick and Richmond (1965)

b. Maniatis *et al.*, (197)

	Plasmid	Other designations	Relevant phenotype	Source
S.aureus	pI258	γ	*blaIblaZ*	R.Novick[a]
	pI258*blai*443	-	constitutive penicillinase	R.Novick[a]
	pII147	β	*blaIblaZ*	R.Novick[a]
	pII147*blai*220	pRN2003	constitutive penicillinase	R.Novick[a]
	pI258Δ*mad→bla*	pRN3173, pI258Δ94	penicillinase deletion	R.Novick
E.coli	pMB9	-	tet^R, single Hind III site	Maniatis

2.1.4 Antibiotics

Benzylpenicillin (penicillin G). at 1600 units/mg., was purchased from Sigma Chemical Co., U.S.A.

Ampicillin (sodium salt) and methicillin (sodium salt) were purchased from Commonwealth Serum Laboratories, Melbourne, Australia.

Erythromycin and tetracycline were gifts from Beecham, Australia.

Cephalosporin 87/312 was a gift of Glaxo Research Ltd., Middlesex. U.K.

Cerulenin was purchased from Makor Chemicals, Jerusalem, Israel.

2.1.5 Biochemicals

All biochemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A., unless otherwise specified.

2.1.6 Chemicals

Chemicals used routinely throughout this work include:

Caesium chloride (optical grade): Harshaw Chemical Company, Cleveland, U.S.A.

Polyethylene glycol (PEG, molecular weight 6000): Union Carbide Corp., New York, U.S.A.

Phenol (Analar AR) was redistilled and stored under nitrogen in the dark at - 15°C.

Bacto-tryptone, Bacto agar, Yeast Extract and Casamino Acids: Difco Laboratories, Detroit, U.S.A.

Scintillation fluid consisted of 3.5 gm of 2,5-dephenyloxazole (PPO) and 0.35 gm of 1,4-bis 2-(5-phenyloxazolyl) benzene (POPOP) dissolved in one litre of toluene.

All other chemicals were of the highest grade available.

2.1.7 Radioisotopes

All tritiated and ^{14}C isotopes were purchased from the Radiochemical Centre, Amersham, U.K.

U-^{14}C amino acid mixture (equimolar), 10m Ci/μmol., 50μCi/ml.

Glycerol-2-^{14}C, 100μCi/ml

l-U-^{14}C lysine monohydrochloride, 340 Ci/mol, 50μCi/ml

L-serine-^{14}C-U, 14.5 mCi/mmol, 50μCi/ml

sodium ^{14}C-acetate, 100 mCi/mmol, 500μCi/ml.

5,6-^{3}H cytidine triphosphate, 28 mCi/mmol, 5 mCi/ml

2.1.8 Miscellaneous materials

GF/A filters were purchased from Whatman Ltd., U.K.

Cellulose phosphate (P11) was purchased from Whatman Ltd., U.K.

Dialysis tubing (8/32 and 18/32) was purchased from Union Carbide, N.Y., U.S.A.

Nitrocellulose was purchased from Satrorious, U.S.A.

2.2 MEDIA AND BUFFERS

All media and solutions were prepared in glass-distilled water, and were sterilized by autoclaving for 25 minutes at 120°C and 15 lb/in^2.

2.2.1 Liquid media for *S.aureus* growth:

a) *S.aureus* was routinely cultured in 5CY broth:

0.5% casamino acids

0.5% yeast extract

0.1M NaCl

0.05 tris-HCl pH 7.8

Sterile glucose (20% w/v) was added to a final concentration of 0.5% after autoclaving.

When plasmid-bearing strains were being cultured, the medium was supplemented immediately prior to use, with the appropriate selective antibiotic to retard spontaneous plasmid loss. For pI258, erythromycin at a final concentration of 15 µg/ml was used. For pII147, ampicillin was added to 25 µg/ml.

b) Exo-enzyme broth (1CYP)

A modified CY broth was used for purification of exo-penicillinase, and for measurement of production of extracellular proteins (Robson and Pain, 1976). This was used as extracellular protein production is more pronounced in media not containing tris buffers (D.Love, personal communication).

1%	Casamino acids
1%	Yeast extract
0.096M	Na_2HPO_4
0.019M	KH_2PO_4
0.001M	$MgSO_4.7H_2O$

The media was supplemented with glucose (0.5% w/v) after autoclaving. The trace metal solution of Novick (1963) did not significantly increase growth rate or penicillinase production, so was omitted.

2.2.2 Liquid media for *E.coli*

E.coli strains were routinely grown in L broth:

1%	Bacto-tryptone
0.5%	Yeast extract
1%	NaCl, pH 7.0

supplemented with glucose (0.1% w/v) after autoclaving. Selective antibiotics were added where necessary, immediately prior to use.

2.2.3 Solid media for *S.aureus*

a) *S.aureus* colonies were routinely cultured on 3CY plates (Novick, 1963)

0.3% Casamino acids

0.3% Yeast extract

0.1M NaCl

0.004 M $CaCl_2$

0.005 M tris-HCl pH 7.8

1.2% agar

b) Penicillinase screening plates.

Colonies were screened for penicillinase production on plates by a method similar to that of Perret (1965). Cultures were first grown on 1CY-starch plates:

1% Casamino acids

1% Yeast extract

0.1M NaCl

0.05 tris HCL pH 7.8

0.2% soluble starch

1.0% agar

then flooded for 30 seconds with 5 ml of

0.016M I_2

0.12M KI

0.05M benzylpenicillin

After removal of excess solution, penicillinase-producing colonies could be identified by the appearance of a clear zone around the colonies, on the dark blue background.

2.2.4 Solid media for *E.coli*

E.coli was grown on L plates

1% Bacto-tryptone

0.5% Yeast extract

1% NaCl pH 7.0

1.5% agar

Plates were supplemented with selective antibiotics when necessary.

2.2.5 Buffers

TM: 0.01M tris-HCl pH 7.1

0.01M $MgSO_4$

TE: 0.01M tris-HCl pH 8.0

0.001M EDTA

BDE (Novick and Bouanchaud, 1971):

1% Brij-58

0.04% Na desoxycholate

0.3M EDTA pH 8.0

2.3 GENERAL METHODS

2.3.1 Storage of bacteria

Short-term storage of bacteria was in 5 ml of Nutrient Agar stabs (Difco), supplemented with antibiotic if required. Stabs were stored at room temperature (*S.aureus*) or 4° (*E.coli*), and were viable for at least a year, although they were discarded several months after first use.

Medium-term storage of bacteria was achieved by diluting a fresh overnight broth culture 1:1 with sterile 80% glycerol, and freezing at -80°C. Long-term stocks were freeze-dried ampoules stored at 4°C, and were viable indefinitely.

2.3.2 Growth of bacterial cultures

A stationary-phase bacterial culture was prepared by inoculating 5 ml of broth with a loopful of agar from a stab, and incubating with shaking, in a New Brunswick gyrotory water bath, at 30° or 37° for 18 hours.

Log-phase cultures (OD_{600} = 0.8) were prepared by inoculating 5 ml of broth with 0.1 ml of a fresh overnight culture, and incubating

with shaking (250 rpm) in a water bath at 30^{o} or 37^{o}. OD_{600} = 0.8 corresponds to approximately 4×10^{8} cfu/ml for *E.coli* and 2×10^{8} cfu/ml for *S.aureus*.

Antibiotics were included in all overnight cultures of plasmid-bearing strains, but not normally in log-phase cultures.

2.3.3 Preparation of dialysis tubing

Dialysis tubing was prepared by firstly, soaking it in distilled water for 30', then boiling for 5' in two changes of distilled water. Tubing was checked for leaks, boiled extensively in TE, then briefly rinsed in the buffer to be used in dialysis.

2.3.4 Preparative isolation of staphylococcal plasmid DNA

Staphylococcal plasmid DNA was purified in large amounts by a method based on that of Novick and Bouanchaud (1971).

2.3.4.1 Growth of cells

Ten x 500 ml of 5CY + glucose, in 2 litre glass flasks, were inoculated with 5 ml each, of a fresh overnight culture of the plasmid-carrying strain,

and grown with gentle shaking at 37° for 18 hours in a Paton orbital Air-shaker. Antibiotic was included in the broth; erythromycin at 15 µg/ml (for pI258) or ampicillin at 25 µg/ml (for pII147).

Cells were harvested by centrifugation (9,000 rpm, 15', 4°C, Beckman JA10), washed once with TM, and the pellet stored at -80°C until used. The yield of cells was about 3 gm (wet weight)/litre.

2.3.4.2 Plasmid preparation

The frozen cell pellet from 5ℓ culture was thawed for 30 minutes at 4°C, then resuspended in 100 ml of 2.5M NaCl + 0.05M EDTA, pH 7.0, at room temperature. Five milligrams of lysostaphin (Sigma, 250 U/mg) was added, and the suspension incubated at 37°, with intermittent shaking. At intervals, 1 ml was withdrawn and added to 1.5 ml BDE, to check on the progress of the protoplasting procedure, as evidenced by the rapid clearing of the cells and increase in viscosity. This usually took about 30'.

Once protoplasting was complete, 150 ml of BDE was added to the cells, and gently mixed.

The viscous mix was immediately centifuged for 30' at 20,000 rpm (Beckman JA20), $4^{o}C$, and the supernatant carefully decanted.

Solid PEG was added to 10% (w/w) to the supernatant, gently dissolved at 4^{o}, and the mix allowed to stand at 4^{o} overnight to precipitate the DNA.

DNA was pelletted by centrifugation (5000 rpm, 10' Beckman JA10), the supernatant discarded, and the large pellet resuspended in a minimal volume (about 30 ml) of 0.01M tris-HCl, pH 8.0, 0.01M EDTA, 0.2M NaCl.

Caesium chloride was added to 48.6% (w/w) and 1/50 the original volume of ethidium bromide (10 mg/ml) was mixed. The final concentration of ethidium bromide is thus about 200 µg/ml, and of CsCl, 47.6% (w/w) (specific gravity 1.54).

The DNA solution was loaded into polyallomer tubes, overlaid with paraffin, and centrifuged at 40,000 rpm for 48 hr at 15^{o}, in a Beckman Ti50 rotor.

DNA bands were visualized under long-wave UV light, and the denser plasmid bands were removed by side-puncture with a hypodermic syringe and needle. Plasmid DNA was pooled, and ethidium bromide was removed by repeated extractions with equal volumes of isoamyl alcohol. At least six extractions were performed.

DNA was then dialysed for 24 hours against 3 x 1ℓ of TE at 4^{o}, re-extracted with an equal volume of buffer-saturated, redistilled phenol, and re-dialysed. For use in the *in vitro* system, this final dialysis was against 1mM tris-acetate pH 8.0, and was followed by concentration of the DNA by rotary evaporation, to about 250 µg/ml. For other purposes, DNA was finally dialysed against TE.

The yield of plasmid DNA was 100-200µg per litre of overnight culture, as estimated by A_{260} readings ($1A_{260}$ = 50µg/ml DNA). Purified DNA was stored at 4^{o}C.

2.3.5 Preparative isolation of *E.coli* plasmid DNA

2.3.5.1 Growth of cells

Ten x 500 ml of L + glucose + antibiotic were inoculated with 5 ml each, of a fresh overnight culture of the plasmid-carrying *E.coli*, and grown with gentle shaking at 37^{o} for 18 hours.

Cells were harvested by centrifugation, washed once with TM, and the pellet stored at -80^{o}C. The yield was about 6 gm cells (wet weight)/litre.

2.3.5.2 Plasmid preparation

Cells from 5ℓ of media were resuspended in 150 ml of 25% sucrose + 0.05 M tris-HCL pH 8.0, at 4^{o}C. Fifty ml of lysozyme (10 mg/ml, in the same buffer) was added, and the culture stirred gently, on ice, for 5'. Fifty ml of 0.5 M EDTA, pH 8.0, was added, and the mix stood on ice for a further 5'.

Cells were lysed by the addition of 125 ml of 1% Triton X100, 0.05 M tris-HCl pH 8.0, 0.06 M EDTA. After 5' on ice, the viscous suspension was centrifuged at 20,000 rpm for 30' at 4^{o}. The supernatant was removed,

4M NaCl added to 0.2M, and solid PEG added to 10% (w/w). After dissolving, the solution was stored at 4^{o} overnight.

DNA was pelletted and resuspended in tris-EDTA-NaCl, as described in section 2.3.3.2.

Solid CsCl was added (x gm to x ml solution), dissolved, and 0.1x ml of 10 mg/ml ethidium bromide added.

Plasmid DNA was banded by centrifugation. removed, extracted and dialysed as described in section 2.3.3.2.

Yields varied from 100-300µg DNA/litre culture, and DNA was stored in TE at 4^{o}.

2.3.6 Preparation of λp*lac*5 DNA

DNA prepared from the hybrid phage λp*lac*5 was used as a template for *in vitro* synthesis of β-galactosidase. The phage was prepared by heat induction and chloroform lysis of the lysogen E233.

2.3.6.1 Isolation of virus

Ten x 500 ml of L broth, in two litre flasks, were inoculated with 5 ml each of a fresh overnight culture of E233, and grown with vigorous shaking in a Paton orbital shaker, at 30°C. The optical density at 600 nm was monitored, and when the culture reached midlog phase (OD_{600} = 0.8), the flasks were transferred to a 42° water bath for 20', then incubated at 37° until the OD_{600} stabilized. Cells were harvested by centrifugation, washed once with TM, and resuspended in TM + 1 mM $CaCl_2$ + 0.06M NaCl (150 ml at 37°). Twenty ml of chloroform was added and shaken for 5'. About 2 mg of pancreatic deoxyribonuclease was added and the mix incubated for 10' at 37° to degrade free chromosomal DNA. The mix was chilled, the chloroform removed, and cell debris removed by centrifugation (9,000 rpm, 20', 4°, Beckman JA 10). The supernatant, containing the free phage, was then centrifuged for 3 hours at 16,000 rpm (Beckman JA20) to pellet phage particles. The pellets were then resuspended in a minimal volume of TM plus $CaCl_2$ and NaCl (as above), CsCl added to 45% (w/w) and the solution centrifuged for 24 hours in a

Ti50 rotor (32,000 rpm, 4°C), to equilibrium. The phage bands were removed by side puncture, and dialysed against 3 x 1ℓ TM. At all steps, phage were assayed by plating dilutions on suppressor strain E541. From 5ℓ of culture, about 5 x 10^{13} pfu could be obtained.

2.3.6.2 Isolation of DNA

The dialysed phage suspension was adjusted to A_{260} = 10, and extracted at 4° at least four times with an equal volume of redistilled phenol equilibrated with TE. In contrast to Zubay (1973), SDS was omitted from the extraction, as this proved difficult to remove during dialysis, and gave no improvement in phase separation.

Extracted DNA was extensively dialysed (4 x 1ℓ) against 0.01M tris-HCl pH 8.0, at 4°. The DNA was then checked for residual protein and phenol by UV-scan, using a Varian spectrophotometer. DNA was considered pure when the ratio $A_{260}:A_{240}$ exceeded 1.8, and there was no "shoulder" at 270 nm. Purified DNA was stored at 4°c.

2.3.8 Assay of penicillinase activity

Two assays of penicillinase activity were used in this work. The chromogenic cephalosporin 87/312 (O'Callaghan, *et al.*,1972) was used for the assay of purified enzyme and enzyme in cultures, but, as well be discussed later, was unsuitable for use with *in vitro* mixes. The acidimetric assay of Rubin and Smith (1973) employed was used for assay of *in vitro* enzyme.

2.3.8.1 Cephalosporin 87/312

Enzyme activity was measured by mixing 0.1 ml of enzyme (e.g. culture, culture supernatant or purified protein) with 0.9 ml of a 57.3 μg/ml solution of 87/312 in 0.05M sodium phosphate buffer, pH 7.0, to give a final concentration of 10^{-4}M. 87/312.

A_{482} was measured at time zero, and again after the development of a significant red colour (ideally about 5'). After subtraction of the appropriate blank values (including OD_{482} of the culture, when cells were being assayed), enzyme activity was expressed as the change in A_{482} per minute per millilitre of enzyme solution.

When enzyme activity of whole cells was being measured, chloramphenicol (100 µg/ml) was included in the assay to prevent residual synthesis of penicillinase.

2.3.8.2 Acidimetric assay

This assay is based on that of Rubin and Smith (1973), with the exception that a substrate concentration of 2mM benzylpenicillin was used, rather than 0.2mM. The assay measures the colour change of the indicator phenol red as the pH of the mix is lowered by the enzymatic production of penillinoic acid.

The reaction mix consists of:

0.65 ml of: .0018% phenol red
2.8 mM benzylpenicillin in
0.4 mM sodium phosphate, pH 7.6

0.25 ml of enzyme in 0.4 mM sodium phosphate, pH 7.6

At zero time, substrate and enzyme solutions were mixed in a 1 ml cuvette, and the A_{558} measured for 3 minutes at 37°, over which time the rate of decolorization is linear (Rubin and Smith, 1973; and personal observation).

Enzyme activity was conveniently expressed as A_{558}/min.

The weak phosphate buffer was always freshly made, as the pH was unstable over long periods of time. The substrate-indicator solution was also freshly made, and allowed to equilibrate for at least 30' before use. pH was checked and, if necessary adjusted immediately before use.

2.3.9 Purification of staphylococcal penicillinase

Extracellular penicillinase was prepared from strain S111, essentially by the method of Robson and Pain (1976). Ten litres of 1CYP broth was inoculated with 200 ml of a fresh overnight culture of S111 in the same medium supplemented with erythromycin (15 µg/ml), and grown at 37° with moderate aeration (5ℓ/min) in a New Brunswick fermenter. The OD_{600} was monitored, and culture supernatant assayed for penicillinase activity with 87/312. When extracellular enzyme activity had reached a peak (approximately 5 hr. after inoculation, activity of 0.3 U/ml), the culture was centrifuged and the supernatant chilled to 4°C.

Two hundred grams of Whatman P11 cellulose phosphate (prepared according to the manufacturer's directions) was added to the supernatant and stirred gently overnight at 4^{o}, at which stage over 98% of penicillinase activity was absent from the supernatant. In comparison, supernatant stored without cellulose phosphate had lost no activity.

The cellulose phosphate was allowed to settle, and the slurry packed into a 6 cm-diameter column, and washed overnight with 0.05M ammonium acetate, at which stage the A_{280} of the eluant was less than 0.1.

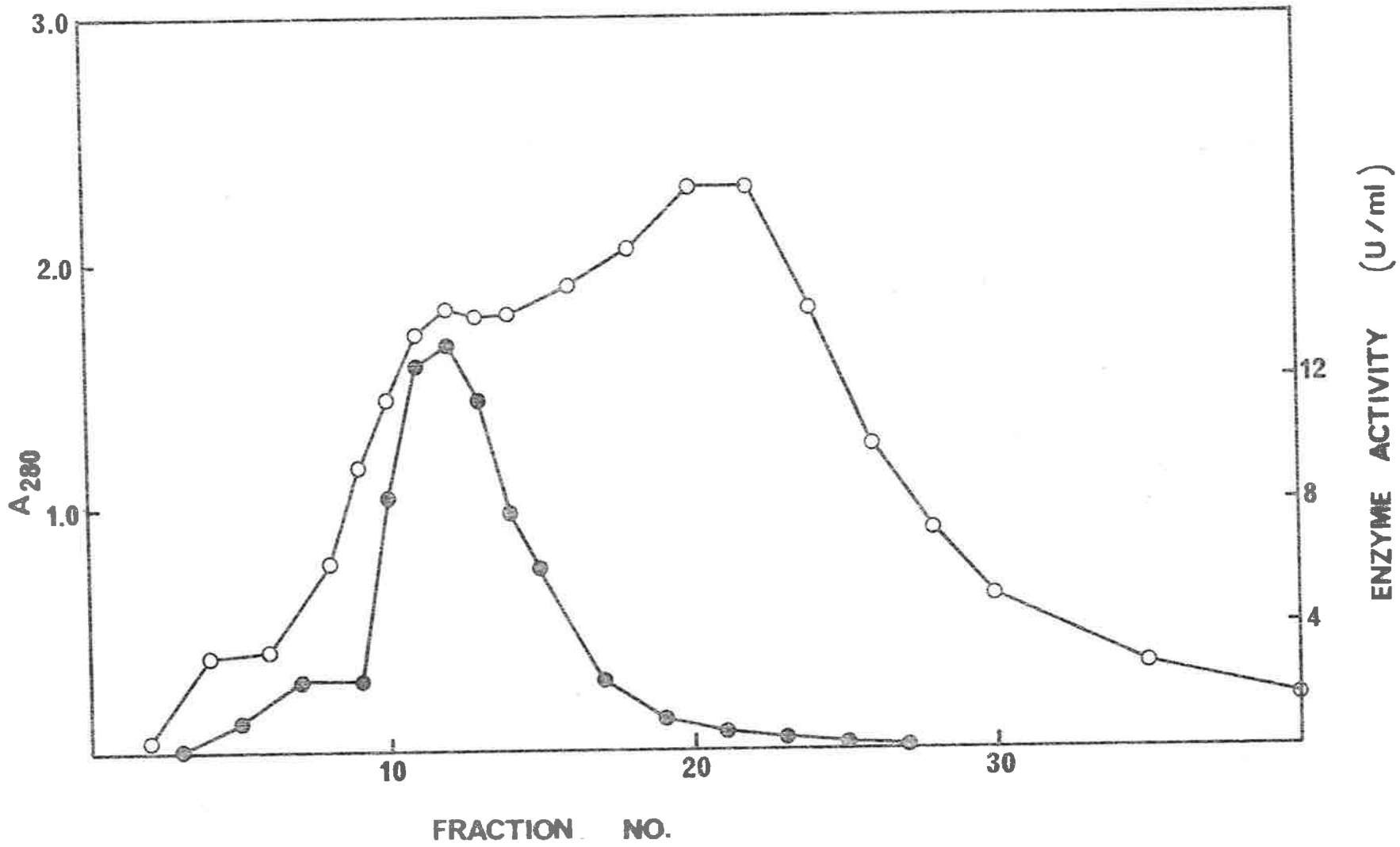
Enzyme was eluted from the column with a linear gradient of 0-80% saturated ammonium sulphate in 0.05M sodium acetate, pH 5.0. Fifty fractions of 15 ml were collected, and their A_{280} and enzyme activity recorded.

Figure 2.1 shows that penicillinase elutes in a single peak, corresponding to a shoulder in the protein elution profile. Fractions containing penicillinase activity (Nos. 10-15) were pooled and dialysed against 0.1M sodium phosphate, pH 7.0, then stored at $-15^{o}C$, in 40% (v/v) glycerol. (For calibration of the

FIGURE 2.1

Partial purification of staphylococcal extracellular penicillinase

Penicillinase was partially purified from the supernatant of a culture of S111 in CYP broth, by adsorption to Whatman P11 cellulose phosphate and elution with ammonium sulphate-sodium acetate, as described in section 2.3.9. 15 ml fractions were collected, and monitored for protein concentration (O), and enzyme activity (●).


3.0
2.0
1.0
A_{280}
10
20
30
FRACTION NO.
12
8
4
ENZYME ACTIVITY (U/ml)

acidimetric assay, aliquots of the enzyme were then dialysed against 0.4 mM phosphate, pH 7.6).

The protein concentration of the stored enzyme was determined by the method of Lowry *et al.*, (1953), and enzyme activity determined by assay with 87/312.

Electrophoresis of a sample of this protein on discontinuous SDS-polyacrylamide gels, followed by staining with Coomassie blue, revealed a single protein band, of M.W. = 29,000 daltons, corresponding to the known molecular weight of staphylococcal penicillinase (Richmond, 1975). Although the elution profile (Fig.2.1) suggests that the enzyme preparation is not pure, the lack of obvious contaminants by direct visualization, and by auto-radiography of labelled protein (as described later) means that this preparation is satisfactory for its use in this work, as a molecular weight standard, and enzyme standard.

As shown in Table 2.4, the total yield of protein was 62 mg, at 620 μg/ml, with an enzyme activity of 1.5U/ml; i.e. the specific activity of the preparation is 2.4U/mg. A considerable amount

TABLE 2.4

PARTIAL PURIFICATION OF PENICILLINASE FROM S111

Enzyme was purified as described in section 2.3.9. The total recovery was about 10% of initial enzyme activity, at a specificity of 2.4U/mg. Units of enzyme activity are derived from assay with 87/312.

TABLE 2.4

	Enzyme activity (U/ml)	Total enzyme (units)	Total recovery of NZ activity	Total protein
Culture supernatant	0.292	1462	100	-
* Supernatant after P-11 adsorption	0.003	15	1*	-
Pooled peaks (15 ml)	9.9	892	61	-
Dialyzed protein, (stored in 40% glycerol)	1.5	150	10	62.5 mg

* i.e. 99% activity was absorbed to the phosphocellulose.

of enzyme activity was lost during elution from the column and during dialysis. This is comparable with Richmond's (1975) experience with penicillinase purification on CM-cellulose, where the best yield during the chromatography step was 66%.

2.3.10 Transduction of staphylococcal plasmids into S138

For some of the experiments described in Chapter 6, penicillinase plasmid DNA was prepared from the enterotoxin-negative strain of *S.aureus* S138 (strain FRI 184, kindly provided by Dr. M. Bergdoll), as a safeguard against the accidental insertion of enterotoxin genes into *E.coli* during recombinant DNA work. This procedure was required by the Australian Academy of Sciences Biohazards Committee. Neither S138, or its plasmid-bearing derivatives produced enterotoxins, and were therefore used as sources of plasmid DNA used for recombination experiments.

The plasmids pI258 and pI258*bla*i443 were inserted into S138 by transduction, mediated by the temperate bacteriophage φ11.

2.3.10.1 Preparation of φ11

φ11 was prepared from S80 (8325-4(φ11)) by UV induction. Five ml of 5CY was inoculated from a fresh overnight culture and grown to OD_{600} = 0.8. Cells were pelleted by centrifugation, and resuspended in 5 ml of phage buffer (Novick and Brodsky, 1972) in a 9 cm petri dish. The suspension was irradiated with UV light (General

Electric germicidal lamp) for 30" at 15 erg/mm^2/sec. 0.5 ml of 10 x 5 CY broth, and 0.05 ml of 0.4 M $CaCl_2$ were added, and the culture incubated at 37^o until visible lysis occurred (about 2 - 3 hours). The culture was sterilized by millipore-filtration, and the free-phage titrated by plating various dilutions on S2, using the standard soft-agar overlay technique. Undiluted phage titres were usually 1 - 4 x 10^{10} p.f.u./ml, and were stored over chloroform at 4^{o}C.

2.3.10.2 Preparation of lysogens of S111 and S112

The plasmid-bearing strains S111 and S112 were made lysogenic by plating dilutions of φ11 on lawns of the cultures in soft agar. After incubation overnight at 37^o, the turbid centres were removed from plaques with a pasteur pipette, and streaked over a 3CY plate. Individual colonies were re-purified by successive streakings, then checked for lysogeny by cross-streaking with φ11, and for plasmid maintenance by growth on 3CY + erythromycin (15 μg/ml).

2.3.10.3 Preparation of transducing lysates

A lysogenic, plasmid-bearing isolate was treated with ultraviolet light and free phage isolated, as described above.

2.3.10.4 Transduction of plasmids to S138

A log-phase culture of S138 in 5CY + 4mM $CaCl_2$ was infected with ϕ11 transducing lysate at a multiplicity of 0.1. After 15' at 37°, dilutions were plated on 3CY + erythromycin plates, and grown at 37° for 48 hr. Individual colonies were purified, and checked for the acquisition of plasmid phenotypes (erythromycin resistance, penicillinase production, cadmium and mercury resistance) by plating on the appropriate solid media.

The approximate rate of transduction was 10^{-7}/p.f.u.

CHAPTER THREE

SYNTHESIS OF PENICILLINASE *in vitro*

3.1 INTRODUCTION

The aim of the work described in this chapter was to establish an *in vitro*, DNA-directed protein-synthesizing system based on that developed by Zubay (1973), that would faithfully transcribe and translate staphylococcal penicillinase DNA.

The Zubay system was chosen because of its comparative simplicity, as it used a crude cell extract (the supernatant of a 30,000 g centrifugation of lysed cells) as the source of RNA polymerase, ribosomes and other components of the protein synthetic machinery. In contrast, other cell-free systems are often more purified. For example, the system of Herrlich and Schweiger (1974) uses a fraction of 200,000 g supernatant, partially purified on DEAE-cellulose, and supplemented with purified ribosomes. While such systems are efficient at protein synthesis, and are particularly useful for examination of various factors involved in protein synthesis, the less purified Zubay system has the advantage of more closely resembling the intracellular environment, so transcription and translation in this system should be more reflective of the *in vivo* situation.

The Zubay system is capable of considerable accuracy in protein synthesis. Not only have a

number of active enzymes been synthesized in it, but genetic control mechanisms such as repression and induction have been shown to act *in vitro* (e.g. see review of Zubay, 1973; Pratt, 1980). At the beginning of this work, no foreign DNA had successfully been transcribed and translated in an *E.coli* based system, so the initial questions were:

1. Will an *E.coli* protein synthesizing system recognise a staphylococcal template, and be able to transcribe and translate it? If so, this would imply that the protein synthetic machinery of the two bacterial species was very similar, including for example the RNA polymerase binding sites, and translation initiation and termination signals. This aspect of the work has become less important since the advent of recombinant DNA technology, but was a significant question early in the work.

2. If the system is able to respond to staphylococcal DNA, does it produce an enzymatically active protein? This question has two significances; firstly, with respect to the fidelity of a foreign DNA, and secondly, as an introduction to the study of the nature of the synthesized

protein, i.e. is the penicillinase produced as a precursor protein?

3. Can the *E.coli* system recognise the genetic control signals of the *bla* operon - i.e. can penicillinase be induced *in vitro* as can occur with *E.coli* operons studies in the system?

3.2 MATERIALS AND METHODS

This section describes those materials and methods used in establishment of *in vitro* protein synthesis. The methods described are typical of those used, although a number of the modifications used will be described in detail later in this chapter.

3.2.1 Glassware

All glassware used in preparation of components of the protein synthesizing system was soaked in 1M KOH in ethanol for at least 2 hours, rinsed in 1M HCl, and then extensively rinsed in distilled water. After drying, all glassware was autoclaved at 120°C for 20' before use.

3.2.2 Storage of chemicals and solutions

All chemicals were stored, in the solid form, at - 15°C.

All stock solutions of chemicals were prepared in glass-distilled water, sterilized by Millipore filtration, and stored at - 80°C.

3.2.3 Preparation of S30 extracts

3.2.3.1 Growth of cells

The bacterial strain used for preparation of S30's was E234. This strain was

provided by R.Mailhamer, who originally obtained it from Zubay. It carries a deletion of the entire *lac* operon (Zubay, 1973)

The cells were grown in a New Brunswick fermenter, at 30°C in KYT broth (Zubay, 1973).

KH_2PO_4	5.6 g/l
K_2HOP_4	28.9 g/l
Yeast extract	10.0 g/l
Thiamine	15 mg/l
Glucose	10 g/l (added as a sterile 20% solution after autoclaving).

$MgCl_2$ was added to 2mM in some preparations, as this was believed to assist in removal of DNA from the S30 extract (M.Heincz, personal communication).

Eight litres of broth was inoculated with 80 ml of a fresh overnight culture of E234, and grown with gentle aeration (6 l/min) until $O.D._{600}$ = 0.8. As will be explained later, it was found that this growth gave the most active S30 extracts.

The culture was chilled by pouring it over two litres of frozen broth, and cells harvested by centrifugation in a Beckman

JA10 rotor (9,000 rpm; 15'; 4°C). Cell pellets were wrapped in aluminium foil and stored overnight at -80°C. The yield of cells is about 6 gm/8 litres of culture.

3.2.3.2 Preparation of S30

The S30 extracts were prepared by the method of Zubay (1973), with some modifications. Ten grams of frozen cells were thawed at 4°C for 30', then suspended in 100 ml of buffer I. (0.01M tris acetate, pH 8.2; 0.014M magnesium acetate, 0.06M potassium acetate, 6mM β-mercaptoethanol). After centrifugation (15,000 rpm; 5'; 4°C, in a Beckman JA-20) the cells were suspended in 40 ml of the same buffer. Cells were again pelleted, and resuspended in 13 ml of buffer II (buffer I, containing 1mM dithiothrietol in place of β-mercaploethanol). The cell suspension was then lysed in an Aminco pressure cell, using an automatic press set at a constant 6000 psi. Twenty micromoles of dithiothreitol was added immediately to the lysate.

The lysate was centrifuged at 20,000 rpm (Beciman JA21 rotor; 4°C) for 30', and the top 10 ml of the supernatant was removed.

centrifugation used by Zubay (1973) was omitted since it did not improve the activity of the S30. The supernatant was mixed with 1 ml of Preincubation Mix (as described in Table 3.1), and incubated at 37° for 80' with occasional gentle shaking, to allow completion of partly translated mRNA. The extract was then dialysed at 4° for 12 hours against three changes of buffer II. Small aliquots (0.3 ml) were dispensed into sterile glass vials, and stored at -80°C.

All steps after the lysis of the cells were carried out in light-protected glassware (R.Mailhamer, personal communication).

The protein concentration of the extract was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Protein concentrations were generally in the range of 22-30 mg/ml.

3.2.4 Stock reaction "cocktail"

The stock protein synthesizing "cocktail" was prepared from stock solutions, as shown in Table 3.2. The stock cocktail was stored at -80° in small aliquots.

TABLE 3.1

Composition of the mix used for preincubation of S30 extracts

The volume given was used for preincubation of 10 ml of extract.

	Stock concentration	Volume used
tris acetate pH 8.2	2M	450
dithiothreitol	0.5 M	20
magnesium acetate	1M	30
amino acids[a]	5mM	20
tyrosine[b]	16.5 mM	7
ATP[c]	0.1 M	75
phospho-enol-pyruvate (Na salt)	0.2 M	420
phospho-enol-pyruvate kinase	10 mg/ml	10
		1032

a. 19 amino acids at 5mM each

b. tyrosine was dissolved in 0.125 M HCl

c. ATP was dissolved in 0.01 M tris acetate pH 8.2

TABLE 3.2

Protein synthesis cocktail

Compsition of the stock protein synthesis cocktail. The cocktail was stored at -80°C, in small aliquots, and used for establishment of the synthesis mix, as described in the text.

TABLE 3.2

	Stock concentration	Volume used in concentration (ml)	Final concentration in synthesis mix
tris acetate pH 8.2	2M	0.500	40 mM
dithiothreitol	0.5 M	0.062	1 mM
potassium acetate	5 M	0.250	50 mM
ammonium acetate	5 M	0.125	25 mM
amino acids[a]	5 mM	1.000	0.2 mM
tyrosine[b]	16.5 mM	0.300	0.2 mM
triphosphates[c]		0.500	2 mM/.5 mM
phosphoenolpyruvate	0.2 M	2.625	21 mM
E.coli tRNA	10 mg/ml	0.250	0.1 mg/ml
pyridoxine-HCl	1 mg/ml		25 µg/ml
p-aminobenzoic acid	1 mg/ml		25 µg/ml
folinic acid	1 mg/ml	0.625	25 µg/ml
NADP	1 mg/ml		25 µg/ml
FAD	1 mg/ml		25 µg/ml
H_2O		0.513	
		6.75 ml	

a. 19 amino acids at 5 mM each

b. tyrosine was dissolved in 0.125 M HCl

c. ATP at 100 mM; CTP, GTP and UTP at 25 mM each; dissolved in 0.01 M tris acetate pH 8.2

3.2.5 Working reaction mix

The working reaction mix, prepared immediately before use, consisted of:

	µl
Cocktail	270
50 mM cAMP (in 0.01 M tris acetate pH 8.2)	10
Mg/Ca stock	10
PEG (150 mg/ml)	100
H_2O	60
	450

Mg/Ca stock solution consisted of 0.6M calcium acetate, and magnesium acetate to give a final, total contribution of approximately 12 mM magnesium ions, as will be described later. Usually this necessitated a stock concentration of about 0.9 M magnesium acetate.

3.2.6 Protein synthesis mix

The protein synthesis mix consisted of:

	µl
Working reaction mix	45
DNA (e.g. 5 µg *plac*5)	
H_2O)	55
s-30 (0.65 mg protein))	
	100

The total volume of the protein synthesis mix was 100 µl, containing DNA, prepared as

described in Chapter 2, S30 to 6.5 mg/ml, and water to 100 µl. Any additions such as radioactive label or protein synthesis inhibitors were added in place of some water.

Immediately after addition of the S30, the mixes, in screw-capped 0.5 ml glass vials, were incubated in the dark at 30°, with rapid shaking, usually for 60'. At the conclusion of the incubation, the flocculent precipitate in the tubes was gently resuspended by shaking.

3.2.7 Assay of β galactosidase synthesized *in vitro*

100 µl of synthesis mix was diluted into 1.5 ml of o-nitrophenol galactopyranoside (ONPG, 0.55 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.3, 0.14 M β-mercaptoethanol, and incubated at room temperature until a significant yellow colour developed. Two drops of glacial acetic acid were added to stop the reaction and precipitate protein, and the assays chilled on ice for 10'. Precipitated protein was removed by centrifugation and 0.5 ml of supernatant was added to an equal volume of 1M sodium carbonate. A_{420} was measured and after subtraction of blank values, enzyme activity was expressed as A_{420}/min.

3.2.8 Assay of penicillinase synthesized *in vitro*

As will be explained in the Results section of

this chapter, the acidimetric assay of Rubin and Smith (1973) was used for detection of penicillinase activity produced *in vitro*. After incubation of the protein synthesis mix, the 100 µl mix was added to 900 µl of ice-cold 0.4mM sodium phosphate buffer, pH 7.6. These were then dialyzed against cold buffer overnight. Usually, about ten 1 ml samples were dialyzed against three one-litre changes of buffer.

0.25 ml of dialysed mix was then assayed for penicillinase activity, as described in Chapter 2.

3.2.9 Incorporation of ^{14}C-lysine into protein

In some experiments, protein synthesis was measured by incorporation of the radioactive lysine into hot TCA-precipitable material. In these experiments, the mix was supplemented with ^{14}C-lysine (50 Ci/ml, 340 mCi/mole). Ten microlitre samples were added to 100 µl of 10% TCA, and chilled on ice for 5'. Samples were heated to 90 - 95^{o} for 15' then chilled for a further 30'. Precipitates were collected on Whatman GF/A filters, which were then batch-washed in three changes of cold 10% TCA, followed by two changes of cold 95% ethanol. After drying, filters were immersed in toluene scintillation fluid and counts recorded as previously described.

3.2.10 Incorporation of ^{3}H-CTP into RNA

RNA synthesis was measured by incorporation of ^{3}H-CTP into TCA-precipitatable material. Protein synthesis mixes were supplemented with 5 µl of 5-^{3}H-CTP (5 mCi/ml, 29 Ci/mmole). Ten microlitre samples were removed and spotted onto GF/A filters. After drying, filters were washed, dried and counted as described.

3.3 RESULTS

3.3.1 Establishment of the *in vitro* protein synthesizing system.

Initially, in setting up an *in vitro* DNA-directed protein synthesizing system, the method of Zubay (1973) was followed strictly. However, repeated failure to synthesize active β-galactosidase in amounts comparable to those reported by Zubay (1973), when priming the system with λ p*lac*5 DNA, made it necessary for the system to be more fully investigated so that optimal synthetic activity could be achieved with β-galactosidase, before applying the system to the study of staphylococcal penicillinase synthesis. This section describes those modifications which were found to increase the activity of the coupled transcription-translation system to acceptable levels.

3.3.1.1 Growth of cells for preparation of the S30 fraction

Zubay (1973) specifies that cells used for the preparation of S30's are grown, in a New Brunswick fermentor, at 28°, for four hours, using a 1/10 inoculum of overnight culture of cells in KYT broth. This yields about 10 g of cells (wet weight) per litre of broth. Zubay claims such a

culture is in mid-log phase, however, when the OD_{600} of such a culture was monitored, the cells appeared to have entered the stationary phase of growth (data not shown). Thus when Zubay's procedure for the preparation of cells is followed, the cells are in a growth-stage probably not optimal for protein synthesis.

Consequently, it was decided to check the activity of S30 preparations from cultures at various stages of growth. Ten litres of KYT, in a New Brunswick fermentor, was inoculated with 100 ml of an overnight culture of E234, and grown at 28^{o} with gentle stirring (200 r.p.m.) and aeration at 6l/min. Aliquots of culture were removed at various times, and the OD_{600} measured (see figure 3.1). Two litre samples were removed at various stages of growth, the cells collected by centrifugation and S30's prepared from them as described earlier in this chapter. The protein synthesizing capabilities of each S30 was then determined by measuring the synthesis of β-glactosidase, using λ *plac*5 DNA, and assayed as described earlier in this chapter. Results are shown in table 3.3 and show that S30's prepared from early-log to mid-log cultures are far more

FIGURE 3.1

Growth of E234 in KYT broth

The optical density of a culture of E234 in KYT broth was measured at 600 nm, using the growth conditions described in Section 3.3.1.1.

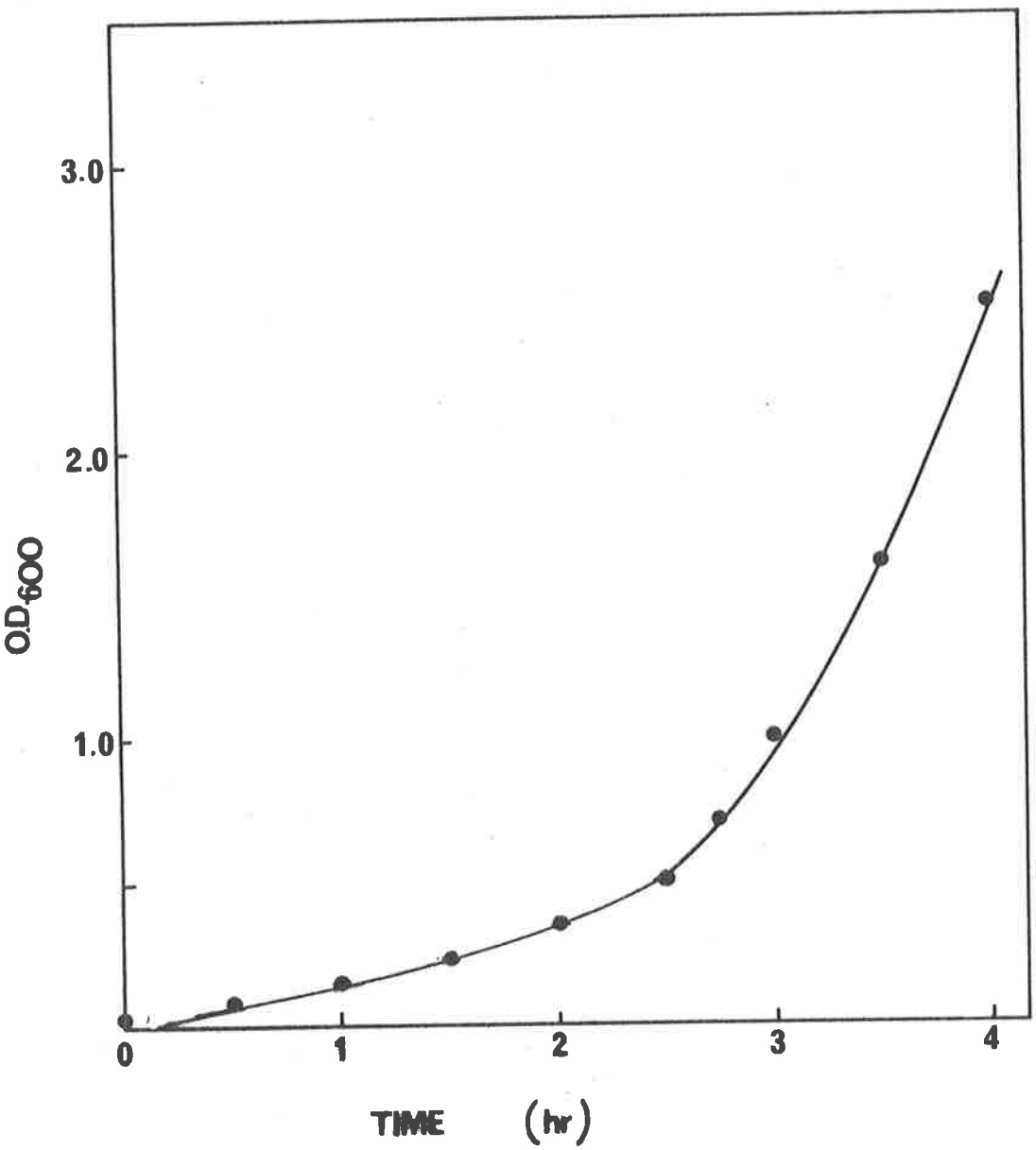

3.0
2.0
1.0
O.D600
0
1
2
3
4
TIME (hr)

TABLE 3.3

Protein synthesizing activity of S30s prepared from cultures of E234 grown to different stages.

S30s were prepared from the harvested cells, as described in the text, and used for the *in vitro* synthesis of β-galactosidase. Protein synthesis mixed contained 12 mM Mg^{2+}, 5μg λp*lac*5 DNA and 6.5 mg/ml S30, and were incubated for 60' and 37° before assay with ONPG.

Cells used for S30s	OD_{600}	Enzyme activity (A420/min)
Early log phase	0.2	0.019
Mid-log phase	0.5	0.028
Mid-log phase	1.0	0.030
Mid-log phase	2.0	0.019
Late-log phase	5.0	0.004

a. Early- and mid-log phase cells were prepared from cultures inoculated 1/100-fold and grown at 20°C. Late-log phase cells were prepared as described by Zubay (1973).

active in the synthesis of β-galactosidase than S30's from late-log to stationary phase cultures.

The maximum levels of synthesis of β-galactosidase are still considerably less than those reported by Zubay in 1973 (about 1.0 A_{420} units in 3-5 minutes), but are obviously considerably enhanced by using cells from cultures in the logarithmic phase of growth - a state not achieved by following Zubay's recipe. Consequently in all further preparations of S30 fractions, cultures were grown from 1/100 inocula, and harvested at an OD_{600} between 0.8 and 1.2

3.3.1.2 Concentration of magnesium and calcium ions necessary for optimal synthesis of β-galactosidase

Zubay (1973, and personal communication) says that the concentration of magnesium acetate added to the protein synthesizing cocktail varies for each S30, but is usually about 14.7 mM. This gives a total concentration of magnesium ions of about 18-20 mM, including that present in the S30 fraction. These values are considerably higher than those used in other *in vitro* protein synthesizing systems. For example, Nirenberg (1963)

recommends a concentration of Mg^{2+} of 8 mM, for his translation system, while Fuchs, (1976) also uses a final concentration of 8 mM Mg^{2+} in a coupled transcription-translation system. Thus a concentration of 18 - 20 mM Mg^{2} seems exceptionally high.

Therefore several S30's were tested for their dependence on magnesium ion concentration. Complete protein synthesizing mixes, primed with λ p*lac*5 DNA, were incubated for 90 minutes with varying concentrations of magnesium acetate, and assayed for β-galactosidase activity. In these experiments, the concentration of calcium acetate was kept constant at 6mM. Typical results are shown in figure 3.2, and clearly show that β-galactosidase synthesis is maximal when the <u>total</u> concentration of Mg^{2+} is 12mM.

For example, a typical S-30 contains 14 mM magnesium acetate, and 30 mg/ml protein, so when used at a final concentration of 6.5 mg protein/ml, will contribute about 3 μ moles of Mg^{2+} to one millilitre of protein synthesizing mix. Therefore 9 μmoles of magnesium acetate must be added to one millilitre of mix, as opposed to the 14.7 moles as recommended by Zubay (1973). This difference of 6 μmoles

of Mg^{2+} has an enormous effect on the synthetic ability of the system, as can be seen in figure 3.2. The magnesium concentrations used here are much more compatible with those of other workers, such as Fuchs (1976), Herrlich and Schweiger (1974), and, more recently, Pratt (1980).

When the total concentration of magnesium ion was kept constant at 12 mM, the synthesizing capabilities of the system were relatively insensitive to the calcium ion concentration, as indicated in figure 3.3. The optimum concentration of Ca^{2+} was consistently 6 mM.

Magnesium and calcium ion concentrations were optimized for each S30 preparation, and were always approximately 12 mM (total Mg^{2+}) and 6 mM (Ca^{2+}).

3.3.1.3 Concentration of S30 protein, and λ*plac*5 DNA.

The concentration of S-30 protein optimal for β-galactosidase synthesis was found to be approximately 6.5 mg/ml, for all S-30's tested, as shown in figure 3.4. In these experiments, total Mg^{2+} concentration was kept constant at 12 mM.

FIGURE 3.2

Effect of total magnesium ion concentration on synthetic ability of the *in vitro* system

In vitro protein synthesis systems, primed with λp*lac*5 DNA, were incubated with varying concentrations of Mg^{2+} (as described in Section 3.3.1.2) to determine the optimum concentration for synthesis of active β-galactosidase. Enzyme activity was measured as described in Section 3.2.7.

o = S30 # 1

● = S30 # 2

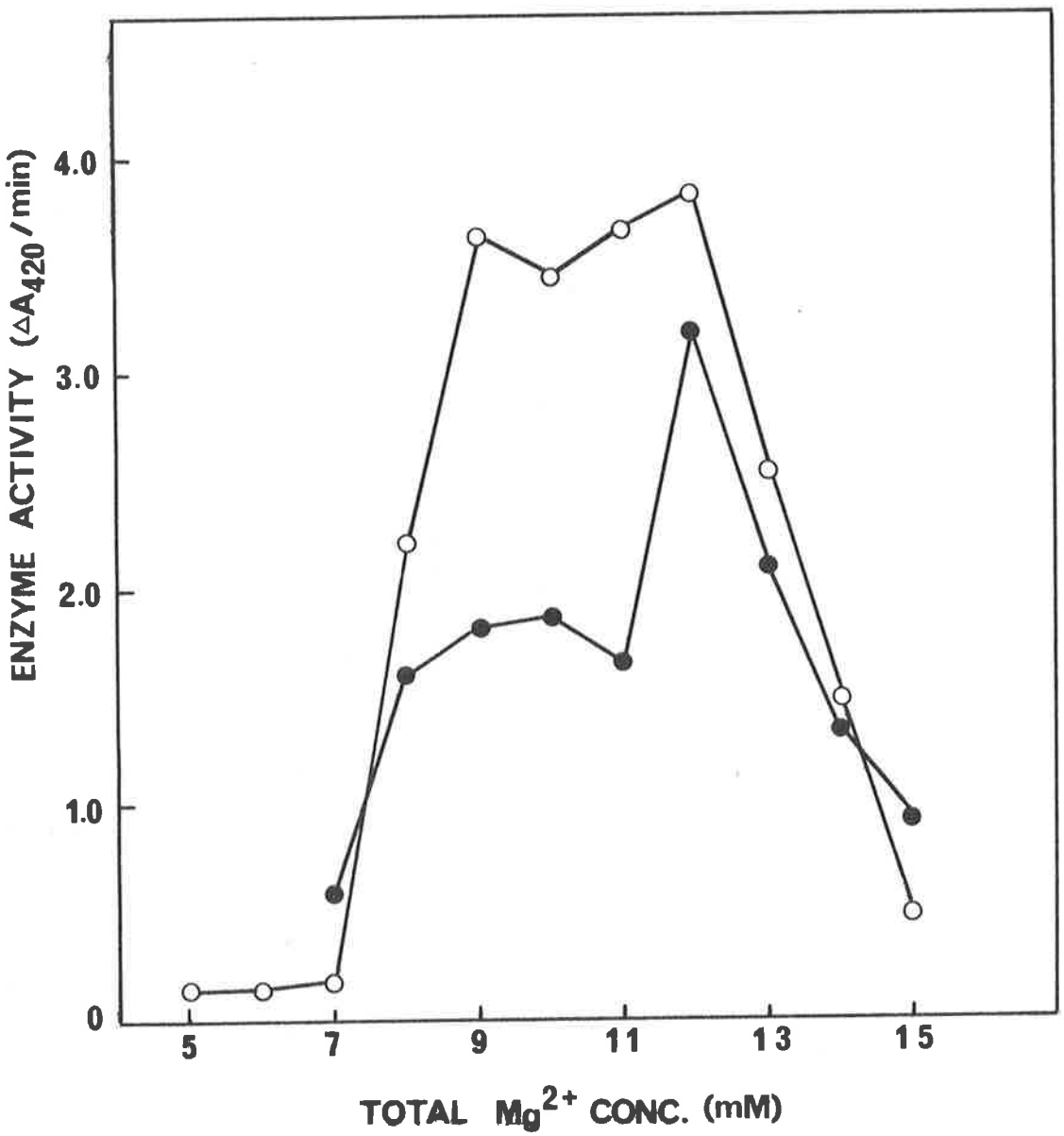

ENZYME ACTIVITY (ΔA_{420}/min)
4.0
3.0
2.0
1.0
0
5
7
9
11
13
15
TOTAL Mg^{2+} CONC. (mM)

FIGURE 3.3

Effect of calcium ion concentration on the synthetic ability of the *in vitro* system.

At a constant Mg^{2+} concentration (12mM) Ca^{2+} concentration was varied, and β-galactosidase assayed as described.

o = S30 # 1

● = S30 # 2

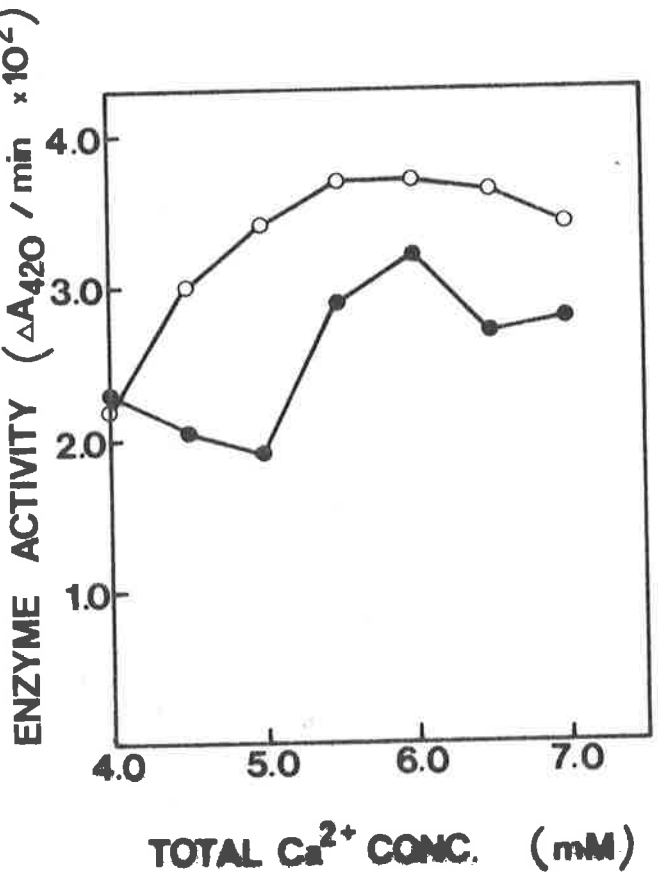
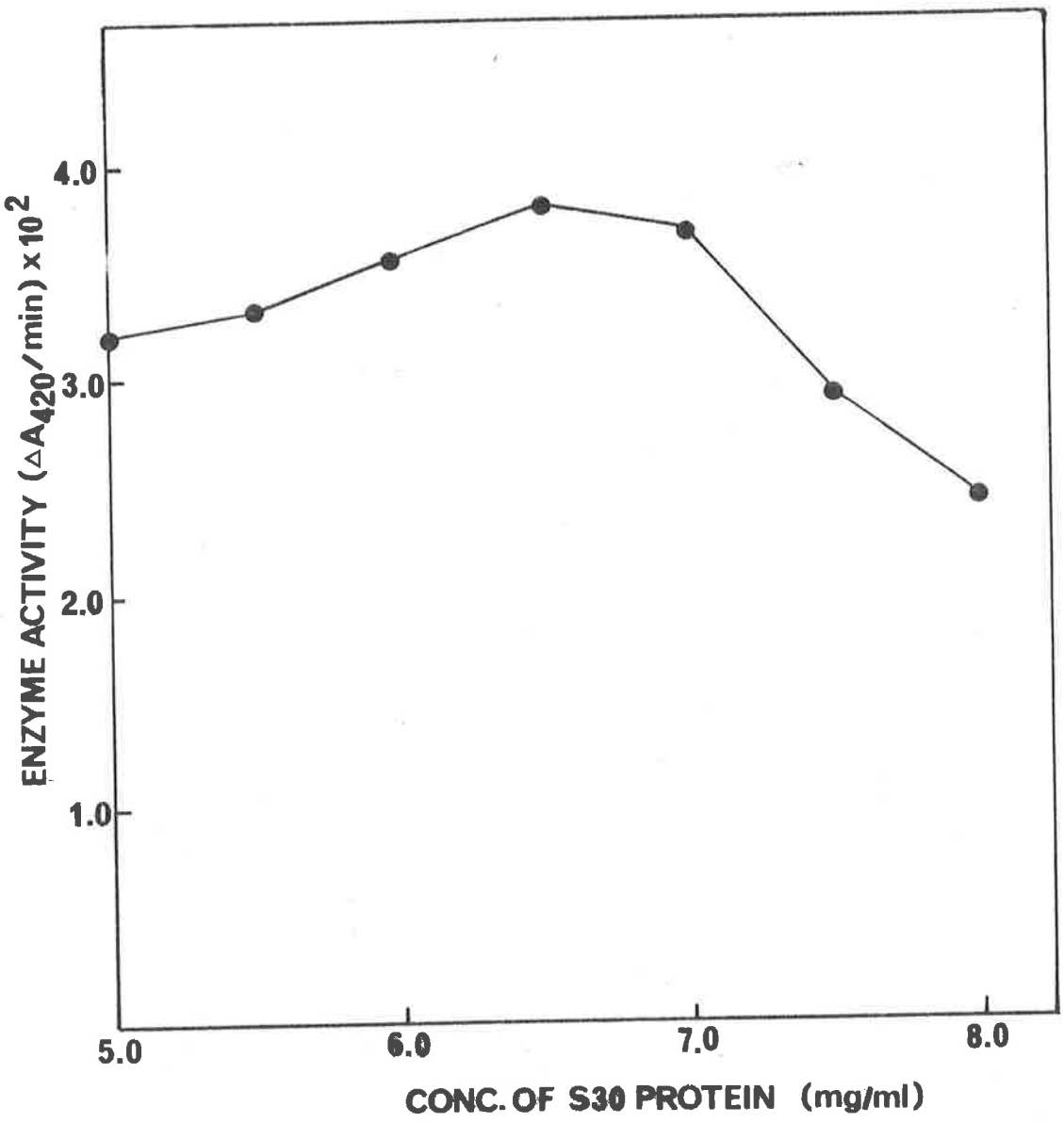

ENZYME ACTIVITY (ΔA_{420} / min $\times 10^2$)
4.0
3.0
2.0
1.0
4.0
5.0
6.0
7.0
TOTAL Ca^{2+} CONC. (mM)

FIGURE 3.4

Effect of concentration of S30 protein on the synthetic ability of the *in vitro* system

At constant (Mg^{2+}) (12mM total) and (Ca^{2+}), (6mM), the effect of differing concentrations of S30 protein were evaluated on the synthesis of β-galactosidase (Section 3.3.1.3).


ENZYME ACTIVITY (ΔA_{420}/min) x 10^2
4.0
3.0
2.0
1.0
5.0
6.0
7.0
8.0
CONC. OF S30 PROTEIN (mg/ml)

Similarly, the concentration of λp*lac*5 DNA was optimal at 50 - 60 μg/ml, as shown in figure 3.5.

Both these figures agree with those of Zubay (1973).

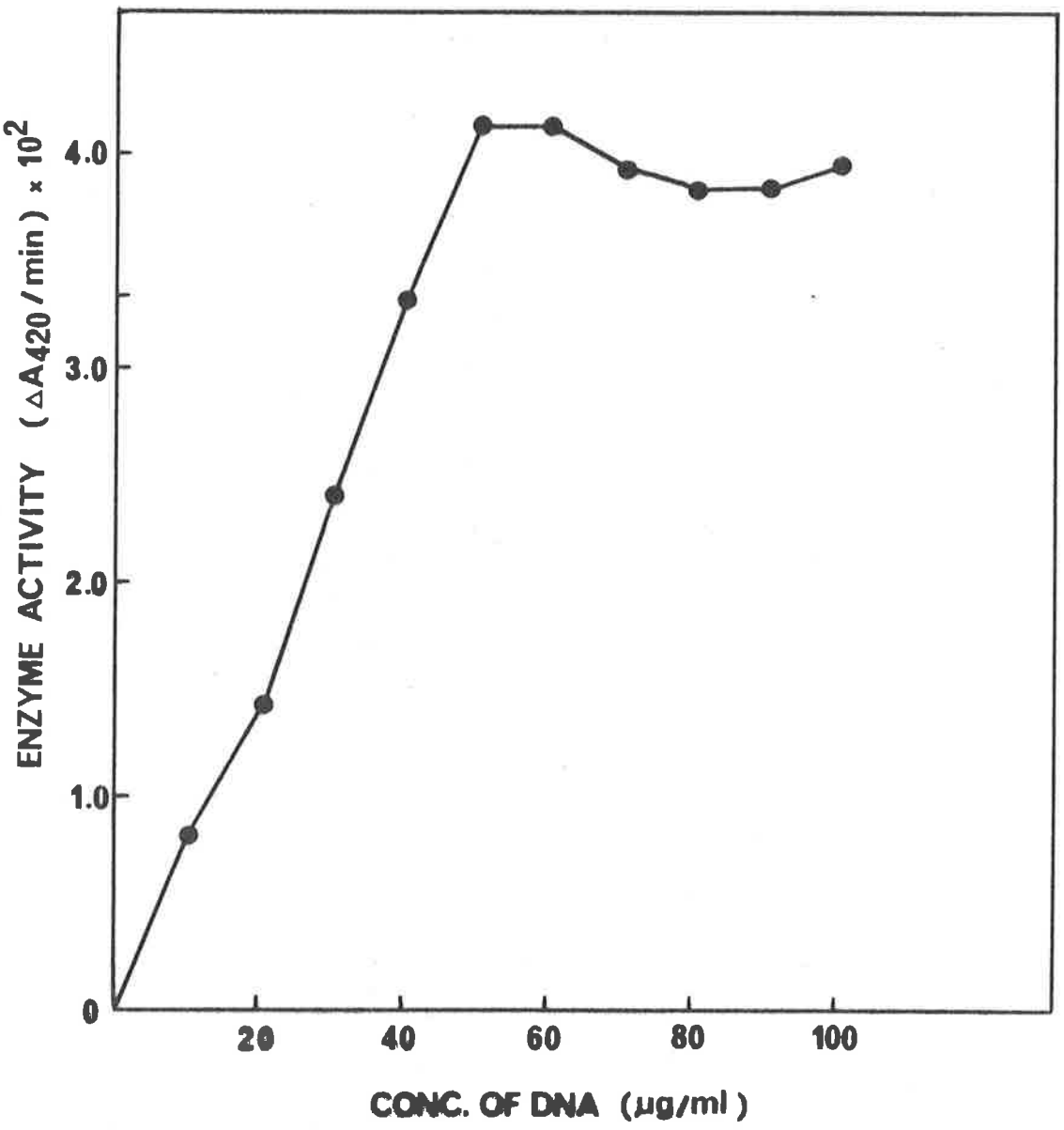
3.3.1.4 Lysis of cells in S-30 preparations

Zubay (1973) lyses cells in a French pressure cell at 4000 - 8000 p.s.i. However, other workers use different pressures, and it has been shown that lysis pressures can have significant effects on the activity of the S30 extracts. For example, Jacogs and Schlessinger (1977) report that extracts made at 3,200 p.s.i. synthesized up to 25-fold more β-galactosidase than extracts made at 7,500 p.s.i., while Pouwels and van Rotterdam (1975) lysed cells at 40,000 p.s.i., and still obtained lysates active in the synthesis of enzymes of the tryptophan operon. In contrast, M.Heincz (personal communication) recommended a lysis pressure of 2,000 p.s.i. Consequently it was decided important to examine the activities of extracts prepared at different pressures. S30's were prepared from log-phase cells as described earlier in this chapter, and used for the synthesis of β-galactosidase, at a concentration of 6.5 mg protein/ml.

FIGURE 3.5

Effect of DNA (λp*lac*5) concentration on synthetic ability of the *in vitro* system

At constant Mg^{2+}, (12mM total); Ca^{2+} (6mM); and S30 (6.5 mg/ml); the concentration of λp*lac*5 DNA optimal for β-galactosidase synthesis was determined (Section 3.3.1.3).


ENZYME ACTIVITY (ΔA420/min) × 10²
4.0
3.0
2.0
1.0
0
20
40
60
80
100
CONC. OF DNA (μg/ml)

As shown in table 3.4, extracts prepared at 6,000 p.s.i. were the most active in β-galactosidase synthesis. Very low pressure (2,000 p.s.i.) yielded an extract too dilute for use, and although the protein concentration could be increased by a second lysis, such a fraction was, under the conditions used here, almost inactive in protein synthesis. Similarly, lysis pressures of 8,000 p.s.i. and above yielded extracts almost devoid of synthetic ability.

Jacobs and Schlessinger (1977) have investigated the effects of Ca^{2+} concentration on the synthetic ability of S30 extracts, and found that high pressure lysates (7,000 p.s.i.) were stimulated by Ca^{2+}, while low pressure lysates (3,200 p.s.i.) were inhibited by that ion. In contrast, however, in the present work it was found that Ca^{2+} was necessary for optimal β-galactosidase synthesis, as shown in table 3.4.

As a result of this experiment, all S30 lysates were prepared at 6,000 p.s.i.

TABLE 3.4

Effect of lysis pressure on the activity of S30.

Cultures were grown to OD_{600} = 0.8, cells harvested and S30s prepared as described in the text. These were used for the synthesis of β-galactosidase, as described, at a concentration of 6.5 mg/ml. The enzyme synthesized represents the average of three experiments.

lysis pressure (psi)	S30 protein conc (mg/ml)	β-galactosidase synthesized (ΔA420/min)	
		6mM Ca^{2+}	No Ca^{2+}
2000	8.4	ND[a]	ND
2 x 2000[b]	17.6	.008	.004
4000	25.1[c]	.030	.019
6000	24.0	.043	.018
8000	25.1	.018	-
12000	25.9	.001	-

a: ND = not determined. Protein concentration was too low for use in protein synthesis.

b: Cells lysed at 2000 psi were re-lysed at 2000 psi

c: Typical concentrations ranged between 21-30 mg/ml

3.3.1.5 Summary

The conditions necessary for optimal synthesis of β-galactosidase were somewhat different to those recommended by Zubay (1973). The growth of cells for S30 production was much more sensitive than suggested in that log-phase cultures gave a far more active extract, although the bacterial strain, the growth medium and even the cultural vessel were the same. A similar requirement has recently been reported by Pratt (1980) and a number of other workers also use log-phase cultures for preparation of protein-synthesizing extracts (e.g. Fuchs, 1976; Randall and Hardy, 1977; Inouye *et al.*, 1977).

The most critical departure from Zubay's procedure, however, was the concentration of magnesium ions present. If, as Zubay recommends, 14-15 mM magnesium acetate is added, the final concentration of Mg^{2+} is 18-20 mM, depending on the volume of S30 used. In comparison, the concentration of Mg^{2+} used by Pratt (1980) and Herrlich and Schweiger (1974) is 12 mM, and in translation systems, a concentration of 8-12 mM is routinely used (e.g. Nirenberg, 1963; Legault-Demare and Chamblis, 1975; Petit-Glatron and Rapoport, 1976). At final concentrations of above 15 mM Mg^{2+} (total), it was found that

β -galactosidase production was severely inhibited.

The pressure of lysis of cells for the S30 extract also affected the activity of the system. Pressures above 6,000 p.s.i. yielded relatively inactive extracts, while lower pressures resulted in dilute S30's unsuitable for use in protein synthesis. In the literature, there is considerable variation in the pressures used; Zubay (1973) used 8,000 p.s.i., Jacobs and Schlessinger (1977) used 3,200 p.s.i., while Pouwells and van Rotterdam (1975) used 40,000 p.s.i.

It is obvious from the considerably varied conditions reported in the literature that it is important to characterize the protein synthesizing system in some detail so that optimum activity can be obtained. Although other parameters could also be investigated (e.g. the stimulatory effect of ppGpp, reported by Smolin and Umbarger, 1975) the characterizations reported here resulted in a system that reproducibly gave significant synthesis of β-galactosidase and so was suitable for the study of staphylococcal penicillinase synthesis.

3.3.2 Synthesis of penicillinase in *in vitro*

Coupled transcription-translation of the staphylococcal penicillinase operon in the *in vitro* system was first attempted using the conditions determined for optimal β-galactosidase described earlier in this chapter. Before characterizing the conditions necessary for optimal synthesis, it was necessary to find a suitable assay for active penicillinase. This section of results will firstly describe the assay systems evaluated, and then the characterization of active penicillinase synthesis, as measured by this assay.

3.3.2.1 Applicability of the 87/312 and acidimetric assays.

It was suspected that the chromogenic cephalosporin 87/312 would not be suitable for assaying penicillinase in the protein synthesizing mix, because 87/312 is degraded by a number of proteins other than penicillinase, in particular by complex protein mixtures such as serum, and by some reducing agents such as β-mercaptoethanol and cysteine (O'Callaghan et al, 1972). This degradation alters the absorption spectrum of the compound, thus masking any specific hydrolysis by β-lactamase. As the complete protein synthesizing mix contains large amounts of

E.coli protein, dithiothreitol and cysteine, it was feared that these could mask specific hydrolysis of 87/312 by penicillinase. This suspicion was confirmed by the following experiment.

Varying amounts of purified penicillinase were added to 0.1 ml of protein synthesis mix (without DNA) and immediately mixed with 0.9 ml of 87/312 in 0.1 M sodium phosphate buffer, pH 7.0 (final concentration of 87/312 = 10^{-4}M). The A_{482} was monitored for several minutes, and after subtraction of the appropriate blank value, ΔA_{482}/min was calculated.

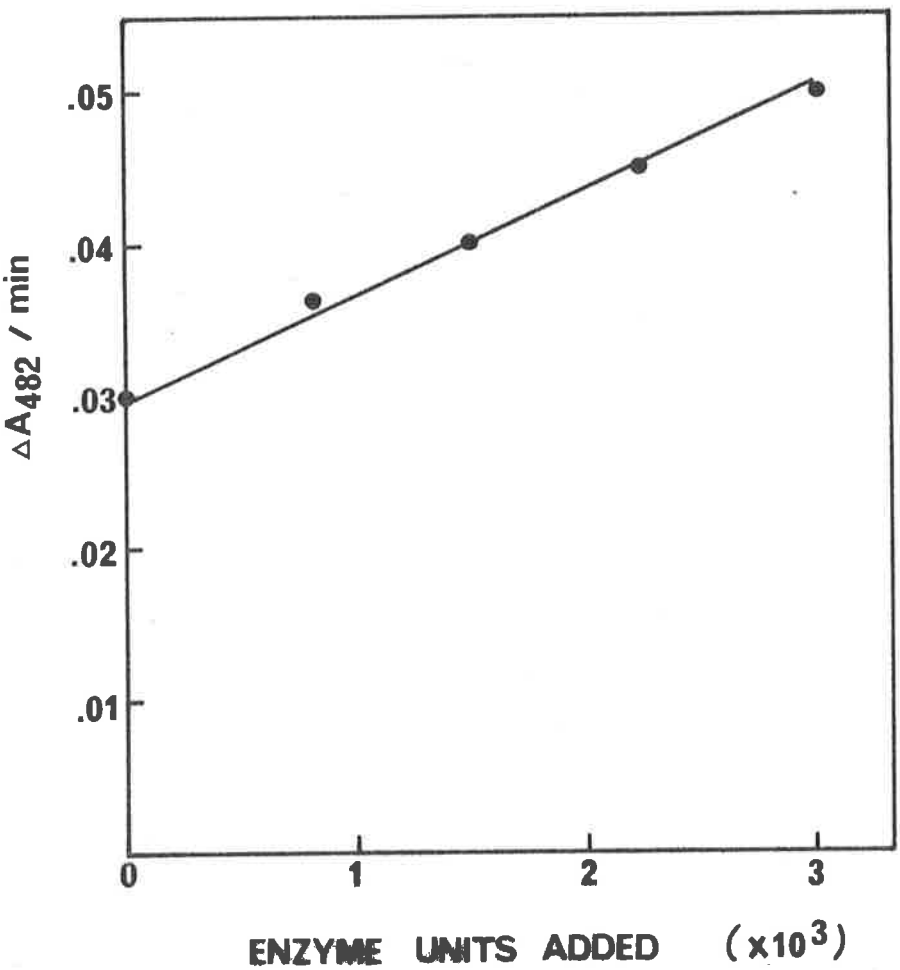
As shown in figure 3.6, even in the absence of added penicillinase, A_{482} rose rapidly, indicating that the protein synthesis mix itself was responsible for most of the colour observed. Although added penicillinase contributed to the rate of substrate hydrolysis, it was felt that the "background" colour change was so high that it could mask any small amounts of penicillinase synthesized in the system.

After dialysis of the mix, which removes dithiothreitol and free cysteine, the rate

FIGURE 3.6

Cephalosporin 87/312 assay of penicillinase activity in the *in vitro* mix

Total protein synthesis mixes plus varying amounts of staphylococcal exopenicillinase, were incubated with 87/312, and the rate of colour change monitored, as described in Section 3.3.2.1.


.05
.04
.03
.02
.01
ΔA_{482} / min
0
1
2
3
ENZYME UNITS ADDED (x10^3)

of colour change in the absence of exogenous penicillinase was unchanged (result not shown), suggesting that the S30 proteins were mainly responsible for the high background levels of colour change.

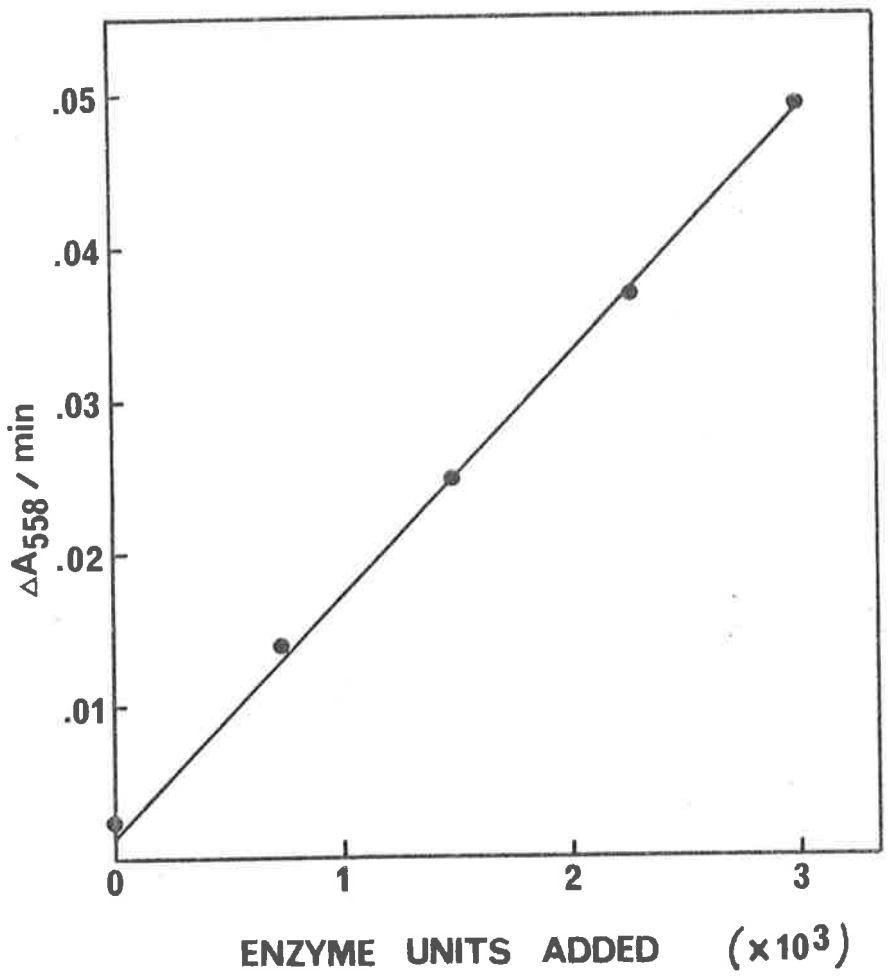
The assay of Rubin and Smith (1973) relies on the lowering of the pH of a weakly buffered solution of benzylpenicillin as it is hydrolysed to penicillinoic acid, which is followed by the lowering of A_{558} of the indicator phenol red. This assay was applied to complete protein synthesis mixes containing exogenous penicillinase, and dialyzed extensively against 0.4 mM sodium phosphate, pH 7.6. When assayed as described in Chapter 2, there is a direct correlation between the concentration of penicillinase in the assay and the initial ΔA_{558}/min, as shown in figure 3.7. More importantly, the sample containing no penicillinase yielded almost no change in A_{558} over the period of assay; i.e. in the absence of penicillinase, the protein synthesis mix does not affect the pH of the assay.

Thus the acidimetric method is a most suitable assay for *in vitro* synthesized penicillinase.

FIGURE 3.7

Acidimetric assay of penicillinase activity in the *in vitro* system.

Total protein synthesis mixes plus varying amounts of added penicillinase, were assayed by the acidimetric method of Rubin and Smith (1973), as modified and described in Section 3.3.2.1.


.05
.04
.03
.02
.01
ΔA_{558} / min
0
1
2
3
ENZYME UNITS ADDED (x10^3)

It is relatively sensitive, by comparison of figures 3.6 and 3.7, and has the advantage of giving no background readings which could mask synthesized penicillinase activity. Its major drawback is that it necessitates extensive dialysis of samples into phosphate buffer prior to assay, however it was felt that this was compensated for by its demonstrated specificity for penicillinase.

Two of the standard iodometric assays for penicillinase were also examined (Sykes and Nordstrom, 1972; and Novick, 1962) but the high rate of background decolorization of the starch-iodine complex, presumably by non-specific iodination of S30 proteins) rendered them unsuitable for direct assay of *in vitro* penicillinase (results not shown).

3.2.2.2 Is penicillinase synthesized *in vitro*?

The coupled transcription-translation system, modified as described, was used to synthesize staphylococcal penicillinase in the following way:

100 µl of total protein synthesis mixes, each containing 10 µg of pI258*blai*443 plasmid DNA, were incubated at 37^{o} for various times and then diluted with 900 µ l of

ice-cold, 0.4 mM sodium phosphate buffer, pH 7.6, dialized and assayed for penicillinase activity as previously described.

Figure 3.8 shows that there is significant penicillinase activity in such mixes. In the absence of plasmid DNA, there is no penicillinase activity over the length of the incubation, nor when λ p*lac*5 DNA is substituted for plasmid DNA, is any penicillinase activity generated. As a further control, the plasmid pI258Δ*mad-bla* was used to prime the system. This plasmid is believed to carry a small deletion covering part of the penicillinase structural gene *blaZ* (Novick, personal communication). *In vivo*, bacteria carrying this plasmid produce no detectable penicillinase. As shown in figure 3.8, this plasmid generates little penicillinase activity *in vitro*.

Thus, penicillinase activity in the protein synthesizing system is generated only in response to a plasmid carrying the complete penicillinase structural gene, which strongly suggests that the system is accurately synthesizing penicillinase, in response to the specific penicillinase gene.

FIGURE 3.8

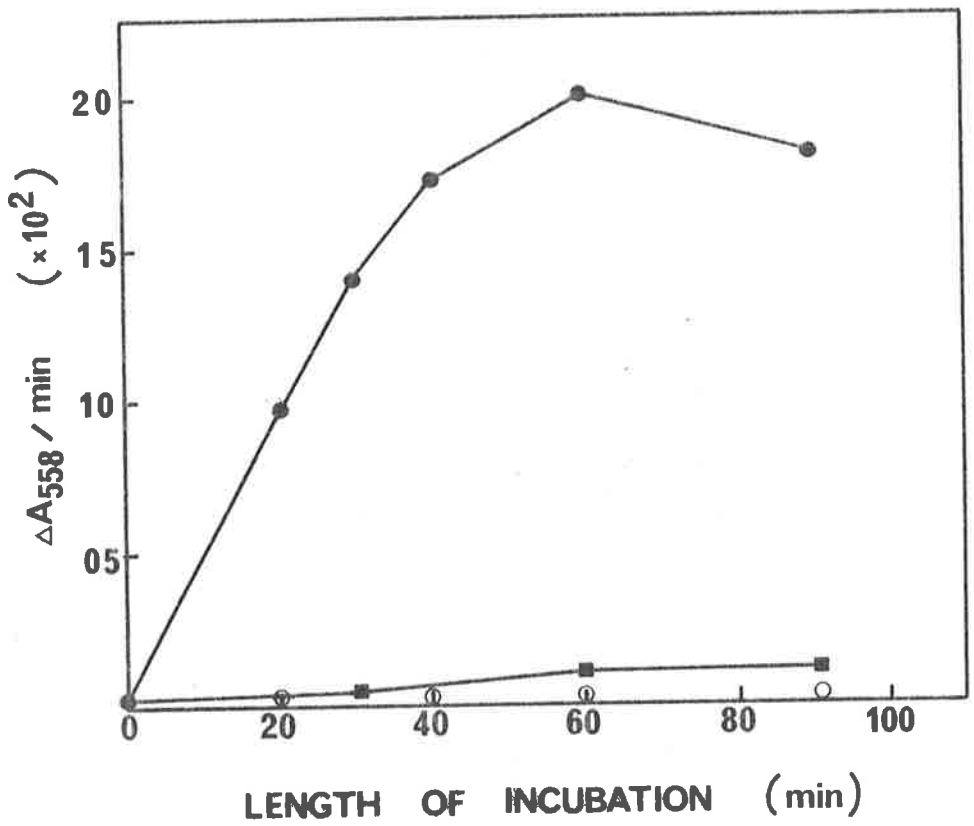
Synthesis of penicillinase *in vitro*

Protein synthesis mixes, primed with different staphylococcal plasmid DNAs, were incubated for varying times, then dialysed and assayed for penicillinase activity, as described in Section 3.3.2.2.

● = system primed with pI258*bla*i443 DNA

■ = system primed with pI258Δ*mad-bla* DNA

o = system primed with no DNA, or λp*lac*5 DNA


20
15
10
05
ΔA_{558} / min ($\times 10^2$)
0
20
40
60
80
100
LENGTH OF INCUBATION (min)

3.3.2.3 Coupled transcription and translation of penicillinase.

That the appearance of penicillinase activity is a result of the transcription and translation of the penicillinase genes was confirmed by treatment of the synthesis mixes at various times with either pancreatic DNAase (20µg/ml) or chloramphenicol (100µg/ml).

Figure 3.9A shows that on addition of either DNAase or chloramphenicol, there is an immediate cessation of penicillinase production. This indicates that destruction of DNA destroys the protein synthesizing potential of the system, and similarly that inhibition of translation by chloramphenicol results in the immediate inhibition of penicillinase synthesis.

When the mix also contains ^{14}C-lysine (0.1µCi/100µl) incorporation of label into protein (measured as described earlier in this chapter) is inhibited by DNAase or chloramphenicol in a manner identical to the inhibition of active penicillinase synthesis (figure 3.9B) - thus implying that the staphylococcal DNA is, in the absence of the inhibitors, being transcribed and translated by the *E.coli* protein synthesizing machinery.

FIGURE 3.9

Effect of disruption of RNA or protein synthesis on the appearance of penicillinase *in vitro*

A. Total protein synthesis mixes, primed with pI258*bla*i443 DNA, were incubated for varying times, with or without added DNAase (20μg/ml) or chloramphenicol (100μg/ml), then assayed for penicillinase activity, as described in Section 3.3.2.3.

B. Total protein synthesis in mixes was gauged by the incorporation of ^{14}C-lysine into hot TCA-precipitable material (Section 3.3.2.3).

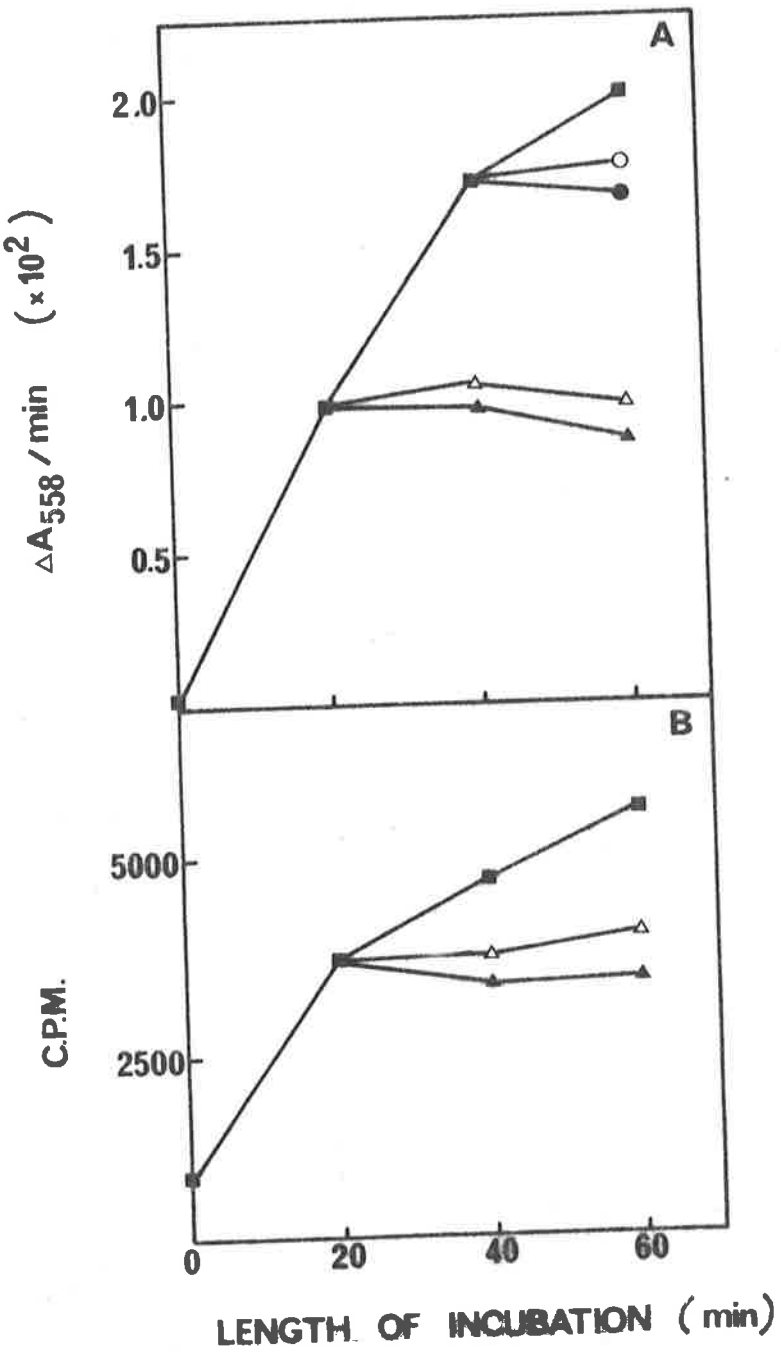
■ : no addition of inhibitor

▲ ● : addition of DNAase

△ ○ : addition of chloramphenicol

△ ▲ : inhibitor added at 20'

○ ● : inhibitor added at 40'


A
2.0
1.5
1.0
0.5
ΔA558 / min (×10²)
B
5000
2500
C.P.M.
0
20
40
60
LENGTH OF INCUBATION (min)

3.3.2.4 Optimization of penicillinase synthesis

It is possible that the optimum conditions for penicillinase synthesis *in vitro* are not the same as those for β-galactosidase synthesis. For example, Fuchs (1976) found that the optimum conditions for the synthesis of bacteriophage T3 and T7 enzymes were not identical, especially in respect to magnesium ion and phospho-enol-pyruvate concentrations. Similarly, Isturiz and Wolf (1975) used only 6 μg/ml DNA in the synthesis of 6-phosphogluconate dehydrogenase in a cell-free system, and omitted cAMP, FAD, NADP, pyridoxine-HCl and p-aminobenzoic acid altogether. Therefore it was considered important to vary concentrations of components of the *in vitro* mix to determine optimal conditions of synthesis. Parameters investigated were total magnesium ion concentration, DNA concentration and the requirement for cAMP, FAD, NADP, pyridoxine-HCl and p-aminobenzoic acid.

Figure 3.10 shows that penicillinase synthesis is equally as sensitive to magnesium ion concentration as β-galactosidase (figure 3.2) and again the optimum concentration was 12 mM.

Figure 3.11 demonstrates the dependence of the system on the concentration of plasmid DNA,

FIGURE 3.10

Effect of total magnesium ion concentration on synthesis of penicillinase *in vitro*

Synthesis of penicillinase *in vitro* was estimated, as described, in the presence of varying concentrations of magnesium ions.

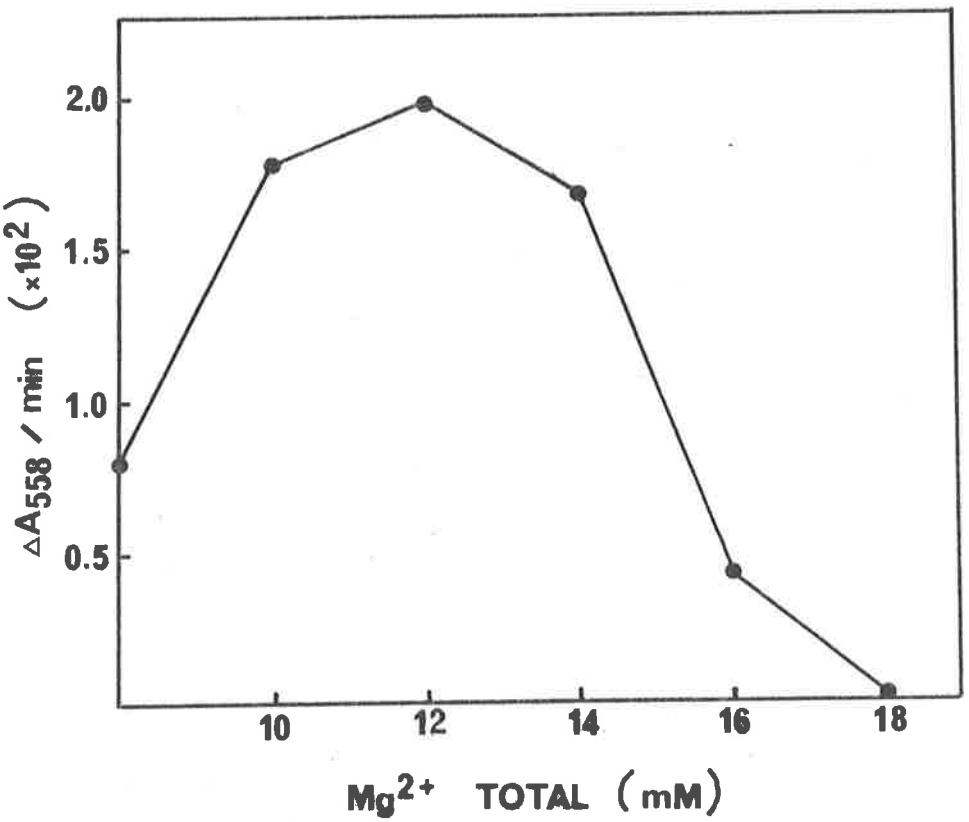
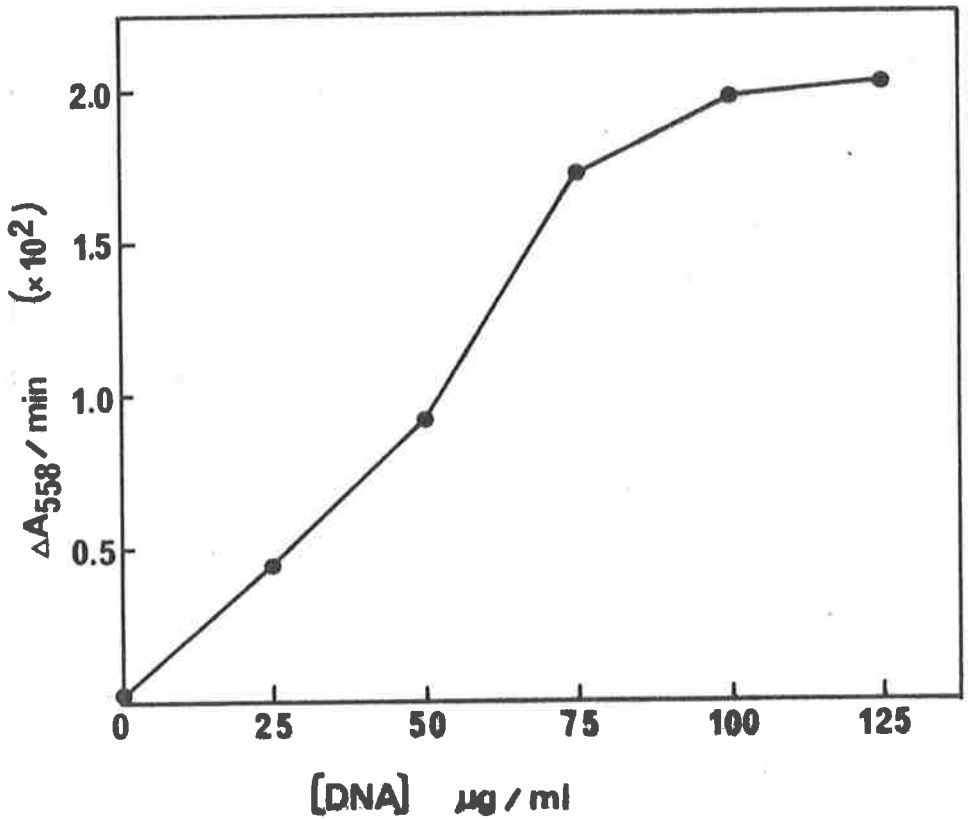

2.0
1.5
1.0
0.5
ΔA_{558} / min ($\times 10^2$)
10
12
14
16
18
Mg^{2+} TOTAL (mM)

FIGURE 3.11

Effect of DNA concentration on the synthesis *in vitro* of penicillinase

Total protein synthesis mixes were incubated with varying concentrations of pI258*bla*i443 DNA, and assayed for enzyme activity as described in the text.


2.0
1.5
1.0
0.5
ΔA_{558} / min ($\times 10^2$)
0
25
50
75
100
125
[DNA] µg / ml

and shows that, unlike λp*lac*5, the optimum concentration of plasmid DNA was about 100 μg/ml. Consequently, this concentration of DNA was used in all experiments.

Table 3.5 shows that the omission of cAMP, or NADP, FAD, pyridoxine-HCl and p-aminobenzoic acid, did not significantly affect the production of penicillinase, a result that is in agreement with the results of Svenningsen (1975), studying the *deo* operon, and Isturiz and Wolf (1975), studying the synthesis of 6-phosphoglucomate dehydrogenase. Although their presence was unnecessary, these ingredients were included in all syntheses; as β-galactosidase synthesis was used as a control in a number of experiments it was decided to maintain the uniformity of the protein synthesizing system.

TABLE 3.5

Omission of various factors from the protein synthesis mix.

Penicillinase was synthesized in mixes with or without either cAMP or NADP, FAD, pyridoxine-HCl and p-aminobenzoic acid, and assayed acidimetrically, as described in the text.

Omission from total protein synthesizing mix	Penicillinase activity (ΔA_{558}/min)
None	.020
cAMP	.018
NADP, FAD[a], pyridoxine-HCl, p-aminobenzoic acid	.022

a: Folinic acid was added separately to the mix.

3.3.2.5 Efficiency of penicillinase production

An estimate of the amount of penicillinase synthesized *in vitro* can be achieved as follows:

Rubin and Smith (1973) have determined that 0.7 µ moles/ml HCl caused a decrease in the A_{558} of their assay mix of one absorbance unit. As one unit of penicillinase is defined as that amount of enzyme that hydrolyzes (i.e. acidifies) 1 µmole of benzylpenicillin per hour, then one unit of enzyme will cause a decrease in A_{558} of 1/0.7 = 1.4, in one hour (or 0.023/min).

As the maximum enzyme synthesized *in vitro* yielded a ΔA_{558} of 0.02/min (figure 3.8) then the amount of enzyme present in the assay is 0.02/0.023 = 0.87 units - i.e. 0.25 ml of diluted, dialyzed protein synthesis mix contains 0.87U of enzyme. Therefore the 1 ml of dialyzed mix, and hence the original 100µl protein synthesis mix, contains about 3.48 units of enzyme.

From Richmond (1975), purified staphylococcal penicillinase A contains 315 units/ µg protein (determined with benzylpenicillin), therefore

the protein synthesis mix contains 3.48/315 = 0.011 μg penicillinase.

The molecular weight of exopenicillinase is 29,600 daltons (Ambler and Medway, 1969), so 0.011 μg represents 3.7×10^{-13} moles, or 2.2×10^{11} molecules of protein.

In 100 μl of synthesis mix there is 10 μg of plasmid DNA, M.W. = 18.6×10^6 daltons (Rush, Novick and Delap, 1975). This is equivalent to 3.2×10^{11} molecules of DNA; thus, over 60 minutes of incubation, each molecule of DNA yields approximately 0.7 molecules of penicillinase.

In comparison, Zubay (1973) estimated that each molecule of λ p*lac*DNA accounts for the synthesis of a 4 β-galactosidase polypeptide chains in a three-hour incubation. When the relative sizes of the peptide chains is considered (penicillinase = 257 amino acids; β -galactosidase = 1147 amino acids), then it can be seen that synthesis of penicillinase is far less than the maximum possible. This is probably due to the fact that the S30s used in this study are less active than those

of Zubay (see section 3.3.1); in fact, it can be roughly estimated that the system used here yields approximately 0.5 β-galactosidase molecules per DNA molecule. If this figure is used, it can be seen that the penicillinase gene accounts for the incorporation of 0.7 x 257 = 180 amino acids into protein, over 60', while the β-galactosidase gene accounts for the incorporation of 0.5 x 1147 = 573 amino acids into protein. Thus penicillinase is produced at about 31% the efficiency of β-galactosidase production.

3.3.2.6 Comparative efficiency of transcription and translation

The relatively low yields of enzymatically active penicillinase are not reflected in the rates of transcription of staphylococcal plasmid DNA.

Protein synthesizing mixes containing 5 μ Ci of ^{3}H-CTP were incubated for 0 - 60 minutes, 10 μl aliquots removed at various times and spotted onto GF/A filters. After TCA precipitation and washing, counts incorporated into RNA were measured. As shown in figure 3.12A incorporation of ^{3}H-CTP into staphylococcal RNA is significantly greater than into λ p*lac*5

FIGURE 3.12

Synthesis of RNA and protein *in vitro*

A. Synthesis of RNA *in vitro*, when primed with various DNAs, was measured by incorporation of ^{3}H-CTP into TCA-precipitable material.

B. Protein synthesis was similarly monitored by the incorporation of ^{14}C-lysine into hot TCA-precipitable material.

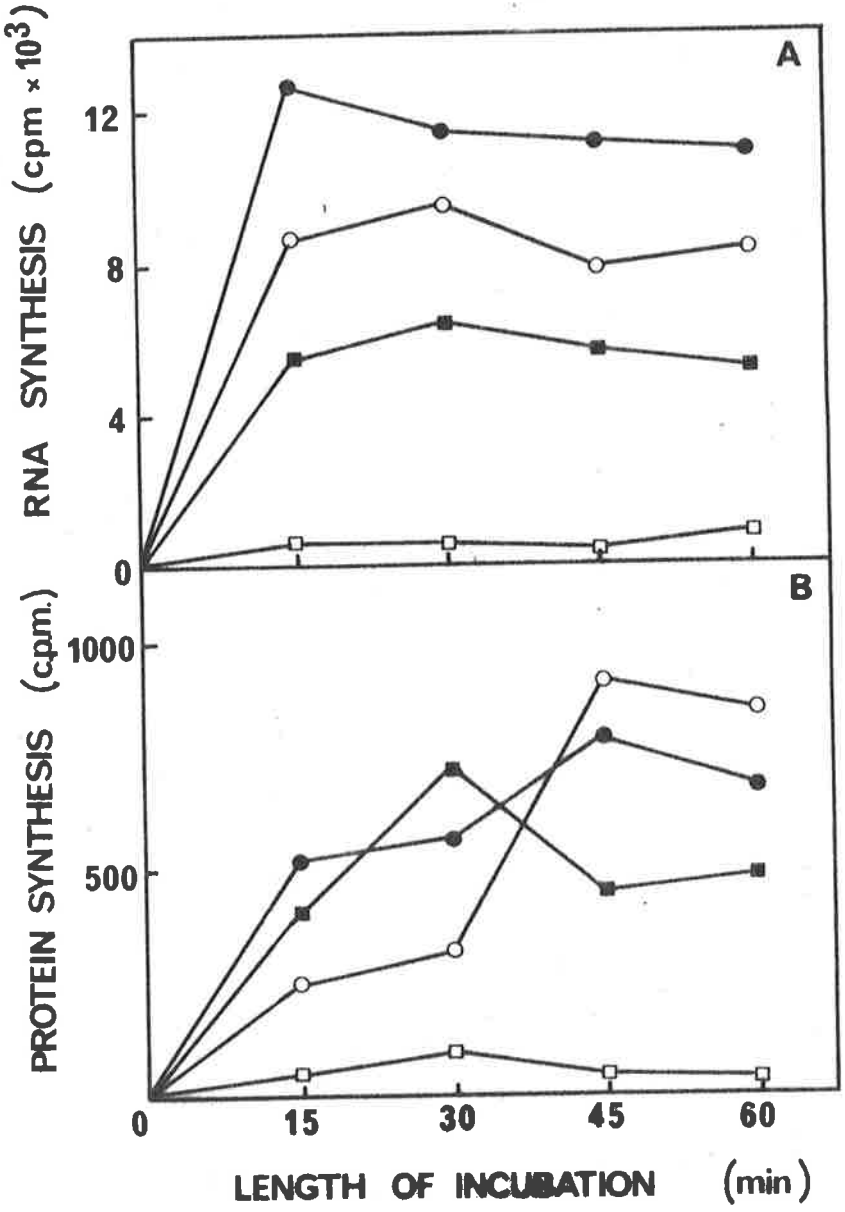
(See Section 3.3.2.6)

● = pI258*bla*i443 DNA (100µg/ml)

○ = pI258*bla*i443 DNA (50µg/ml)

■ = λp*lac*5 DNA (50µg/ml)

□ = no added DNA


A
B
RNA SYNTHESIS (cpm × 10³)
PROTEIN SYNTHESIS (c.p.m.)
12
8
4
0
1000
500
0
15
30
45
60
LENGTH OF INCUBATION (min)

when the optimal concentrations of DNA are used (i.e. 100 µg/ml plasmid, 50 µg/ml λp*lac*5). Incorporation into staphylococcal RNA at 50 µg/ml plasmid is almost identical to λp*lac*5 at the same concentration. Thus transcription of both DNA's are about equally efficient, both in absolute yields, and in the pattern of incorporation (implying that RNA synthesis is completed at about 30'), indicating that the lower yields of penicillinase, and the necessity for a higher concentration of plasmid DNA necessary, are not due to a lowered rate of transcription.

When ^{14}C-lysine incorporation into hot TCA-precipitable material is measured, there was no significant difference between the rates of translation of the two DNA sources, or between different concentrations of plasmid DNA.

The significance of these results will be discussed later in this chapter.

3.3.3 Genetic control of penicillinase synthesis *in vitro*

The experiments described so far were performed with the plasmid p1258*bla*i443, which carries an inactive repressor gene (Novick, 1965) and so is constitutive for penicillinase production; a situation which

is reflected in the synthesis of the enzyme *in vitro*. Protein synthesizing systems such as that used here have been shown to reflect accurately the genetic control mechanisms of several *E.coli* operons (e.g. Zubay, Schwartz and Beckwith, 1970; Pouwells and van Rotterdam, 1975; Kelker *et al.*, 1976; Pratt, 1980). As staphylococcal penicillinase is expressed in *E.coli* cells (Chang and Cohen, 1974), and in *E.coli in vitro* systems (as described in this section) it would be informative to examine whether the *in vitro* system was capable of interpreting the control signals of the wild-type penicillinase operon, i.e., can penicillinase production be induced in the *in vitro* system primed with wild-type penicillinase plasmid? Therefore the system primed with wild-type plasmid pI258, in the absence or presence of methicillin (0.5 µg/ml), which is an effective inducer of penicillinase production in *S.aureus* (Richmond, 1975).

As shown in figure 3.13, systems containing this plasmid produced some penicillinase regardless of the presence of the inducer, suggesting firstly, that the *in vitro* system was not subject to the normal *in vivo* control mechanisms, and secondly, that penicillinase could be synthesized in the presence of (i.e. the transcription and translation of) the repressor gene.

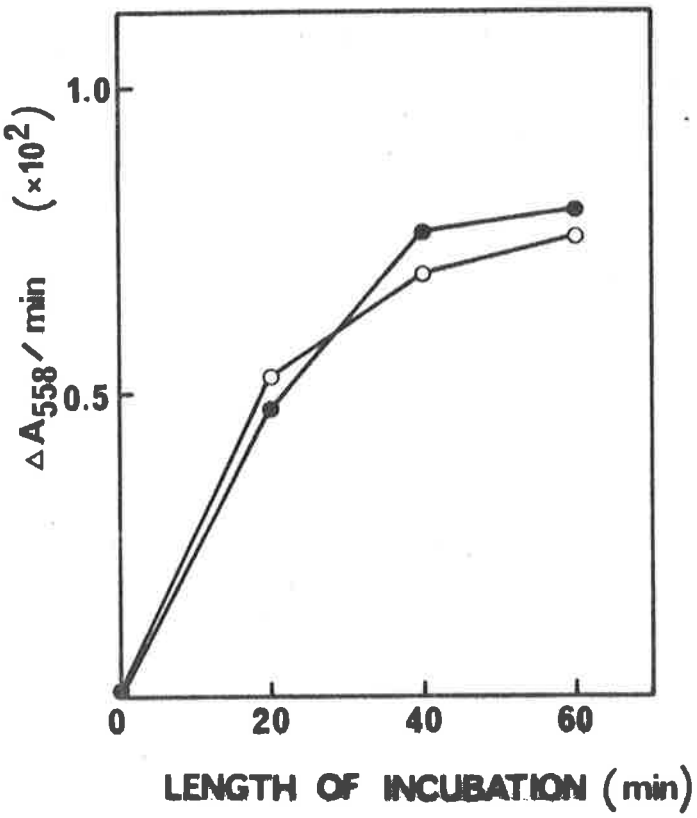
FIGURE 3.13

Induction of penicillinase synthesis *in vitro*

Protein synthesis mixes were incubated with pI258 (wild type) DNA, at 100µg/ml, in the presence or absence of inducer (methicillin, 0.5µg/ml), and assayed for enzyme activity as previously described.

● = plus methicillin (0.5 g/ml)

o = no methicillin


1.0
0.5
ΔA_{558} / min ($\times 10^2$)
0
20
40
60
LENGTH OF INCUBATION (min)

Thus the implications of this result will be discussed in detail, later in this chapter. However, it is worth noting the behaviour of the wild-type penicillinase gene when residing in the *E.coli* cell. Although *E.coli* cells carrying the penicillinase operon are resistant to penicillin antibiotics (Chang and Cohen, 1974) there is little evidence of induction of the enzyme in such cells. When *E.coli* E392 carrying the plasmid pSC113 (Chang and Cohen, 1974) was grown in the presence of methicillin (0.5 g/ml), there was no obvious increase in the activity of periplasmic penicillinase in *E.coli* spheroplasts (results not shown). Presumably, in these cells residual penicillinase production is sufficient to confer resistance to β-lactam antibiotics in these strains.

3.4 DISCUSSION

This chapter describes the establishment and characterization of an *in vitro*, DNA directed protein synthesizing system, based on that of Zubay (1972), which synthesizes enzymatically active penicillinase when primed with staphylococcal plasmid DNA.

The system was first optimized for the synthesis of *E.coli* β-galactosidase (summarized in section 3.3.1.5), which served as a basis for the study of penicillinase synthesis, and will also be used as a comparison between intracellular and extracellular protein synthesis. When this work was begun, no "foreign" DNA had been both transcribed and translated in an *E.coli* coupled system. A number of workers had successfully translated foreign mRNA in an *E.coli* system (e.g. Legault-Demare and Chambliss, 1975; Petit-Glastron and Rapoport, 1976) and mitochondrial DNA had been both transcribed, and the product translated, by *E.coli* cell free extracts, although not in a coupled system (Scragg and Thomas, 1975). Therefore we considered the coupled transcription-translation of a staphylococcal gene by *E.coli* extract was important. Not only was the staphylococcal mRNA correctly translated by the *E.coli* protein synthesizing machinery, but the staphylococcal DNA was obviously correctly

transcribed by the host RNA polymerase. This then implied that there is considerable similarity between the DNA polymerase binding sites of two widely separated bacterial species, sufficient to allow faithful and relatively efficient transcription of one by the machinery of the other - i.e. that transcription control signals are as conserved as are translation signals.

The importance of this result has been superseded in recent years by the development of recombinant DNA technology. It is now possible to clone into *E.coli* many foreign genes (within certain size limits) and to have them expressed in the cell. In fact, the same staphylococcal gene used in this study was one of the first to be so cloned (Chang and Cohen, 1974) and is expressed *in vivo*, so demonstrating the universality of transcription-translation mechanisms, at least among bacteria.

The optimal conditions for β-galactosidase and penicillinase synthesis were found to be almost identical in this work, particularly the optimum concentration of magnesium ions - a factor responsible for consideration variation in cell-free systems. Cyclic AMP was not necessary for penicillinase syntheses. Its necessity for β-galactosidase synthesis is related to the actions of the CAP protein

(Zubay, Schwartz and Beckwith, 1970) so it was not surprising that it was not required for penicillinase production. The concentration of staphylococcal DNA which yielded maximum synthesis of penicillinase was 100 μg/ml, twice that of λ p*lac*5 DNA (figure 3.11). In contrast, the rates of transcription and translation of staphylococcal DNA are similar to those of λ p*lac*5 (figures 3.12A and B), so that the necessity for the higher concentration of staphylococcal DNA is not due to an impaired ability of the protein synthesizing machinery to respond to the foreign DNA. Figure 3.12B shows that incorporation of ^{3}H-CTP into staphylococcal RNA proceeds at a greater rate than incorporation into λ p*lac*5 RNA, suggesting that there is no deficiency of transcription in such systems. However, these data do not indicate how much of this represents completed, accurate mRNA molecules. It is possible that some of the RNA is synthesized as randomly initiated and/or terminated fragments, due for example, to differences in RNA polymerase binding sites between *E.coli* and *S.aureus*, or to the effects of residual ethidium bromide intercalated into the plasmid.

Similarly, the levels of incorporation of ^{14}C-lysine into protein do not reflect the levels of active penicillinase synthesized. In figure 3.12B, both 50 μg/ml and 100 μg/ml plasmid yield

levels of lysine incorporation identical to those for λp*lac*5. Amino acid incorporation is not an accurate measure of active β-galactosidase synthesis (Zubay, 1973), so these figures may suggest that synthesis of completed penicillinase molecules is greater in the presence of the higher rate of RNA synthesis (100 μg/ml plasmid).

The relatively low yields of active penicillinase (section 3.3.2.5) may represent inaccuracy in transcription or translation, yielding a percentage of untranslatable mRNA or of incomplete or enzymatically inactive molecules.

Alternatively, there could be some partial degradation of completed polypeptides, by endogenous proteases. The latter alternative is a distinct possibility, as when protein synthesizing mixes are incubated for 90 - 120 minutes (in contrast to the usual 60'), there is a gradual drop in their penicillinase activity (result not shown) suggesting that there is some degradation of penicillinase occurring. In contrast to β-galactosidase, for example, (where no such drop in activity occurs) staphylococcal penicillinase contains no cysteine residues (Ambler, 1975), and hence no internal disulphide bridges; this presumably facilitates passage of the peptide chain into and through the cell membrane, but also yields a more "flexible"

molecule (Robson and Pain, 1976), which may be more susceptible to proteolytic degradation, especially as, *in vitro*, the penicillinase is in an "intracellular" environment, unlike its location *in vivo*. Another possibility is that some of the penicillinase molecules are synthesized in an enzymatically inactive form, such as a precursor. This will be the subject of the work of Chapter 4.

The experiments on enzyme synthesis in response to wild-type plasmid pI258 yield two interesting results. Firstly, that some penicillinase is synthesized in such a system, presumably in the presence of synthesized repressor protein. Thus the *in vitro* system does not respond to the genetic control of penicillinase production. In contrast, *in vivo*, there is little penicillinase synthesized in the absence of inducer (Richmond, 1975), i.e. the repressor molecule effectively prevents enzyme production *in vivo*. This suggests that the cell-free system is not synthesizing enough repressor to bind to the penicillinase operator-promoter and so inhibit penicillinase production. If the repressor molecule is being synthesized approximately as efficiently as the active enzyme is (i.e. about 0.5 molecules/DNA molecule) then obviously there will not be enough repressor produced to totally prevent transcription of the structural gene. Similarly, it has been postulated that, in

Bacillus the direction of transcription of the penicillinase operon is *bla*Z → *bla*I (Kelly and Brammar, 1973), that is the structural gene is transcribed before the repressor gene, so there will always be a low level of enzyme synthesized in these cells. If the staphylococcal operon is arranged similarly, then, given the low level of enzyme produced *in vitro* it is apparent that the repressor molecules will not appear in the mix until after production of the enzyme.

Secondly, the addition of methicillin, an effective inducer of penicillinase *in vivo*, did not alter the level of enzyme *in vitro*. This result is in agreement with the work of Imsande (1973) and Imsande and Lilleholm (1976) on the nature of penicillinase induction. Imsande (1978) postulates that inducer molecules (i.e. β-lactam antibiotics) do not bind directly to the repressor molecule, but to an "antirepressor" molecule, during synthesis of that molecule. This form of the antirepressor then binds to the repressor protein, inactivating it and allowing transcription of the penicillinase operon. As genetic analysis of the staphylococcal operon suggests that it contains only the structural and repressor genes (reviewed by Imsande, 1978) such an antirepressor must be coded for by a gene not in the operon (see,

for example Cohen and Sweeney, 1968; Cohen, Vernon and Sweeney, 1970, for supporting genetic evidence); possibly the antirepressor is in fact a protein involved in cell wall synthesis, and thus able to bind β-lactams (Blumberg and Strominger, 1974; Kozarich and Strominger, 1978), so that the penicillinase operon utilizes the sensitivity of the cell-wall synthesizing machinery to β-lactams, as the mode of induction. Certainly, Imsande (1978) argues convincingly that the penicillinase operon contains only the two genes (*blaZ* and *blaI*), plus the operater-promoter region.

Given this information, it is explicable that no enzyme induction was observed *in vitro*. If, as Imsande suggests (1978), induction involves binding of β-lactam to *de novo* synthesized antirepressor protein, it would not be expected to occur in the cell-free system, where there would be no synthesis of that protein. In fact, if such a protein is associated with cell-wall synthesis, then there would be very little in the S30, as most cell-wall and membrane material is removed during centrifugation. Thus the inability to get penicillinase induction *in vitro* provides some indirect support for the theory of Imsande on the nature of penicillinase regulation.

CHAPTER FOUR

THE PROTEIN PRODUCTS OF THE *in vitro* SYSTEM

4.1 INTRODUCTION

This chapter describes studies which utilize the *in vitro* system to examine the nature of the penicillinase produced in Chapter 3.

Initially, it was expected that the system would produce a precursor form of the protein, as has been reported by other workers (e.g. Inouye and Beckwith, 1977; Chang, Blobel and Model, 1978; Dancer and Lampen, 1975). When this was not the case, several modifications were introduced to the system, based on the knowledge that some or all of the processing procedure is membrane-associated (Inouye and Beckwith, 1977; Chang, Model and Blobel, 1979), and hence the possibility that the S30 fraction contains membrane fragments. In particular, attempts were made to perturb membrane structure, and to determine whether penicillinase is preferentially associated with the membrane, either the protein, or the polysomes translating the penicillinase mRNA.

Despite these modifications, and attempts to inhibit any proteolytic activity in the mix, there was no evidence of an intermediate, precursor form of the enzyme, nor was there any necessity for the presence of membrane material to achieve synthesis of penicillinase (mature size).

These results suggest either:

1) penicillinase is not produced as a precursor. This contrasts with the results of Lampen and his colleagues on *Bacillus* penicillinase, and in particular with Dancer and Lampen (1975), although the validity of their precursor has been questioned (see review in Chapter 1); and with the DNA-sequencing data of Sutcliffe (1978) on *E.coli* β-lactamase.

2) Penicillinase is rapidly and efficiently processed independently of membranes, at least *in vitro*, i.e. the *in vitro* system may not be truly representative of the *in vivo* situation.

3) The *blai* operon used in this work may also contain a cryptic mutation in the structural gene, which affects either the size of the precursor, or the nature of its processing.

4.2 MATERIALS AND METHODS

4.2.1 Electrophoretic analysis of proteins produced *in vitro*

4.2.1.1 Labelling of proteins synthesized *in vitro*

For analysis by electrophoresis, proteins were synthesized *in vacuo*, in the presence of 1μCi/100μl of ^{14}C-lysine. Lysine was first evaporated *in vacuoo* to dryness, then resuspended in 5μl H_2O before adding it to the protein synthesis mix.

Lysine was chosen as a suitable label because of the high lysine content of staphylococcal penicillinase (Richmond, 1975 a).

4.2.1.2 Polyacrylamide gels

Samples were analysed by electrophoresis on 10% polyacrylamide gels, 14 cm x 14 cm x 0.2 cm, using the discontinuous system of Laemmli (1970).

Separating gel: 10% acrylamide
0.15% N,N'-*bis*-methylene-acrylamide
0.375 M tris-HCl pH 8.8
0.1% SDS

Stacking gel: 3% acrylamide
0.1% bis-acrylamide
0.125M tris-HCl pH 6.8
0.1% SDS

Electrode buffer: 0.025M tris-HCl pH 8.3
0.192M glycine
0.1% SDS

The separating gels (about 10 cm deep) were polymerized by the addition of tetra-methylethylene-diamine (TEMED) to 0.025%, and ammonium persulphate to 0.1%. After polymerization, the stacking gel was overlaid, a perspex comb containing 1 cm-wide teeth was inserted, and the gel allowed to polymerize, after which the comb was carefully removed, and the wells filled with electrode buffer.

4.2.1.3 Sample preparation

50µl of protein synthesis mix was added to an equal volume of 10% TCA and chilled for 15'. Precipitate was collected by centrifugation, washed twice with acetone-ether (1:1), and resuspended in a minimal volume of Loading Buffer.

0.0625M tris-HCl, pH 6.8
2% SDS
10% glycerol
5% β-mercaptoethanol
0.001% bromophenol blue

4.2.1.4 Electrophoresis

30µl samples of protein were immersed in boiling water for 15', then rapidly chilled, and loaded into wells with a drawn capillary.

Electrophoresis was at 30 mA, until the tracking dye approached the bottom of the gel (aout 3 - 4 hr).

4.2.1.5 Fixing and fluorography of gels

The gel was removed from the glass plates, and fixed overnight in a 10% acetic acid, 25% isopropanol, followed by a 4 hour wash in 10% acetic acid.

The gel was then immersed twice in 500 ml of dimethylsulphoxide (DMSO) for 30' each, then impregnated with PPO (22% w/v in DMSO) for 3 hr. The gel was then soaked in 500 ml H_2O for 1½ hr, then laid on Whatman 3 MM paper, and dried under vacuum.

Gels were then fluorographed at -80°C, with Fuji X-ray film, using an Ilford Intensifying Screen. Fluorography was for 3-7 days.

4.2.1.6 Molecular weight markers

^{14}C-methylated molecular weight markers used were purchased, as a mix, from Amersham.

lysozyme	14.3 kilodaltons (kd)
carbonic anhydrase	30 kd
ovalbumin	46 kd
bovine serum albumin	69kd
phosphorylase b	92.5 kd
myosin	200 kd

1µl of markers was added to 30µl of S30 proteins in loading buffer, prior to application to the gel.

Staphylococcal exo-penicillinase, purified in Chapter 2, was labelled with tritium by the method of Kumarasamy and Symons (1979). 1µl of the preparation was loaded, in S30 + loading buffer, as described for the molecular weight markers.

4.2.2 Preparation of S70 fraction of *E.coli*

S70 (70,000 g supernatant) was prepared from E234 by an identical procedure to the preparation of S30's as described in Chapter 3, except that the 30,000 g centrifugation was replaced by a 70,000 g centrifugation (Ti50, 34,000 rpm, 20').

4.2.3 Analysis of polysomes of the *in vitro* system.

Polysomes formed during *in vitro* protein syntheses were analysed on 5-40% sucrose gradients.

4.2.3.1 Preparation of gradients

5 ml sucrose gradients were prepared in KOH-washed cellulose nitrate centrifuge tubes (12 ml capacity), from 20% sucrose in 0.01 M tris acetate, pH 8.2, 0.01 M Mg acetate, 0.06 M potassium acetate, by the freeze-thaw technique of Davis and Pearson (1978).

After three freeze-thaw cycles, the gradients were underlaid with 7 ml of 60% sucrose in the same buffer, and used immediately.

4.2.3.2 Centrifigation, fractionation, and concentration of polysome peaks

200 µl of synthesis mix, as detailed in the Results of this chapter, were overlaid on the gradients, and the tubes centrifuged for 75' at 27,000 rpm in a SW41 rotor.

The gradients were then analysed by the collection of 0.5 ml fractions generated on an Isco Fractionater. A_{260} of the fractions was measured.

The desired fractions were pooled, diluted 1/10 with 0.01 M tris acetate pH 8.2, 0.06 M K acetate, 0.01 M Mg acetate, and polysomes precipited by centrifugation at 120,000 g for 90' (45,000 rpm, Ti50).

Pellets were then gently resuspended in 50µl of 0.01 M tris-acetate, 0.06 M K acetate.

4.2.3.3 Translation of polysomes *in vitro*

100µl of *in vitro* protein mixes were set up as described, except that DNA was omitted and replaced with 30µl of the appropriate polysome preparation. The mix also included 1µl of 4 mg/ml pancreatic DNAase to destroy residual transcription activity. ^{14}C-lysine (1µCi) was included to allow fluorographic identification of proteins translated by the polysomes.

4.3 RESULTS

4.3.1 Is penicillinase synthesized as a precursor?

As described in Chapter 3, the DNA-directed protein synthesizing system produces enzymatically active penicillinase in response to staphylococcal plasmid DNA. Whether this protein was produced in the mature form, or as a larger precursor, was investigated by incubating the mixes with 1μCi/100μl of ^{14}C-lysine, then electrophoresing treated samples on 10% polyacrylamide gels, as described earlier in this chapter.

Figure 4.1 shows that, at all times, penicillinase appears in a form indistinguishable from the extracellular *in vivo* form, on fluorographed gels. Track 1 carries a sample of the extracellular enzyme, purified as described in Chapter 2, and labelled with tritium by the method of Kumarasamy and Symons (1979). By comparison with molecular weight markers, the protein is about 30,000 daltons (30 kd), which agrees well with the published figure of 29.6 kd (Richmond, 1975 a).

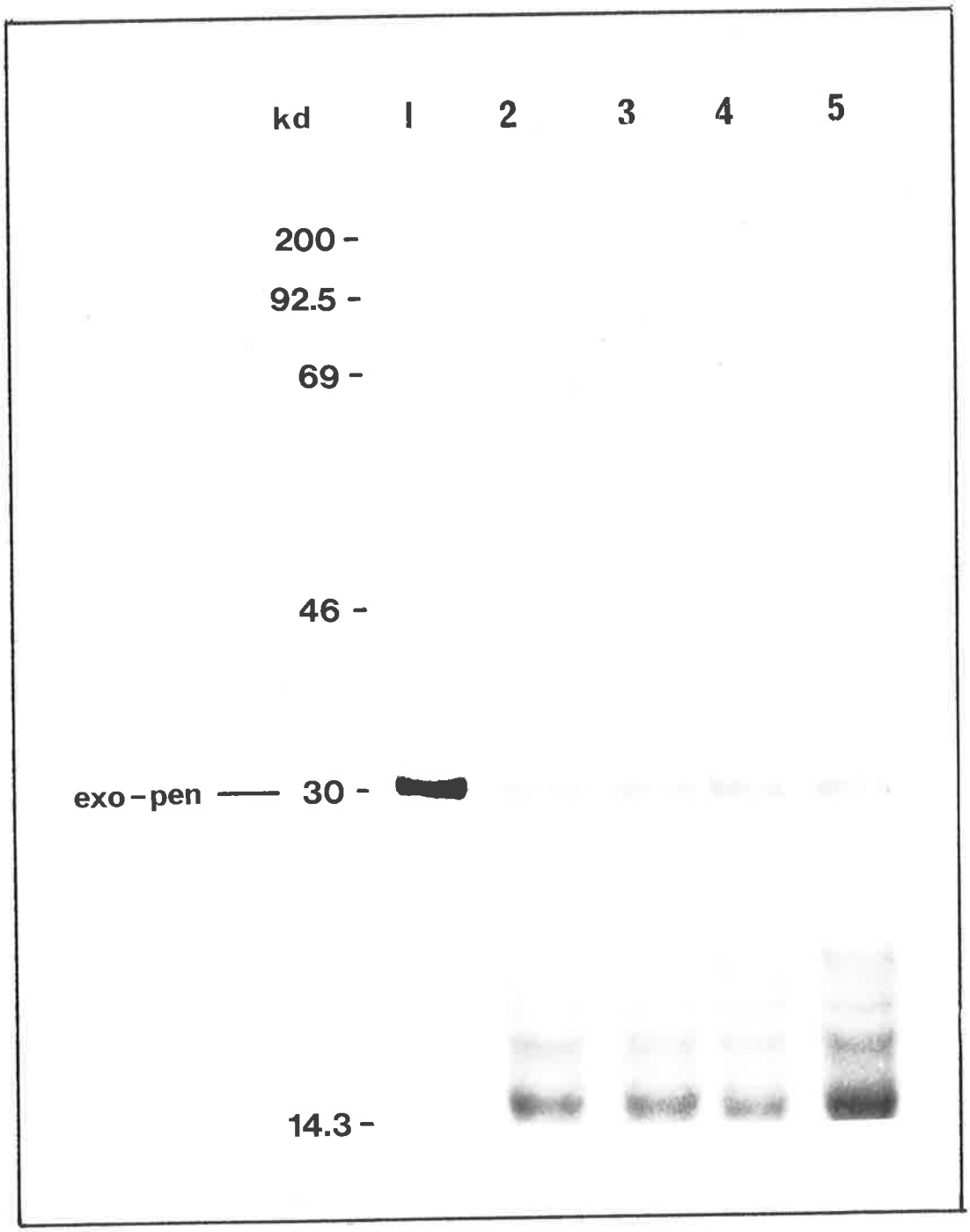
Tracks 2-5 contain samples of the protein synthesis mix after 10, 20, 30 and 60 minutes' incubation respectively. There are several major proteins visible in these tracks:

FIGURE 4.1

Time-course of *in vitro* synthesis of proteins directed by pI258*bla*i443.

Samples of protein synthesis mix, primed with pI258*bla*i443 DNA, were incubated with 1 µCi/100µl of ^{14}C-lysine. At various times, samples were removed, treated and electrophoresed, as described in the text. Molecular weight markers are described in the Materials and Methods of this chapter.

Track	
1	labelled extracellular penicillinase
2	10' incubation
3	20' "
4	30' "
5	60' "


kd
1
2
3
4
5
200 -
92.5 -
69 -
46 -
exo-pen
30 -
14.3 -

Band A: has an approximate molecular weight of 38.9 kd

Band B: M.W. ≑ 30 kd

Band C: M.W. ≑ 15 kd

There are a number of smaller weight bands whose size cannot be accurately determined from these gels.

When the system is incubated in the absence of exogenous DNA, none of these bands appear in fluorographs (results not shown).

Band B corresponds in size exactly to the purified penicillinase in track 1.

When the *in vitro* system is primed with pI258Δ*mad→bla* DNA, then this band is essentially absent, as shown in figure 4.2. As shown in Chapter 3, this DNA does not produce penicillinase enzyme activity.

Thus it appears that protein band B in figure 4.1 represents the penicillinase protein synthesized *in vitro*. The interesting information provided by these fluorographs is that there is no evidence of a higher molecular weight, precursor form of penicillinase present in the synthesis system.

FIGURE 4.2

In vitro protein synthesis primed with pI258Δ*mad→bla*

exo-pen —

This contrasts markedly with the results of other workers. For example, Inouye and Beckwith (1977) synthesized *E.coli* alkaline phosphatase in a system similar to the one used here, and found that the protein was produced in a higher molecular weight form than that found *in vivo*, in the periplasm. The authors later showed (Chang, C.N. *et al.*, 1980) that inclusion of an inner membrane preparation of *E.coli* in the synthesis mix resulted in the co-translational conversion of that precursor to the mature form of the protein. Similarly, the f1 coat protein (a membrane protein) is produced as a precursor *in vitro*, but in the presence of membrane fragments, the precursor was co-translationally converted to the mature protein and incorporated into the membrane, as occurs *in vivo* (Chang, Blobel and Modol,1978). Recently, Smith (1980) has shown that crude mRNA fractions of *Corynebacterium diphtheriae* and *E.coli*, translated *in vitro*, yield precursors of diphtheria toxin and alkaline phosphatase respectively, unless inner membranes were included in the mix, when the proteins appeared in a mature form.

Thus the synthesis of penicillinase *in vitro* is not typical of the synthesis of other extracellular proteins in similar systems. There are several possible explanations for this result:

1. Staphylococcal penicillinase is not produced as a precursor - i.e. it is not a typical extracellular protein.

2. The processing of penicillinase is different to that of other secretory proteins, such that the *in vitro* mix is able to process it - i.e., again, penicillinase must be, by comparison with other enzymes, somewhat atypical in its processing requirements.

3. The *in vitro* system used in this work retains the capacity to process secretory protein precursors.

The remainder of this chapter investigates these possibilities.

4.3.2 Effect of protease inhibitors on the synthesis of penicillinase

If the *in vitro* system is able to process a penicillinase precursor, then it must presumably contain the endopeptidase which is believed to remove the amino-terminus of the precursor (Aiyappa, Traficante and Lampen, 1977; Chang, Blobel and Model, 1978).
To test this, the effects of several protease inhibitors on the production of penicillinase were investigated by including them in the synthetic mix,

then analysing the resultant proteins on 10% polyacrylamide gels.

The protease inhibitors tested were phenylmethyl-sulphonyl fluoride (PMSF), L-tosylamido-lysyl-chloro-methyl ketone (TLCK) and the biological inhibitor chymostatin.
Initially, their effect on the synthesis of enzymatically active penicillinase was determined and compared with their effects on the synthesis of β-galactosidase in systems primed with *λlac5* DNA. As seen in figure 4.3, the inhibition of enzyme synthesis by these inhibitors is essentially the same for both β-galactosidase and penicillinase - thus there is no evidence that the inhibitors preferentially suppress the appearance of the secretory protein.

Based on these results, penicillinase was synthesized in the presence of the following concentrations of inhibitors:

PMSF	3μg/ml
TLCK	10μg/ml
chymostatin	10μg/ml

These were chosen as concentrations which were high enough to have a noticeable effect on the synthetic capacilities of the system, without drastically inhibiting protein synthesis.

FIGURE 4.3

Inhibition of β-galactosidase synthesis by protease inhibitors

Protease inhibitors (TLCK, PMSF, chymostatin) were checked for their inhibitory effect on the synthetic capabilities of the *in vitro* system, in order to determine suitable concentrations for use in penicillinase synthesis mixes

A: TLCK

B: PMSF

C: chymostatin

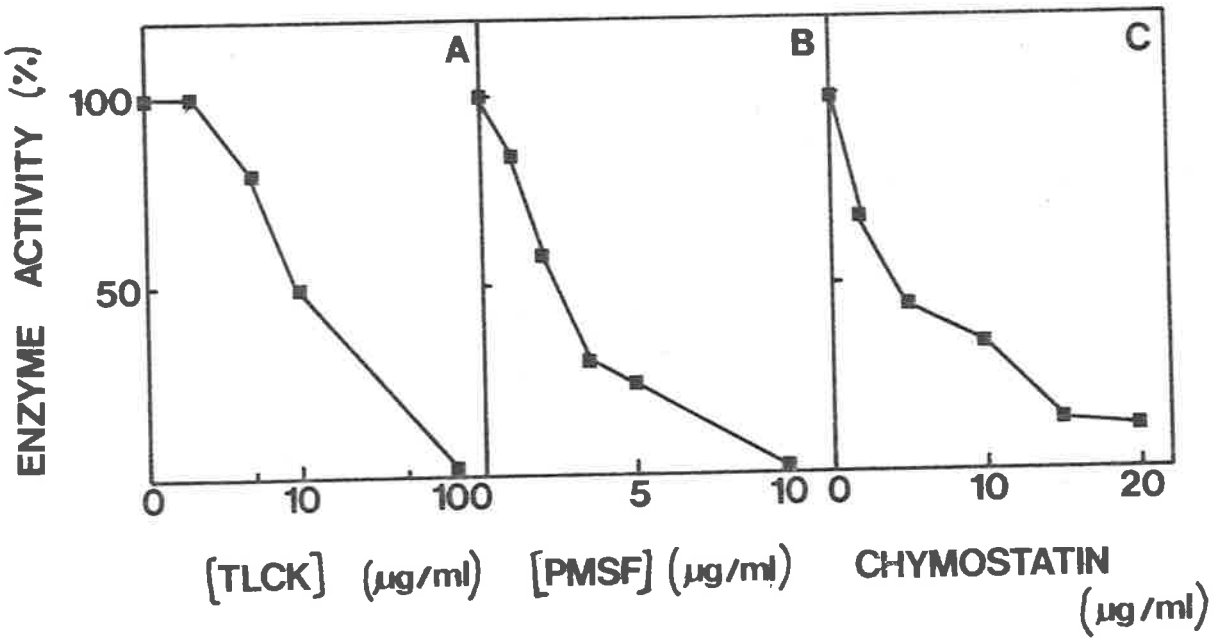

ENZYME ACTIVITY (%)
100
50
A
B
C
0
10
100
5
10
0
10
20
[TLCK] (µg/ml)
[PMSF] (µg/ml)
CHYMOSTATIN
(µg/ml)

Figure 4.4 shows that the proteins synthesized in the presence of these concentrations of inhibitors are not altered, in comparison to the control mix. In particular, the penicillinase band remains unchanged, implying that the protease inhibitors have not prevented processing of a penicillinase precursor.

4.3.3 Is the *in vitro* synthesis of mature penicillinase due to the presence of membranous material in the S30?

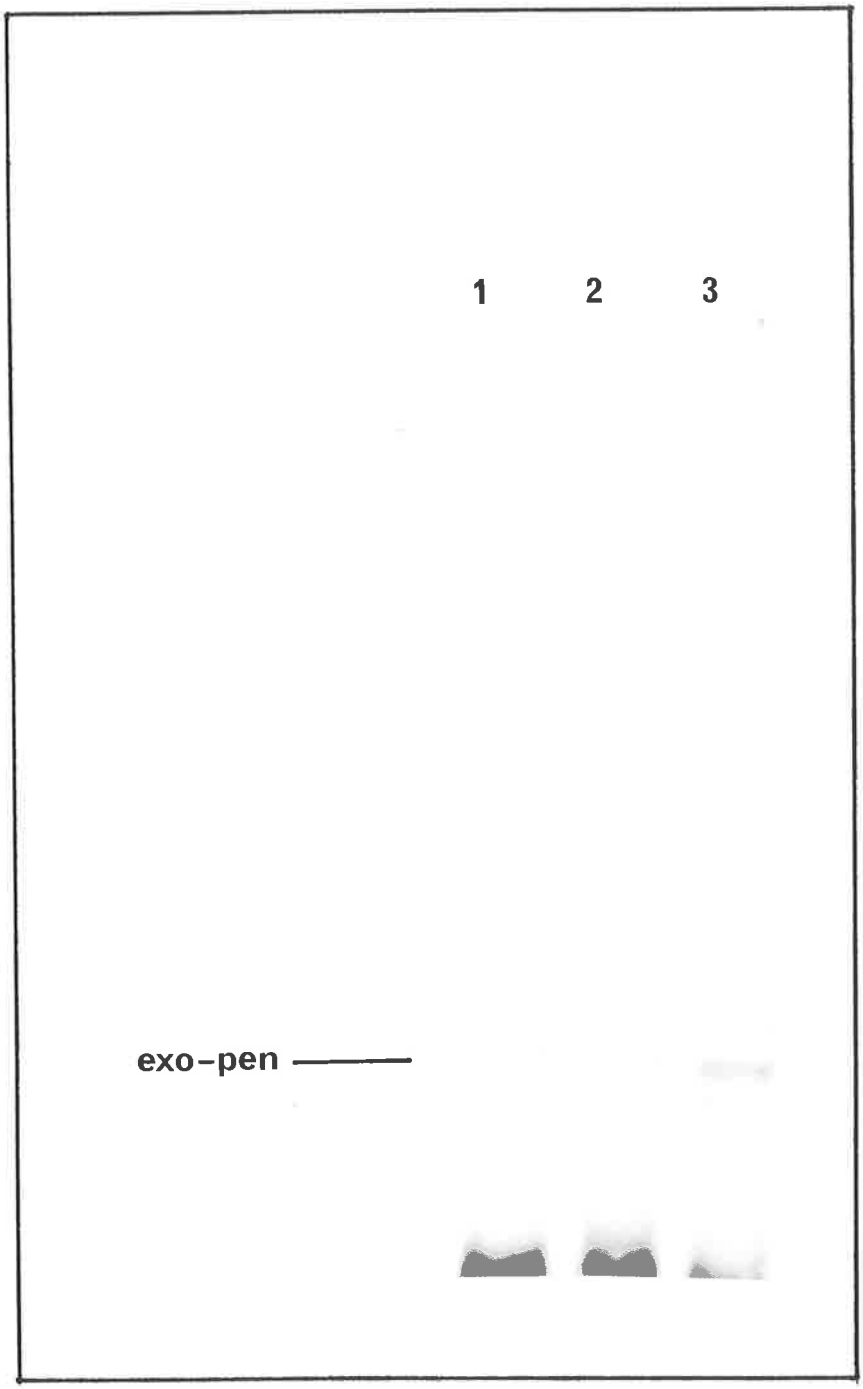
The common theories on the synthesis of a secretory protein suggest that to be processed, the peptide chain must be incorporated into the cell membrane during translation, to enable cleavage of the molecule by the membrane-associated endopeptidase. The fact that penicillinase appears to be synthesized *in vitro* as the mature protein suggests that the protein synthesizing mix may contain membrane material, such as inverted inner membrane vesicles, to which the polysomes translating the penicillinase mRNA become attached, thus allowing the "correct" synthesis and processing of the peptide. This section describes experiments which investigate this possibility, and suggest that such membranes play a role in the *in vitro* synthesis of penicillinase.

FIGURE 4.4

Effect of protease inhibitors on the synthesis of penicillinase

Track

1	Synthesis in the presence of TLCK (10µg/ml)
2	" " " " " PMSF (2µg/ml)
3	" " " " " chymostatin (10µg/ml)


1
2
3
exo-pen

4.3.3.1 Location of penicillinase produced *in vitro*

After 60' incubation, 500 µl of protein synthesis mixes, primed with either λ*plac*5 or pI258*bla*i DNA, were subjected to a 20' centrifugation at 70,000 g, to pellet any membrane vesicles that may be present in the mix.

After centrifugation, the supernatant was removed, and the small pellet resuspended either in 500 µl of 0.1 M phosphate buffer, pH 7.3 (λ*plac*5) or 500 µl of 0.4 mM phosphate buffer, pH 7.6 (pI258i$^-$). The fractions were then assayed for either β-galactosidase or penicillinase activity, using the procedures described in Chapter 3.

Table 4.1 shows that a significantly large proportion of penicillinase is found in the 70,000 g pellet, in contrast to β-galactosidase, most of which remains in the supernatant. This result suggests:

1. that the S30 contains a significant amount of membranous material (including, possibly, inverted vesicles) which can be sedimented by a 70,000 g centrifugation, and;
2. that synthesized penicillinase preferentially associates with this material.

TABLE 4.1

Location of enzyme activity produced *in vitro*

After completion of synthesis, *in vitro* mixes were centrifuged at 70,000 g for 20' then the supernatants and pellets assayed for either β-galactosidase or penicillinase activity.

Enzyme assayed	% enzyme activity in S70 supernatant	S70 pellet
β-galactosidase	73	27
penicillinase	39	61

4.3.3.2 Penicillinase synthesized by an S70 fraction of *E.coli*

Fuchs (1976) used a 70,000 g fraction ("S70") of lysed *E.coli* as a source of protein synthetic machinery, in contrast to the S30 fraction used by Zubay (1973). Should this faster centrifugation remove membranous material, the one would expect that either the S70 fraction would be unable to synthesize penicillinase, or that it would synthesize the enzyme on free ribosomes (equivalent to cytoplasmic ribosomes *in vivo*), in the unprocessed, "precursor" form.

To investigate this, an S70 fraction of lysed E234 cells was prepared, using the same procedure as for preparation of the S30 (Chapter 3), and substituted, at the same final protein concentration, and under the same conditions of synthesis.

This system is about 50% as efficient in the synthesis of β-galactosidase, as shown in Table 4.2, although ^{14}C-lysine incorporation into protein was almost identical.

The system was primed with penicillinase DNA, in the presence of ^{14}C-lysine, and the protein

TABLE 4.2

Synthetic capabilities of the S70 protein fraction

β-galactosidase was synthesized in either S30 or S70-systems, then 100µl mix assayed with ONPG.

Total protein synthesis was estimated by measuring the incorporation of ^{14}C-lysine (1µCi/100µl) into 5µl of synthesis mix protein.

Protein fraction	β-galactosidase activity (ΔA_{420}/min)	^{14}C-lysine incorporation (cpm)
S30	0.031	22985
S70	0.017	20015

products analysed on 10% polyacrylamide gels. As shown in Figure 4.5, the S70 fraction produces penicillinase in a form identical to the S30 - i.e. the same size as the extracellular protein. Thus although the results of the previous section imply that penicillinase is preferentially associated with the S70-sedimentable material, it can be produced equally as well, and in the same final form, in the absence of such material.

4.3.3.3 Disruption of membrane structure in the S30

Halegoua, Hirashima and Inouye (1976) have shown that perturbation of the structure of the cell membrane by exposure to 1% toluene, allows the accumulation of alkaline phosphatase precursor. When complete protein synthesis mixes were incubated in the presence of 1% toluene, or when the S30 was pre-incubated for 30' with 1% toluene prior to addition to the synthesis mix, there was no difference in the appearance of penicillinase, or in its size (data not shown).

4.3.3.4 Polysome profile during penicillinase synthesis

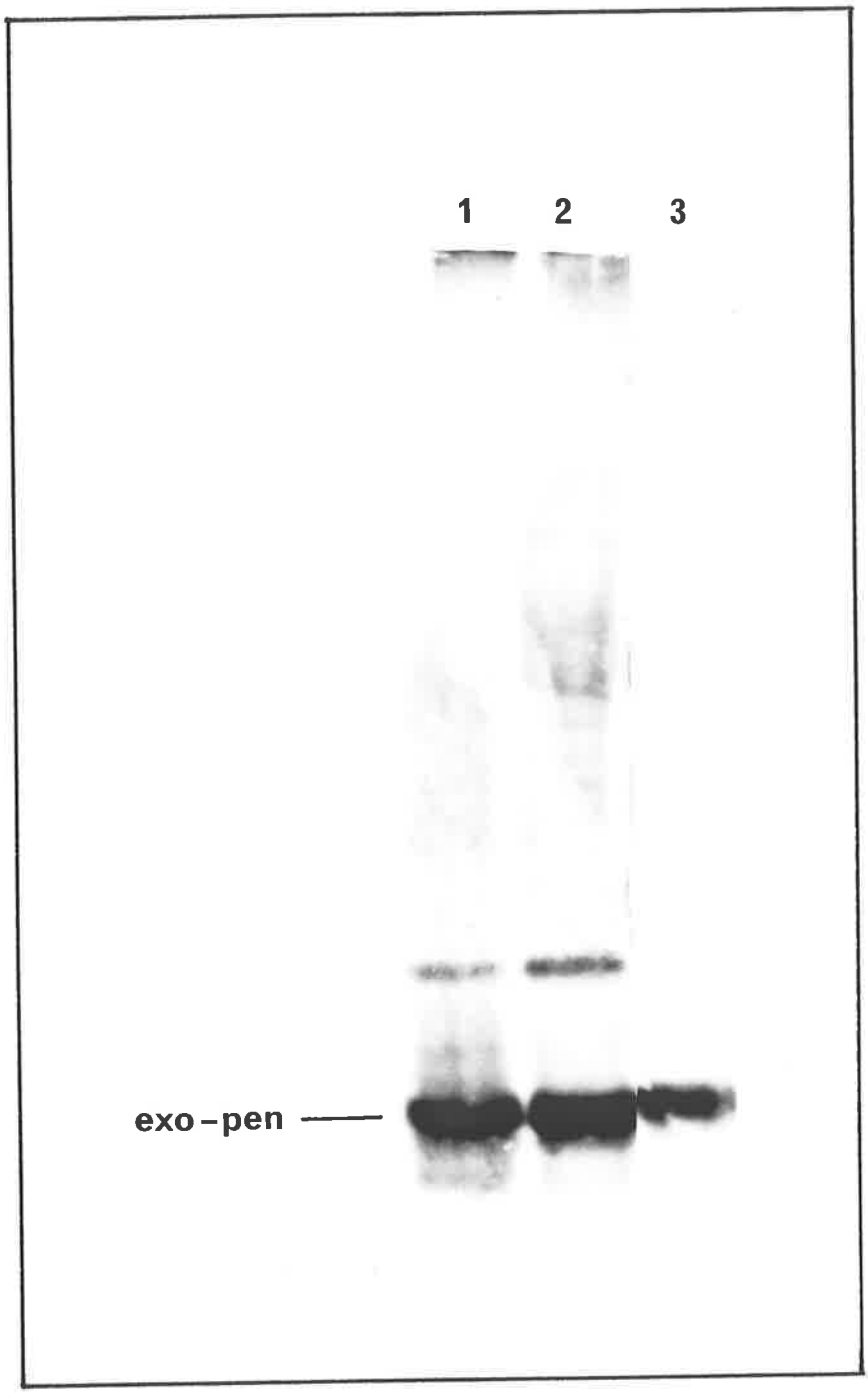
When 200 µl of protein synthesis mix is incubated for 20', with either λp*lac*5 or pI258*bla*i^{-} DNA, and then centrifuged through a 5-40% sucrose gradient (SW41, 27,000 rpm, 75') as described in the Materials and Methods of this chapter, there is a significantly different pattern of distribution

FIGURE 4.5

Protein synthesis by S30 and S70 extracts

pI258*bla*i443 proteins were synthesized by either S30 or S70 extracts, as described in the text.

Track	
1	S30 extract
2	S70 "
3	extracellular penicillinase


1
2
3
exo-pen

of A_{260} material, as shown in Figure 4.6. Randall and Hardy (1977) analysed the polysome profile of lysed cells, and showed that alkaline phosphatase was produced almost exclusively on polysomes associated with membrane material. The results of this experiment are very similar. When the mixes are primed with penicillinase DNA, there appears in the A_{260} profile of the centrifuges material, a second peak. In contrast, λp*lac*5-primed systems yield only one, less dense peak. These peaks correspond to the free-polysome and membrane-polysome peaks described by Randall and Hardy (1977).

The peak fractions were pooled, as indicated in Figure 4.6, and polysomes sedimented by a 90', 120,000 g centrifugation, resuspended in 50 µl of 0.01 M tris-acetate, pH 8.2 and added to complete protein synthesis mixes (without added DNA), After incubation for 60', samples derived from λp*lac*5 polysomes were assayed for β-galactosidase activity, while samples derived from pI258i$^-$ polysomes were electrophoresed on 10% polyacrylamide gels.

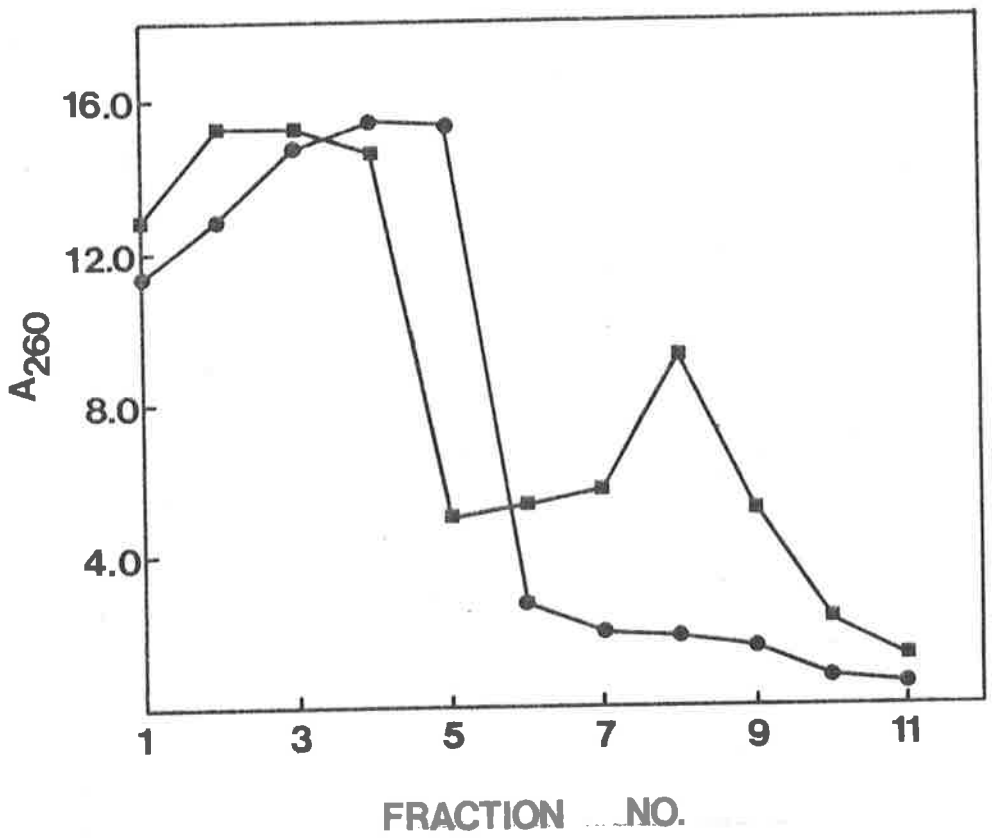
Table 4.3 shows that all β-galactosidase activity is found in the "free polysome" fraction (L-1),

FIGURE 4.6

Polysome profile of *in vitro* mixes during protein synthesis

Polysomes from protein synthesis mixes were analysed by sucrose gradient centrifugation, and 0.5 ml fractions collected. The A_{260} of the fractions (or their dilutions) was measured, and peaks were pooled as follows:

	Peak designation	fractions
λp*lac*5 mix (●)	L-1	2-4
	L-2	7-9
penicillinase mix (■)	P-1	3-5
	P-2	7-9


16.0
12.0
8.0
4.0
A260
1
3
5
7
9
11
FRACTION NO.

implying that β-galactosidase-producing polysomes are found exclusively in this fraction. As controls, complete mixes including polysomes were assayed at time zero, and indicate that all enzyme activity detected in the incubated samples was produced during the incubation. The inclusion of DNAase in the mixes ensures that all enzyme activity is derived from polysomes, not from residual DNA that may be in the polysome preparation (see Figure 3.9).

TABLE 4.3

Synthesis of β-galactosidase directed by fractionated polysomes

Pooled ribosomal fractions were used to direct protein synthesis *in vitro*, as described in the text, then assayed for β-galactosidase activity.

L-1 = fractions 3-5

L-2 = fractions 7-9.

fraction	β-galactosidase activity (ΔA_{420}/hr)
L-1	0.011
L-2	0.000

4.4 DISCUSSION

Other extracellular proteins have been synthesized by *in vitro* systems, and have always been produced as precursors - i.e. proteins of a higher molecular weight than their extracellular counterparts. For example, in a Zubay-type system primed with alkaline phosphatase DNA, the gene product is of a higher molecular weight (Inouye and Beckwith, 1977) and can be processed co-translationally, by added membranes, to the periplasmic form (Chang, C.N., *et al.*, 1980). The authors interpret these results as supporting the details of the signal hypothesis, as reviewed in Chapter 1 - i.e. that the polysome-membrane association is essential for the processing of the translated amino-terminus of the polypeptide.

Similary, the membrane protein, f1 coat protein, has been shown to be produced *in vitro* as a precursor which can be co-translationally converted to its final form by added *E.coli* inner membranes (Chang, Blobel and Model, 1978; Chang, Model and Blobel, 1979).

In contrast, staphylococcal penicillinase has been shown here to be produced as a protein of the same size as the extracellular protein, implying either that penicillinase is not produced as a precursor, or that it is being processed rapidly (cotranslationally?) by some

component of the *in vitro* mix. If the *in vitro* system does process a precursor, such processing would be expected to be rapid. Recently, Crowlesmith, Gamon and Henning (1981) have shown that the half-lives of two outer membrane protein precursors is only about 30". If the *in vitro* system reflects this behaviour, then it is not surprising that no precursor was observed. Perhaps synthesis at a lower temperature would allow its detection, as is the case for their system.

Treatment of the protein synthesis mixes with protease inhibitors did not result in the appearance of a precursor (figure 4.4), however, ideally, a far wider range of inhibitors should be tested; unfortunately, many others examined severely limited the synthetic capabilities of the system, so making it impossible to accurately measure their effects on penicillinase production (results not shown).

The most likely explanation for the non-appearance of a precursor is that the S30 contains significant amounts of membrane material, particularly inverted inner membrane vesicles, which can be generated by pressure lysis (E.McMurchie, personal communication). These have their cytoplasmic surface exposed, so could allow attachment of polysomes, and incorporation of penicillinase molecules into or through them, with concomitant processing.

This chapter suggests that this may be the case, and the results of Chapter 6 also support the idea that there is considerable lipid material in the S30.

When completed mixes are centrifuged at 70,000 g, a significant proportion of penicillinase activity is found in the pellet, in contrast to β-galactosidase (Table 4.1), which is compatible with the protein being associated with membrane vesicles. Similarly, the polysome profile of mixes is significantly different when they are producing penicillinase, which implies that at least some polysomes are associated with membranes, forming the denser peak (Figure 4.6).

However, these results are not conclusive. Firstly, in the absence of membranes (when an S70 fraction is substituted for S30), mature penicillinase is still produced (Figure 4.5). Secondly, disruption of membrane integrity with toluene does not alter the appearance of penicillinase *in vitro*, unlike its effects on alkaline phosphatase *in vivo* (Halegoua, Hirashima and Inouye, 1976). Thirdly, although the polysome profile of the penicillinase synthesis mix implies that there are membrane-polysome complexes formed in the mix, most penicillinase was still produced, as mature polypeptides, in the "free ribosome" fraction (Figure 4.7).

Thus there is no firm evidence for the necessity of a membrane association for the synthesis of a mature-sized staphylococcal penicillinase. Rather, although there is some evidence that a membrane attachment does occur, and that penicillinase activity is preferentially associated with the membrane, it appears that membranes are not essential for penicillinase processing. It is worth noting that Chang, Blobel and Model (1978) observed processing of f1 pre-coat protein in the absence of added membranes, but in the presence of detergent, implying that their protein synthesizing fraction does contain some fairly specific peptidase activity. Likewise, outer membrane preparations have been shown (Inouye and Beckwith, 1977) to have some precursor-cleaving properties. There is thus some evidence that processing is not exclusively performed by membrane associated protease, at least *in vitro*. This could be enhanced by the methods used in preparation of the S30. In contrast, Randall, Hardy and Josefsson (1978) isolated membrane-polysomes which synthesized a number of periplasmic proteins as precursors, despite their attachment to membranes.

However, the question still remains as to why staphylococcal penicillinase is not appearing as a precursor, despite the modification tested. It is possible that the *blaZ* gene used here has been

modified during mutagenesis, and selection of the macroconstitutive operon pI258*bla*i, so that the precursor is not noticeably larger than the final product. This would best be investigated by

a) DNA sequencing of the operon,

b) comparison with other secretory proteins, and other penicillinase operons, *in vitro*.

c) amino-terminal analysis of the *in vitro* and extracellular proteins

d) identification and characterization of the membrane-associated form of the enzyme *in vivo*.

CHAPTER FIVE

DISRUPTION OF LIPID SYNTHESIS BY CERULENIN - ITS EFFECT ON THE SYNTHESIS OF PENICILLINASE

5.1 INTRODUCTION

This chapter describes studies on the effect of modifying the lipid state of the cell on extracellular protein synthesis.

As discussed fully in Chapter 1, there are some substantial differences between the sensitivity of synthesis of intracellular and extracellular proteins, in particular to various antibiotics, and to disruption of the cell membrane (e.g. Fishman, Rottem and Citri, 1980). Sensitivity to the lipid state of the cell is understandable because of the intimate association between the cell membrane and polysomes producing extracellular proteins, and because of the necessity for such proteins to pass into and through the membrane. However, there has also been considerable speculation that exo-protein synthesis is intimately associated with lipid synthesis rather than simply to the lipid state of the cell. In particular, studies using the antibiotic cerulenin have suggested that specific inhibition of lipid synthesis effectively depresses synthesis of exo-proteins (e.g. Altenbern, 1977; Fishman, Rottem and Citri, 1978; Berkeley et al., 1978). Cerulenin irreversibly binds to fatty acid synthetase (Omura, 1976), preventing the synthesis of fatty acids, and hence of lipids. Cells treated

with cerulenin often have dramatically reduced extracellular protein synthesis. This inhibition can be reversed by the addition of exogenous fatty acid, thus suggesting that the inhibition of exo-protein synthesis is due directly to the inhibition of lipid synthesis.

The work described in this chapter was prompted by the initial observation, studied in full in this chapter, that constitutive penicillinase synthesis was not inhibited by cerulenin. Consequently, a detailed examination of the effects of lipid synthesis on penicillinase production was undertaken - using cerulenin (Chapter 5), and a mutant defective in lipid synthesis (Chapter 6).

The results suggest that lipid synthesis *per se* is not necessarily an obligatory partner of extracellular penicillinase synthesis, but more likely affects enzyme synthesis via the state of the cell membrane.

5.2 METHODS

5.2.1 Treatment of cells with cerulenin

Cerulenin (Makor Chemicals) was dissolved in 95% redistilled ethanol at 20 mg/ml, and this stock solution stored at -20°C.

Cells to be treated with cerulenin were grown as follows: CYP broth was inoculated with a 1/50 dilution of a fresh overnight culture of the relevant bacterial strain, and grown at 37°, with shaking, to OD_{600} = 0.8. Cells were then pelleted, washed once with CYP broth, and resuspended in fresh broth at OD_{600} = 0.8. Cerulenin stock solution was added to the desired final concentration, and the cultures incubated at 37° with shaking. Initially, control cultures containing equivalent volumes of ethanol were included in all experiments, and showed that ethanol had no effect on the growth or behaviour of the cultures (data not shown).

The time of addition of cerulenin is "time zero" in all experiments.

5.2.2 Fatty acid and lipid synthesis in *S.aureus*

5.2.2.1 Fatty acid synthesis

A culture of S2 was grown to OD_{600} = 0.8 washed and resuspended in CYP plus cerulenin, as

described above. Sodium ^{14}C-acetate was added to cultures at 2.5 μCi/ml.

At various times, 0.25 ml of culture was withdrawn and added to 1 ml of cold 1M sodium acetate (unlabelled). Cells were centrifuged and resuspended in 0.2 ml 1 M sodium acetate.

Lipid material was extracted essentially by the method of Kates (1972). To the 0.2 ml of washed cells was added 0.75 ml of methanol-chloroform (2:1), the phases mixed, and the mixture left at room temperature overnight. Particulate matter was removed by centrifugation, and to the supernatant was added 0.5 ml water and 0.5 ml chloroform. The mixture was vortexed thoroughly and the phases separated by a brief centrifugation. The lower chloroform phase was removed and washed once with 0.5 ml water. The pellet from the original extraction was resuspended in 0.2 ml water and re-extracted as above.

The two chloroform phases were pooled and evaporated, *in vacuo*, to dryness. The residue was solubilized in 5 ml toluene scintillation fluid, and counts measured in Packard scintillation counter.

5.2.2.2 Lipid synthesis

Lipid synthesis was monitored by incorporation of ^{14}C-glycerol into methanol-chloroform soluble extracts.

After growth to OD_{600} = 0.8, cells were washed and resuspended in CYP + 0.1% glycerol with 0.5 Ci/ml ^{14}C-glycerol, and cerulenin if required.

0.25 ml aliquots of culture were withdrawn and lipid material extracted as described above.

5.2.3 Protein synthesis

Total protein synthesis in cerulenin treated cultures was estimated by measuring incorporation of ^{14}C-amino acids (equimolar) into hot TCA-precipitable material. After washing and resuspending in CYP broth, cells were grown at 37^{o} in the presence of 0.5 µCi/ml of labelled amino acids. At various times, 50 µl of culture was withdrawn and added to 2 ml of ice-cold 10% TCA + 1% casamino acids. After 30' on ice, the mix was heated to 95^{o} for 20', then chilled for 30'. The precipitate was gathered by filtration, under gentle vacuum, through a Whatman GF/A filter. Each filter was washed with 50 ml of cold 10% TCA, then 5 ml ethanol. After drying at 65^{o}, filters were placed in 5 ml of toluene scintillation

fluid, and counted in a Packard Scintillation Counter.

Extracellular protein synthesis was measured by spotting 50 µl of culture, containing 5 µCi/ml ^{14}C-amino acids directly onto a GF/A filter. When dried, filters were batch-washed in ice-cold 10% TCA + 1% casamino acids (four changes, with 20 ml per filter per wash), followed by two washes in 95% ethanol. Filters were dried and counted as described above.

5.2.4 RNA synthesis

RNA synthesis was measured by monitoring incorporation of ^{3}H-uridine into TCA-precipitable material.

Fifty microlitres of a log phase culture was withdrawn, added to 2 µ Ci of ^{3}H-uridine and incubated at 37^{o}, usually for one minute. Ten microlitres of cells were spotted directly onto a GF/A filter, dried, washed and counted as described above.

Preliminary experiments (described later) indicated that incorporation of uridine was linear for 1-2 minutes under these conditions, when cells untreated with cerulenin were used.

5.2.5 DNA-RNA hybridization

A. Preparation of labelled RNA.

Total ^{3}H-labelled RNA was prepared essentially as described by Zouzias *et al.* (1973). S112 was grown in CYP broth, plus or minus penicillinase inducer (methicillin, 0.5 µg/ml) to OD_{600} = 0.8, at which time cerulenin was added to cultures if required. At various times 10 ml of culture was removed, added to 100 µCi of ^{3}H-uridine, and incubated at 37^o for 2 minutes, the time which yielded maximum incorporation of ^{3}H-uridine into RNA (see sections 5.2.4; 5.3.1.5). Sodium azide was added to 20 mM, the cultures chilled rapidly on dry ice-ethanol, and cells pelleted by centrifugation at 4^{o}C.

Pellets were resuspended in 0.2 ml of 0.1M NaCl, 0.05M EDTA, 0.01M tris-Hcl (pH 7.9), 0.02M NaN_3, containing 100 µg/ml lysostaphin, and incubated at 37^o for 10'. SDS was added to 0.2%, and the lysed cells extracted with an equal volume of 60^{o}C phenol, saturated with buffer. The mixes were chilled on ice, phases separated by centrifugation, the aqueous phase removed, and re-extracted with phenol twice more. Residual phenol was removed with chloroform/isoamyl alcohol (24:1) and nucleic acid precipitated at -20^o overnight with 2 volumes of ethanol.

After pelleting the resulting precipitate by centrifugation, the supernatant was discarded, the pellet washed with ethanol and dried, then resuspended in 200 µl of 0.01M $MgCl_2$, 0.01M tris-HCl (pH 7.9), containing DNAase I (40 µg/ml), and incubated at 37° for one hour. After further hot phenol and chloroform/iso-amyl alcohol extractions, the RNA was re-precipitated with ethanol, and resuspended in 100 µl of 2 x SSC (1 x SSC is 0.15M NaCl,0.015M trisodium citrate, pH 7.4).

One microlitre was spotted onto a GF/A filter, and after TCA precipitation and washing, filters were counted to determine the yield of labelled RNA.

B. Preparation of DNA for hybridization.

The DNA used for hybridization was the recombinant plasmid pPG122, a clone of the Hind III fragment of pI258*bla*i443 spanning the penicillinase gene, into the single Hind III site of the *E.coli* vector plasmid pMB9 (Maniatis *et al.*, 1976). The isolation of this clone will be described in Chapter 6.

The plasmid DNA was linearized prior to hybridization by digestion with EcoRl. 100 µg of DNA (700 µl) was incubated with 50 units of EcoRI

(Biolabs) using the conditions specified by the manufacturer, for 3 hours at 37°, in a total volume of 1 ml. An aliquot electrophoresed on a 1% agarose gel showed that the DNA was completely linearized at this time. The DNA was purified by 3 cold phenol extractions, precipitated with 2 vol. ethanol at -80°C for 30', then resuspended in 300 µl of 0.1 mM EDTA, pH 8.0.

DNA was denatured by the addition of 100 µl of 1M NaOH. After standing at room temperature for 15', the DNA was chilled on ice, and neutralized by the addition of 400 µl of 2M NH_4 acetate, and immediately applied to the nitrocellulose filters.

C. Preparation of nitrocellulose filters.

Hybridization was performed with Sartorius nitrocellulose filters, 24 mm in diameter, essentially by the technique of Kafatos *et al.* (1979). A 10 cm thick wad of tissue paper was overlaid with two sheets of wet Whatman 3MM paper and one sheet of nitrocellulose. Individual filters, presoaked in sterile H_2O for 30', were placed on the nitrocellulose, and when all moisture had been absorbed from them, washed with 50 µl of 1 M NH_4 acetate.

Denatured DNA (5 µg in 40 µl) was applied to each filter (initial experiments determined that this

represents an excess of DNA - see later). After all liquid was drawn through the filter, each was washed with 50 µl of 1 M NH_4 acetate, then removed from wad and dried for 1 hour at room temperature. Filters were then baked at 80°C, in vacuum, for 6 hours.

D. Hybridization.

The technique of Kafatos *et al.* (1979), as modified by Bill Kalionis of this laboratory, was used.

Loaded filters were pretreated for one hour at 42°C in:

50% deionized formamide
5 x SSC
5 x Denhardt's solution (Denhardt, 1966)
50 mM sodium phosphate pH 6.5
100 µg/ml *E.coli* tRNA

Filters were then placed in individual siliconized, sterilized scintillation pots containing 0.5 ml of:

50% deionized formamide
5 x SSC
1 x Denhardt's solution
20 mM sodium phosphate pH 6.5
100 µg/ml *E.coli* tRNA

and 200,000 cpm of the appropriate labelled RNA.

Hybridization was for 24 hrs at 42°C, after which filters were washed 3 times in 2 x SSC, 0.1% SDS, for five minutes each at room temperature, followed by 2 times in 0.1 x SSC, 0.1% SDS, at 50°, for 15' each.

Following a brief rinse in 95% ethanol, filters were dried at room temperature, then counts recorded in toluene scintillation fluid.

5.2.6 Penicillinase assay

Penicillinase activity on cells, and on culture supernatant, was measured with cephalosporin 87/312, as described in Chapter 2.

5.2.7 Assay of extracellular DNAase activity

Extracellular DNAase production was assayed by the method of Ryden (1973). Fifty microlitres of S2 supernatant grown in CYP as previously described, was added to 0.2 ml of DNA solution (0.025 M glycine, pH 8.6, 2mM $CaCl_2$, 1.5 mg/ml calf thymus DNA) which had previously been denatured at 100°C for 15', then chilled on ice. The mix was incubated at 37° for exactly 30', then the reaction stopped with 0.5 ml of ice-cold 10% TCA and chilled at 0° for 30'. 0.5 ml H_2O was added, and precipitate was removed by centrifugation (5' in an Eppendorf centrifuge).

The supernatant was diluted to 1/10 with water, and A_{260} recorded.

After subtraction of appropriate blank values (TCA and supernatant added simultaneously), one unit of DNAase activity was defined, for the purposes of this work, as a change in A_{260} of 1.0 during the 30' assay.

5.3 RESULTS

5.3.1 The effect of cerulenin on the growth of *S.aureus*

In order to examine the effect of cerulenin and so of lipid synthesis, on the production of staphylococcal extracellular proteins, especially penicillinase, it was first important to characterize its effect on the general metabolism of *S.aureus*.

5.3.1.1 Culture growth in the presence of cerulenin

Figures 5.1 and 5.2 show the alterations on the growth of S2 in the presence of various concentrations of cerulenin.

Cells from a log-phase culture of S2, in CYP broth, were washed by centrifugation and re-suspended in fresh broth containing various concentrations of cerulenin. The growth of the cultures was monitored for three hours, and the final growth density measured 24 hours after exposure to cerulenin.

Figure 5.1 shows that concentrations of 2 - 25 µg/ml cerulenin did not significantly alter the final growth density of S2 cultures. In fact, 2 and 10 µg/ml cerulenin consistently stimulated growth of the cells. Significant inhibition of culture growth occurred at antibiotic concentrations of 25 µg/ml and

FIGURE 5.1

Effect of cerulenin on the growth of *S.aureus* S2

Logarithmic cultures of S2 were washed and resuspended in fresh CYP broth containing various concentrations of cerulenin as described in Section 5.3.1.1. The OD_{600} was monitored at various times after exposure to cerulenin.

□ = 0 µg/ml cerulenin

○ = 2 µg/ml cerulenin

■ = 10 µg/ml cerulenin

▲ = 25 µg/ml cerulenin

△ = 50 µg/ml cerulenin

● = 75 µg/ml cerulenin

▼ = 125 µg/ml cerulenin

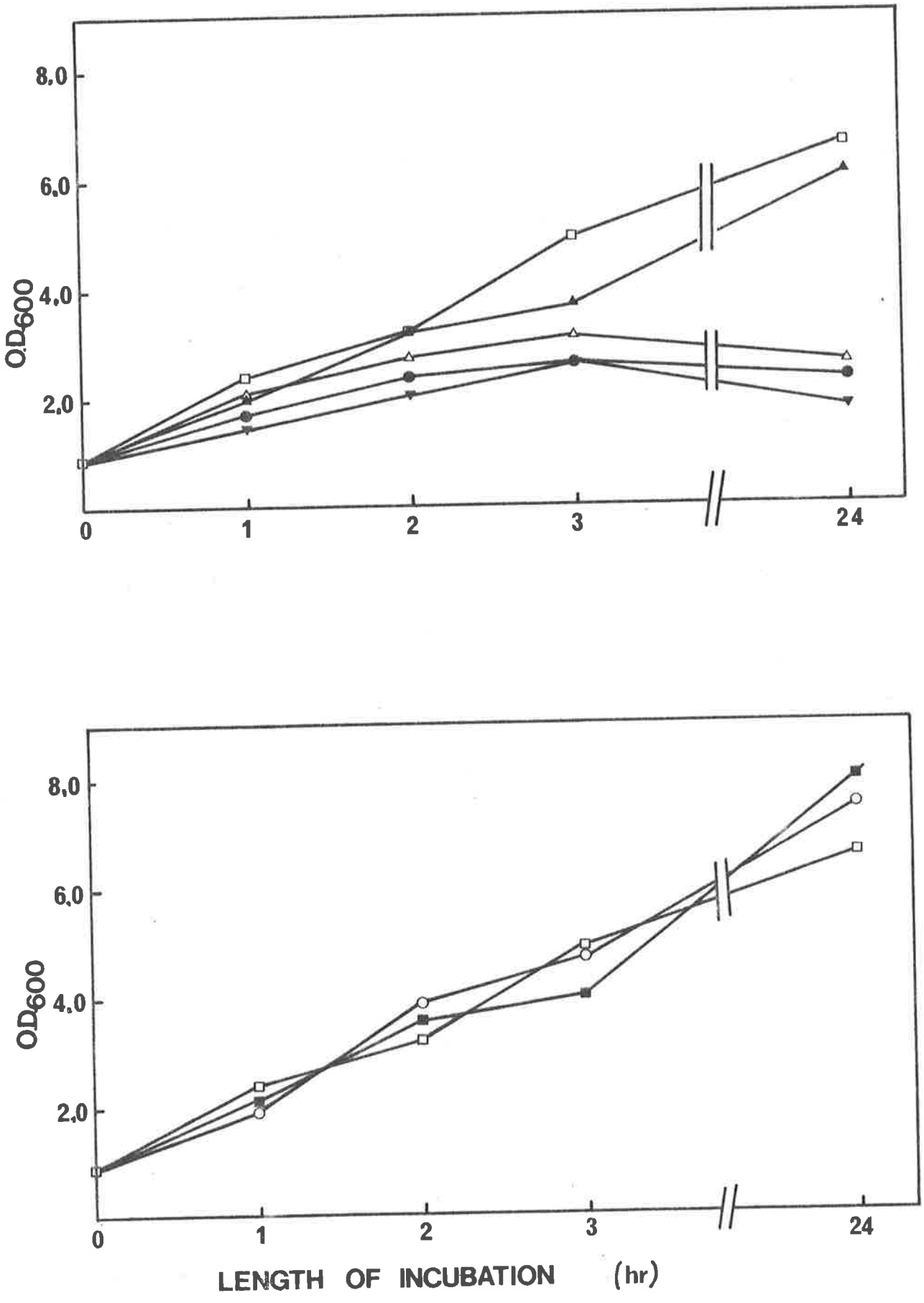
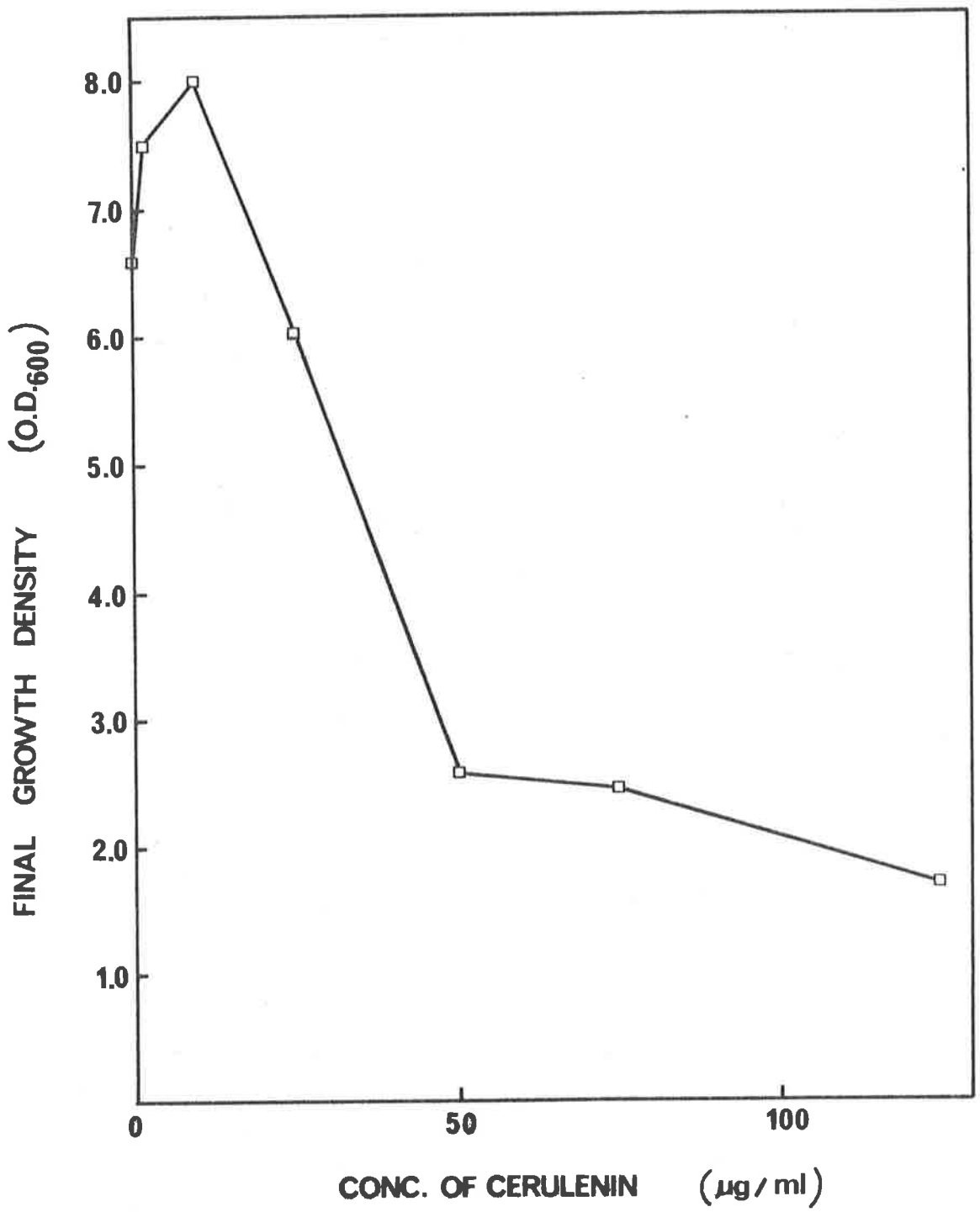

8.0
6.0
4.0
2.0
OD600
0
1
2
3
24
8.0
6.0
4.0
2.0
OD600
0
1
2
3
24
LENGTH OF INCUBATION (hr)

FIGURE 5.2

Effect of cerulenin on final density of *S.aureus* cultures

The final growth densities (OD_{600}) of cultures of S2 are here plotted against the concentration of cerulenin to which they were exposed for 24 hr. The points are those derived from the data in Figure 5.1.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
FINAL GROWTH DENSITY (O.D.600)
0
50
100
CONC. OF CERULENIN (μg / ml)

greater - a level which is compatible with the results of Altenbern (1977) and Berkeley *et al.* (1978).

This inhibition of growth does not occur immediately on exposure to cerulenin, as can be seen from figure 5.2. It is apparent from this graph that, at low concentrations (0-25 µg/ml) the growth rate is slightly retarded, although the final growth density is not affected greatly. At higher concentrations of cerulenin, where the final growth density is affected, the inhibition does not become apparent until 1 - 2 hours after addition of the antibiotic. This result could be interpreted as evidence for a "pool" of lipid precursors in the cell; that is when fatty acid synthesis is inhibited by inactivation of the fatty acid synthesis system, there is enough residual fatty acid in the cell to enable one or two cell divisions to take place before the cells are starved of lipid. The existence of such a pool has been suggested by Beacham, Taylor and Youell (1976) after studying glycerol-requiring strains of *E.coli*, and may have important implications in the synthesis and secretion of extracellular proteins.

5.3.1.2 Fatty acid synthesis in cerulenin-treated cells.

Fatty acid synthesis was measured by monitoring the incorporation of ^{14}C-acetate into methanol-chloroform soluble material, as described earlier in this chapter.

Figure 5.3 shows that fatty acid synthesis is slightly stimulated in the presence of low levels of cerulenin (2 µg/ml) during the first three hours of the experiment. At higher concentrations of the antibiotic, fatty acid synthesis is dramatically and immediately inhibited. As cell division continues for some time in the presence of cerulenin (figure 5.2) this result implies that some lipid synthesis can occur in the absence of fatty acid synthesis.

In figure 5.4, it can be seen that total acetate incorporation over 24 hours is inhibited by all concentrations of the antibiotic. The data on this graph have been expressed in two ways: the total counts incorporated, and these figures expressed as counts incorporated per OD_{600} unit of the appropriate culture, at 24 hours. This enables us to compare the counts incorporated per cell independently of the actual number of cells in the culture, and will be a useful form of comparison when looking at other cell

FIGURE 5.3

Fatty acid synthesis in cerulenin-treated cultures of S2

Fatty acid synthesis in cultures of S2 exposed to various concentrations of cerulenin, was measured by the incorporation of ^{14}C-acetate into methanol-chloroform extracts, as described in Section 5.3.1.2, at various times after exposure of the cells to the antibiotic.

□ = 0 µg/ml cerulenin

○ = 2 " "

■ = 10 " "

▲ = 25 " "

△ = 50 " "

● = 75, 125 " "

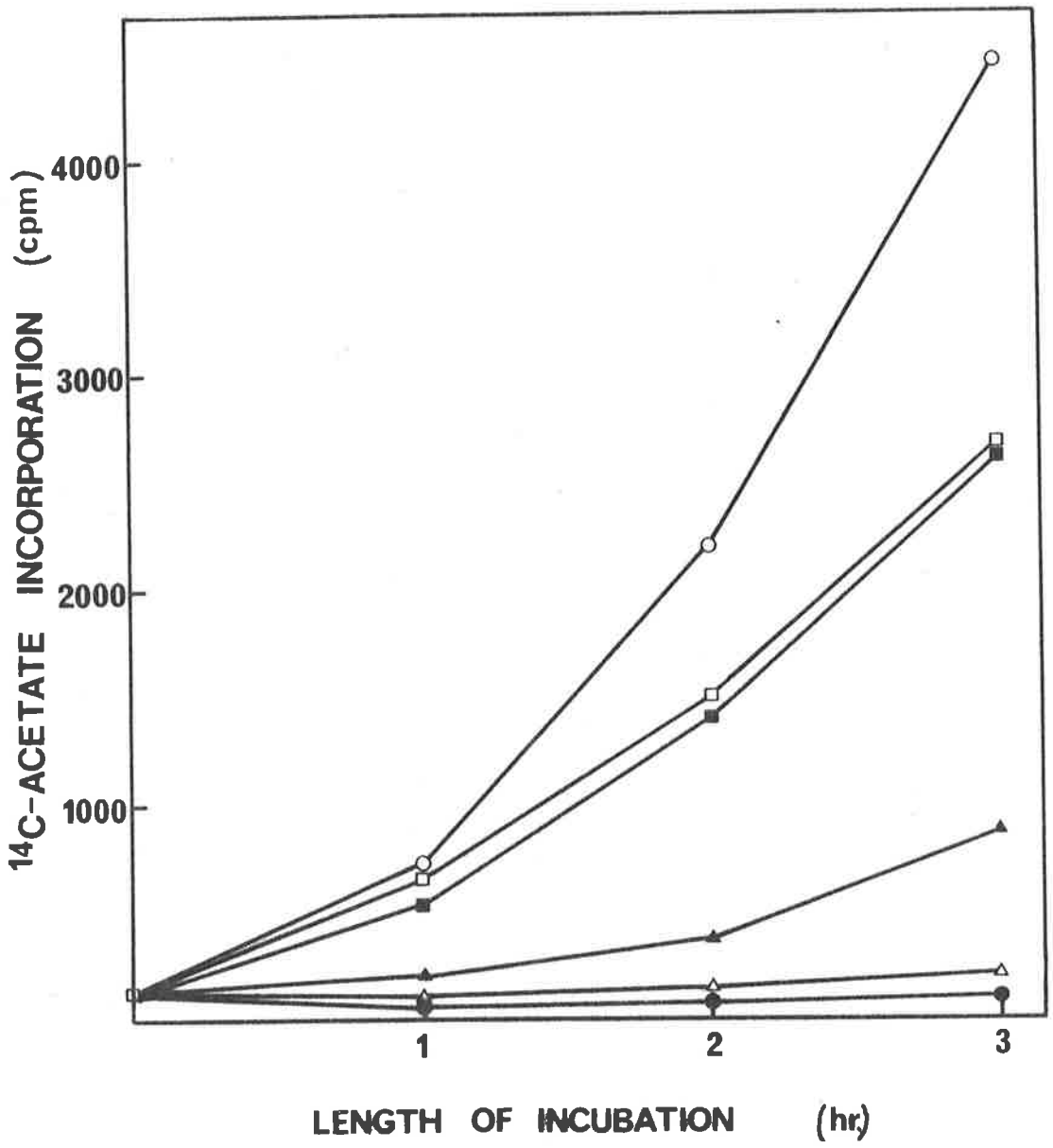

^{14}C-ACETATE INCORPORATION (cpm)
4000
3000
2000
1000
1
2
3
LENGTH OF INCUBATION (hr)

FIGURE 5.4

Incorporation of ^{14}C-acetate into S2 after 24 hours' exposure to cerulenin

Cultures of S2 exposed to cerulenin, in the presence of ^{14}C-acetate for 24 hours were assayed for total fatty acid synthesis as described in Section 5.3.1.2.

● = total ^{14}C-acetate incorporation

□ = ^{14}C-acetate incorporation/OD_{600}

^{14}C-ACETATE INCORPORATION (×10^{-3})
30
20
10
0
50
100
CERULENIN (μg / ml)
4
3
2
1
^{14}C-ACETATE INCORPORATION / OD$_{600}$ (×10^{-3})

functions which may be affected by cerulenin.

In the case of fatty acid synthesis, the inhibition of ^{14}C-acetate incorporation is closely related to the inhibition of cell growth (figures 5.1 and 5.2). The most noticeable fact is that at 25 µg/ml, cerulenin inhibits fatty acid synthesis by almost 90%, whereas cell growth is unaffected (figures 5.4 and 5.1 respectively). Thus the cell can continue to divide in the presence of only 10% of its normal fatty acid synthesis (presumably with a concommitent decrease in the lipid content of the membrane). It will be important to note later whether this level of cerulenin is critical in the production of extracellular proteins; i.e. can the effects of cerulenin on extracellular protein production be correlated with disruption of membrane lipid content, rather than with lipid synthesis itself?

5.3.1.3 Lipid synthesis in cerulenin-treated cultures

Lipid synthesis, as distinct from fatty acid synthesis, should not be directly inhibited by cerulenin. If there exists a pool of fatty acids available for incorporation into lipids, then there should be some synthesis of lipids even in the absence of fatty acid synthesis. This can be inferred from the data on the incorporation of ^{14}C-glycerol into methanol-

chloroform extracts, presented in figure 5.5. Glycerol continues to be incorporated for at least one hour after the addition of cerulenin, even at concentrations (50 μg/ml) which totally prevent fatty acid synthesis (figure 5.3).

Even after 24 hours (figure 5.6), cerulenin has not depressed glycerol incorporation to the same extent as acetate incorporation. This result implies that, although cerulenin effectively inhibits fatty acid synthesis, there may still be some lipid synthesis. This could happen if there is a "turnover" of lipids in the cell; that is, the cell may be degrading lipids to free fatty acids, and then re-synthesizing lipids using available glycerol (either exogenous or synthesized). Thus even though there is no fatty acid synthesis in a culture, there may be some qualitative synthesis of lipids (without a net increase in lipids), using fatty acids generated from partial degradation of existing lipid.

5.3.1.4 Total protein synthesis in cerulenin-treated cultures

Total protein synthesis in S2 was measured by the incorporation of ^{14}C-amino acids into hot TCA-precipitable material, as described earlier in this chapter. Protein syntheses at one and

FIGURE 5.5

Lipid synthesis in cerulenin-treated cultures of S2.

Lipid synthesis in cultures of S2 exposed to various concentrations of cerulenin, was measured by the incorporation of ^{14}C-gylcerol into methanol-chloroform extracts, as described in Section 5.3.1.3, at various times after exposure of the cells to the antibiotic.

□ = 0 µg/ml cerulenin

○ = 2 " "

■ = 10 " "

▲ = 25 " "

△ = 50 " "

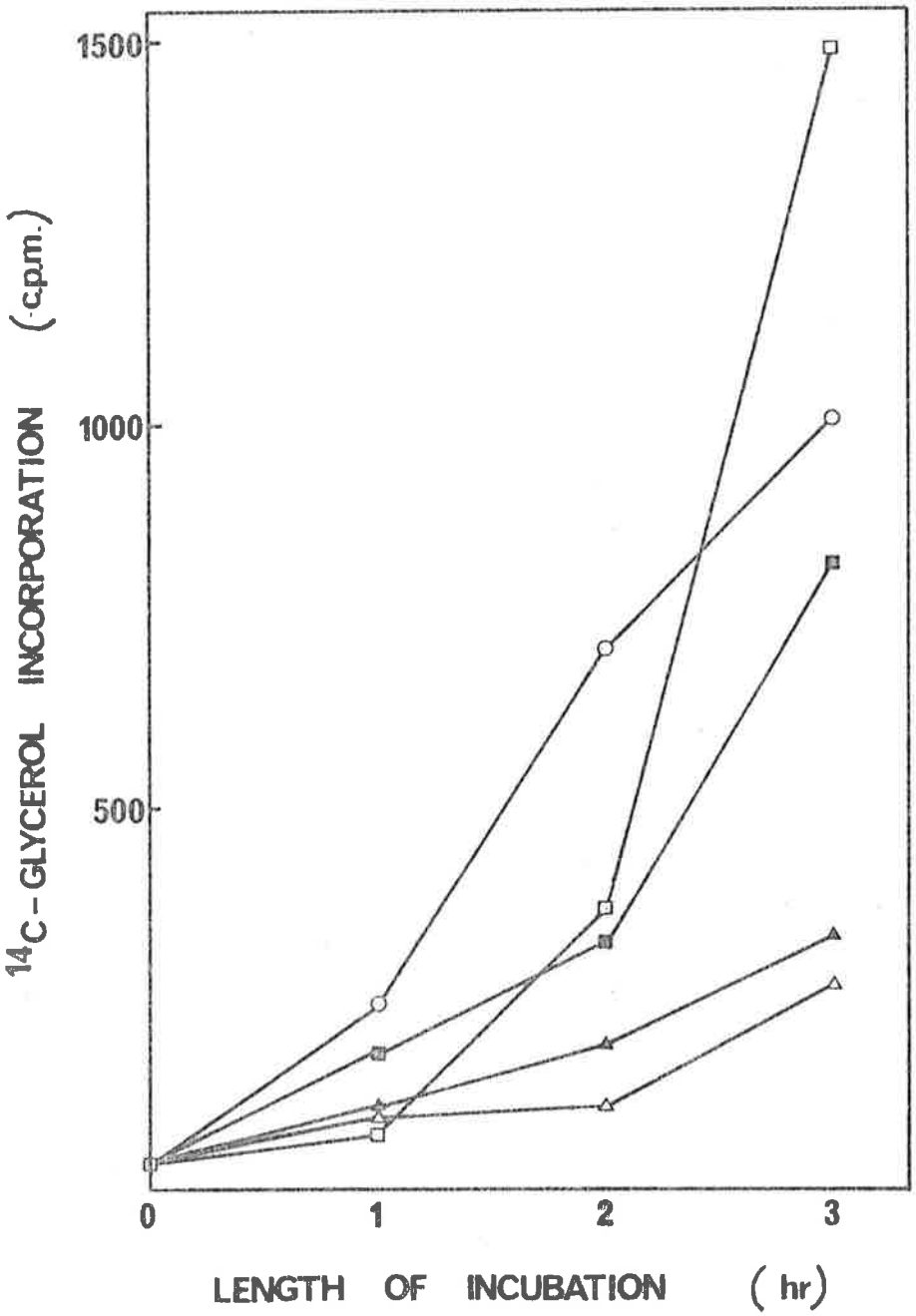
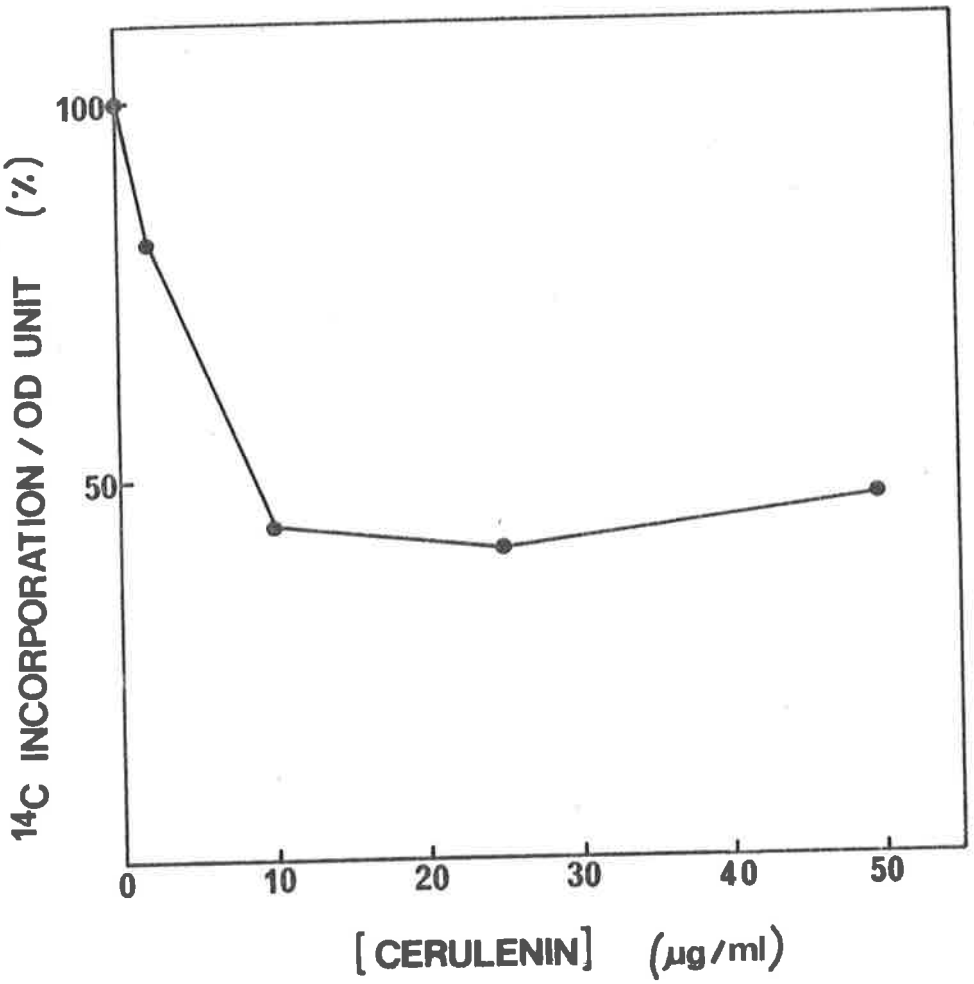

1500
1000
500
0
1
2
3
^{14}C-GLYCEROL INCORPORATION (c.p.m.)
LENGTH OF INCUBATION (hr)

FIGURE 5.6

Incorporation of ^{14}C-glycerol into S2 after 24 hours' exposure to cerulenin.

Cultures of S2 exposed to cerulenin, in the presence of ^{14}C-glycerol for 24 hours, were assayed for total lipid synthesis, as described in Section 5.3.1.3.


100
50
0
10
20
30
40
50
^{14}C INCORPORATION / OD UNIT (%)
[CERULENIN] (μg/ml)

24 hours of cerulenin treatment are shown in Figure 5.7, expressed as counts incorporated per OD_{600} units, as a percentage of the control culture. The effects of cerulenin on protein synthesis are generally compatible with its effects on cell growth - after one hour's exposure, there is some stimulation of cellular activity at low concentration of cerulenin, and little inhibition at higher concentrations, while at 24 hours, there is a marked inhibition of protein synthesis at higher concentrations of antibiotic. The level of protein synthesis at 25 µg/ml cerulenin is not directly compatible with the final growth density of this culture (figure 5.1) - an explanation of this will be offered later.

5.3.1.5 RNA synthesis in cerulenin-treated cultures.

Total RNA synthesis in cerulenin-treated cells, measured by one-minute pulses with 3H-uridine, as described earlier in this chapter, is also initially stimulated by low concentrations of cerulenin (figure 5.8).

In contrast, however, to the other cellular functions examined, there is some inhibition of uridine incorporation at 25 and 50 µg/ml cerulenin in the first hour of treatment. Whether this represents a drop in RNA synthesis is

FIGURE 5.7

Total protein synthesis in cerulenin-treated cultures of S2

Total protein synthesis in cultures of S2, exposed to various concentrations of cerulenin, was monitored by the incorporation of ^{14}C-amino-acids into protein material, as described in Section 5.3.1.2.

● = 1 hours' exposure to cerulenin

■ = 24 hours' exposure to cerulenin

(Incorporation is expressed as a percentage of the control incorporation, per OD_{600} unit of the cells).

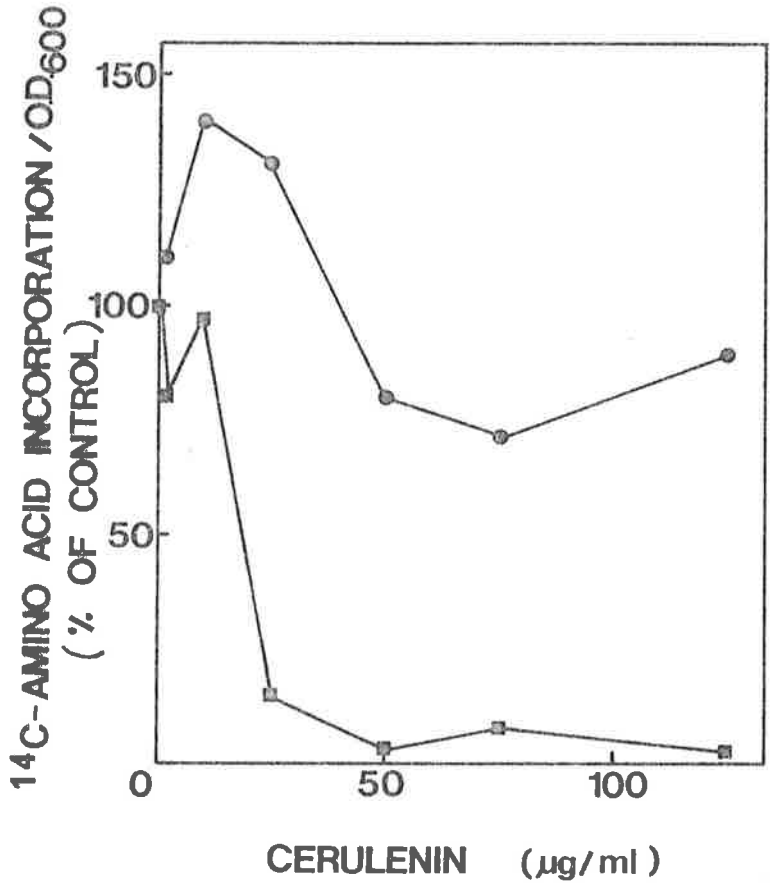

^{14}C-AMINO ACID INCORPORATION /OD$_{600}$
(% OF CONTROL)
150
100
50
0
50
100
CERULENIN (μg/ml)

FIGURE 5.8

RNA synthesis in cerulenin-treated cultures of S2.

Total RNA synthesis in cultures of S2 exposed to various concentrations of cerulenin was measured by the pulse-incorporation (1') of 3H-uridine into cell extracts after varying lengths of exposure to cerulenin, as described in Section 5.3.1.5.

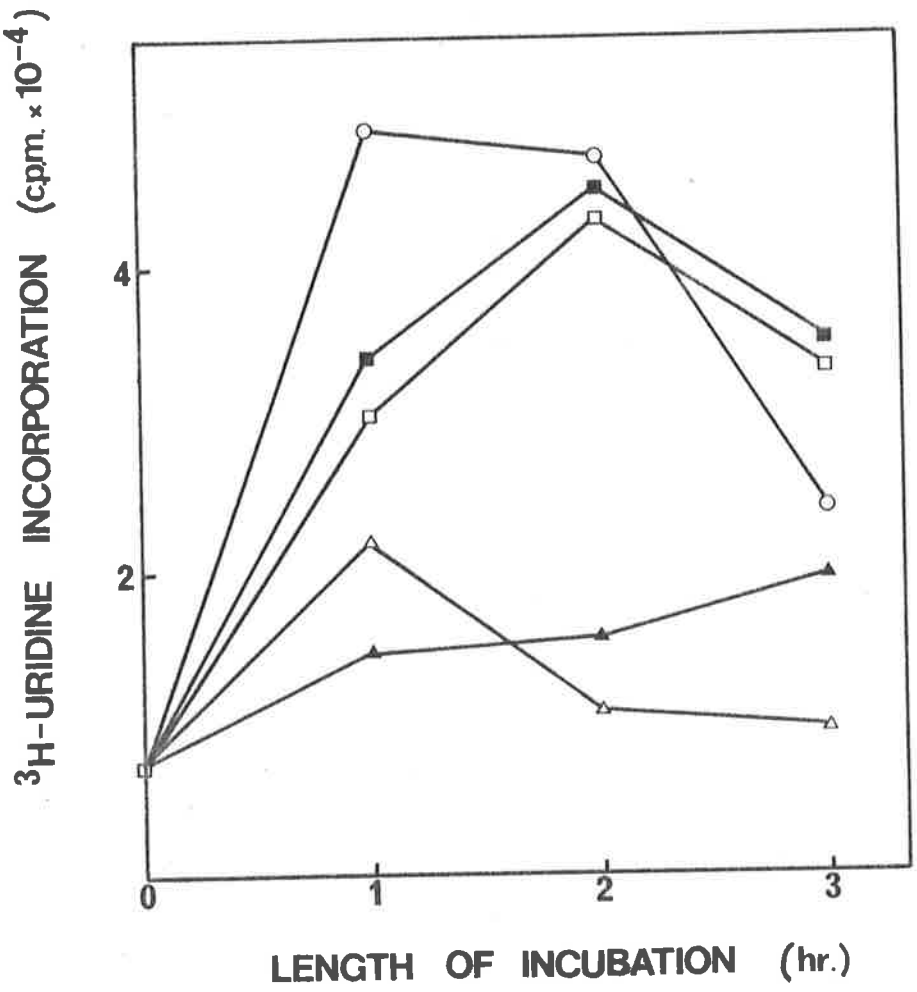
□ 0 µg/ml cerulenin

○ 2 µg/ml cerulenin

■ 10 µg/ml cerulenin

▲ 25 µg/ml cerulenin

△ 50 µg/ml cerulenin

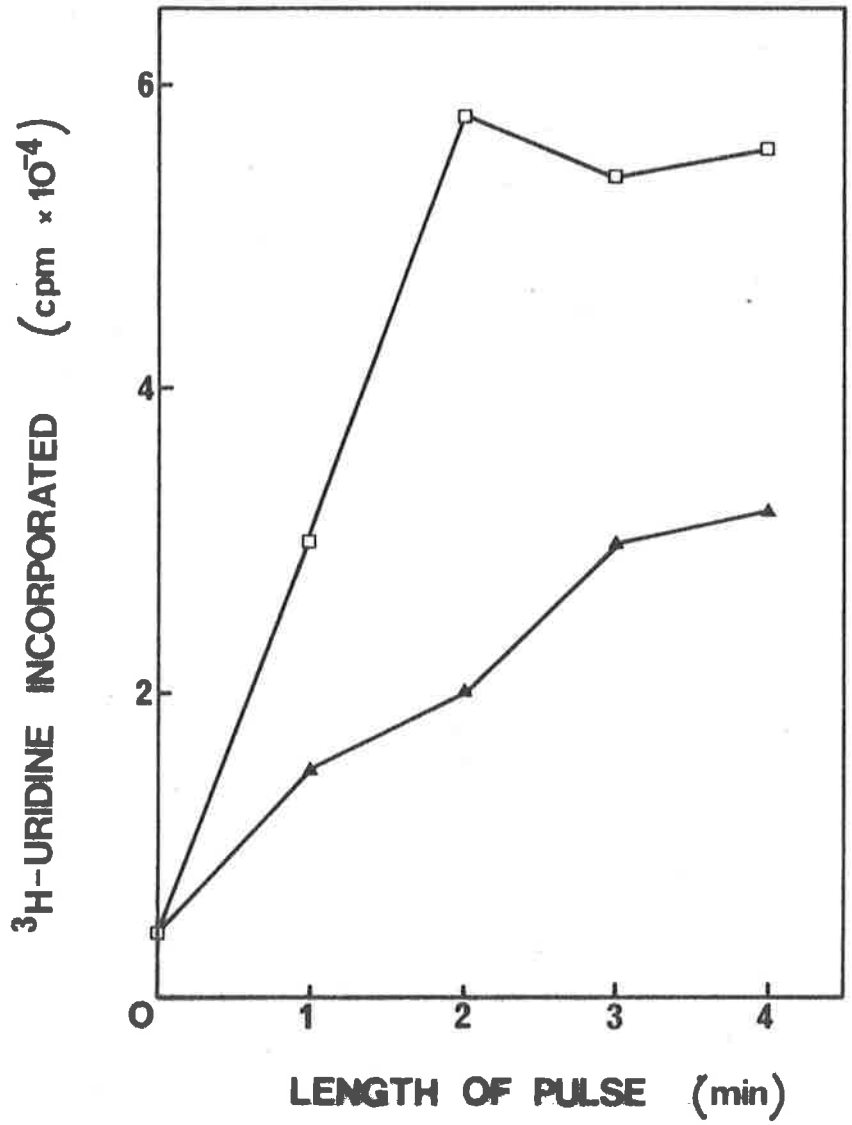

^{3}H-URIDINE INCORPORATION (c.p.m. × 10^{-4})
4
2
0
1
2
3
LENGTH OF INCUBATION (hr.)

questionable - the short exposure (one minute) of the cells to the label means that any deficiency in the uptake of uridine would be reflected in the counts incorporated into RNA. Thus if, for example, cerulenin disrupts the cell membrane, either by inhibiting lipid synthesis, or by direct interaction, it could interfere with the efficient uptake of uridine, producing artifactually low data on incorporation. In fact, there is some evidence that there is a lowered rate of uptake of uridine in cerulenin-treated cultures. Figure 5.9 compares the incorporation of 3H-uridine into RNA in 0 and 25 µg/ml cerulenin cultures (after 2 hr exposure to the antibiotic) over four minutes. In the absence of cerulenin, incorporation is linear for about two minutes, then becomes somewhat unpredictable, presumably representing turnover of RNA in the cell. At 25 µg/ml cerulenin, uridine incorporation into RNA continues linearly for about three to four minutes. The rate of incorporation is obviously greater, and linear incorporation briefer, in untreated cultures, thus implying a lowered rate of uptake of label, and/or a lowered rate of turnover of RNA, in the cerulenin-treated culture. So, although cerulenin obviously disrupts RNA synthesis, it is not possible to determine whether this is due

FIGURE 5.9

Rate of incorporation of ^{3}H-uridine into RNA of S2

Cultures of S2 exposed to either 0 µg/ml (□) or 25 µg/ml (▲) cerulenin for 2 hours were pulse-labelled with ^{3}H-uridine for up to four minutes, then assayed for incorporation into RNA, as described in Section 5.3.1.5.


6
4
2
0
1
2
3
4
^{3}H-URIDINE INCORPORATED (cpm x 10^{-4})
LENGTH OF PULSE (min)

to an altered rate of synthesis, impaired uptake of label or an altered rate of turnover of RNA.

5.3.2 Effect of cerulenin on synthesis of extracellular proteins

The aim of the work described in this chapter was to examine the effects of inhibition of lipid synthesis on the production of extracellular proteins of which penicillinase was chosen as a typical, and easily measurable example. This section of results describes experiments which imply that penicillinase may not be a typical exo-protein, or else that disruption of lipid synthesis does not necessarily disrupt extracellular protein production.

5.3.2.1 Effect of cerulenin on the synthesis of extracellular penicillinase

The production of extracellular penicillinase in the presence of cerulenin was measured, as described earlier in this chapter.

Figure 5.10 shows that the amount of penicillinase secreted by cerulenin-treated cultures of SIII is not nearly as affected by cerulenin as would be suggested by the data presented earlier. While 25 - 50 µg/ml cerulenin inhibits cell growth and lipid, protein and

FIGURE 5.10

Exo-penicillinase synthesis in the presence of cerulenin

Logarithmic-phase cultures of S111 were washed and resuspended in fresh CYP broth, containing various concentrations of cerulenin. At times after the initial exposure to the antibiotic, aliquots of the supernatant were assayed for the amount of penicillinase present, and expressed on the graph as a fraction of the optical density of the culture of that time and antibiotic concentration.

□ = 0 µg/ml cerulenin

○ = 2 µg/ml cerulenin

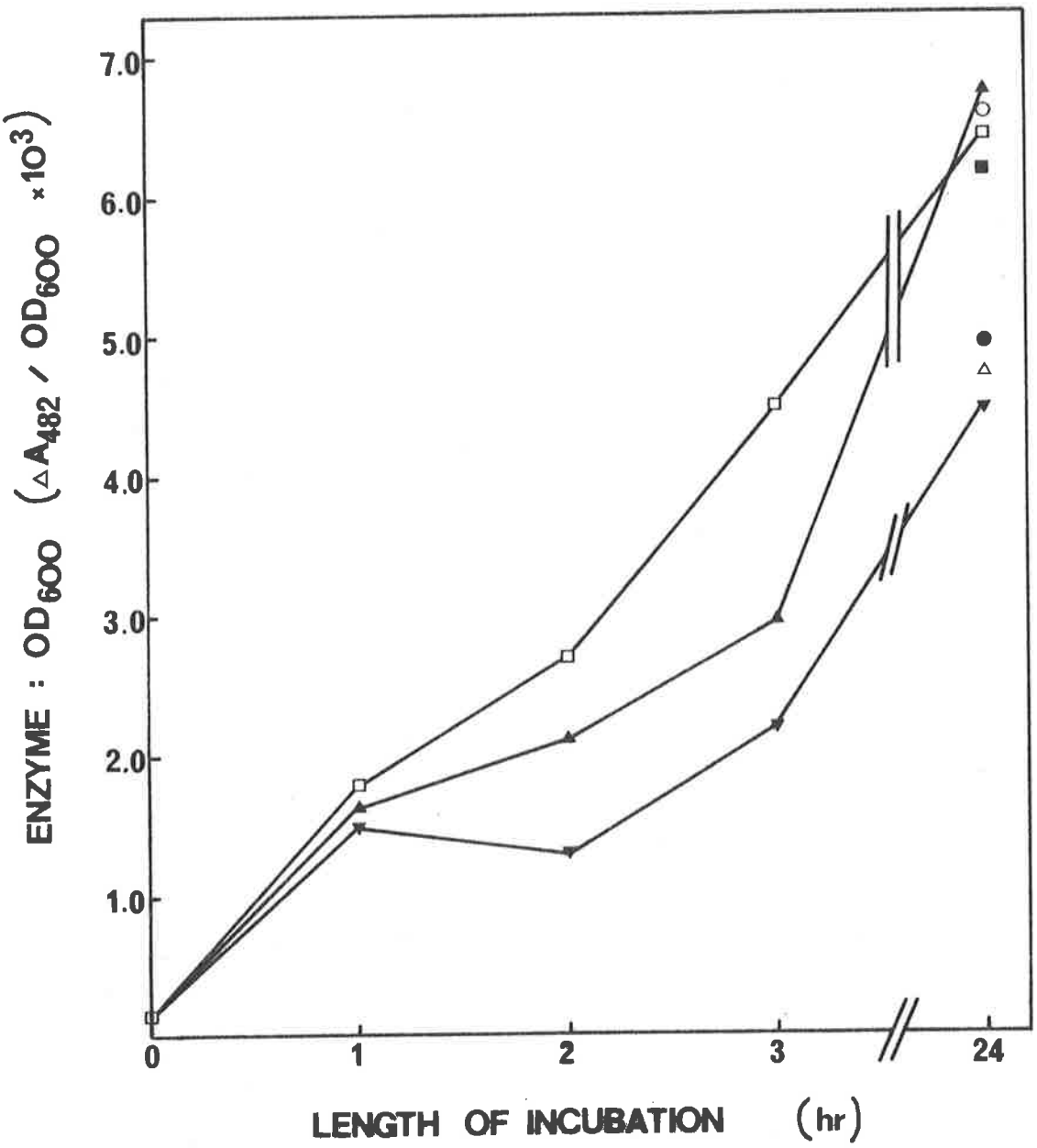
■ = 10 µg/ml cerulenin

▲ = 25 µg/ml cerulenin

▼ = 125 µg/ml cerulenin

● = 50 µg/ml cerulenin

Δ = 75 µg/ml cerulenin


ENZYME : OD_{600} (ΔA_{482} / OD_{600} $\times 10^3$)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
24
LENGTH OF INCUBATION (hr)

RNA synthesis, cerulenin at 25 µg/ml did not affect penicillinase production at any time, and 50-125 µg/ml cerulenin resulted in only a 25% reduction in supernatant enzyme levels.

In the light of the work of Altenbern (1977), where cerulenin at 4 µg/ml completely inhibited enterotoxins B and C formation in *S.aureus*, and Berkeley *et al.* (1978), where 10 µg/ml inhibited enterotoxin A by 80%, this result was unexpected. Despite the fact that cerulenin inhibits most cell functions, penicillinase continues to be produced at levels (per cell) comparable to the control, thus implying that there is no differential inhibition of extracellular penicillinase.

5.3.2.2 Total extracellular protein production in the presence of cerulenin.

As cerulenin did not inhibit penicillinase production as much as expected, it was thought important to examine its effects on other extracellular proteins. Total extracellular protein production in S2 (ie. in a strain not producing penicillinase), was measured by following the incorporation of ^{14}C-amino

acids into culture supernatant proteins, as shown in figure 5.11, which shows the counts incorporated per OD unit of cells. Antibiotic at 2 and 10 µg/ml causes a large (57% and 83% respectively) drop in supernatant protein production after twenty four hours of growth; the inhibition becoming apparent about two hours after beginning the experiment. Fifty µg/ml cerulenin causes an immediate drop in supernatant protein production.

Thus, total extracellular protein production is strongly inhibited by low concentrations of cerulenin, as also found by Altenbern (1977) and Berkeley *et al.* (1978), but in contrast to the results on penicillinase production.

5.3.2.3 DNAase production in the presence of cerulenin

As an example of an extracellular enzyme, staphylococcal DNAase was assayed in the supernatant of cerulenin treated cultures of S2. Figure 5.12 shows that 10 µg/ml cerulenin inhibits the production by over 90% in 24 hour cultures, while cultures containing 25 µg/ml or more produced no detectable DNAase (Control experiments showed that cerulenin did not affect DNAase activity,

FIGURE 5.11

Total extracellular protein production in the presence of cerulenin.

Total extracellular protein production in the presence of cerulenin was measured by following the incorporation of ^{14}C-amino acids into the TCA precipitable supernatant of cultures of S2, as a fraction of the optical density of the culture, as described in Section 5.3.2.2.

□ = 0 µg/ml cerulenin

○ = 2 µg/ml cerulenin

■ = 10 & 25 µg/ml cerulenin

△ = 50 µg/ml cerulenin

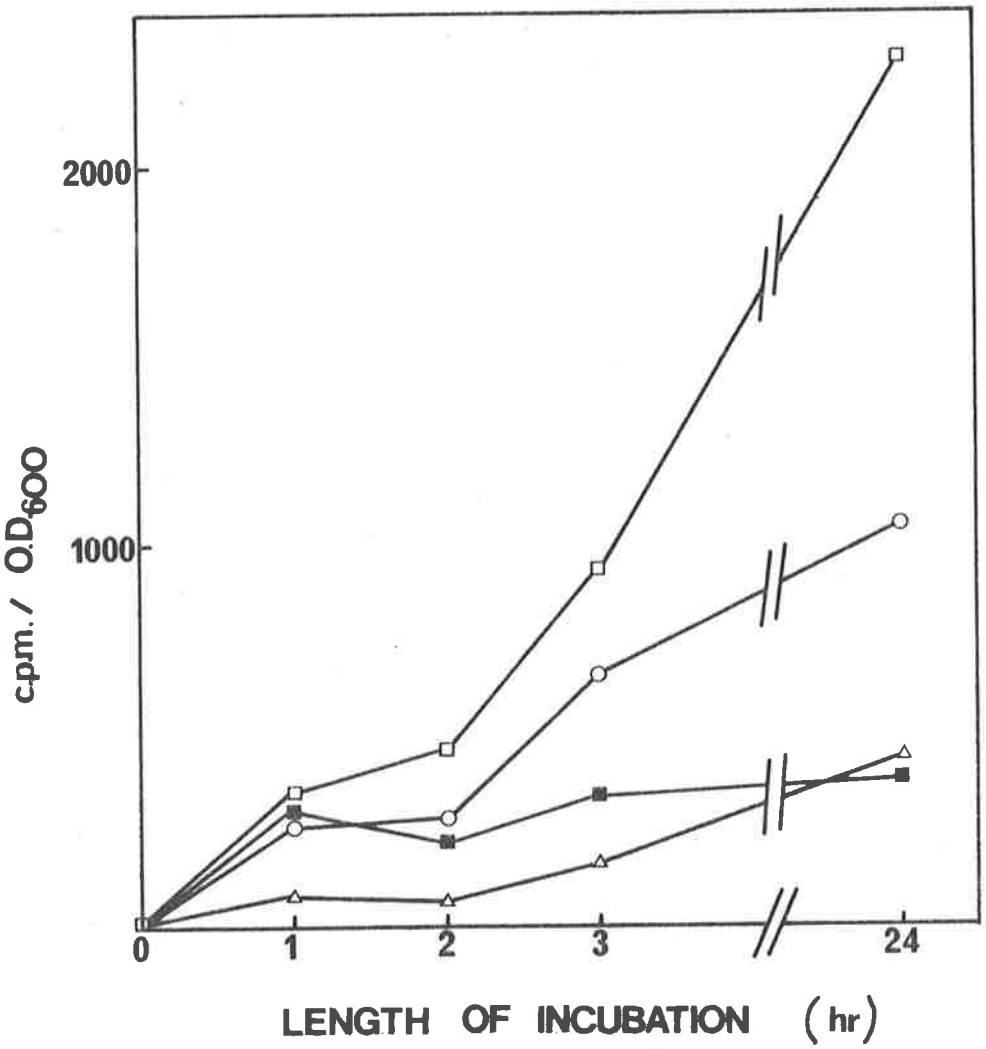

2000
1000
c.p.m. / O.D600
0
1
2
3
24
LENGTH OF INCUBATION (hr)

FIGURE 5.12

Production of extracellular DNAase in the presence of cerulenin

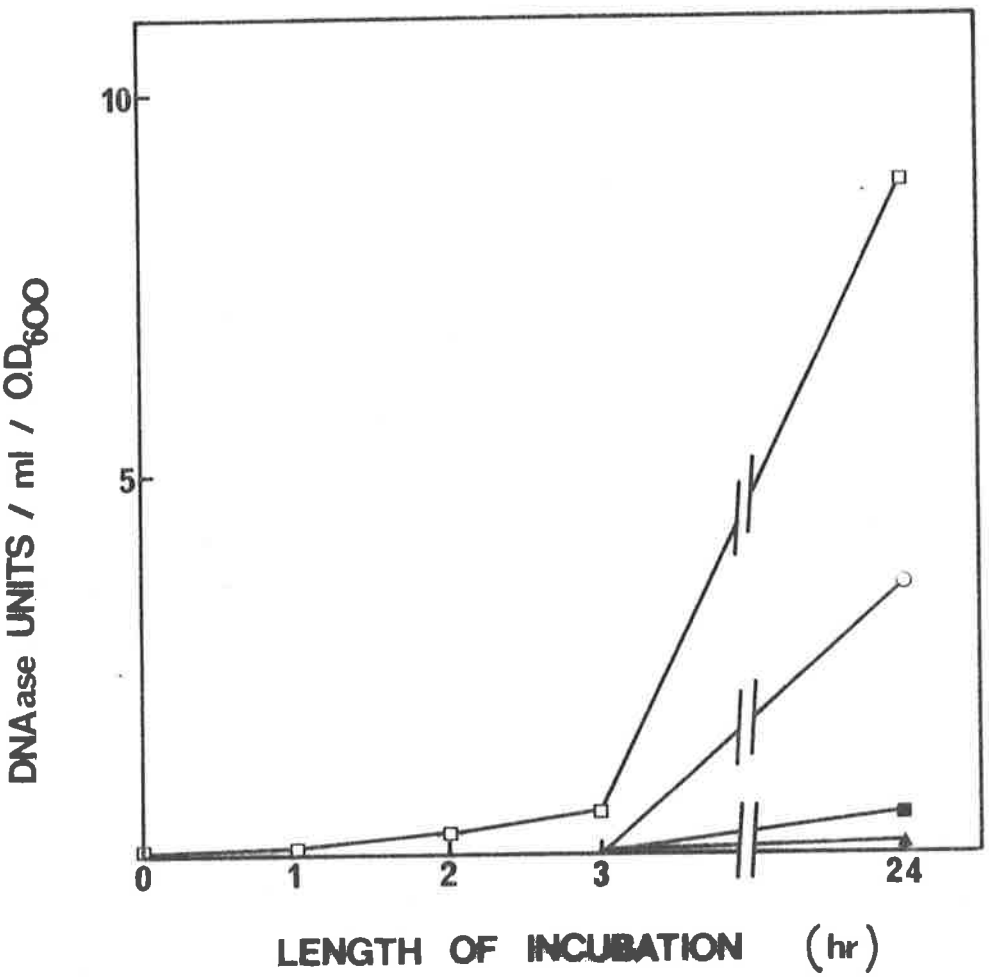
Extracellular DNAase production was measured on the supernatant of cultures of S2 exposed to various concentrations of cerulenin for differing times, and expressed as a fraction of the optical density of the culture, as described in Section 5.3.2.3.

□ = 0 µg/ml cerulenin

○ = 2 µg/ml cerulenin

■ = 10 µg/ml cerulenin

▲ = 25 µg/ml cerulenin


10
5
0
1
2
3
24
DNAase UNITS / ml / O.D600
LENGTH OF INCUBATION (hr)

nor did ethanol, at the concentrations used, affect DNAase production or activity).

These results are very similar to those of Altenbern (1977) and Berkeley *et al.* (1978), in that subinhibitory concentrations of cerulenin, such as 2 - 10 µg/ml, specifically depress extracellular protein synthesis.

Why then is penicillinase such an obvious exception?

5.3.2.4 Effect of cerulenin on inducible penicillinase

Altenbern (1977) found that while 4 µg/ml cerulenin inhibited the formation of enterotoxins B and C, enterotoxin A was not greatly affected by the antibiotic. In contrast, Berkeley *et al.* (1978) showed that enterotoxin A production was inhibited by low levels of cerulenin (2.5 µg/ml). The difference between their results could be explained by the fact that Altenbern used a mutant strain of *S.aureus*, which produced extremely high levels of enterotoxin A, whereas Berkeley *et al.* used a "wild-type strain". So there is a possibility that the inducibility or otherwise of a particular enzyme may influence its susceptibility to cerulenin.

Figures 5.10 and 5.11 of this section were derived from a strain of *S.aureus* carrying the plasmid pI258*bla*i443, a macroconstitutive mutant of the penicillinase repressor gene (Novick, 1963). It was considered important to see whether *S.aureus* carrying the wild-type penicillinase operon (i.e. the inducible enzyme on pI258) was just as insensitive to cerulenin.

Therefore, penicillinase in cultures of S112 in CYP broth were induced with 0.5 μg/ml methicillin, grown to OD_{600} = 0.8; cells were washed and resuspended in fresh CYP + methicillin plus various concentrations of cerulenin. Supernatant penicillinase was assayed as before, and the results shown in figure 5.13.

From this figure, it can be seen that even low levels of cerulenin caused significant inhibition of extracellular penicillinase within the first two hours of the experiment. This contrasts dramatically with the effect of cerulenin on constitutive penicillinase production, in figure 5.10, but is more compatible with the data on total extracellular protein, and DNAase production, shown in

FIGURE 5.13

Synthesis of induced penicillinase in the presence of cerulenin

Fully induced cultures of S112 were washed and exposed to varying concentrations of cerulenin. The culture supernatant was assayed for penicillinase activity, expressed as activity/OD_{600}, as described in Section 5.3.2.4.

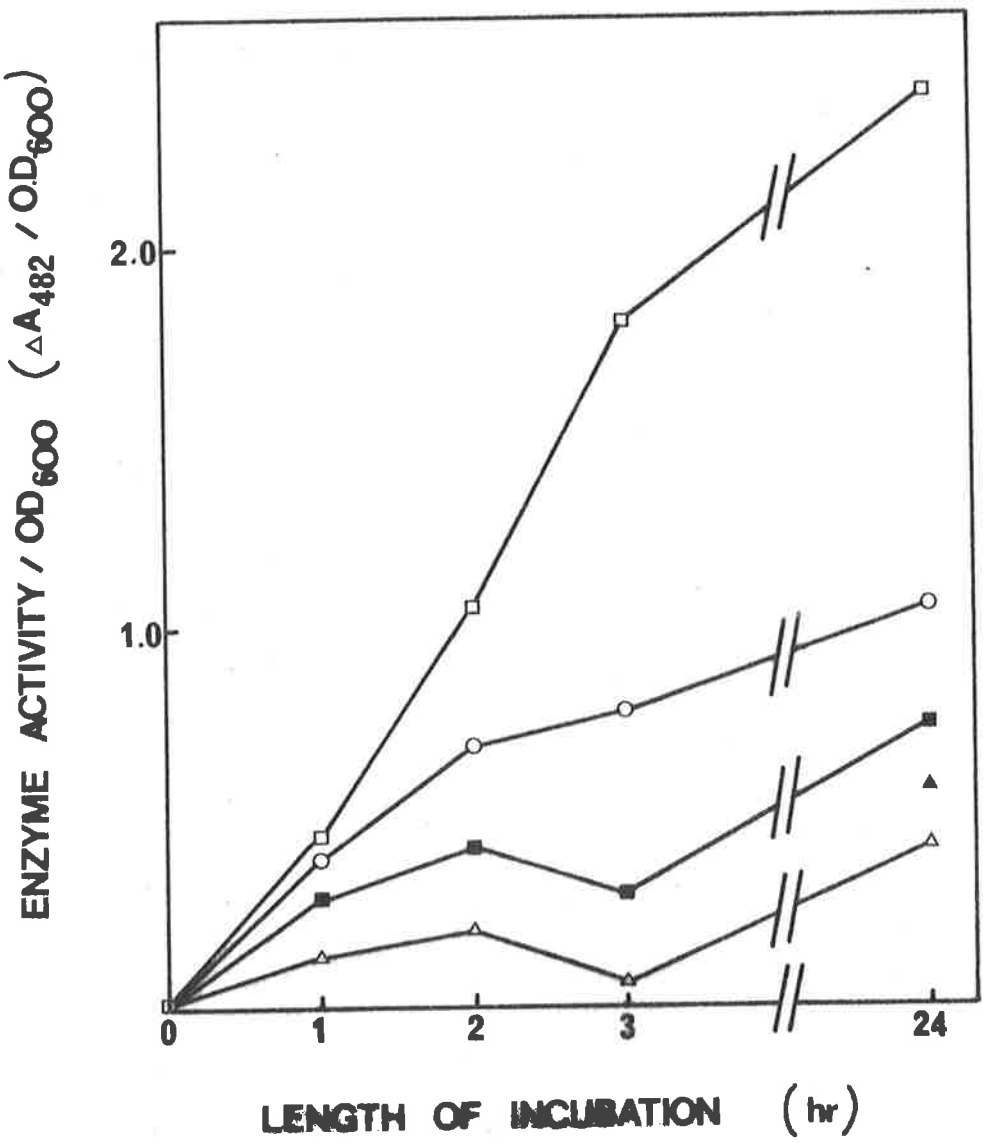
□ = 0 µg/ml cerulenin

○ = 2 µg/ml cerulenin

■ = 10 µg/ml cerulenin

▲ = 25 µg/ml cerulenin

△ = 50 µg/ml cerulenin


ENZYME ACTIVITY / OD600 (ΔA482 / O.D600)
2.0
1.0
0
1
2
3
24
LENGTH OF INCUBATION (hr)

figures 5.11 and 5.12. This raises the possibility that cerulenin is acting, not at the level of synthesis or secretion of extracellular proteins, as is predicted by its mode of action (e.g. Fishman, Rottem and Citri, 1978; Berkeley *et al.*, 1978), but on, for example, the control of enzyme production, such as its inducibility. This possibility will be discussed later.

5.3.2.5 Is pII147 penicillinase similarly affected by cerulenin?

The data on the pI258 penicillinase suggest that constitutive enzyme production is far less sensitive to cerulenin inhibition than inducible enzyme production. To further check this possibility, the effect of cerulenin on the production of penicillinase C, the genes of which are carried on the plasmid pII147, was measured in strains S150 (carrying the wild type, inducible penicillinase) and S151, carrying the constitutive operon - pII147*bla*i220 (Novick, 1965).

Figure 5.14 shows that extracellular penicillinase C production is disrupted in a way identical to that of penicillinase A. The constitutive penicillinase of strain S151

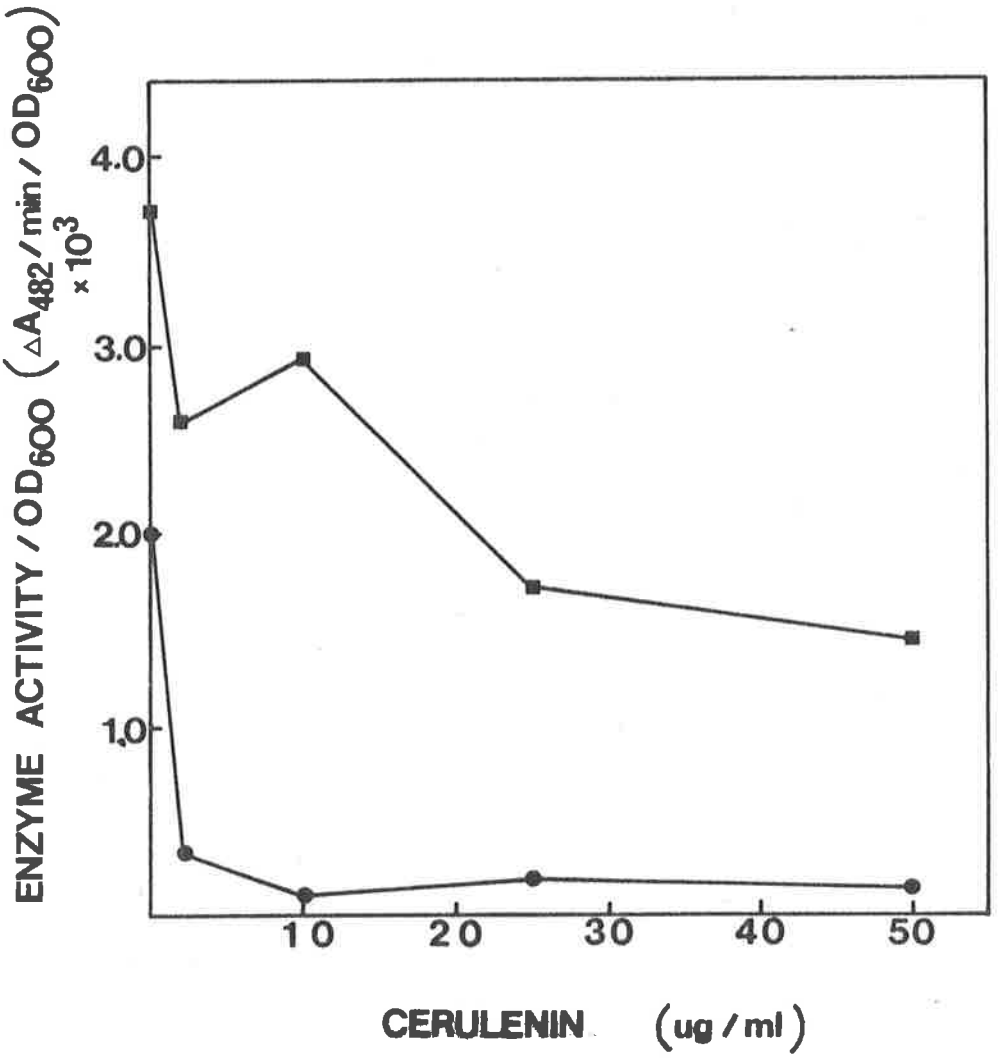
FIGURE 5.14

Effect of cerulenin on the penicillinase of pII147

Cultures of S150 (fully induced with methicillin) and S151, were washed and grown in the presence of cerulenin for 24 hr. The supernatant was assayed for enzyme activity (expressed here as activity per $O.D._{600}$) as described in Section 5.3.2.5.

● = S150 penicillinase

■ = S151 penicillinase


ENZYME ACTIVITY / OD600 (ΔA482 / min / OD600) x 10^3
4.0
3.0
2.0
1.0
10
20
30
40
50
CERULENIN (ug / ml)

is produced at about 40% of controls in the presence of 50 µg/ml cerulenin, while 2 µg/ml cerulenin inhibits inducible enzymes by 85%.

Thus for both enzymes, cerulenin appears to act, not by interfering with synthesis or secretion of penicillinase, but, in some way, on the control of its induction.

5.3.2.6 Does cerulenin affect release of membrane-associated penicillinase?

It is possible that, rather than inhibiting synthesis of an extracellular protein, cerulenin inhibits its release from the cell membrane. Table 5.1 compares the relative percentages of penicillinase that is found in the supernatants of 24 hour cultures of S1-1, S112, S150 and S151, and it can be seen that penicillinase release is not significantly affected by the antibiotic. That is, enzyme that is synthesized, and incorporated into the membrane is then being secreted normally, so when cerulenin is inhibiting the appearance of supernatant penicillinase, it is doing so at a stage earlier than at the putative protease-release mechanism. A similar result was

TABLE 5.1

Percentage of total penicillinase that is extracellular

Cultures of S111, S112, S150 and S151 were grown in the presence of cerulenin, and methicillin (0.5µg/ml) for 24 hr, then penicillinase activity determined for the culture supernatant and supernatant plus cells.

% extracellularity is expressed as

$$\frac{\text{enzyme activity of supernatant}}{\text{total enzyme activity}} \times 100$$

% extracellular

plasmid	pI258*bla*i443	pI258	pII147*bla*i220	pIII147
cerulenin (µg/ml)				
0	74	41	1.8	2.1
2	85	42	1.6	2.8
10	78	46	1.9	1.4
25	80	44	2.8	2.6
50	62	46	1.8	2.3
125	60	41	1.2	1.6

noticed by Fishman, Rottem and Citri (1978) with *Bacillus licheniformis* penicillinase.

5.3.3 Hybridization of staphylococcal RNA from cerulenin-treated cells.

Other workers have postulated that cerulenin specifically interferes with extracellular protein synthesis by inhibiting phospholipid synthesis and so, either by restricting attachment of phospholipid to the protein leader sequence, or by interfering with the cell membrane structure. However the evidence presented in this chapter suggests that there may be some other mode of action, as the effects of cerulenin are different on inducible and non-inducible penicillinase. As inducible penicillinase is not produced in the presence of cerulenin (section 5.3.2.4) it is possible that the drug interferes with the induction process. To test this possibility, the effects of cerulenin on the synthesis of penicillinase mRNA were examined by DNA-RNA hybridization studies. Using the recombinant plasmid pPG122 (described in Chapter 6), which contains a Hind III fragment of p1258*bla*i443 that spans the penicillinase gene, the relative amounts of penicillinase mRNA in induced S112 were determined after various exposures to cerulenin.

This section describes the isolation of the labelled staphylococcal mRNA, and hybridization of this to pPG122 DNA.

5.3.3.1 Isolation of labelled RNA.

Using the procedure described in section 5.2.5, RNA was prepared from induced and uninduced cultures of S112, after 0, 1, 2 and 3 hours' exposure to cerulenin (0, 10 or 50 µg/ml).

Table 5.2 shows the total yields of RNA (in counts per minute) from 5 ml of culture pulse-labelled for 2 minutes with 10 µCi/ml of ^{3}H-uridine. Yields of RNA are greatest in 1 and 2 hour cultures, when cells are in log-phase, and at relatively high densities, while RNA synthesis drops at 3 hours, when the cells are entering late-log to stationary phase.

5.3.2.2 Hybridization to pPG122.

To determine the amount of DNA necessary to give complete hybridization of a given amount of RNA, filters were loaded with varying amounts of linearized denatured pPG122 DNA, and probed with a constant amount of RNA (2×10^{5} cpm). The RNA used was from induced S112, grown in the absence of cerulenin

TABLE 5.2

Total counts of ^{3}H-uridine incorporated into 100µℓRNA, prepared from 10 ml of treated cells

Figures represent counts per minute ($\times 10^{-5}$) determined by counting 1µℓ samples of the final RNA preparation.

inducer	cerulenin	length of exposure to cerulenin (hr)		
		1	2	3
-	0	5.8	6.8	2.9
	10	4.4	4.8	2.8
	50	5.6	4.2	1.7
+	0	6.8	6.7	2.9
	10	10.9	8.0	4.3
	50	7.4	3.2	1.8

("1 hour" in table 5.2). As shown in figure 5.15, maximum hybridizable counts were achieved when 4 - 5 µg of DNA was used per filter. All future hybridizations were conducted using 5 µg DNA per filter. As 2×10^5 cpm of RNA was probed, the maximum hybridizable counts, 216 cpm, represents about 0.1% of the newly synthesized RNA. The validity of this figure will be discussed later in this chapter.

5.3.3.3 Hybridization of RNA from cerulenin-treated cultures

2×10^5 cpm of RNA from cells exposed to cerulenin (10 or 50 µg/ml) for between 0 - 3 hours was hybridized to 5 µg of pPG122 DNA, as described. As controls, filters were also probed with RNA from uninduced cultures, incubated for identical times with or without cerulenin. All test hybridizations were performed in duplicate, and the values from the corresponding control (representing non-specific binding of RNA to the filters, or to pMB9 DNA) substracted from the average.

From figure 5.16, it appears that cerulenin does have some effect on the synthesis of penicillinase mRNA. RNA prepared from both 10 and 50 µg/ml cerulenin cultures contained

FIGURE 5.15

Hybridization of ^{3}H-mRNA to varying amounts of PG122 DNA

0 - 6 µg of pPG122 DNA was bound to nitrocellulose filters, and probed with a constant amount (2×10^{5} cpm) of ^{3}H-RNA prepared from a penicillinase-induced culture of S112, as described in Section 5.3.3.1.

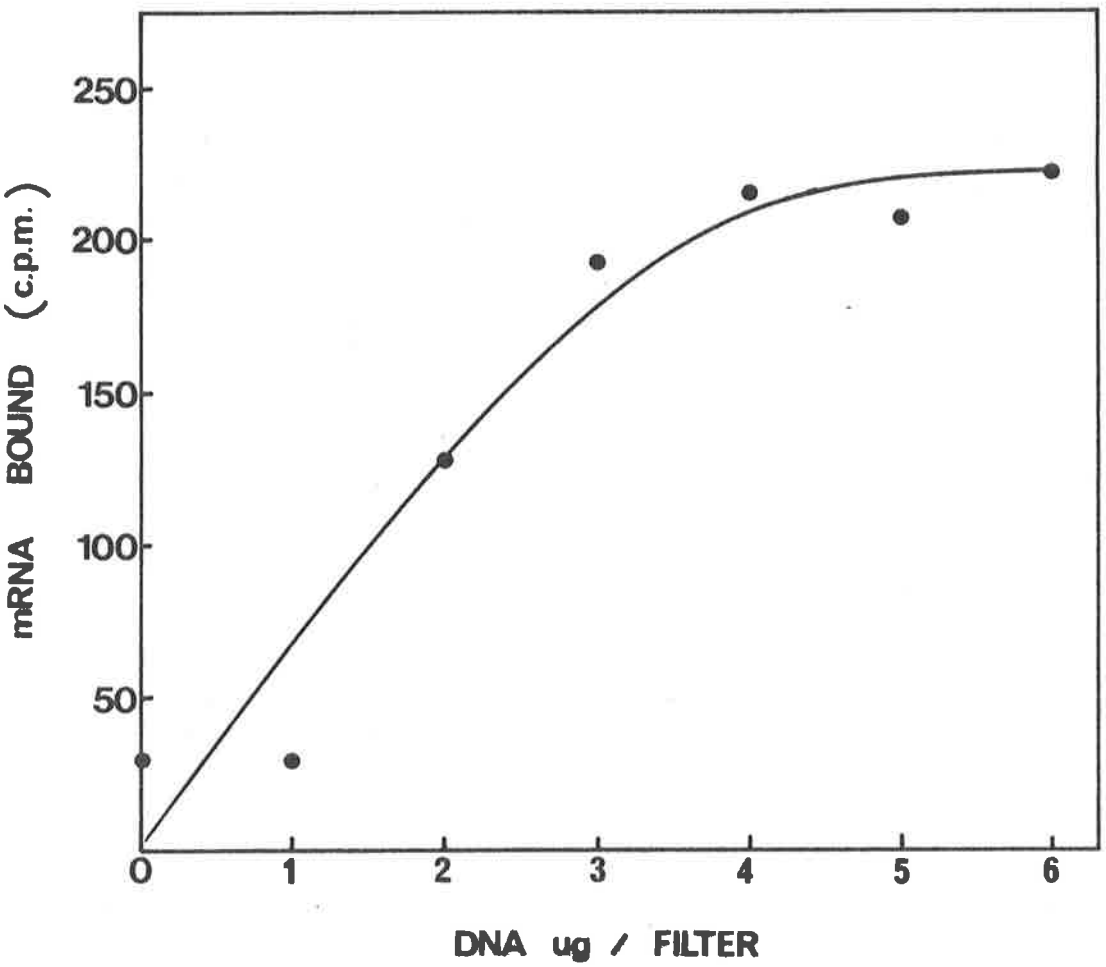

250
200
150
100
50
0
1
2
3
4
5
6
mRNA BOUND (c.p.m.)
DNA ug / FILTER

FIGURE 5.16

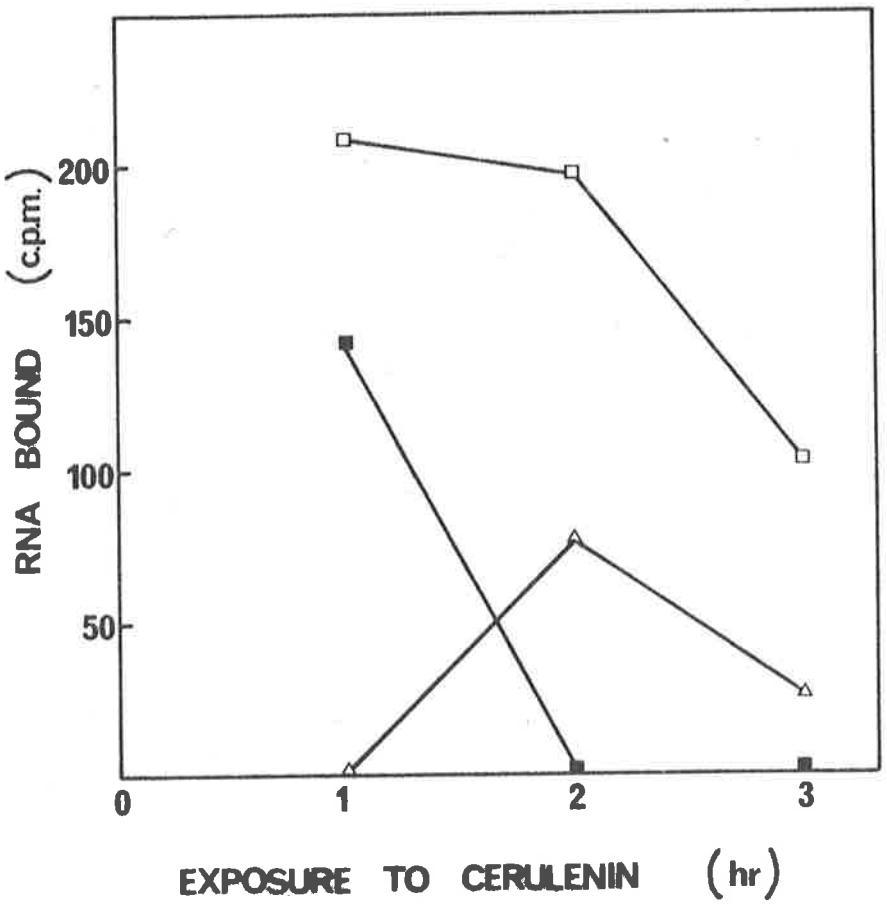
Hybridization of mRNA to pPG122.

^{3}H-RNA (2×10^{5} cpm) from various cultures of S112, induced with methicillin and exposed to cerulenin, was hybridized to 5 µg per filter of pPG122, to determine the fraction of counts belonging to penicillinase mRNA, as described in Section 5.3.3.3.

□ = 0 µg/ml cerulenin

■ = 10 µg/ml cerulenin

△ = 50 µg/ml cerulenin


RNA BOUND (c.p.m.)
200
150
100
50
0
1
2
3
EXPOSURE TO CERULENIN (hr)

less hybridizable counts, at all times examined. This supports the idea that cerulenin is acting not simply by retarding translation or secretion of penicillinase, but by selectively depressing the synthesis of the mRNA.

These results are not unequivocal. The drop in hybridizable counts from the three-hour control culture does not agree with the appearance of the enzyme (figure 5.13) nor do the cerulenin-treated samples show any direct correlation with the appearance of enzymes in these cultures (figure 5.13). This is presumably due to the very low amount of hydridizable RNA present in the isolates. The maximum of 216 cpm RNA hydridized represents about 0.1% of the counts applied. Zousias *et al.* (1973) estimate that about 1% of the total cellular RNA is plasmid specific, in preparations from an strain of *S.aureus* carrying the pI258 penicillinase gene on a hybrid pI258-pII147 plasmid. As the penicillinase gene represents about 3.5% of the whole plasmid (about 1 kilobase/28 kilobases), then in uninduced cells, about 0.036% of RNA will be penicillinase mRNA. Thus a value of 0.1% is compatible with induced enzyme. This value does not take into account

an estimate of the efficiency of the hydridization procedure, which may be only 50% (Zouzias *et al.*, 1973). It would be informative to fully quantitate the hybridization procedure and results, but this was not considered necessary for this work.

However, the results in Figure 5.16 do suggest that cerulenin has caused depression of penicillinase mRNA production.

5.4 DISCUSSION

This chapter describes studies on the effects of disruption of lipid synthesis on the production of staphylococcal extracellular protein synthesis, using the antibiotic cerulenin, which specifically inhibits the synthesis of fatty acids, the precursors of lipids (Omura, 1976).

The work of Lampen (1978) has suggested that a phospholipid (phosphatidylserine) is attached to the amino-terminus of the *B.licheniformis* penicillinase precursor, and that plays a role in directing the precursor towards the membrane, where it is incorporated and/or processed to the extracellular form. Obviously, it is tempting to suggest that lipid metabolism will play an important role in the ability of a cell to produce extracellular proteins.

Inhibition of phospholipid synthesis by cerulenin has been shown to selectively inhibit the formation of extracellular protein in a number of bacterial systems, for example, the staphylococcal enterotoxins A (Berkeley *et al.*, 1978), B and C (Altenbern, 1977), *B.licheniformis* penicillinase (Fishman, Rottem and Citri, 1978) and *B.amyloliquefaciens* amylase and proteases (Paton, 1979), lending support to the theory that lipid synthesis and extracellular protein synthesis are intimately linked.

These results were partly confirmed by this work, but with some important exceptions. For comparison, important data from this chapter have been summarized in table 5.3.

The effects of cerulenin on the growth of *S.aureus* were first determined. In agreement with Altenbern (1977), it was found that the minimum inhibitory concentration of cerulenin was in the range of 25-50 µg/ml (figures 5.1 and 5.2), concentrations which also greatly inhibit fatty acid synthesis (figures 5.3 and 5.4). Although there is little fatty acid synthesis in cultures treated with 25 µg/ml cerulenin, their final growth density is not greatly affected, suggesting that cells can continue to grow in the absence of fatty acid synthesis. However, in these cultures, there is still some synthesis (or turnover) of lipids, as evidenced by the continuing incorporation of ^{14}C-glycerol into lipid (figures 5.5 and 5.6). This supports the results of Hsu and Fox (1970), that some turnover of phospholipids occurs in cells with disrupted lipid synthesis, and of Mindich (1971), that the polar region of phospholipids is turned over during glycerol deprivation of glycerol auxotrophs. Thus, at this level of cerulenin, the cell has no absolute requirement for fatty acid synthesis, either for growth or for lipid turnover.

TABLE 5.3

Comparison of effects of cerulenin on various cell functions

The figures are derived from graphs in this chapter. All are expressed as % of control, per OD_{600} unit, and are taken from 24 hour cultures.

(cerulenin) g/ml	cell growth	fatty acid synth.	lipid synth.	total protein synth.	exo-protein synth.	inducible penicillinase (supernatant)	constitutive penicillinase (supernatant)	DNAase (supernatant)
0	100	100	100	100	100	100	100	100
2	115	88	82	81	46	44	103	41
10	123	76	44	97	17	31	96	6
25	92	9	41	13	17	24	105	1
50	42	4	48	4	19	17	74	0
75	41	1.2	N.D.*	8	N.D.	10	77	0
125	27	0	N.D.	3	N.D.	5	70	0

* N.D. = not determined

Presumably, though, such cells would have significantly altered membrane lipid compositions reflecting the lack of a net increase in lipids.

At 25 µg/ml cerulenin, total protein synthesis over 24 hours is strongly inhibited (suggested by figure 5.7), despite the relatively normal OD_{600} of the culture. As shown in figure 5.11 there is little extracellular protein produced in these cultures; while this would account for about 30% of the missing counts (see Abbas-Ali and Coleman, 1977), the incorporated counts may be lowered by the ability of the cell to re-use amino acids from un-secreted, degraded proteins originally destined for export from the cell, so reducing the cell's need for exogenous amino acids.

For similar reasons, total RNA synthesis, as measured by pulse-labelling, may not exactly reflect the true levels of RNA synthesis, as discussed in section 5.3.1.5.

The synthesis of extracellular proteins is dramatically affected by sub-inhibitory concentrations of cerulenin, as shown in figures 5.11, 5.12 and 5.13. These results are in complete agreement, both empirically and quantitatively, with those of Altenbern (1977), Berkeley *et al.* (1978), and Fishman, Rottem and Citri (1978).

Unexpectedly, however, the synthesis of constitutive penicillinase was almost unaffected by cerulenin, so cells carrying this operon continue to produce significant amounts of enzyme in the absence of other extracellular protein production, and even in the absence of total protein and RNA synthesis and cell growth (e.g. at concentrations of 50 µg/ml cerulenin and greater). A similar situation occurred with the penicillinase of pII147 (figure 5.14). Combined with the observation of Altenbern (1977), wherein a high enterotoxin A producer was not susceptible to cerulenin, and the passing comment of Fishman, Rottem and Citri (1978) on *Bacillus* penicillinase production it is obvious that the simple explanation that lipid synthesis is necessary for protein secretion is inadequate.

Disruption of lipid synthesis could directly affect protein secretion in several ways:

(i) By interference with the attachment of a phospholipid to the amino-terminus of the protein precursor, as has been suggested by Yamamoto and Lampen (1975), for *Bacillus* penicillinase.

(ii) By disrupting the incorporation of the protein into the membrane, or its passage through the membrane. For example, inhibition of lipid synthesis in glycerol auxotrophs of *S.aureus*

causes changes in the membrane-associated lactose permease system (Mindich, 1971). Although the proteins were incorporated into the membrane in the absence of lipid synthesis, their enzymatic activity was reduced, suggesting that lipid synthesis was necessary for their full activity. Conceivably then, interaction of the extracellular protein precursor with the membrane could be affected by a lack of lipid synthesis, e.g. by altering the lipid content of the membrane, reducing their accessibility to the protease release mechanism, or by altering their intra-membrane conformation.

(iii) Similarly, by causing conformational changes to the membrane-associated ribosome-binding proteins. Such proteins exist in eukaryotes (Blobel and Dobberstein, 1975 a,b), but possibly not in prokaryotes (Smith, Tai and Davis, 1978). If they do exist, their ability to bind ribosomes could be impaired, similarly to the impairment of lactose permease activity (Mindich, 1971), so preventing correct synthesis of extracellular proteins.

(iv) Again, by disruption of the membrane associated protease which removes the leader peptide.

All of these possibilities can be criticised, partly on the results in this chapter.

A. The hybridization data presented in figure 5.16 suggests that there is some inhibition of mRNA synthesis in the presence of cerulenin. This argues against possibilities (i), (ii) and (iv), wherein cerulenin would act either co-translationally, or post-translationally. Although the hybridization data presented here is not unequivocal, due to the low levels of hybridization (a maximum of 0.1%), they do suggest strongly that penicillinase mRNA synthesis is inhibited by cerulenin in inducible strains of *S.aureus*.

B. Table 5.1 suggests that there is no accumulation of active membrane penicillinase in cerulenin treated cultures. This argues aginast possibility (iv) - although membrane penicillinase synthesized in the presence of cerulenin may not be enzymatically active (as for lactose permease synthesized in the absence of lipid synthesis (Mindich, 1971); this could best be investigated either by quantitating membrane penicillinase with anti-penicillinase antibodies, or by seeing if resumption of fatty acid synthesis leads to a rapid increase in membrane-penicillinase activity (as is the case for lactose permease; Mindich, 1971).

C. Table 5.3 shows that there is still considerable lipid turnover in cells not producing penicillinase,

contradicting possibility (i), and arguing against a direct link between lipid synthesis and protein secretion.

D. Even when lipid synthesis is disrupted (by high levels of cerulenin), constitutive strains continued to secrete newly synthesized penicillinase, again contradicting the direct link between the two.

The important point is that two completely independent constitutive penicillinases are resistant to cerulenin, whereas their inducible parents are as sensitive as other exo-proteins; enterotoxin A may show a similar reaction (compare Altenbern 1977 and Berkeley *et al.*, 1978). Both constitutive penicillinase mutations are in the repressor gene (Novick, 1965), so the structural genes, and hence the proteins, are probably unmutated, and not somehow intrinsically more resistant to lipid inhibition. It therefore seems likely that cerulenin is not acting directly on the protein, but on, for example, induction of the enzymes.

A hypothetical scheme is as follows: staphylococcal cell wall synthesis is brought about by membrane-associated enzymes (Archibald, 1972), some of which bind penicillinase (Blumberg and Strominger, 1974; Kozarich and Strominger, 1978). It is

possible that one of these is the penicillinase "antirepressor" protein, which, it is postulated (Imsande, 1978) inactivates the repressor gene when bound with penicillin. Mindich has shown (1971) that staphylococcal lactose permease is synthesized and incorporated into the membrane in the absence of lipid synthesis, but has little enzymatic activity, suggesting that it is not in the correct conformation, presumably through the altered lipid state of the membrane. Should the conformation of the antirepressor protein be altered by the lack of lipid synthesis, such that it can no longer bind penicillin (but may still be enzymatically active), then there can be no induction (or "derepression") of the penicillinase operon in Imsande's model (1978), or if already induced, progressive denaturation of the antirepressor would lead to progressive de-induction of the operon.

Such a theory agrees with the data presented here, and accommodates the difference between inducible and constitutive penicillinases (the latter need no antirepressor protein for expression), unlike theories which postulate that lipid synthesis has a direct effect on exoenzyme synthesis.

The genetics of other staphylococcal exo-proteins, and the mode of their induction are not well understood, which makes it difficult to examine their sensitivity to cerulenin. It is well known, however, that many are expressed co-ordinately (see Forsgren, *et al.*, 1971; van der Vijvers, Es-Boon and Michel, 1975 a,b), and usually in stationary phase (e.g. Jeljaszewicz, 1972). Ryden, Lindberg and Philipson (1973) studied protease-negative mutants of *S.aureaus*, which had concommitantly lost a number of other extracellular proteins, and the ability to take up many carbohydrates (a membrane-associated function). As revertants to wild type carbohydrate utilization also regain extracellular enzyme function, they suggested that there is a single locus controlling enzyme secretion and carbohydrate uptake. Further, Uduo and Ichikawa (1978) have shown that acetylmethylcarbinol, an end-product of pyruvate metabolism in the presence of high sugar levels, can inhibit formation of α-toxin and nuclease, suggesting, for example that suppression of exo-enzyme production can be achieved by sugar levels (conversely, could low sugar levels lead to induction of extracellular enzyme production?). It is possible,then that DNAase, for example, is induced not by a lack of available nucleotides, but by the presence of some unknown substance in the culture medium (e.g. a concentration of some

cell waste product) which co-ordinately induces a number of extracellular proteins. Thus cerulenin could act in preventing induction of these proteins in an analogous manner to that described above, i.e. by disruption of the cell's method of recognizing an inducer.

Such a theory is hypothetical and merely reflects the evidence that lipid synthesis *per se* does not directly influence extracellular protein synthesis, but rather could act by interfering with the cells' ability to recognize the need for extracellular protein synthesis. Neither is the theory necessarily meant to be universal, but it does seem applicable to staphylococcal penicillinase and possibly for Altenbern's (1977) results with enterotoxin A.

It is also worth noting that other antibiotics have differential effects on extracellular protein synthesis, despite not affecting lipid production. Gemmell and Shibl (1975) have shown that sub-inhibitory concentrations of lincomycin inhibit staphylococcal coagulase, α-toxin, lipase and formation, and Glenn (1976) discusses a number of examples from other bacterial species.

A recent paper (Michel, Stessman and Stessman, 1980) has similarly shown that subinhibitory

concentrations of erythromycin clindmycin and pristinamycin can depress the appearance of extracellular penicillinase in some strains of *S.aureus*, although having no effect, or raising the level, in other strains. This is despite the fact that all three antibiotics have similar modes of action on the 50S ribosomal subunit. The reason for these sensitivities is unknown. Thus despite the attractiveness of associating lipid synthesis and exo-protein production, results from cerulenin studies may be partly artifactual.

Similarly, it has recently been shown (Fishman, Rottem and Citri, 1980) that a number of membrane-mofidying chemicals can preferentially suppress exoenzyme formation. As cerulenin is a fatty acid analogue, (Omura, 1976), it may be acting by direct disruption of the membrane, rather than by its effect on lipid synthesis. This would again necessitate a hypothesis such as the one presented to explain the difference between inducible and constitutive penicillinases.

CHAPTER SIX

INHIBITION OF LIPID SYNTHESIS AND ITS EFFECT ON PENICILLINASE SYNTHESIS

6.1 INTRODUCTION

The results in Chapter 5 suggest that inhibition of extracellular protein synthesis by cerulenin may be, at least partly artifactual. To investigate further the role of lipid synthesis in extracellular protein synthesis, it was decided to study the production of penicillinase in a host genetically deficient in lipid synthesis, and to compare enzyme production under these conditions to its production in the presence of cerulenin.

The simplest lipid synthesis mutants to isolate are glycerol auxotrophs. Glycerol is the precursor of L-α-phosphatidic acid, itself the precursor of phospholipids. Mutants unable to synthesize glycerol are thus dependent on an exogenous source of glycerol for lipid synthesis. Such mutants have been used to study the production of membrane-associated and extracellular proteins in several bacterial species (Hsu and Fox, 1970; Mindich, 1970, 1971; Beacham, Taylor and Youell, 1976).

It was therefore decided to isolate a glycerol-requiring strain of *S.aureus* S2, and use it as a host for penicillinase plasmids. When this was unsuccessful, a glycerol-dependent mutant of *E.coli* was isolated, and a recombinant plasmid, containing the *bla* operon

of pI258*bla*i443 was inserted, so that some information could be gained about the effect of disruption of lipid synthesis on penicillinase production. This plasmid (designated pPG122) is an insertion of a Hind III fragment of the staphylococcal plasmid into the single HindIII site in the *tet* gene of the vector pMB9 (Maniatis *et al.*, 1976). It was isolated as one of a number of recombinants containing various staphylococcal *bla* DNA fragments, for use in sequence analysis of the DNA coding for the leader peptide of the protein precursor. The aim of this is to compare the leaders of penicillinases with different degrees of extracellularity (e.g. the enzymes of pI258 and pII147), to determine the effect, if any, that this sequence has on the efficiency of processing. This work is underway, but is not reported here.

A supplementary experiment was suggested by the results of Chapter 4, wherein it has been shown that penicillinase is produced *in vitro* as the mature protein. This implies that the enzyme precursor is being processed co-translationally. The question can then be asked: is there lipid synthesis in the *in vitro* system? The answer to this question would offer useful information in determining the necessity for lipids in extracellular protein synthesis.

NOTE:

As *S.aureus* is a pathogen, all recombinant DNA experiments were performed under C-O laboratory conditions, as specified by the Adelaide University Biohazards Committee, and the Australian Academy of Science regulations.

The work was approved by these bodies prior to its commencement, and the experimenter was licensed by them for this work.

Staphylococcal DNA was purified from strain S138(pI258*blai*443), as this strain produces no enterotoxins (see Chapter 2). The plasmid was transduced, as described in Chapter 2, into S138, from S111, using ϕ11 bacteriophage, and the purified strain was checked for enterotoxin production by the Microbiology Section of Kraft Foods, Victoria. S138 (pI258*blai*443) produced no enterotoxins, thus implying that the plasmid carried no expressable enterotoxin genes.

6.2 MATERIALS AND METHODS

6.2.1 Restriction endonucleases

The following restriction endonucleases were used in this work:

Bgl II

Xba I

Hind III

All enzymes were purchased from Biolabs (Massechussetts, U.S.A.), and were stored at -15^{o} in the storage buffers recommended by the manufacturers, at a concentration of 0.25 units/µl.

6.2.2 Digestion of DNA with restriction enzymes

For analytical purposes, 1 µg DNA was digested in a minimal volume containing 0.25 units of restriction enzyme. A typical digestion consisted of:

	µl
1µg DNA	20
10 x enzyme buffer	3
H_2O	6
Restriction enzyme	1
	30

Restriction was for 1 hour at 37^{o}. The enzyme buffers used were those recommended by Biolabs, with the exception that NaCl was omitted from the Xba buffer, as this was found to increase the efficiency of digestion.

At the completion of the incubation, protein was removed from the mix by extraction with an equal volume of cold, TE-saturated phenol, followed by an extraction with ether.

For gel electrophoresis, the DNA was then mixed with loading buffer (as described later), and loaded onto the gel. For ligation, DNA was precipitated with ethanol (2 volumes), at a NaCl concentration of 0.2M, pelleted, washed with cold ethanol, briefly dried and resuspended in TE at an approximate concentration of 1µg/10µl.

6.2.3 Ligation of restricted DNA

Ligations were performed at 10°C., for 18-24 hr.

	$\frac{1}{50}$
5 µg restricted donar DNA	
0.5 µg restricted vector DNA	5
10 x ligation cocktail	7
1 unit T4 ligase (Biolabs)	1
H_2O	$\frac{7}{70}$

10 x Ligation cocktail:

0.1M $MgCl_2$

0.66 M tris-HCl pH 7.5

1mM ATP

0.01M EDTA

0.4M NaCl

After ligation, the aliquots were diluted to concentrations of vector DNA of 0.1, 0.01 or 0.001 µg/100µl, in 0.1 M tris-HCl pH 7.5, and used for transformation as described later.

Ligation activity was monitored by electrophoresis of a small aliquot of the mix on 1% agarose gels, to observe the generation of high molecular weight DNA.

6.2.4 Transformation of plasmids

6.4.2.1 Growth of cells

50 ml of L-broth was inoculated with 0.5 ml of a fresh overnight culture of the recipient bacterial strain (E392 for recombinant plasmid isolation, E234 for the GLY^- studies described later in this chapter), and grown with vigorous shaking at 37^{o}, to an OD_{600} = 0.8.

The culture was chilled on ice, and the cells pelleted by centrifugation, washed with 25 ml of ice-cold 0.1M $MgCl_2$, and resuspended in 2.5 ml of ice-cold 0.1M $CaCl_2$.
The suspension was kept on ice for at least 30' to achieve competence.

6.2.4.2 Transformation and selection of recombinants.

0.2 ml of competent cells were added to 0.1 ml DNA in 0.1 M tris-HCl, pH 7.5. For plasmids untreated with restriction enzymes, 0.001 µg of DNA was used, while for restricted and/or ligated DNA, a range of dilutions was used (0.1, 0.01 and 0.001 µg vector DNA). The mix was chilled for 30', heat-treated as 42° for 2' and chilled for a further 30'.

After warming to room temperature, 0.3 ml of L-broth was added, and the mix incubated at 37° for 30', to allow the establishment of the plasmids. The mix was then divided into three 0.2 ml aliquots. To each was added 3 ml of molten 0.7% agar, in L-broth, which was then poured over an agar plate containing L agar, plus the appropriate selective agent. For selection of recombinant plasmids, ampicillin (25 µg/ml) was used, while for selection of vector plasmids, tetracycline at 20 µg/ml was used.

Solidified plates were incubated at 37° for 24-48 hr. Potential transformants were removed, and colonies purified by three successive single colony isolations.

6.2.5 Mini-screening for potential recombinant plasmids

Ampicillin-resistant colonies isolated from the transformation mixes were examined for recombinant plasmids by partial purification of the plasmid.

Cells from a 1.5 ml overnight culture in L + ampicillin broth were pelleted, washed and resuspended in 150 µl of:

15% sucrose

50 mM tris-HCl pH 9.0

50 mM EDTA

50 µl of lysozyme (4 mg/ml in the same buffer) was added and the cells incubated for 15' at room temperature, and 30' at 0°.

200µl of ice-cold H_2O was added, and the mix heated up to 72° for 15', to lyse cells.

Debris was pelleted at 18,000 rpm in a Beckman JA 21 rotor, for 30' at 4°. Supernatant was removed, extracted three times with cold, TE-saturated phenol, and once with ether. Sodium chloride was added to 0.2 M, and DNA ethanol-precipitated.

DNA was ethanol-washed, dried and resuspended in 50 µl H_2O and an aliquot digested with restriction

enzyme and examined by agarose gel electrophoresis. Cells containing the desired plasmid were then used for large-scale plasmid preparation, as described in Chapter 2.

6.2.6 Agarose gel electrophoresis

DNA was analysed on vertical 1% agarose gels, prepared by dissolving 0.64 gm of agarose (Sigma type I) in 80 ml of Gel Electrophoresis buffer:

40 mM tris-acetate pH 8.2
20 mM sodium acetate
1 mM EDTA

The molten agarose was poured between two scratched glass plates (14 x 14 cm), separated by two 0.2 cm thick perspex spacers. A comb containing ten one-centimetre teeth was inserted into the open top of the gel to form wells in the molten agarose. After solidification at room temperature, the comb was carefully removed and the gel fitted to the electrophoresis apparatus. The wells and the reservoirs were filled with gel electrophoresis buffer.

DNA samples (usually about 1 µg DNA in 30 µl) were mixed with 8 µl of 5 x Loading Buffer; (0.005% bromophenol blue, 50% glycerol, 5 x electrophoresis buffer), and layered in the wells with a drawn capillary tube.

Electrophoresis was for about 5 hours at 30 mA, after which the gel was removed from the plates, and DNA stained with gel buffer containing 15 μg/ml ethidium bromide for 20'. After destaining for 20' in buffer, the DNA bands were visualized under UV light, and the gel photographed with Kodak Recording Film 2475, using a red filter.

6.2.7 Isolation of glycerol auxotrophs of E234

Glycerol-requiring mutants of E234 were isolated by nitrosoguanidine (NNG) mutagenesis, followed by penicillin enrichment.

6.2.7.1 Nitrosoguanidine mutagenesis

5 ml of L broth supplemented with 0.2% (v/v) glycerol was inoculated with 0.2 ml of an overnight culture of E234 and grown to OD_{600} = 0.8. Cells were pelleted by centrifugation, washed once with 0.1 M sodium citrate buffer (pH 5.5) and resuspended in 4 ml of that buffer. One ml of NNG (1 mg/ml, freshly prepared in citrate buffer) was added, and the culture incubated for 30' at 37^{o}.

Cells were pelleted and washed twice with citrate buffer then resuspended in 5 ml of fresh L+ glycerol broth. Dilutions plated

at this stage show that this procedure kills 99% of cells.

The culture was then grown overnight at 37° till cells had reached stationary phase.

6.2.7.2 Penicillin-enrichment for glycerol auxotrophs.

5 ml of fresh L + glycerol broth was inoculated with 0.2 ml of the overnight, mutagenized culture, and grown to OD_{600} = 0.8. Cells were washed twice with L broth (no glycerol), resuspended in 5 ml of L broth and incubated for 90' at 37°. This allows time for depletion of intracellular glycerol in auxotrophs (Beacham, Taylor and Youell, 1976).

Benzylpenicillin (1600 U/mg) was added to 1 mg/ml, and the culture incubated with gentle shaking at 37°. Lysis of the cells was monitored, and when completed (OD_{600} < 0.1), the surviving cells were harvested by Millipore filtration, washed on the filter to remove traces of penicillin, resuspended in 5 ml of fresh L + glycerol broth and grown to OD_{600} = 0.8 (4-5 hr. at 37°).

6.2.7.3 Isolation of auxotrophs

The penicillin-enriched culture was diluted 10^{-5} into TM, and 0.1 ml samples plated onto L + glycerol plates. After 24 hours at 37^{o}, the colonies were replicated, using velvet pads onto L + glycerol, and L plates.

After 48 hours' incubation, potential glycerol auxotrophs were identified by their inability to grow on L plates. The colonies were purified by three single-colony isolations on L + glycerol plates, and checked for their glycerol requirement.

Glycerol auxotrophs are identified in this work as GLY^{-}, as the precise loci involved were not determined.

6.2.8 Assay of penicillinase produced in *E.coli*

In gram-positive organisms, synthesis of extra-cellular proteins is easily measured, as they exist in the culture supernatant. In contrast, in gram-negative organisms extra-cellular proteins are located primarily in the periplasmic space. To quantitate such proteins, it is desirable to ensure that they are freely

accessible to the appropriate protein assay. In the case of staphylococcal penicillinase in *E.coli*, most enzyme activity is found in the periplasm, and can only be assayed by cephalosporin 87/312 in cells that have been protoplasted prior to the assay (results not shown).

To obtain protoplasts, 100 µl of cell culture was centrifuged and the cells resuspended in 100 µl of 0.01M tris-HCl, pH 8.0, 20% sucrose. To this was added 1 µl of 0.1 M EDTA and 1 µl of 10 mg/ml lysozyme. After incubation at 37^{o} for 15', the mix was assayed with 87/312 as previously described. Control incubations were always performed, to determine the background turbitity of the assay.

6.2.9 Incorporation of labelled chemicals into lipid materials.

The possibility that lipid synthesis occurs *in vitro* was investigated by measuring incorporation of various radioactively labelled chemicals into methanol-chloroform extracts of the system. The labels used were:

^{14}C-acetate, ^{14}C-glycerol, ^{14}C-serine,

at a final concentration of 1 µCi/100µl mix.

α-^{32}p-ATP (prepared by Dr. R. Symons, of this department), at a final concentration of 1 Ci/100 µl mix.

When used, all labels were dried under vacuum, then resuspended in 5 µl H_2O, before adding to the protein synthesizing mix.

Lipid material was extracted and assayed as described in Chapter 5.

6.3 RESULTS

6.3.1 Isolation of pMB9-pI258 recombinants

Cloning of Hind III fragments of pI258*bla*443 into pMB9 was carried out as described earlier in this chapter, and ampicillin-resistant transformants were isolated on L + ampicillin plates. From 0.1µg of ligated vector DNA was isolated 225 ampicillin resistant colonies of which all produced penicillinase when screened by the starch-iodine method described in Chapter 2.

After miniscreening of these isolates, to ensure that they carried the desired recombinant plasmid, one colony was purified and used as a source of plasmid DNA. This plasmid is designated pPG 122.

6.3.2 Identification of the components of pPG 122.

pPG 122 DNA, purified by caesium chloride-ethidium bromide centrifugation, was digested with the restriction enzymes Hind III and/or XbaI, and the fragments resolved by electrophoresis on 1% agarose gels, as shown in Figure 6.1.

FIGURE 6.1

Analysis of DNA restriction fragments on 1% agarose gels.

Restriction of DNA's, and gel electrophoresis was as described in the text.

Track

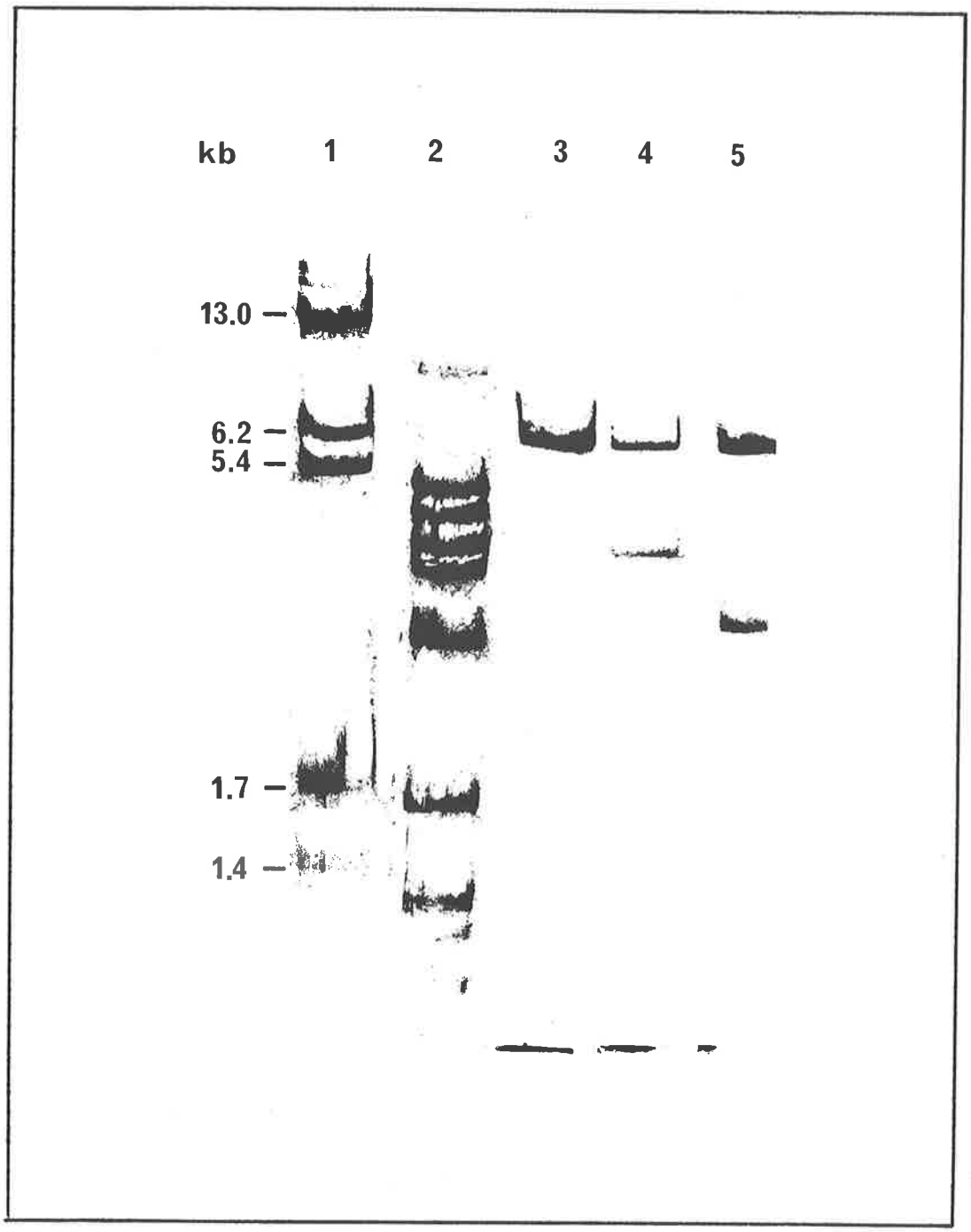
1 Bgl II digestion of pI258. Sizes of fragments are derived from Shalita, Murphy and Novick (1980)

2 Hind III digestion of pI258

3 Hind III digestion of pMB9

4 Hind III digestion of pPG122

5 XbaI/Hind III digestion of pPG122


kb
1
2
3
4
5
13.0
6.2
5.4
1.7
1.4

Track 1, of Figure 6.1 contains, as size markers, a BglII digestion of pI259 DNA. The visible bands have sizes of 13.0, 6.2, 5.4, 1.7 and 1.4 kilobase-pairs (kb) as determined by Shalita, Murphy and Novick (1980). The 0.5 kb fragment of the plasmid is not visible on this gel.

Using these standards, the sizes of the major Hind III bands of pI258 can be estimated (figure 6.2). Nine bands are visible, in track 2 of figure 6.1, and, from figure 6.2, have approximate sizes of:

Band	kb
A	5.0
B	4.5
C	3.8
D	3.5
E	2.7
F	1.7
G	1.3

The lowest two bands (H and I) cannot be sized accurately on this gel. The sum of these fragments is 22.5 kb, which compares with the known size of

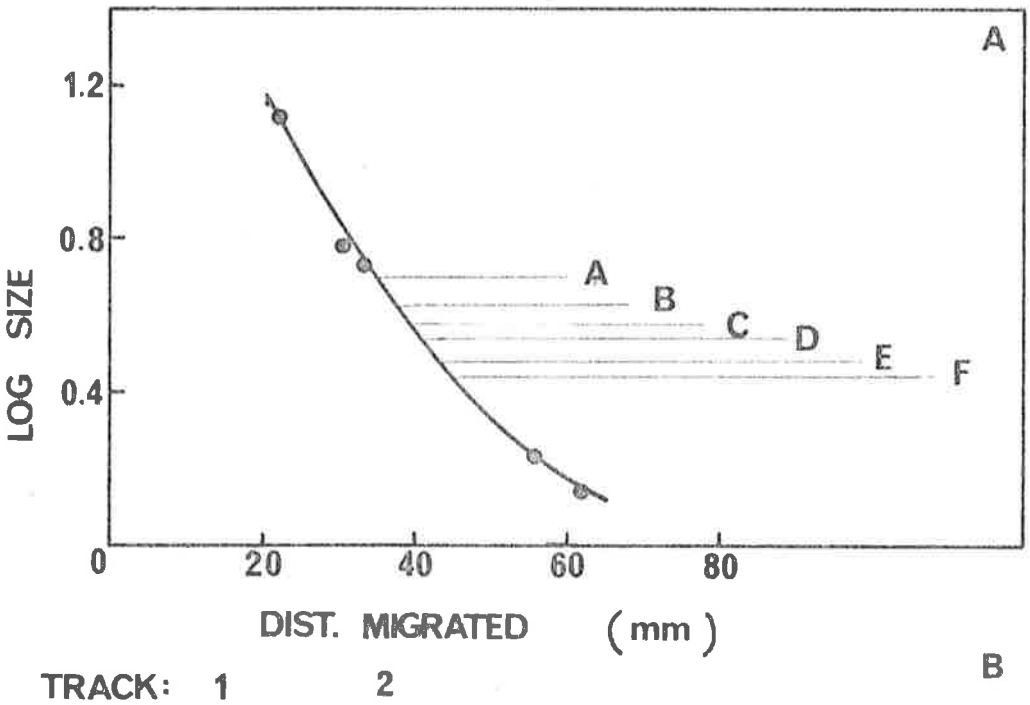
FIGURE 6.2

Sizes of Hind III restriction fragments of pI258

A. Using the Bgl II fragments of pI258 as size standards, the major fragments (designated A-I in order of decreasing size) has been sized as:

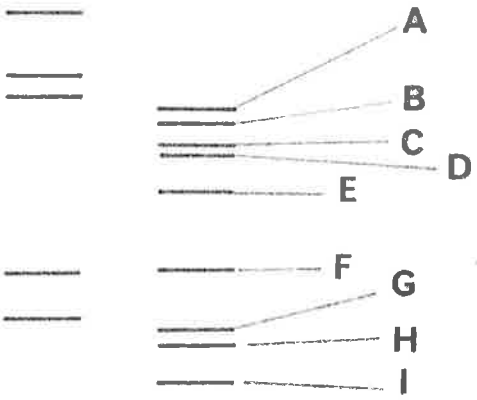
Band	Size (kb)
A	5.0
B	4.5
C	3.8
D	3.5
E	2.7
F	1.7
G	1.3
H	not determinable
I	not determinable

B. Pictorial representation of the Bgl II and Hind III restriction fragments of pI258, as determined from Figure 6.1.


A
1.2
0.8
0.4
0
LOG SIZE
A
B
C
D
E
F
20
40
60
80
DIST. MIGRATED (mm)

B

TRACK: 1 2


A
B
C
D
E
F
G
H
I

the pI258 plasmid (28.2 kb), as determined by Shalita, Murphy and Novick (1980), suggesting either that there are a number (totalling about 5.7 kb) of very small sized Hind III fragments not resolvable on this gel, or that at least one of the larger bands is in fact a doublet, although this is not apparent on this gel.

Track 3 carries a Hind III digestion of pMB9, yielding a single (linear) band of about 6.0 kb - slightly larger than the size quoted by Maniatis *et al.* (1976).

Track 4, of figure 6.1 shows the Hind III pattern of pPG122. Two bands are visible - one equivalent to linear pMB9 and the other equivalent to the Hind III C fragment of pI258 (3.8 kb). This implies that the *bla* determinant of pI258 resides on the 3.8 kb Hind III fragment. That this band carries the *bla* genes is further suggested by the fact that it carries a Xba site (track 5, figure 6.1), as it is known (Murphy and Novick, 1979) that there is a Xba site close to, or in, the *bla* gene of pI258. Digestion of pPG122 with Xba destroys the 3.8 kb Hind III band, yielding a 2.9 kb fragment, and, presumably, a 0.9 kb fragment, although this is not readily visible in figure 6.1. Similarly,

pSC113 (Chang and Cohen, 1974), which carries two Eco fragments of pI258, including the *bla* operon, also has a 3.8 kb Hind III fragment which is cleaved by Xba; this is the only Xba site on the pSC113 molecule (result not shown).

In summary, pPG122 contains a single Hind III fragment of pI258*bla*i443, almost certainly carrying the *bla* genes. The Hind III digestion pattern of pI258 is obviously fairly complex, and it is not possible to map the restriction sites accurately, especially as there are apparently a number of small fragments, less than 1 kb in size, not resolvable on 1% agarose gels. However, it is sufficient for this work, and probably also for DNA sequence analysis of the *bla* operon, to have a clone of the operon in a readily purifiable form.

6.3.3 Isolation of GLY$^-$ mutants of *S.aureus*

Ideally, investigation of the effect of inhibition of lipid synthesis on penicillinase production would be performed in the plasmid's natural host. However it was not possible to isolate glycerol auxotrophs of *S.aureus* using the technique described in this chapter. Approximately 60,000 colonies from mutagenized cultures of S2 were screened, but although several were isolated that showed a slight requirement for glycerol, none were absolutely

dependent on it. This is in contrast to Mindich (1971), who readily obtained glycerol auxotrophs of *S.aureus* using a similar technique. Several variations on the enrichment procedure were tried, such as the use of higher concentrations of penicillin or substitution of methicillin or cycloserine, however no GLY$^-$ mutants were isolated.

It was therefore decided to use the recombinant plasmid pPG 122, in a GLY$^-$ strain of *E.coli* to examine the role of lipid synthesis on penicillinase production.

6.3.4 Isolation of GLY$^-$ mutants of *E.coli*

Three glycerol-requiring mutants of E234 were isolated amongst approximately 4,000 colonies screened after penicillin enrichment. All three behaved identically with respect to their dependence of glycerol. One of these, designated E234GLY-1, was used in this work.

Figure 6.3 shows that E234GLY-1 is dependent on exogenous glycerol (0.2% v/v) for normal growth. Cultures of the mutant and its parent were grown in L $\pm$ glycerol to an OD_{600} = 0.8, cells pelleted and washed twice with L broth, and resuspended in L$^{\pm}$ glycerol. The optical density of the culture

was monitored, and it can be seen from figure 6.3 that while E234 is unaffected by glycerol, GLY-1 requires glycerol for normal growth. In the absence of glycerol, GLY-1 continues to grow for approximately one generation, after which growth ceases abruptly. This behaviour is similar to that of the glycerol auxotrophs of Beacham, Taylor and Youell (1976), and similar mutants found in *B.subtilis* (Mindich 1970) and suggest that GLY-1 is exhausting intracellular glycerol during the first hour of deprivation.

Total protein synthesis in these cells is not affected by glycerol deprivation, as shown in figure 6.4. This also agrees with the results of Beacham, Taylor and Youell (1976).

There is, however, an immediate drop in the incorporation of ^{14}C-acetate into lipid material in the glycerol-deprived culture (figure 6.5). Beacham, Taylor and Youell (1976) measured phospholipid by incorporation of ^{32}P into lipid, and found that there was an immediate inhibition in phospholipid synthesis on removal of glycerol. Similarly, the results in figure 6.5 suggest that in the absence of glycerol (i.e. lipid synthesis), there is also a depression of fatty acid synthesis, as directly measured by ^{14}C-acetate incorporation.

FIGURE 6.3

Growth of E234 GlY-1 and E234 in the presence or absence of glycerol

Log-phase cultures of E234 GLY-1 and E234 were grown in the presence of 0.2% glycerol; cells were washed and resuspended in fresh broth plus or minus glycerol. The cell density was measured at various times.

● ▲ : E234 GLY-1

○ △ : E234

▲ △ : + glycerol

● ○ : - glycerol

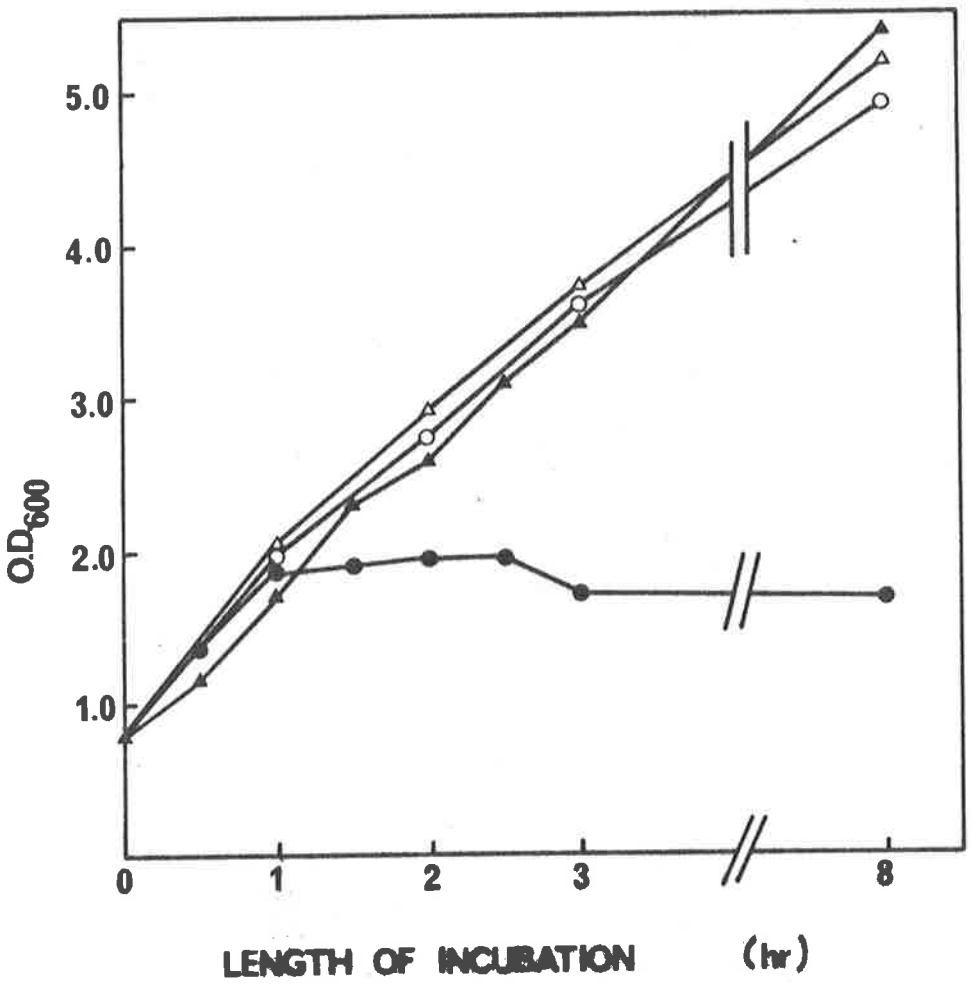

5.0
4.0
3.0
2.0
1.0
O.D600
0
1
2
3
8
LENGTH OF INCUBATION (hr)

FIGURE 6.4

Protein synthesis in E234 GLY-1

Incorporation of ^{14}C-amino acids (1 μCi/ml) into protein was measured in aliquots of E234 GLY-1 grown in the presence or absence of glycerol.

▲ : + glycerol

● : - glycerol

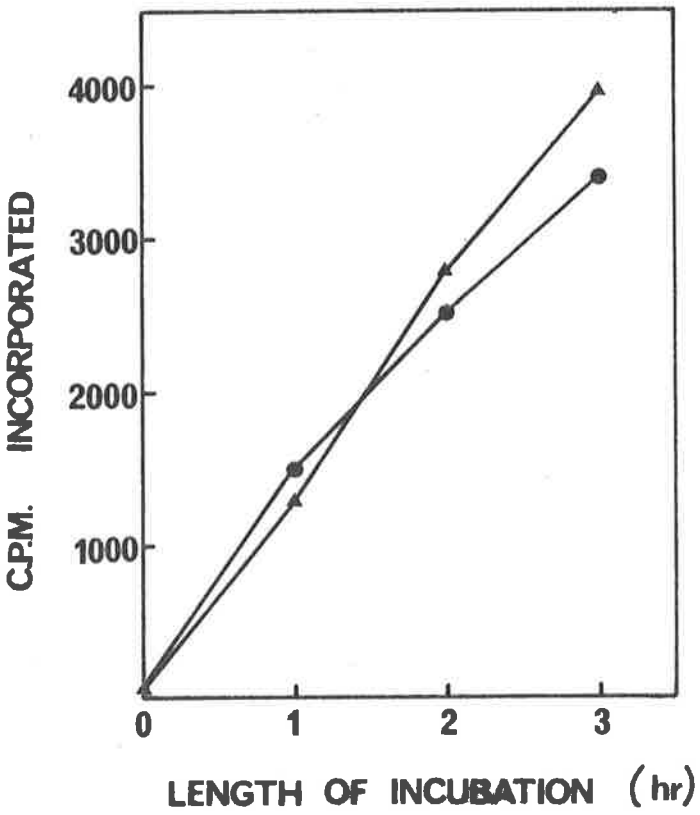

4000
3000
2000
1000
C.P.M. INCORPORATED
0
1
2
3
LENGTH OF INCUBATION (hr)

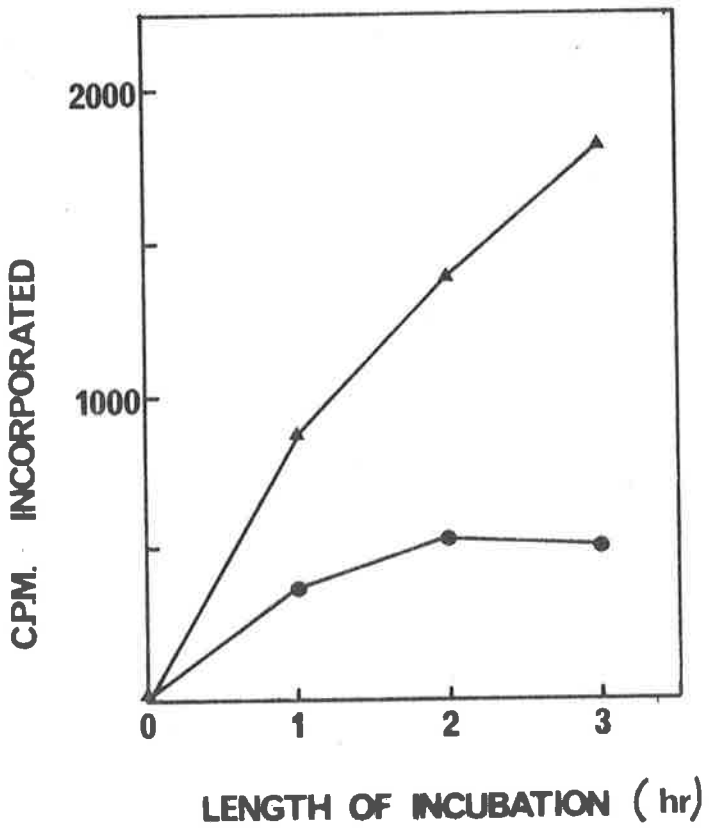
FIGURE 6.5

Fatty acid synthesis in E234 GLY-1

Fatty acid synthesis was measured in E234 GLY-1 grown in the presence or absence of glycerol, by monitoring incorporation of ^{14}C-acetate into methanol-chloroform extracts of the cells, as described in the text.

▲ : + glycerol

● : - glycerol


2000
1000
0
1
2
3
C.P.M. INCORPORATED
LENGTH OF INCUBATION (hr)

6.3.5 Behaviour of staphylococcal penicillinase in E234GLY-1

The aim of isolating the glycerol auxotroph was to complement the studies presented in chapter 5 on the inhibition of lipid synthesis by cerulenin, by using instead a mutant deficient in lipid synthesis. Once this strain was isolated, the plasmid pPG122 was transformed into it, using the procedure described earlier in this chapter (except that transformants were isolated on L + ampicillin plates supplemented with glycerol). A successful transformant was identified by the plasmid miniscreen procedure described (results not shown).

This strain, E234GLY-1 (pPG122), was then grown to OD_{600} = 0.8 in L + glycerol. Cells were pelleted, washed and resuspended in L broth $\pm$ glycerol, and penicillinase activity monitored in protoplasts (see 6.2.8).

The results are presented in figure 6.6, and show that the GLY-1 strain continues to produce significant amounts of penicillinase for at least 8 hours after glycerol deprivation. Thus the production of penicillinase is compatible with the total protein synthetic activity of glycerol-deprived cells (see Figure 6.4); i.e. there is no evidence that depression of lipid synthesis affects the production of at least one extracellular protein. This result

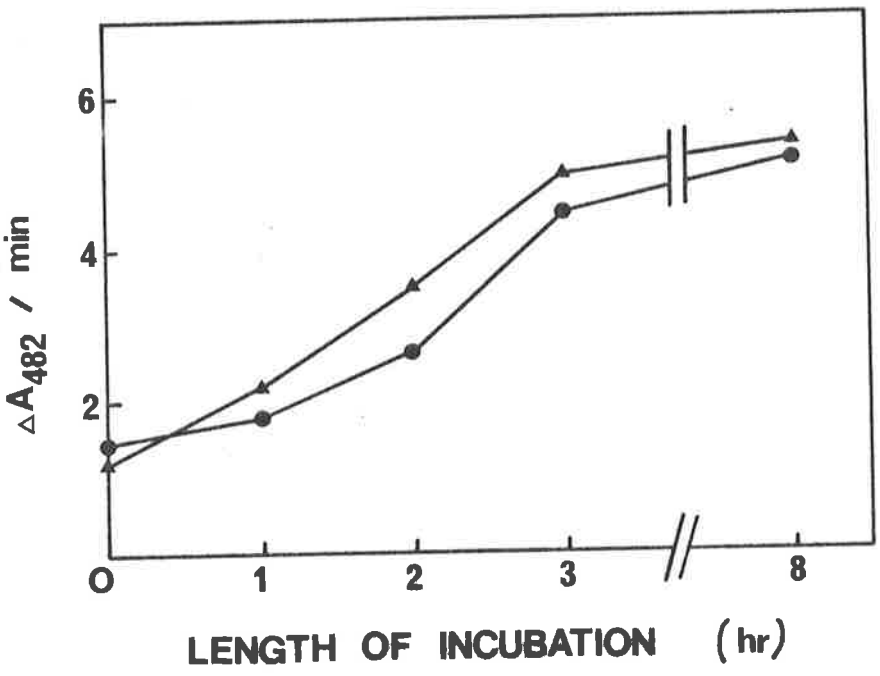
FIGURE 6.6

Penicillinase synthesis in E234-GLY1 (pPG122)

E234-GLY1 (pPg122) was grown in the presence or absence of glycerol, as described in the text. At various times, samples were removed, protoplasted and assayed for penicillinase activity (Section 6.2.).

▲ : + glycerol

● : - glycerol


6
4
2
O
1
2
3
8
$\triangle A_{482}$ / min
LENGTH OF INCUBATION (hr)

confirms confirms those of other workers (e.g. Hsu and Fox, 1970; Mindich, 1970, 1971; Beacham, Taylor and Youell, 1976), using a number of glycerol-dependent mutants to study the synthesis of extracellular and membrane-associated proteins. The implications of these results will be discussed later in this Chapter.

6.3.6 Is there lipid synthesis *in vitro*?

As shown in Chapter 4, staphylococcal penicillinase is synthesized *in vitro* as a protein identical to that found extracellularly *in vivo*. Should, as has been suggested, lipid synthesis be an important adjunct of extracellular protein production *in vivo*, then it would be informative to determine whether or not such lipid synthesis is occurring during *in vitro* synthesis of penicillinase.

To examine this complete *in vitro* protein synthesizing mixes containing pI258*blai*$^-$ DNA (as described in Chapter 3), were incubated in the presence of one of several labelled precursors of lipids:

γ-^{32}P-ATP; which is the donor of the phosphate moiety of phospholipids.

^{14}C-glycerol; the precursor of triglycerides and phosphatidic acid (see White, Handler and Smith, 1968).

^{14}C-serine; which can be incorporated into

phosphatidylserine - possibly the amino-terminus of the *Bacillus* penicillinase precursor peptide (Yamamoto and Lampen, 1977).

^{14}C-acetate; as a measure of fatty acid synthesis. For measurement of incorporation of acetate into lipids, the *in vitro* mix was modified by replacing all sources of acetate ions (with the exception of those provided by the DNA solution) with an equimolar amount of chloride ions (e.g. Herrlich and Schweiger, 1974).

At various times of incubation, 50 µl aliquots were removed, diluted with 50 µl H_2O, and extracted with methanol-chloroform (2:1) as described in Chapter 5. After washing and drying of the extracts, radioactive counts incorporated were measured. As shown in figure 6.7, there was no incorporation of ^{14}C-acetate, ^{32}P, or ^{14}C-glycerol into lipid material, suggesting that there is no *de novo* synthesis of fatty acids, triglycerides or glycerophosphate, in the *in vitro* system.

In contrast, there was considerable incorporation of ^{14}C-serine into lipid material. In the synthesis of phosphatidylserine, the addition of the serine molecule to the phospholipid is the final synthetic step - it does <u>not</u> necessarily involve *de novo* synthesis of lipids (White, Handler and Smith, 1968),

FIGURE 6.7

Lipid synthesis *in vitro*

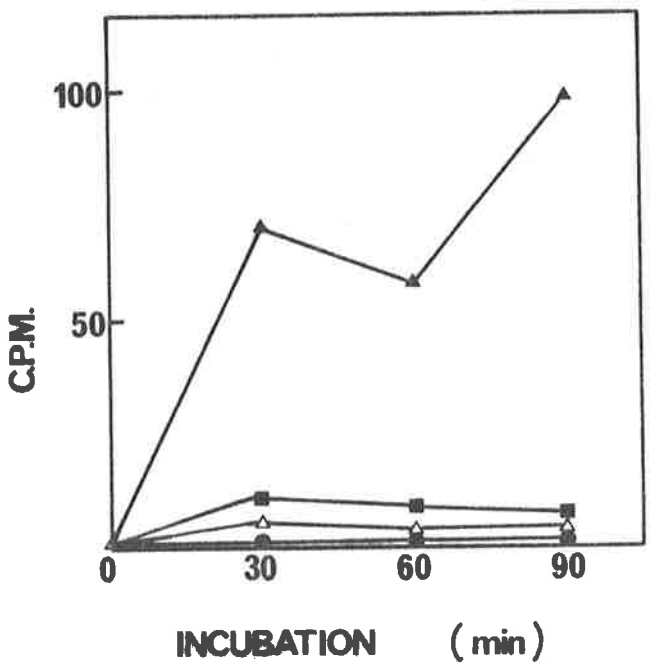
Lipid synthesis in the *in vitro* protein synthesizing mix was estimated by monitoring the incorporation of labelled lipid precursors into methanol-chloroform extracts of the mix (see Section 6.3.).

Δ : α-^{32}P-ATP

▲ : ^{14}C-serine

● : ^{14}C-acetate

■ : ^{14}C-glycerol


100
50
C.P.M.
0
30
60
90
INCUBATION (min)

but could occur by a "turnover" of lipid material, some of which may then become phosphatidylserine.

Thus although there is no evidence of gross lipid synthesis in the *in vitro* system, there is some evidence that there is synthesis of at least one important phospholipid derivative, phosphatidylserine, implying that there could be some lipid turnover in the system. This result reinforces the idea discussed in Chapter 5, and later in this chapter, that there is no absolute requirement for lipid synthesis in achieving efficient production of extracellular proteins, but leaves open the possibility that some high-level phospholipid activity (theoretically necessary for attachment of a lipid moiety to the amino-terminus of a penicillinase precursor) contributes to extracellular production.

6.4 DISCUSSION

This chapter describes preliminary experiments using a strain of *E.coli* dependent on glycerol for its normal growth; judging by the incorporation of ^{14}C-acetate into lipids, this dependence is due to inhibition of lipid synthesis when the cells are deprived of glycerol. This mutant was then used to examine the synthesis of staphylococcal penicillinase, in the presence and absence of glycerol (i.e. lipid synthesis).

During glycerol deprivation, the mutant strain continues to grow for about one generation (figure 6.3) although incorporation of ^{14}C-acetate into lipid material ceases immediately (figure 6.5). Total protein synthesis (figure 6.4), and penicillinase synthesis continue, however, at a comparable rate regardless of the presence or absence of glycerol. The implication of these results is that there is no tight association between *de novo* lipid synthesis and extracellular protein synthesis; penicillinase continues to accumulate in the periplasm even in the absence of cell division and lipid synthesis, at a rate commensurate with the total rate of protein synthesis.

This result is in agreement with other studies in *E.coli* which suggest that both membrane-associated

proteins (Hsu and Fox, 1970) and periplasmic proteins (Beacham, Taylor and Youell, 1976) are synthesized in the absence of lipid synthesis. Similarly, the results in this chapter agree with those in Chapter 5, wherein lipid synthesis was inhibited by the disruption of fatty acid synthesis, yet did not affect production of constitutive penicillinase. These results lend further weight to the idea, discussed fully in Chapter 5, that there is no direct link between lipid synthesis and extracellular protein synthesis.

Ideally, this study should also examine the effects of lipid synthesis disruption on the production of inducible penicillinase, as was done in Chapter 5. Should production of this protein be upset by glycerol starvation, it would provide further evidence for the ideas put forward in Chapter 5. Unfortunately, it was not possible to induce staphylococcal penicillinase in *E.coli*. When the recombinant plasmid pSC113 (Chang and Cohen, 1974), carried in strain E392, was exposed to methicillin (0.5μg/ml), there was no increase in periplasmic penicillinase activity (data not shown). This is despite the fact that pSC113 confers resistance to β-lactam antibiotics; presumably this is due to low level of production of the enzyme.

Interestingly, when studying synthesis of alkaline phosphatase in glycerol-deprived auxotrophs, Beacham,

Taylor, and Youell (1976) did not use a strain inducible for the enzyme, but one in which the enzyme was induced by a temperature shift, i.e. it was essentially a constitutive producer of alkaline phosphatase, under their assay conditions. This may represent another example of the independence of constitutive exo-protein production and lipid synthesis.

There is no evidence of *de novo* lipid synthesis in the *in vitro* system (figure 6.6), either of fatty acids, triglycerides or phospholipids. There is, however, some evidence for the incorporation of serine into phospholipids (phosphatidylserine). This suggests that there is some conversion of existing phospholipid into new forms, which could play a role in the production and/or processing of penicillinase.

These results reinforce the idea that lipid synthesis *per se* is not a prerequisite for extracellular protein synthesis, although they do not rule out the possibility that lipids have some direct role (as distinct from affecting the overall state of the cell membrane) in the process. Further experiments are necessary to examine the part played by specific lipids (e.g. phosphatidyl serine) in the processing of penicillinase. While the results of chapter 5, and this chapter, argue against a direct role, it is

still possible that specific lipids are somehow involved in the secretory process. Ideally such studies should use the natural host of staphylococcal penicillinase; the fact that it appears uninducible in *E.coli*, while consistent with the behaviour of similar enzymes normally resident in *E.coli* (Richmond, 1975 b), does suggest that its behaviour is not the same in both species.

CHAPTER SEVEN

GENERAL DISCUSSION

The major question asked at the beginning of this work was: what are the requirements for the synthesis of a bacterial secretory protein? This question was prompted by the knowledge of the process in eukaryotes, which centres around the membrane-bound polysome, believed to be the sole source of secretory protein synthesis. This project intended to examine the process in bacteria, initially by utilizing an *in vitro* DNA-directed protein synthesizing system - a technique which has been proven to reproduce accurately and faithfully many *in vivo* aspects of protein synthesis.

It was shown (Chapter 3) that an *E.coli*-based system was quite capable of synthesizing enzymatically active staphylococcal penicillinase, although not as efficiently as the production of the *E.coli* enzyme β-galactosidase. Unexpectedly however, there was no evidence that penicillinase was synthesized as a higher molecular weight precursor, a result predicted by comparable studies on other secretory or membrane-associated proteins (e.g. Inouye and Beckwith, 1977; Chang, Blobel and Model, 1978). As discussed in Chapter 4, this result indicates one of several possibilities:

1. Staphylococcal penicillinase is not a classical extracellular protein - i.e. it is not first translated as a precursor protein. This is unlikely, considering the genetic similarities

between the staphylococcal and *Bacillus* enzymes (compare Imsande, 1978; Kelly and Brammer, 1973), and their physical similarities (Richmond, 1975 a).

2. The particular mutant used for the *in vitro* studies of Chapters 3 and 4 is no longer a representative extracellular protein. Kelly and Brammar (1973) reported a polar mutation which affected both the structural penicillinase gene and its control gene. As pI258*bla*i443 was chosen for both its constitutive phenotype, and for its high level of enzyme production (Novick, 1965), it is conceivable that the operon contains more than a single mutation, or a pleiotropic mutation; such that its precursor form is altered. For example, the size of the precursor may have been altered by reduction of the leader sequence, making it difficult to differentiate from the mature form, whereas the wild-type precursor would be expected to be about 20-25 amino acids (about 10%) longer than the extracellular protein. Alternatively, the extracellular form of this protein may be slightly longer than its wild-type counterpart. These possibilities should be investigated by examining the synthesis of other staphylococcal penicillinases *in vitro*, and by DNA sequence analysis of the relevant operons.

3. The *in vitro* system used in this study may be different to those used by other workers, and be quite capable of efficient and rapid processing, probably co-translationally, of the penicillinase precursor. This is the most likely possibility. Certainly the S30 component of the system contains membrane and lipid material (Chapters 4 and 6), which it is believed are essential for the synthesis-processing-secretion procedure. However, as shown in Chapter 4, the presence of membranes was <u>not</u> essential for the appearance of the mature-sized penicillinase (remembering the reservations expressed in point 2. about the operon used), suggesting that the protein can be processed in the absence of membranes, or rather, the membrane-associated protease. This processing may not be representative of the *in vivo* situation - it has been shown that f1 pre-coat protein can be processed in the absence of exogenous membranes (Chang, Blobel and Model, 1978) and that an <u>outer</u> membrane preparation can catalyze some processing of alkaline phosphatase precursor (Inouye and Beckwith, 1977) although is is questionable whether these represent truly the *in vivo* sequence of events. Similarly it has been reported that the membrane associated penicillinase of *Bacillus* can be cleaved by a membrane-protease after translation, although this too may not reflect the situation (especially as gram-positive cells

do not normally convert membrane-penicillinase to the extracellular form) (Coles and Gross, 1967, a,b; 1969; Crane *et al.*, 1973).

It is now becoming apparent that the sequence of events in the production of an extracellular protein is not necessarily as straightforward as predicted by the signal hypothesis, or its variations (Blobel and Dobberstein, 1975 a,b; Inouye and Halegoua, 1980). Membrane-associated *Bacillus* penicillinase has been found in a number of forms (Simons, *et al.*, 1978) - a fact incompatible with the sequence of events predicted by the signal-vectorial extrusion-cotranslational processing hypothesis. A recent paper by Koshland and Botstein (1980) has shown that the carboxyl-terminus of the TEM-lactamase is necessary for its correct secretion in *Salmonella typhimurium*. Although synthesized with an intact amino-terminal leader sequence (see Sutcliffe, 1978), mutant enzymes lacking some C-terminal amino acids were not secreted, but found in the cytoplasm, implying that an intact carboxyl-terminus is also necessary for the correct processing of this enzyme, despite the apparently correct processing of the amino-terminal leader sequence. Incidentally, this result supports the earlier suggestion of Kelly and Brammar (1973) that an intact carboxy-terminus is

necessary for the production of extracellular *Bacillus* penicillinase. The notion that the carboxyl-terminus plays a role in protein secretion implies that the polypeptide must be virtually completed prior to its secretion (regardless of events at the amino-terminus), which is difficult to reconcile with the co-translational, vectorial extrusion models.

Similarly, Randall, Josefsson and Hardy (1980) have shown, by pulse-labelling of *in vitro* protein synthesis mixes, that there are specific pauses in the translation of maltose binding protein mRNA, which may play a role in the secretion procedure, perhaps by allowing folding of the peptide in the membrane (see von Heijne, 1980). The authors also note that some maltose binding protein is synthesized on cytoplasmic polysomes - an interesting analogy with the results of Chapter 4.

Smith (1980) has shown that the proteolytic activity which processes alkaline phosphatase resides on the *cytoplasmic* surface of inner membrane vesicles of *E.coli*. This is not completely consistent with the current theories of protein secretion - it implies cleavage of the leader sequence while most of the protein is still in the cytoplasm

(either the protein or the translation machinery producing it). Possibly this indicates that the protein is assembled in the membrane prior to its cleavage, although this is inconsistent with the work of Smith, Tai and Davis (1978).

The *in vitro* system is ideally suited to the study of the secretion of penicillinase, and a number of experiments can be suggested by the results of Chapters 3 and 4. For example, the identification and characterization of the repression-induction mechanisms of the operon deserve to be investigated. Imsande (1978) has presented a theoretical model of the induction process, and the *in vitro* system may allow this theory to be investigated. The repressor protein could be identified (for example, by transcribing deletion mutants of the repressor gene) and challenge with various potential "anti-repressor" proteins. More importantly, the nature of the penicillinase peptide found in this system and nature of its precursor, must be investigated. This can be done by purifying and dissecting the *in vitro* system, for example by using the more purified system of Herrlich and Schweiger (1974). This may assist in identification of the membrane-associated components involved in the secretion process, such as the putative processing endopeptidase, and the cytoplasmic activities which also appear

to process penicillinase (Chapter 4). If indeed a precursor does exist, it is essential that it be found - pulse labelling during synthesis may assist in this (as done by Randall, Hardy and Josefsson, (1980), and Crowlesmith, Gamon and Henning (1981), in their examinations of extracellular protein synthesis). Likewise, other staphylococcal extracellular proteins should be examined in the *in vitro* system, including other penicillinases and other genetic variations of the pI258 penicillinase operon.

The role of lipids in the secretion process was also studied *in vivo*, as described in Chapters 5 and 6, and the significance of these results has been discussed in detail in these chapters. The results suggest that, in contrast to the conclusions of other workers, lipid synthesis is not necessary for extracellular protein synthesis. The production of constitutive penicillinase in the presence of the fatty acid synthesis inhibitor cerulenin, is almost unchanged, whereas inducible penicillinase is as sensitive to that inhibition as are extracellular proteins examined by other workers. It is likely that by disrupting membrane structure, (either directly, or by inhibiting lipid synthesis), cerulenin is affecting induction mechanisms mediated by membrane-proteins, rather than directly affecting enzyme synthesis itself. This conclusion was supported by

the results of Chapter 6, wherein lipid synthesis was inhibited by starvation of a glycerol auxotroph of *E.coli*. Constitutive staphylococcal penicillinase continues to be produced under these conditions. Again, more extracellular proteins should be examined in this system.

The demonstration that there is some lipid synthesis, or turnover, *in vitro* (Chapter 6) confirms the presence of membranous material in the S30 (Chapter 4), and leaves open the possibility that some direct phospholipid-extracellular protein interaction occurs as part of the secretion process.

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