TRANSPORT AND PROCESSING OF STAPHYLOCOCCAL α-LYSIN AND δ-LYSIN

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To my parents and
my sisters for so
many things.

PREFACE

This thesis was the original work of the author.

Ker Yin Lee

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SUMMARY

The main object of the investigation described in this thesis was to determine whether staphylococcal α-lysin and δ-lysin are synthesized as larger precursor molecules from which signal sequences are processed during transmembrane translocation.

δ-lysin was first detected at the mid-logarithmic phase of growth of S. aureus NCTC 10345, and production, which appeared to obey exponential kinetics, continued well into the stationary phase of growth. It appeared as a very diffuse band just behind the dye front when supernate proteins were separated by SDS-PAGE.

Rifampicin, at a concentration of 200μg ml^{-1}, rapidly blocked the incorporation of [^{3}H] uridine into the RNA of S. aureus. The same concentration of rifampicin required considerably longer time to inhibit cell growth at both the mid-logarithmic and late logarithmic-early stationary phases of growth; growth of the bacteria stopped after 1 and 2 h respectively in each phase. Addition of rifampicin to the late logarithmic-early stationary phase culture had less effect on growth than the mid-logarithmic phase culture. The stability of mRNA for the staphylococcal δ-lysin was determined by measuring the residual lysin synthesis after inhibition of DNA-dependent RNA polymerase activity with rifampicin. At the late logarithmic-early stationary phase of growth the δ-lysin mRNA was very stable with a half-life of about 20 min.

Total cellular RNA was extracted from cells from late logarithmic-early stationary cultures phase of S. aureus NCTC 10345 and translated with an E. coli S-30 extract (cell-free system). The rate of amino acid incorporation was linear for 15 min and the staphylococcal mRNA stimulated the translation to a level approximately 20-fold above the background. Protein synthesis was almost abolished by chloramphenicol (50 μg ml^{-1}), was

dependent on an exogenous energy source (ATP, GTP, PEP) and was barely activated by the protease inhibitor, PMSF (1mg ml^{-1}) or the RNase inhibitor, heparin (1mg ml^{-1}). δ-lysin was identified amongst the translation products by immunoprecipitation and immunoabsorption. The δ-lysin synthesized *in vitro* was of a size similar to mature δ-lysin and seemed not to require a signal sequence for secretion from the cell.

Phenethyl alcohol (PEA), at the maximum concentration which did not inhibit growth (0.3% v/v) inhibited the production of α-lysin and exoproteases but not that of δ-lysin in *S. aureus* Wood 46. The inhibition of α-lysin was reversible and transient accumulation of cell-associated α-lysin occurred in the presence of PEA.

A precursor of α-lysin, of molecular weight approximately 3,000 daltons (peptide elongation of about 20-30 amino acids) larger than extracellular α-lysin, was immunologically detected in the SDS extracts of membranes and whole cells of PEA-treated *S. aureus* Wood 46 cultures. Also, a degraded form of α-lysin , of molecular weight approximately 27,000 daltons was only detected in membranes prepared from cells lysed by lysostaphin but not in membranes from cells lysed with an X-press or SDS extracts of whole cells.

It was concluded from these results that α-lysin is synthesized with a 3 kdal N-terminal signal sequence which is removed during transmembrane translocation whereas δ-lysin seems not to require a signal sequence for secretion from the cell.

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PUBLICATIONS

1. *In vitro* synthesis of the delta-lysin of *Staphylococcus aureus*. Infection and Immunity, 44:434-438.

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ABBREVIATIONS

A	amps
$A_{y\ nm}$	absorption value of a solution at a wavelength of y nm
ATP	adenosine triphosphate
bis	N,N'-methylenebisacrylamide
Bq	becquerel
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CDS	citrate dextrose saline
cpm	count per minute
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	disintegration per minute
EC	Enzymic Commission
EDTA	ethylene diamine tetra acetate
EGTA	ethyleneglycol-bis(β-aminoethyl ether) N,N'-tetra-acetic acid
g	gram
g	force of gravity
HU_{50}	haemolytic unit
l	litre
LD_{50}	median lethal dose
M.W.	molecular weight
mRNA	messenger RNA
MSC	maximum subinhibitory concentration
NCTC	National Collection of Type Cultures
NP-40	Nonidet P-40

NSP	N-succinimidyl propionate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEA	phenethyl alcohol (2-phenylethanol)
pI	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
poly(A)	poly(adenylic)acid
POPOP	phenyloxazolylphenyloxazolylphenyl
PPO	2,5-diphenyloxazole
R_f	relative electrophoretic mobility
RNA	ribonucleic acid
RNase	ribouclease
rRNA	ribosomal RNA
S_{20w}	sedimentation coefficient corrected to 20 ^{o}C
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N -tetramethylethylenediamine
Tris	tris(hydroxymethyl)amino methane
tRNA	transfer RNA
v/v	volume to volume
WHO	World Health Organisation
w/v	weight to volume

INTRODUCTION

A. STAPHYLOCOCCAL INFECTION AND EXTRACELLULAR PRODUCTS

In the one hundred years that have elapsed since the staphylococcus was first discovered in human pus and described by Koch (1878), we have learnt much about this bacterium and its ability to cause a wide variety of disease syndromes, but even today it continues to reveal new features to taunt both the clinician and the microbiologist.

Staphylococcus aureus, as well as occurring as a harmless commensal in the nose and on the skin of many healthy persons, is also the cause of a diverse array of both superficial and systemic infections (Table 1). It has been reported (Kaplan and Tenenbaum, 1982) to be the most common cause of severe infection in the noncompromised patient. The high incidence of staphylococcal infections reflects the magnitude of this problem in the industrialized world. In under developed countries, staphylococcal infection is even more serious, with untreated disease destroying limbs, mutilating faces, blinding children and adults and often leading to fatal bacteraemia.

Staphylococcal infections have been reviewed by Shulman and Nahmias (1972) and Musher and McKenzie (1977). The skin is the most common site of infection and, once virulent staphylococci have gained a foothold in deeper tissue of the body, their multiplication causes necrosis and eventual abscess formation. In severe infections the organisms may break through the localised barrier of the lesion(s) and invade the lymphatics and bloodstream. If bacteraemia becomes established, metastatic foci frequently develop (Table 1).

Staphylococci produce a large number of extracellular toxins (exotoxins) and enzymes during growth (Table 2). The concept of a relationship between virulence and extracellular products is very old.

Table 1. Spectrum of Staphylococcal Infections*

Local Infections

Site	Infection
skin	Carbuncles, boils, abscesses, impetigo, toxic epidermal necrolysis.
eye	conjunctivitis.
nose and throat	pharyngitis, sinusitis.
lung	primary pneumonia
gastrointestinal tract	enterocolitis.
Vagina	cervicitis, pelvic abscesses.

Metastatic Infections

bloodstream	bacteraemia.
bones and joints	osteomyelitis.
lungs	secondary pneumonia.
skin and muscle	abscesses.
heart	endocarditis, myocarditis, pericarditis.
central nervous system	brain abscesses, cerebritis.
others	intra-abdominal visceral abscesses- spleen, liver, pancreas.

* Adapted from Shulman and Nahmias (1972)

Table 2. Extracellular Proteins from Staphylococcus aureus

Toxins*	Enzymes**
Membrane-Damaging Toxins (Lysins)	Coagulase**+
α-lysin	
β-lysin	Bacteriolytic Enzymes
γ-lysin	Endo-β-N-acetylglucosaminidase
δ-lysin	Endopeptidase
	N-acetylmuramyl-L-alanine amidase
Panton-Valentine leucocidin	
	Hyaluronate lyase
Epidermolytic Toxins	
A (Exofoliatin A)	Penicillinase
B (Exofoliatin B)	
	Proteinase
Enterotoxins	Metallo-proteinase
A, B, C_1, C_2, D, E and F	Serine-proteinase
	Thiol-proteinase
Pyrogenic Exotoxins*+	
A, B and C	Staphylokinase
	Phosphatase
	Lipase
	Lysophophatidase
	Phosphatidylcholine:cholesterol o-acyl-transferase

* For references see Freer and Arbuthnott (1983).

*+ For reference see Schlievert et al. (1979).

** For reference see Arvidson (1983).

**+ For reference see Jeljaszewicz et al. (1983).

The ability of culture filtrates of S. aureus to cause inflammatory reactions after inoculation into experimental animals was first reported in 1888 (cited by Wiseman, 1975). Although the number and quantity produced may vary, Bernheimer and Schwartz (1961) detected 12-14 different proteins by analytical starch gel electrophoresis; more recent studies by isoelectric focusing in polyacrylamide gels have shown that certain strains of S. aureus gave as many as 25-30 bands while others gave only 4-10 bands (Wadstrom et al., 1974). Excellent reviews on the extracellular products of S. aureus are by Gladstone (1966), Bernheimer (1970), Wiseman (1970, 1975), Jeljaszewicz (1972), Woodin (1972), Rogolsky (1979), Bergdoll (1983), Freer and Arbuthnott (1983), Arbuthnott (1983) and Arvidson (1983).

There is general agreement that synthesis of exoproteins in S. aureus occurs in a biphasic manner in liquid batch cultures. Low levels of exoproteins are produced during exponential growth and a marked increase in production occurs just before the onset of the slower rate of growth prior to stationary phase. This biphasic pattern of production has been directly or indirectly revealed in work on α-lysin (Duncan and Cho, 1971; Abbas-Ali and Coleman, 1977 a,b), β-lysin (Low and Freer, 1977), staphylokinase (Arvidson et al., 1973a), staphylococcal serine-proteinase (Bjorklind and Arvidson, 1978), endo-β-N-acetyl-glucosaminidase (Arvidson et al., 1970), enterotoxin B (Morse and Baldwin, 1971), nuclease (Carpenter and Silverman, 1976) and staphylococcal metallo-proteinase (Arvidson, 1973b). Both the availability of RNA-polymerase following the "switching off" of rRNA synthesis at the end of the exponential growth phase and an increase in the nucleotide pool resulting from rRNA "turnover" have been proposed by Coleman et al. (1975) as controlling parameters. In support of the "competition" modes for regulation of exoprotein synthesis, Abbas-Ali and Coleman (1977b) demonstrated that the

increased rate of exoprotein secretion at the end of the exponential growth phase was accompanied by an increase in intracellular nucleotide concentration. They concluded that at higher growth rates and accompanying greater rates of cellular protein synthesis, there was correspondingly less of the biosynthetic machinery available for extracellular protein synthesis.

Evidence that the formation of extracellular proteins in S. aureus is controlled by a common mechanism comes from the isolation of mutants which have simultaneously lost the ability to produce several exoproteins but with no indication that secretion of extracellular proteins was altered (Yoshikawa et al., 1974; Katsuno and Kondo, 1973 ; Bjorklind and Arvidson, 1980). Proteins which were repressed in the mutant all belong to the group of exoproteins (serine-proteinase, α-lysin, leucocidin, metallo-proteinase, staphylokinase, nuclease and acid phosphatase) produced at an increased rate during the post-exponential growth phase; protein A and coagulase are produced only during the exponential phase (Bjorklind and Arvidson, 1980). The repression of protein A and coagulase synthesis at the end of the exponential growth phase thus coincides with the increased synthesis of other exoproteins. By assuming the existence of protein A and coagulase repressor substances , they suggested that the efficient transcription of exoprotein genes requires the presence of an activator protein (EX) and a co-activator (S). Although the exact mechanisms of exoprotein regulations have not yet been established, it seems clear that the synthesis of many extracellular proteins from S. aureus is subject to co-ordinate control. The fact that the synthesis of most extracellular proteins is "induced" when bacterial growth becomes nutrient limited at the end of the exponential growth phase, strongly supports the idea that

one of their main functions is to provide the bacteria with low molecular weight nutrients.

B. MEMBRANE-DAMAGING TOXINS (LYSINS)

a. Discovery

By the early 1900's, the toxic properties of *S. aureus* culture filtrates were well recognized, and the existence of more than one toxin in culture filtrates was first suggested by Walbum (1921) who described a haemolysin which did not lyse goat erythrocytes at 37 °C but caused rapid lysis on cooling to 0 °C. This so-called hot-cold haemolysin was subsequently reinvestigated by Bigger *et al.* (1927) and found to be active on sheep erythrocytes. Later, Glenny and Stevens (1935) showed this haemolysin to be immunologically distinct from the haemolysin which lysed rabbit erythrocytes and they termed the rabbit haemolysin alpha (α-), and the sheep haemolysin beta (β-), a terminology which is still widely used.

The suggestion that there were two serologically distinct rabbit haemolysins was favoured by several authors. Morgan and Graydon (1936) designated their toxins α_1 and α_2, while Smith and Price (1938) named their second rabbit haemolysin gamma (γ-). These additional rabbit haemolysins, the α_2 of Morgan and Graydon (1936) and the γ of Smith and Price (1938), appeared to share certain similarities. Not until the studies of Plommet and colleagues (See review by Freer and Arbuthnott, 1983) was the existence of staphylococcal γ-lysin well established.

In 1947, Williams and Harper observed that some strains of *S. aureus* grown on sheep blood agar produced a haemolytic agent which was not neutralized by either α- or β- antitoxin; this they named delta (δ-) toxin. Elek and Levy (1950) suggested that the previously named α_2 and γ

lysins were in fact identical to δ-haemolysin but it is now realized that they could not have detected γ-haemolysin by using blood agar plates, as its haemolytic activity is inhibited by agar (Mollby and Wadstrom, 1971). Marks (1951) reported that γ-haemolysin was a separate entity, antigenically different from α-haemolysin. The picture was even more confused by the suggested existence of a further haemolysin. Elek and Levy (1950) claimed that 95% of 77 coagulase-negative skin strains of staphylococci produced a "wide zone" haemolysin which they designated epsilon (ε-). In 1968, Kleck and Donahue claimed to have demonstrated that δ- and ε- haemolysin are identical and the existence of the haemolysin is still in doubt. There is now general agreement that four haemolysins are produced by S. aureus, namely α, β, γ and δ. An additional haemolysin which lyses equine erythrocytes has been shown in canine strains of S. aureus (Turner and Pickard, 1979). Although it has not been characterised, it appears to be different from a further haemolysin produced by coagulase positive staphylococci of canine origin and designated zeta (ζ-) by Fraser (1964).

b. Nomenclature

The term "haemolysin" was widely employed by early workers who investigated the haemolytic agents of Staphylococcus aureus. The haemolysins were differentiated on the basis of their lytic activity for different species of erythrocytes. As knowledge about the haemolysins grew, it became evident that these toxins were active on cells other than erythrocytes. In order to define the spectrum of the biological activity of the haemolysins more accurately, Bernheimer (1974) proposed the term "cytolytic toxins" or "cytolysins", which were simply defined as bacterial products capable of causing physical dissolution of a variety of

cells in vitro. However, Thelestam and Mollby (1975 a,b) discovered that low concentrations of cytolytic toxins induced permeability changes in the membranes of intact susceptible cells without causing lysis. Therefore, McCartney and Arbuthnott (1978) suggested that "membrane-damaging toxin" is a more appropriate term than cytolytic toxin to describe α-, β-, γ- and δ- toxins. In any event, either cytolytic toxin or membrane-damaging toxin is a more appropriate term that haemolysin in describing the biological activity of these toxin. Therefore, Rogolsky (1979) suggested that the term "haemolysin" in reference to these toxin, should be abandoned. However, Mollby (1983) suggested that the most suitable designation would still be "haemolysin" since the haemolytic capacity is the only effect shared by all the staphylococcal haemolysins. (Table 3).

c. Alpha-Lysin

1. Production of α-Lysin

α-lysin is produced by the majority of coagulase-positive staphylococci, but one particular strain, Wood 46, has been most commonly employed since the time of Burnet (1929) for the studies on α-lysin. In 1974, Goode and Baldwin suggested that α-lysin produced by different strains was very similar in its biological and physicochemical properties.

The types of media used and the effect of various factors on α-lysin production have been extensively reviewed by Arbuthnott (1970), Wiseman (1975) and Mollby (1983). Factors such as CO_2 tension and glucose concentration both affected the yield of α-lysin. However, there is still a dearth of information on environmental factors controlling its production. Low levels of penicillin, uninhibitory to growth, stimulated α-lysin production (Hällander et al. 1972).

Small amounts of α-lysin are produced during exponential growth with

Table 3. Comparison of Toxic Effect of Staphylococcal Membrane-Damaging Toxins*

Toxic Effects	Designation	Staphylococcal α-lysin	Staphylococcal β-lysin	Staphylococcal γ-lysin	Staphylococcal δ-lysin
Lysis of erythrocytes	Haemolysin	+	+	+	+
Membrane damage to cells	Membrane Damaging Agent	+	(+)	(+)	+
Damage to cells	Cytotoxin	+	-	-	+
Lysis of karyotic cells	Cytolysin	-	-	-	+

(+) Denotes weak effects

* Adapted from Mollby (1983).

an increase occurring with the onset of stationary phase (Hendricks and Altenberg, 1968; Duncan and Cho, 1971; McNiven and Arbuthnott, 1972). In 1983, Totake and Ichikawa reported an inverse correlation between α-lysin production and growth rate in S. aureus. There is no evidence that α-lysin accumulates in any appreciable quantities intracellularly prior to its release, a fact which is entirely compatible with current ideas on synthesis and release of exported proteins (Davis and Tai, 1980).

In 1961, Blair and Carr showed that toxigenicity could be conferred on non-toxigenic strains by lysogenisation with phages from toxigenic strains. However, Hendricks and Altenberg (1968) failed to find evidence of the lysogenic conversion. More recently, Kehoe et al. (1983) reported that the α-lysin determinant was localized to a small region of S. aureus DNA of approximately 1,620 bp.

2. Purification of α-lysin

It is clear that highly purified lysin preparations are required for the study of its biological properties as Colacicco and his colleagues (1977) admitted that lysis of sphaeroplasts and protoplasts by an α-lysin preparation was probably due to contamination by δ-lysin. Numerous methods for the purification of α-lysin are available and this topic has been reviewed most recently by Freer and Arbuthnott (1983) and Mollby (1983). In recent years, attention has been concentrated on reducing the complexity of the procedures and improving the purity of lysin preparations. An elegant and simple method was presented by Cassidy and Harshman (1976a); this involved chromatography on controlled-pore glass followed by ion-exchange chromatography. Saleh and Freer (1984) later suggested that it was necessary to substitute isoelectric-focusing in a broad pH gradient for the ion-exchange chromatography step in order to remove all detectable proteolytic activity as well as acidic proteins from

the final product. Another simplified purification procedure was presented by Fussle et al., (1981b) who exploited the binding of α-lysin to rabbit erythrocyte membranes as a concentration step.

3. Physicochemical Properties of α-Lysin

α-lysin is a protein, consisting of one polypeptide chain without carbohydrate residues (Watanaba and Kato, 1974), molecular weight estimates have ranged from 1 to 4 x 10^4 daltons (Table 4), perhaps due to different methods of estimation employed by different investigators. Six and Harshman (1973b) suggested that the molecular weight of α-lysin fell within the range 2.6 to 3.1 x 10^4 daltons by four different methods of assessment on a single product. Sedimentation coefficients for active lysin in the range 2.85 - 3.15S have been reported frequently although additional minor components in the range 10S - 16S (due to aggregation or polymerisation of 3S form) have also been reported (see Table 4). The existence of so-called "multiple forms" of α-lysin has been reported by many workers (Bernheimer and Schwartz, 1963; Wadstrom, 1968; McNiven et al., 1972 ; Dalen, 1975); these possibly arise during purification procedures but possess similar biological activities (Wadstrom, 1968; Bernheimer, 1970; McNiven et al., 1972).

One remarkable property of α-lysin is the Arrhenius effect, i.e. while inactivated at 60 °C it regains part of its activity upon further heating to 100 °C. There is no haemolytic activity nor membrane-damaging activity left upon heat-inactivation; however, the mitogenic activity of the α-lysin is quite intact upon heating to 60 °C (Petrini and Mollby, 1981).

An amino acid analysis of α-lysin was first published by Bernheimer and Schwartz (1963), who showed the complete absence of half-cysteine.

Table 4. Some Characteristics of Purified α-Lysin

Reference	Molecular Weight	Sedimentation coefficient ($S_{20,w}$)	Isoelectric point (pI)
Kumar et al. (1962)	$1.0-1.5 \times 10^4$	1.45	-
Bernheimer & Schwartz (1963)	4.4×10^4	3.0[+]; 12.0[*]	-
Lominski et al. (1963)	-	3.1	-
Cooper et al. (1966)	-	2.8	-
Coulter (1966)	2.1×10^4	2.8	-
Arbuthnott et al. (1967)	-	3.0	-
Wadstrom (1968)	-	-	8.5[+]
Forlani et al. (1971)	3.3×10^4	2.8	-
McNiven et al. (1972)	3.6×10^4	-	-
Six & Harshman (1973a,b)	2.8×10^4	3.0(A);3.0(B)	7.2(A);8.4(B)
Coode & Baldwin (1973)	-	3.0[+]; 10.5	8.65[+] ; 5.8
Watanabe & Kato (1974)	3.6×10^4	-	7.98 ± 0.05
Dalen (1975, 1976b)	3.9×10^4	-	8.6[+] ; 7.4
Fussle et al. (1981b)	3.6×10^4	-	-

[+] Major component

[*] Inactive aggregate

There is overall similarity in the results from different laboratories (Coulter, 1966; Fackrell and Wiseman; 1976 ; Six and Hashman, 1973b; Watanabe and Kato, 1974; Kato , 1982), in that half-cysteine is absent and there are large amounts of lysine, aspartic and glutamic acids.

In 1978, Watanabe and Kato reported that trypsin treatment of purified α-lysin yielded two components. A heavy chain fragment (molecular weight 17,000) had lethal activity in mice but lacked haemolytic and dermonecrotic activities; a light fragment (molecular weight 14,000) had unstable haemolytic activity which was reduced to 10% of the original value. Both fragments, though immunologically distinct, showed partial identity to native lysin. Amino acid analysis indicated different contents of lysine, histidine, threonine, methionine and tyrosine in the two fragments (kato, 1982) and the lethal toxic fragment was distinctive in containing only one histidine residue localized at the C-terminal moiety of the native lysin molecule.The amino acid sequence of α-lysin, recently determined by Gray and Kehoe (1984) did not show region corresponding to the partial sequences found by Watanabe and Kato (1978).

4.Modes of Membrane Damaging Action of α-Lysin

Although reviewed extensively, the mode of action of α-lysin is still disputed (Arbuthnott, 1970; Jeljaszewicz, 1972; Bernheimer, 1974; Freer and Arbuthnott, 1976, 1983; McCartney and Arbuthnott, 1978; Harshman, 1979; Wadstrom, 1983). Nevertheless, the primary site of action is generally accepted as being the plasma membrane and the many biological activities of α-lysin (see Table 5) can be explained as secondary effects resulting from cell membrane damage (Wadstrom, 1983).

The study of α-lysin-membrane interactions has involved three main targets, whole cultured cells, isolated membranes and artificial membranes. While results from one system are not directly comparable with those from

Table 5. Biological Effects Attributed to Staphylococcal α-Lysin*

1. *Lethal for man and animals*

2. *Effects on nervous system*
   Action on function of the system, disturbance of polarization and depolarization of neuronal membrane.
   Functional damage to reticular system of the hypothalamus and visual-sensory region of the cerebral cortex.
   Injury of isolated vagus nerve sheaths.

3. *Effects on smooth muscle*
   Increased permeability of small blood vessels inhibited by antihistamine agents.
   Liberation of histamine and serotonin from perfused organs.
   Spastic contraction of large blood vessels and intestinal tract.

4. *Effects on the circulatory system*
   Hypertension due to catecholamine liberation, ECG changes.

5. *Effects on cells*
   Haemolytic and leucocidal according to species and type of cell.
   Destroys blood platelets.

6. *Other effects*
   Renal cortical necrosis
   Embryotoxicity
   Skin necrosis
   Leucocytosis accompanied by liberation of granulocyte reserve pool from bone marrow
   Amino acid activation in the aorta

* From Wadstrom (1983)

another, a combination of results from all three will be necessary to determine the action at the molecular level. Studies aimed at elucidating the mode of action of α-lysin have focused mainly on the following three aspects : (1) the possible enzymic activity of α-lysin, (2) morphological and biochemical studies for characterization of the structural membrane damage induced by the lysin, and (3) studies involving binding of the lysin to possible specific receptors on the surface of sensitive cells.

Wiseman and Caird (1970, 1972) and Wiseman et al., (1975) suggested that once bound to the membrane the lysin is activated by endogenous proteolytic activity in the membrane to become a protease and that such activity is more abundant in rabbit than in other red cells. However, Freer et al. (1973) were unable to verify a proteolytic action of α-lysin. The fact that the lysin is consumed in the lytic process (Madoff et al., 1964; Cassidy and Harshman, 1976b) and is able to cause structural membrane damage to artificial membrane systems lacking proteins (Freer et al., 1968) provide further evidence against an enzymatic mode of action. In similar work, Dalen (1976a) reported proteolytic activity associated with purified α-lysin, and suggested that autodigestion may explain the observed changes and heterogeneity in molecular weight. However, such changes could also result from interaction of the lysin with extraneous proteases.

There have been many reviews published in recent years covering aspects of interaction of α-lysin with isolated membranes and liposomes (Harshman, 1979; Rogolsky, 1979; Freer and Arbuthnott, 1983; Thelestam, 1983) and this work can be summarized as follows:-

1. The lysin has pronounced surface activity; it spreads as a film at an air-water interface in the absence of lipid (Buckelew and Colacicco,

1971), penetrates mixed lipid films (Freer et al., 1968; Buckelew and Colacicco, 1971) and releases marker molecules from liposomes (Freer et al., 1968).

2. α-lysin can penetrate the hydrophobic region of the membrane; at relatively high concentrations (10-100 μg ml^{-1}) the toxin disrupts liposomes composed of various mixtures of phospholipids and cholesterol, and the interaction with liposomes is accompanied by loss of haemolytic activity, as is the interaction with erythrocyte ghosts (Weissman et al., 1966; Freer et al., 1973; Cassidy et al., 1974). Freeze-etching studies showed that α-12S rings penetrate the hydrophobic region of the platelet membrane (Bernheimer et al., 1972), and that treatment of rabbit erythrocyte membranes with α-lysin induces marked changes in the hydrophobic fracture plane (Freer et al., 1973). The specificity of the lysin for certain membranes does not solely depend on lipid composition of the membrane as Cassidy et al. (1974) found no difference in sensitivity of liposomes prepared from rabbit or human erythrocyte lipids.

Reports concerning specific plasma membrane damage of cultured cells after exposure to highly purified α-lysin are relatively rare. An indication of the type of membrane damage caused to human embryonic lung diploid fibroblasts was provided by the studies of Thelestam and Mollby (1975a,b, 1979). They suggested that α-lysin caused small "functional holes" in the cytoplasmic membrane. More recently, Thelestam (1983) suggested that highly purified α-lysin increased the membrane permeability of epithelial-like cells more effectively than that of fibroblasts. However, the observations on cultured mammalian cells so far do not seem to contradict the idea of surface activity as the main determinant for the lysin's membrane-damaging effect.

On the basis of the surface-active properties of α-lysin, it is

believed by many authors that disruption of susceptible biomembranes may be explained solely by a direct interaction with membrane lipid. However, no variation in sensitivity was detected in liposomes prepared from membrane lipid extracts of erythrocytes of different species (Cassidy et al., 1974) which does not explain why the rabbit erythrocyte is at least a hundred times more sensitive to α-lysin than are other erythrocytes or other cells. This observation, which infers some specificity of the rabbit red cell membrane other than its lipid composition, stimulated a series of studies on the interaction of α-lysin with rabbit erythrocytes aimed at defining the receptor of α-lysin. High affinity toxin-receptors have been reported but their nature remains uncertain (see Table 6). In contrast, Phimister and Freer (1984) found α-lysin binding to both rabbit and horse erythrocytes to be non-specific and not to be affected by concanavalin A. They suggested that the sensitivity of rabbit red blood cells is due to peculiarities in lipid transitions in the membrane rather than to the existence of specific receptors for α-lysin. Also, Thelestam et al. (1983) showed, by use of photolabelling techniques, that α-lysin penetrated into the hydrophobic region of the outer lipid layer of the rabbit erythrocyte-membrane , providing further evidence that α-lysin binds to the erythrocyte membrane, rather than to specific receptors.

Two different binding mechanisms in lysin-membrane interactions, specific and non-specific, were proposed by Maharaj and Packrell (1980) to account for the considerable sensitivity to α-lysin-induced haemolysis of erythrocyte species which apparently lack high affinity receptors on their membranes. Non-specific "hydrophobic" binding by lysin was also recognized by Cassidy and Harshman (1976b). Thus, the mode of membrane interaction of α-lysin with various targets may differ due to different

Table 6. Possible Receptors of Staphylococcal α-Lysin

Nature of Receptor	Pronase digestion sensitivity	References
Protein	+	Cassidy & Harshman (1976a,b,c; 1979)
NAN-Gal-GlcNAc-containing glycolipid and/or glycoprotein	+	Kato (1982)
Band 3 minor glycoprotein	+	Maharaj and Packrell (1980)
Glycophorin	?	Bernheimer & Avigad (1980)

sensitivity of the targets to α-lysin. Speculations on the mode of membrane interaction of α-lysin on various targets have been summarized (Table 7) by Thelestam (1983).

d.Delta-Lysin

1.Production of δ-Lysin

δ-lysin is produced by 97% of pathogenic strains of coagulase-positive staphylococci (Elek, 1959; Jeljaszewicz, 1972), and a high proportion (50-70%) of coagulase-negative isolates (Gemmell et al., 1976).

Two methods have been employed for production of δ-lysin; (1) The cellophane-on-agar technique, and (2) the liquid shake culture in modifications of casein hydrolysate-yeast extract diffusate medium (Gladstone and van Heyningen, 1957). The latter has been commonly employed by recent investigators (Yoshida, 1963; Kayser and Raynaud, 1965; Heatley, 1971; Kapral and Miller, 1971; Kreger et al., 1971; Kantor et al., 1972; Turner, 1978a; Fitton et al., 1980). Optimal conditions of production have not been thoroughly investigated but adequate aeration is important (Yoshida, 1963; Turner, 1978a ; Mollby, 1983).

2.Purification of δ-Lysin

Methods for the purification of δ-lysin have been reviewed recently by Mollby (1983). Some common purification methods have met with little success due to the anomalous behaviour of δ-lysin in aqueous solutions but the lysin has been purified by adsorption of lysin to inorganic matrices of alumina (Kantor et al., 1972) or hydroxylapatite (Kreger et al., 1971), or by exploiting the unusual differential solubility of the lysin in organic solvents of differing polarity (Heatley, 1971).

Although Lee et al. (1976) claimed that δ-lysin purified by the methods of and Kreger et al. (1971) and Kantor et al. (1972) contained

Table 7. Speculations Concerning Modes of Membrane Interaction of α-Lysin on Various Targets*

Target	Specific receptor	Concentrations needed ($\mu g\ ml^{-1}$)	Possible mode of action
Rabbit erythrocyte	Yes	0.1-0.2	1. General hydrophobic membrane perturbation, due to increased local concentration after binding (Harshman, 1979). 2. Transmembrane pores formed by amphiphilic hexamers (Fussle et al., 1981a). 3. Lysin-receptor (band 3) interaction could lead to a change of ion balance across the membrane, which could result in osmotic lysis. (Barzilay et al., 1979). 4a. Lateral redistribution of lysin-receptor-complex, causing membrane disorganization. 4b. Transmembrane destabilization of cytoskeletal elements which link to receptor.
Human erythrocyte, Cultured mammalian cells, liposomes	No	10-100	1. General hydrophobic membrane perturbation (Buckelew and Colacicco, 1971; Freer et al., 1973; Thelestam et al., 1983). 2. Transmembrane pore (Thelestam and Mollby, 1975a,b, 1979; Thelestam 1983).

*Adapted from Thelestam (1983).

varying but low levels of other toxic and enzymic activities, it is important to note that their study was done with a different strain of S. aureus and different procedures for the preparation of the cell-free extract. Similarly, Fackrell and Wiseman (1974) claimed that a sample of δ-lysin provided by Kreger contained both α- and γ-lysins. Using the method of Kreger et al. (1971), Chao and Birkbeck (1978) produced δ-lysin with a high haemolytic specificity towards fish erythrocytes and neither α-, β - nor γ -lysins were detected in this preparation (Birkbeck, personal communicaton). Smith and Shaw (1981) compared three methods of purification of δ-lysin of S. aureus RN 25 : (1) selective desorption from alumina C_γ gel (Kantor et al., 1972), (2) chromatography on hydroxylapatite (Kreger et al., 1971), and (3) the solvent transfer method of Heatley (1971). The method of Heatley (1971) was found to be superior in terms of recovery and purity of the product. More recently, Nolte and Kapral (1981a) reported on purification by adsorption to the hydrophobic gels octyl-sepharose and phenyl-sepharose. Hydrophobic gel chromatography was also used by Fitton et al. (1980) to improve the purity of the product obtained by the method of Heatley (1971).

3. Physicochemical Properties of δ-Lysin

A very wide range of values of molecular weight (2962 to 21 x 10^4) have been reported (Table 8) and the earlier studies suggesting that native δ-lysin was likely to have molecular weight >100,000 probably reflect varying degrees of aggregation of the lysin. The most reliable value for the minimum molecular weight of δ-lysin is 2,962, calculated from the sequence of this 26 amino acid residue peptide from strain 186X (Fitton et al., 1980,1984). Aggregation of monomers in various ways, perhaps in part influenced by cultivation conditions and strain variation may explain the different reported molecular weight and isoelectric points (Table 8).

Table 8. Some Characteristics of Purified Staphylococcal δ-Lysin

Reference	Molecular Weight	Sedimentation coefficient ($S_{20,w}$)	Isoelectric point (pI)
Yoshida (1963)	$6.8\text{-}15\times10^4$	6.1	-
Kayser & Raynaud (1965)	1.2×10^4	1.4; 5.5	-
Hallander (1968)	20×10^4	-	-
Caird & Wiseman (1970)	20×10^4	2.8; 9.8	-
Maheswaran & Lindorfer (1970)	-	-	i) 3.32 ii) 3.75 iii) 8.45
Mollby & Wadstrom (1970)	-	-	9.6
Heatley (1971)	-	4.9	-
Kreger et al. (1971)	-	4.9; 11.9	i) 9.5 ii) 5.0
Kantor et al. (1971)	10.3×10^4	6.04	i) 4.65 ii) 6.7 iii) 9.0
Turner (1978a)	8.2×10^4 1×10^4	-	4.5; 9.5 7.8
Birkbeck & Whitelaw (1980)	21×10^4	-	-
Stearne & Birkbeck (1980)	-	-	4.9
Fitton et al. (1980)	2962	-	-

The "anionic and cationic" forms of δ-lysin reported by Kreger et al. (1971) may also result from electrofocusing conditions as they do not arise when a valme trough is used (Stearne & Birkbeck, 1980).

The amino acid analyses of δ-lysin from several laboratories (Table 9) are in broad agreement, with the exception of Fackrell and Wiseman (1976), and demonstrate the absence of arginine, histidine, proline, cysteine and tyrosine. The high content of hydrophobic amino acids was noted by Bernheimer (1974), who suggested that concentration of these residues in one region of the molecule may result in a polypeptide with linear amphipathicity similar to that found with the melittins (Bernheimer, 1974). However, from the published amino acid sequences of δ-lysin (Fitton et al., 1980,1984), there appear to be no such hydrophobic regions (Fig. 1). Based on the known sequence of δ-lysin, Freer and Birkbeck (1982) proposed that this 26 residue peptide adopts an α-helical rod-like configuration with separate hydrophobic and hydrophilic faces. (Fig. 2). Similar secondary structure analysis led Fitton (1981) to propose that the lysin possesses two helical domains joined by a flexible hinge region and thus has a secondary structure similar to melittin; although no homologies exist between δ-lysin and melittin at the primary structure level, Fitton (1981) claimed that similarities at the secondary and tertiary structures levels between δ-lysin and melittin gave similarities in the cytolytic effects.

δ-lysin also differs from the other lysins of S. aureus in being relatively thermostable (no loss of activity after 80 °C for 1 h; Kreger et al., 1971), highly surface active (Colacicco et al., 1977) and adsorbing quickly and strongly to glass and polypropylene (Heatley, 1971). In addition, δ-lysin is amphipathic, from its ability to dissolve in both

Table 9. Amino Acid Composition of Staphylococcal δ-Lysin

Amino Acid	Percent of total weight of amino acids							
	Yoshida (1963)	Kreger et al. (1971)	Heatley* (1971)	Kantor et al. (1972)	Fackrell & Wiseman** (1976)	Whitelaw (1978)	Turner+ (1978a)	Fitton et al (1980,1984)
Lysine	16.55	16.66	13.60	16.20	2.60	16.60	18.40;12.92	15.6 ; 10.8
Histidine	0.41	-	trace	-	0.40	-	0.97; 1.68	- ; 1.5
Arginine	0.94	-	-	-	2.80	-	1.75; 3.35	- ; 2.3
Aspartic acid	12.53	13.46	10.90	13.60	16.20	17.90	14.85;10.25	16.0 ; 9.6
Threonine	6.98	8.02	7.50	9.40	10.60	10.90	6.94; 4.59	11.4 ; 5.4
Serine	5.12	2.46	3.20	4.30	7.50	2.90	4.33; 3.22	4.6 ; 4.2
Glutamic acid	7.99	4.90	5.20	4.60	7.20	4.80	6.06;13.10	4.2 ; 11.5
Proline	0.45	-	-	-	1.20	-	1.04; 2.22	- ; 2.3
Glycine	3.73	3.60	8.10	6.20	6.30	2.30	3.47; 4.06	4.6 ; 6.5
Alanine	4.06	3.09	5.90	4.80	5.00	2.40	3.72; 5.25	4.2 ; 7.3
Half cysteine	-	0.09	-	-	-	-	0.26; 0.31	- ; -
Valine	4.55	6.64	5.50	6.70	6.60	7.10	6.12; 6.90	8.8 ; 8.1
Methionine	4.88	4.56	4.30	4.00	-	2.20	0.26; 0.19	3.0 ; 3.1
Isoleucine	9.71	18.17	10.50	17.10	9.90	19.60	12.99;10.97	17.1 ; 11.5
Leucine	7.41	6.04	6.30	4.90	5.90	4.30	7.25; 8.92	4.2 ; 8.1
Tyrosine	1.68	-	-	-	1.20	-	3.37; 2.18	- ; 1.5
Phenylalanine	10.40	7.93	7.60	5.50	6.50	5.20	6.64; 8.25	3.8 ; 6.2
Tryptophan	2.61	3.30	4.00	2.50	10.00	3.70		2.7 ; -
Ammonia	1.63	1.08	7.40				1.57; 1.66	

* percentages calculated from the data presented, calculated from E_{280} data assuming 4% tryptophan

** percentages calculated from the data presented, calculated from E_{280} data assuming 10% tryptophan

\+ results of δ-haemolysin preparations from two different strains of S. aureus

Figure 1. The Primary Structure of Staphylococcal δ-Lysin*

C-DHL (MW 2962)

	5				10				15				20				25
f-Met	Ala	Ala	Asp	Ile	Ile	Ser	Thr	Ile	Val	Glu	Phe	Val	Lys	Leu	Ile	Ala	Glu	Thr	Val	Glu	Lys	Phe	Ile	Lys	Lys

	5				10				15				20				25
f-Met	Ala	Gln	Asp	Ile	Ile	Ser	Thr	Ile	Gly	Asp	Leu	Val	Lys	Trp	Ile	Ile	Asp	Thr	Val	Asn	Lys	Phe	Thr	Lys	Lys

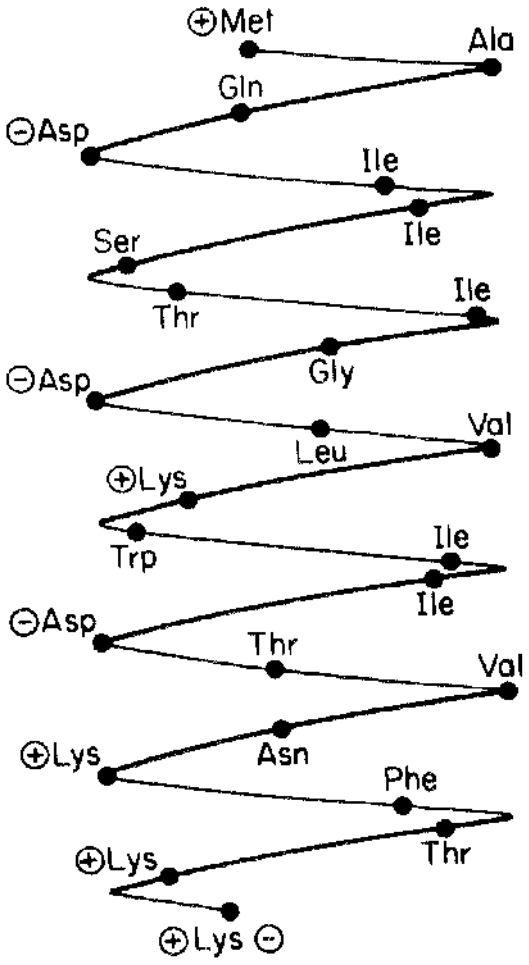
DHL (MW 2962)

* From Fitton et al. (1980, 1984)

C-DHL = Canine δ-lysin

DHL = Human δ-lysin

Figure 2. Diagrammatic Representation of the Proposed α-Helical Structure of δ-Lysin*



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aqueous (Heatley, 1971; Kreger et al., 1971; Colacicco, 1972) and organic solvent (Marks and Vaughan, 1950; Jackson and Little, 1958; Heatley, 1971; Kreger et al., 1971: Colacicco et al., 1977). It was concluded that δ-lysin has the structural characteristics of a membranophilic protein that can behave simultaneously as a lipid and as a protein and readily insert itself into hydrophobic membrane structures (Colacicco et al., 1977).

Neutralization of δ-lysin by normal serum from various animals was first reported by Marks and Vaughan (1950). Formation of a precipitin line has also been found between normal serum and δ-lysin on immunodiffusion (Gladstone and Yoshida, 1967) and immunoelectrophoresis (Kantor et al., 1972). Only recently, Whitelaw and Birkbeck (1978) have identified α- and β-lipoproteins as the inhibitory factors present in normal serum. In addition, δ-lysin is also inactivated by many phospholipids (Kreger et al., 1971; Kapral, 1972; Whitelaw and Birkbeck, 1978) and fatty acids with long chain length (Kapral, 1976).

4. Immunogenicity of δ-Lysin

Early reports on the immunogenicity of S. aureus δ-lysin were conflicting and there was no evidence that non-specific lipoprotein inhibitors had been removed from the sera. More recently, however, the lysin has been shown to be immunogenic in rabbit by Packrell and Wiseman (1974), Heatley (1977), Turner (1978a), Birkbeck and Whitelaw (1976, 1980) and Nolte and Kapral (1981b). The immunogenicity of δ-lysin, normally very poor, may be markedly enhanced by toxoiding with formaldehyde (Stearne and Birkbeck, 1980). Purified anti-δ-lysin IgG has been used in indirect immunofluorescence staining to bind to lysin-treated erythrocytes and ghosts. This indicated both that δ-lysin binds to erythrocyte membranes and that at least part of the antibodies formed were not directed against

the binding sites of the lysin (Nolte and Kapral, 1981b).

5. Biological Activities of δ-Lysin

δ-lysin has a wide haemolytic spectrum (Wiseman, 1970; Jeljaszewicz, 1972) and Gladstone (1966) stated that all erythrocytes tested were susceptible to δ-lysin. Human erythrocytes have generally been used to detect haemolytic activity, but, Chao and Birkbeck (1978) showed that cod erythrocytes are more sensitive than mammalian erythrocytes to the action of δ-lysin (Table 10).

In vitro, δ-lysin exerts a strong cytopathic effect on various cell lines (Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967; Thelestam et al., 1973), leucocytes (Jackson and Little, 1957; Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967) and bacterial protoplasts and sphaeroplasts (Bernheimer et al., 1968; Kreger et al., 1971). Cellular organelles such as lysosomes and mitochondria (Bernheimer and Schwartz, 1964; Gladstone and Yoshida, 1967; Evans and Lack, 1969; Kreger et al., 1971; Kantor et al., 1972; Rahal, 1972) and lipid spherules (Freer et al., 1968; Kreger et al., 1971) are also disrupted by δ-lysin. In addition to being cytotoxic, δ-lysin can also inhibit water absorption (Kapral et al., 1976) and increase cyclic AMP (cAMP) levels in the guinea pig ileum (O 'Brien and Kapral, 1976). The considerably delayed increase in cAMP after administration of the δ-lysin was different from the action of cholera toxin and the mechanisms of elevation of cAMP probably differ for the two toxins. This suggestion was later supported by a complete lack of any cAMP-mediated morphological changes in Y-1 adrenal cells and chinese hamster ovary cells after treatment with δ-lysin (O'Brien and Kapral, 1977). A rapid increase in ion flux suggests that intracellular rather than transcellular movement of

Table 10. Relative Sensitivity of δ-Lysin of Erythrocytes of Different Species

Species	Haemolytic activity compared to that of humans (%)				
	Wiseman (1970)		Kreger et al. (1971)	Kantor et al. (1972)	Chao & Birkbeck (1978)
	Strain Newman	Strain E-Delta	W46M	W46M	NCTC 10345
Human	100	100	100	100	100
Rabbit	25	25	200	50	60
Sheep	25	12.5	40	50	20
Horse	3	6			100
Guinea pig	6	12.5	50		
Pig	12.5	12.5	40		
Calf/ Bovine			40		
Goat			20		
Cat			20		
Chicken			20		
Monkey		50			
Cod					400

ions across the mucosa may be stimulated by δ-lysin (O 'Brien et al., 1978). Since staphylococcal enterotoxin B studied in various intestinal animal models was always found to cause negative reactions in similar ion flux experiments, Carpenter (1980) suggested that δ-lysin is the only staphylococcal exoprotein which may be defined as an enterotoxin.

The toxicity of δ-lysin in vivo has been studied by several workers (Table 11). It must, however, be emphasised that the lethal and dermonecrotic effects observed with the large doses shown in Table 11 could well be due to contamination with α-lysin which is considerably more toxic than δ-lysin. Highly purified δ-lysin also causes erythema when given intradermally in rabbits, but very high doses are required to cause dermal necrosis inside the erythematous zone (Kreger et al., 1971; Turner, 1978b); increase in vascular permeability also results from intradermal injection of this lysin (Birkbeck, 1982).

6. Modes of Membrane Damaging Action of δ-Lysin

Several physicochemical properties of δ-lysin and its interaction with membranes (Table 12) have led to the suggestion that δ-lysin affects membrane by a detergent-like action (Kreger et al., 1971; Bernheimer, 1974; Thelestam et al., 1983). The similarities between δ-lysin and melittin, the major lytic peptide of bee venom, have been noted in several publications (Thelestam and Mollby, 1975a,b; Freer and Arbuthnott, 1976) and there have been suggestions that similarities in properties result from a common mode of membrane damage by these two agents (Bernheimer, 1974; McCartney and Arbuthnott, 1978). The amino acid sequence of δ-lysin (Fitton et al., 1980, 1984) differs from that of melittin, but Fitton (1981) has suggested that the 26 amino acids are ordered in a rather similar way to those of melittin (Dawson et al., 1978). The latter authors postulated that the membrane-damaging mechanism for melittin

Table 11. Effects of δ-Lysin in Animals

Animal	Dose ($mg\ kg^{-1}$) Lethal	Dose (mg) Dermonecrotic	Reference
Mouse	110 (MLD)	-	Kreger et al. (1971)
	10 (LD_{50})	-	Gladstone (1966)
	>4 (LD_{50})	>0.1	Fackrell & Wiseman (1976b)
Rabbit	-	0.5-1.0	Kreger et al. (1971)
	-	≤1	"
	-	0.005-0.5	Gladstone (1966)
	5000 (LD_{50})	-	Wadstrom & Mollby (1972)
Guinea pig	30 (MLD)	0.5-1.0	Kreger et al. (1971)
	-	≤1	"
	>4 (LD_{50})	>0.1	Fackrell & Wiseman (1976b)

Table 12. Characteristics of δ-Lysin and its Interaction with Membranes Suggesting a "Detergent-Like" Mode of Action*

Characteristics	References
1. High content of hydrophobic amino acids	Fitton et al. (1980, 1984)
2. High degree of surface activity	Colacicco et al. (1977)
3. Rapid and temperature-independent membrane damaging effect	Thelestam et al. (1973)
4. Low specific cytolytic activity	Bernheimer (1974)
5. Broad spectrum of cytolytic activity	Bernheimer (1974)
6. Inhibition of cytolytic activity by phospholipids	Kapral (1972)
7. Ability to remove some membrane proteins	Thelestam et al. (1983)

* Adapted from Thelestam (1983).

involves initial ionic binding to the acidic groups on membrane surface followed by a configurational change, possibly induced by the low dielectric constant at the immediate surface of the membrane, which results in a wedge-like insertion of the molecule into the hydrophobic region of the membrane. A considerable body of evidence suggests that membrane damage caused by δ-lysin differs from that caused by melittin. Fitton (1981) proposed that δ-lysin exists as tetramers or oligomers under physiological conditions whereas melittin is found as monomers or tetramers in aqueous solution (Habermann, 1972; Gauldie et al., 1976). According to the patterns of leakage of differently sized cytoplasmic markers induced from prelabelled human lung fibroblasts by these two proteins, Thelestam and Mollby (1979) classified them in different groups and suggested that neither δ-lysin nor melittin could be assigned to the detergent-group comprising true detergents such as tritons, SDS and sodium deoxycholate.

Also, Durkin and Shier (1980) observed that the susceptibility of 3T3 mouse fibroblasts to δ-lysin-induced lysis was cell-cycle dependent; synchronized populations of cells in mitosis and early G1 phase exhibited a resistance to δ-lysin at concentrations which were lytic to interphase cells (up to 80μg ml^{-1}). However, melittin was reported by the same authors to lyse mitotic and interphase 3T3 cell with equal efficiency. More recently, Bhakoo et al. (1982) observed that δ-lysin showed little or no specificity in its interactions with all types of lipid films studied, whereas melittin showed preferential interaction with films of acidic lipid.

In 1982, Freer and Birkbeck noted the periodicity of distribution of charged residues in δ-lysin and suggested that δ-lysin adopts an α-helical rod-like configuration with separate hydrophilic and hydrophobic faces.

Association of 6 such monomers in a cell membrane may result in the formation of a transmembrane "pore" lined by the hydrophilic faces of the monomers. Such transmembrane pores could constitute the primary membrane damage, permitting leakage of low-molecular weight markers such as α-amino isobutyric acid (Freer, 1982). This marker was shown by Thelestam and Mollby (1979) to be released rapidly from human diploid fibroblasts treated with δ-lysin. It is conceivable then that the pores could coalesce when present in a large enough number, permitting the escape of higher molecular weight markers and finally disrupting the membrane integrity (Freer et al., 1984) (Fig. 3).

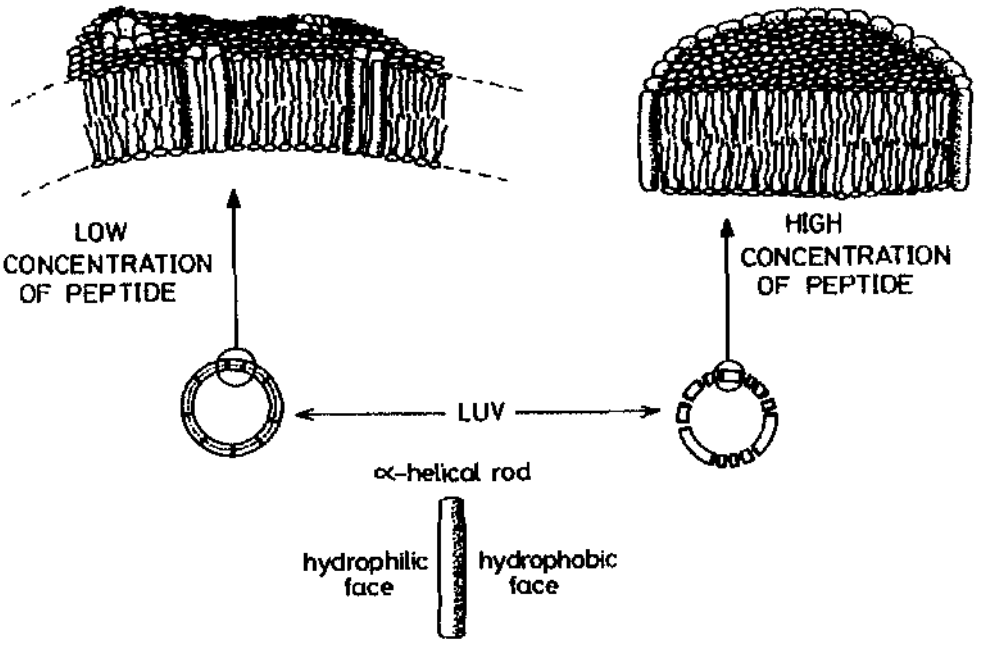
An alternative mode of action for δ-lysin was suggested by Shier (1979) who proposed that self-destruction of the target membrane was a mechanism of action of several cytolytic toxins including δ-lysin and melittin. Activation of endogenous phospholipase A_2 due to the membrane perturbing action of δ-lysin at the plasma membrane could lead to membrane destruction by (1) a direct breakdown of structurally important membrane phospholipids and (2) the production of lysolecithin which in itself exerts a detergent action on the membrane. These observations were supported by those of Umezawa et al. (1980) who found that δ-lysin at concentrations of 1-10 μg ml^{-1} (no cytolytic effect) rapidly inhibited the binding of epidermal growth factor (EGF) to its cell surface receptors in rat embryo fibroblast and in HeLa cells and induced arachidonic acid (hydrolysis of lecithin leads to the production of arachidonic acid and lysolecithin) release.

C. PROTEIN SECRETION ACROSS MEMBRANE

a. Historical Introduction

The basic principles and details of protein synthesis have long been

Figure 3. Hypothetical Scheme for Mechanism of Membrane Pertubation by δ-Lysin*



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LUV = Large Unilamellar Vesicles

established and in recent years there has been increasing interest in the mechanism of protein secretion. This began with the discovery (Palade, 1955) that animal cells contain membrane-bound ribosomes as well as ribosomes free in the cytoplasm. Subsequently, various secretory proteins were shown to be synthesized on the bound ribosomes and various cytoplasmic proteins on the unbound ribosomes (reviewed by Palade, 1975). This distribution suggested that secreted proteins might cross membranes as growing chains rather that after completion of synthesis. Such a mechanism was supported by the observation of Redman and Sabatini (1966) that even incomplete polypeptide chains were not located on the cytoplasmic side of the membrane. Sabatini and Blobel (1970) showed that microsomes protected nascent chains of secretory but not of cytoplasmic protein from proteolytic degradation and the concept that the growing polypeptide chain is translocated through the membrane of the endoplasmic reticulum during polypeptide synthesis was then recognized. These authors also suggested that the mRNA codes for a special sequence near the beginning of the polypeptide chain which might be recognised by a membrane-binding factor; the chain would then be secreted whilst still growing (Blobel and Sabatini, 1971).

The attachment of bound polysomes to membranes via the amino-terminal region of the growing polypeptide chain was first shown by Milstein et al. 1972) in their studies on the translation of immunoglobulin light chain mRNA in a cell-free system. They showed that a larger precursor containing extra amino acids at the NH_2-terminus was synthesized in the rabbit reticulocyte lysate but not in the presence of microsomal membranes. This finding was later confirmed by Mach et al. (1973) and extended by Schechter (1973) who presented the first data on the amino acid sequence of the NH_2-terminal segement. Milstein et al. (1972) had already used the

term "signal" for this region and speculated about its possible role in binding polysomes to the endoplasmic reticulum shortly after the onset of protein synthesis. Thus, these assumptions were raised to the status of a widely accepted scientific theory through the work of Blobel and Dobberstein (1975a,b) who presented the decisive experimental data which led to the formulation of the signal hypothesis with the following predictions : (1) secretory polypeptides contain an amino-terminal signal peptide that binds to the endoplasmic reticulum and initiates vectorial discharge (transmembrane translocation) of the growing polypeptide chain; (2) this transport must start early, before the chain reaches a certain length, and be strictly coupled to protein synthesis (co-translational secretion), and (3) signal peptides are transient entities that are cleaved before chain termination. Cotranslational secretion, although widespread, is not the only mechanism for protein export. Later studies on the biosynthesis of some eukaryotic nuclear DNA-specified proteins destined for localization in mitochondria (Neupert and Schatz, 1981; Schatz and Butow, 1983), chloroplasts (Dobberstein et al, 1977) and peroxisomes (Robbi and Lazarow, 1978; Goldman and Blobel, 1978), led to the conclusion that transfer across membranes can also occur after completion of polypeptide chains by post-translational mechanism (post-translational secretion).

Despite the fact that bacteria are generally considered to be simple organisms, investigators working with eukaryotic systems have led the field with respect to biochemical studies. Nevertheless, both cotranslational and post-translational secretion have been directly demonstrated in bacteria (Davis and Tai, 1980). Eukaryotes and prokaryotes appears to share some common mechanism of protein export as shown when the gene for a eukaryotic secreted protein, preproinsulin, was introduced into E. coli.

The eukaryotic signal sequence of preproinsulin promotes transfer of the polypeptide across prokaryotic cytoplasmic membrane (Talmadge et al., 1980b) and the signal peptide is cleaved at the correct position to yield proinsulin (Talmadge et al., 1980a). Ovalbumin may also be secreted by E. coli (Fraser and Bruce, 1978). In the reverse situation, when the gene for a prokaryotic β-lactamase was introduced into yeast cells, the β-lactamase was also correctly processed (Randall et al., 1978). The use of bacterial systems has allowed new approaches to the study of protein secretion based on the accessibility of the relevant bacterial membrane surface and the isolation of bacterial mutants altered in secretion.

Knowledge of signal sequences for secretion has important practical applications and to increase the production of insulin in recombinant bacteria, the insulin gene was fused with the gene for the E. coli periplasmic β-lactamase (Villa-Komaroff et al., 1978). More recently, Netzer (1983) suggested that further work on protein export will lead to new methods of immunization by placing a functional enzyme or functional fragment of an enzyme onto the surface of E. coli and develop a "biocatalytic bed".

b.Models for Protein Export

Various models for the mechanism of protein export have been presented (Blobel and Dobberstein, 1975a,b; DiRienzo et al., 1978; Von Heijne and Blomberg, 1979; Wickner, 1979), but it is unlikely that a single model will be applicable to the export of all bacterial proteins. According to the signal hypothesis (Blobel and Dobberstein, 1975a,b), a protein destinated to be secreted from cells is synthesized initially as a larger precursor with 15 to 30 additional amino acids at the NH_2-terminal end of the molecule; this peptide extension (signal sequence) initiates binding

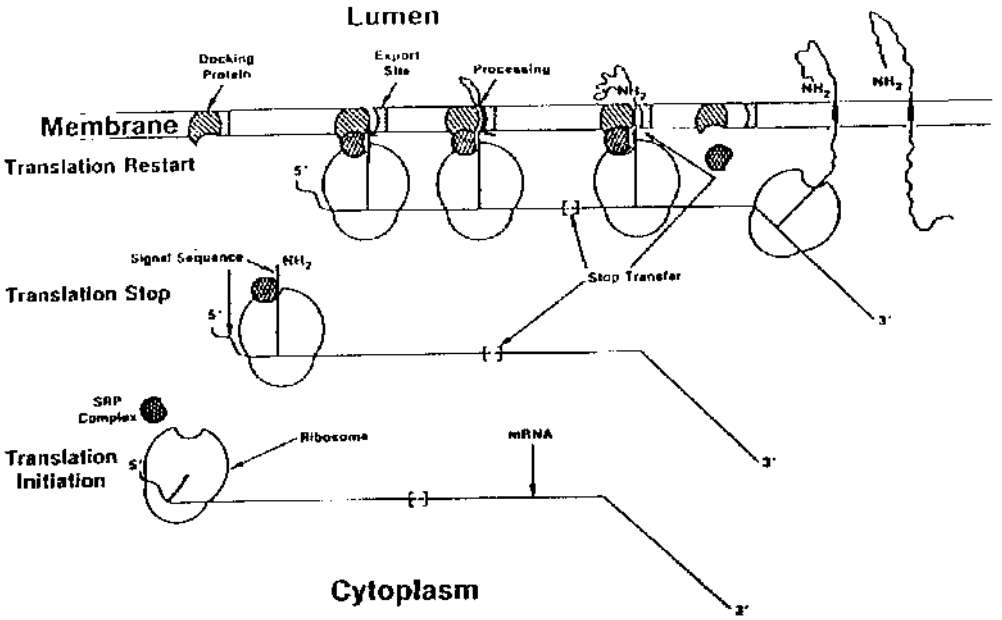
of the translation complex to the membrane, resulting in the formation of a transient pore through which the nascent peptide chain passes as synthesis proceeds. At some point the signal sequence is cleaved off, and the mature protein ultimately folds outside the cell (Fig. 4). Further, the existence of "stop-transfer" sequences interrupting the translocation process has been postulated for transmembrane proteins (Blobel, 1980). A very different model which does not require an ordered vectorial export process nor any special cellular export machinery has been proposed by Wickner (1979). This "membrane trigger" hypothesis emphasizes the role of self-assembly of proteins in mediating their own transport. It allows for synthesis of a complete protein molecule before its export begins. The basic function of the signal sequence is to promote the folding of the newly made precursor into a conformation that is soluble and competent for export. Upon reaching the membrane the protein is "triggered" into a new conformation that allows it to spontaneously insert into or through the phospholipid bilayer unaided by any sort of pore. Cleavage of the signal sequence would facilitate this conformational change and render it irreversible. Whereas the loop model of DiRenzo et al. (1978) and the direct transfer model of Von Heijne and Blomberg (1979) are different from the original signal hypothesis, it boil down to the question of whether the nascent chain does or does not encounter a non-polar (hydrophobic) environment during its passage through the membrane.

c. Structure and Function of Signal Sequence

1. Demonstration of Precursor Proteins

Since the precursor form of an exported protein contains extra amino acids at the amino terminus, its molecular weight is greater than that of the corresponding mature protein. Thus, precursor species can be detected

Figure 4. A Schematic Illustration of Signal Hypothesis (Co-Translational Export)*



* Reproduced with permission of T.J.Silhavy, S.A.Benson and S.D.Emr. Microbiol. Rev. 47 (1983) p316.

by SDS-PAGE, which separates proteins according to size. In general, four methods have been used in bacterial systems to demonstrate precursor forms of exported proteins (Table 13). In eukaryotic system, ovalbumin, the major secretory protein of hen oviduct, is the only well-characterized secretory protein to be synthesized and secreted without a transient signal sequence (Palmiter et al., 1978) and an internal signal-like sequence may be located within the molecule (Lingappa et al., 1979; Meek et al., 1982). In E. coli, colicin E1 colicin E3 and colicin E3 immunity protein are reportedly secreted in the form in which they are synthesized (Jakes and Model, 1979; Yamada et al., 1982) and Yamada and Nakazawa (1984) showed that colicin E1 contain internal signal-like sequence near the COOH-terminus.

Almost all characterized periplasmic and outer membrane proteins of E. coli contain an NH_2-terminal signal sequence that is removed during the export process (Michaelis and Beckwith, 1982; Silhavy et al., 1984). In contrast, inner membrane proteins present a more complicated picture with respect to NH_2-terminal signal sequence. The major and minor f1 (M13) coat proteins (Schaller et al., 1978; Sugimoto et al., 1977), penicillin-binding proteins PB5 and PB6 (Pratt et al., 1981) and inner membrane protein Tsr (Boyd et al., 1983) are apparently made in precursor form. However, the lactose permease (Ehring et al., 1980) and two subunits (b and c) of the Fo complex of ATPase (Nielsen et al., 1981) are not made in precursor form.

2. Structure of Signal Sequences

Signal peptides vary considerably in both length and structure (Kreil, 1981) but when the degree of polarity is considered there is a striking similarity among all signal peptides. Garnier et al (1980), in an analysis of the amino acid sequences of 22 signal peptides, found all

Table 13. Methods Used in Bacterial System to Demonstrate Precursor Forms of Exported proteins*

Method	Protein+	Reference
A. In vivo pulse-labelling	λ receptor protein	Josefsson & Randall (1981a)
	Maltose-binding protein	Josefsson & Randall (1981b)
	TEM-1 β-lactamase	Koshland & Botstein(1982)
	Enterotoxin B of S.aureus	Tweten & Iandolo(1983)
B. Invitro synyhesizing system		
1. transducing Φ or plasmid containing the cloned structure gene	Alkaline phosphatase	Inouye & Beckwith(1977)
	Penicillinase of B.licheniformis	Sarvas et al.(1978)
2. membrane-bound polysome	Maltose-binding protein	Randall et al.(1978)
	Arabinose-binding protein	Randall et al.(1978)
3. RNA extracts	Diphtheria toxin of C.diphtheriae	Smith(1980)
C. Mini or maxicells system	TEM-1 β-lactamase	Koshland & Botstein(1982)
	Protein H	Oudega et al.(1984)
D. Inhibitors		
1. agents altering membrane: fluidity		
i. ethanol	Exotoxin A of P.aeruginosa	Lory et al.(1983)
ii. phenethyl alcohol	Matrix protein	Halegoua & Inouye(1979)
iii. procaine	Alkaline phosphatase	Lazdunski et al.(1979)
iv. growth of a fatty acid auxotroph in the presence of eliadate	Tol G	DiRienzo & Inouye(1979)
2. energy uncouplers: (such as carbonyl cyanide m-chloro-phenylhydrazone or dinitrophenol)	α-Lysin of S.aureus	Tweten et al.(1983)
	Leucine-specific binding protein	Daniels et al.(1981)
	β-lactamase	Daniels et al.(1981)
	Coat protein of M13 coliphage	Date et al.(1980)
	B subunit of heat-labile enterotoxin	Palva et al.(1981)

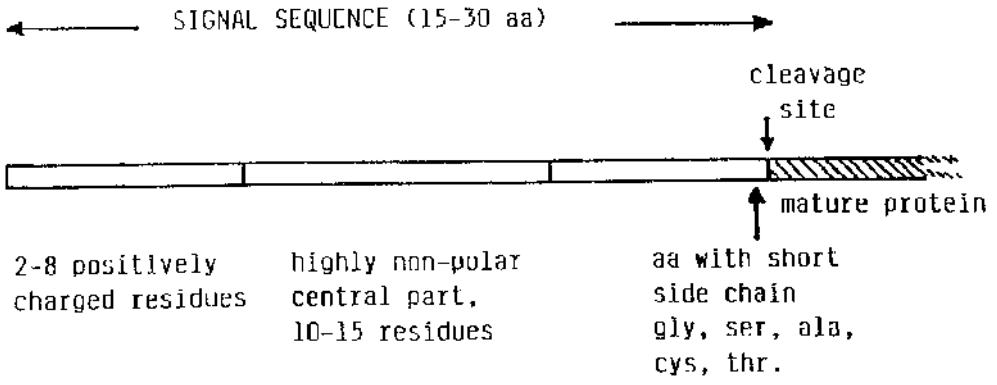
* This listing is not intending to be exhausive for precursor forms of exported proteins listed in the method used.

+ E.coli proteins unless otherwise stated

signal peptides to contain a central region of hydrophobic residues with a minimal length of about 9 residues. A sample of 34 hydrophobic core-segments from known signal peptides have been analysed by Von Heijne (1982) with regard to the mean hydrophobicity at each position in the segment. These core-segments were not uniformly hydrophobic; instead, the more hydrophobic residues were concentrated around the midpoints of the segments. A similar analysis of 12 membrane-spanning segments from transmembrane proteins indicated that the hydrophobic residues were much more evenly distributed along the segment (Von Heijne, 1982). A decrease in hydrophobicity, achieved by replacing leucine with β-hydroxyleucine in the *in vitro* translational system, resulted in an inhibition of translocation and processing of the growing polypeptide chain (Hortin and Boime, 1980). Also, Emr *et al.* (1980) identified a series of mutations within the signal peptide of the phage λ receptor, which prevented the export of the protein and led to accumulation of the precursor in the cytoplasm. Sequence analysis revealed that most of these mutations resulted from single amino acid substitutions, usually the exchange of a hydrophobic residue for a charged one. Similar results were obtained from the maltose binding protein (Bedouelle *et al.*, 1980), underlining the importance of extended regions of uncharged residues in initiating membrane penetration.

Most signal peptides also contain, besides the α-amino group, at least one or more charged residue(s) near the end, frequently an arginine or lysine. Moreover, the last residue before the cleavage site always has a short side chain (glycine, serine, alanine, cysteine or threonine) (Inouye and Halegoua, 1980). The common features in primary structure of signal peptides are shown in Fig. 5.

Figure 5. A Schematic Illustration of the Common Structural Features of Signal Sequences



Attempts have also been made to look for common elements in the secondary structure of signal peptides and some similarities emerged for the study of 22 signal peptides by Garnier et al. (1980), who claimed that the signal peptides exhibited a high potential for making α-helix and, to a lesser degree, extended or β-sheet conformation. Rosenblatt et al. (1980) also proposed that the signal part is entirely α-helical except for a β-turn near the carboxyl end. Several conclusions can be drawn from the similarities of predicted secondary structures. Firstly, it is likely that the cleavage site is governed by the secondary structure of the signal sequence. Secondly, at the time that cleavage takes place folding of the amino-terminal portion of the nascent chain has already taken place. Lastly, the potential of the central residues for adopting α- or β-structure may be important in the way that interaction with the membrane occurs.

3. Interaction between Membrane and Signal Peptide

Several models have been proposed to relate the structure of the signal peptide to its function. One of these, the loop model of Inouye and Halegoua (1980), proposes that the basic, positively-charged amino-terminal region of this peptide allows attachment of nascent precursor to the negatively-charged inner surface of the cytoplasmic membrane by ionic interaction. Subsequently, the hydrophobic core region can insert into the hydrophobic interior of the bilayer, forming a loop structure that initiates the export process. Other investigators (Bretscher, 1973; Wickner, 1979; Engelman and Steitz, 1981; Von Heijne and Blomberg,1979; Garnier et al., 1980; Steiner et al., 1980) also postulated a primary interaction of the signal sequence (due to its hydrophobic nature) with the lipid bilayer rather than with specific protein receptors, although it may rely on the participation of proteins. More recently, Hall and Schwartz

(1982) described a unique mutation affecting the hydrophilic segment of the λ-receptor signal sequence and suggested that the hydrophilic portion of the signal sequence mediates the initial interaction between a nascent secreted protein and the membrane. In addition, they proposed the existence of a so-called "stop translation" sequence early in the precursor that extends past the signal sequence. Once synthesized, it binds to the ribosome and prevents further translation unless export is initiated. The role of this sequence is to stop translation in order to allow sufficient time for the hydrophilic portion of the signal sequence to initiate export by interacting with the membrane.

The alternative view requires the existence of specific protein assemblies within the lipid bilayer which are responsible for initial binding of signal peptide and for facilitating the motion of protein across the membrane during protein synthesis (Blobel and Dobberstein, 1975 a,b; Blobel, 1980). Prehn et al (1980) have shown that preproinsulin, but not proinsulin, bind to stripped membranes of the rough endoplasmic reticulum and that this binding can be abolished by pretreatment of membranes with protease. This agrees with earlier observations made by Warren and Dobberstein (1978) and Walter et al. (1979) that translocation is highly susceptible to protease treatment and/or high salt washes. Furthermore, competition experiments (Lingappa et al., 1978) have shown that different secretory proteins compete for the translocation machinery of endoplasmic reticulum. In 1980, Walter and Blobel purified an 11S protein complex from dog pancreas microsomal membrane. This complex, called signal-recognition protein (SRP, later renamed the signal-recognition particle; Walter and Blobel, 1982) has been shown to function by binding to the signal sequence as it emerges from the ribosome and

halting futher translation (Walter and Blobel, 1981 a,b; Walter et al., 1981). This translational block is relieved when the complex (ribosomal, SRP and nascent polypeptide chain) interacts with the membrane-associated docking protein at the export site (Meyer et al., 1982). They claimed that the initial events leading to translocation and providing its specificity do not involve protein-lipid interaction (signal sequence - lipid bilayer), but protein-protein interactions (signal sequence - ribosome - SRP - docking protein) (see Fig.4). Very recently, Kumamota et al. (1984) speculated upon the occurrence of an SRP-like model in bacteria.

d. Ribosome Binding and Export Machinery

The binding of ribosomes to the membrane of the endoplasmic reticulum through their large subunits is well documented (Palade, 1975; Unwin, 1979). As depicted in the signal hypothesis, ribosome binding would occur shortly after interaction of the membrane with the signal peptide. Two proteins, ribophorins I and II have been invoked as being responsible for ribosome binding and are only present in the rough endoplasmic reticulum (Kreibich et al., 1978a,b). A functional attachment was also suggested by the finding that the membrane-bound polysome fraction of E. coli produces more of a secretory protein (alkaline phosphatase) than does the free fraction (Cancedda and Schlessinger, 1974). Similarly, Randall and Hardy (1977) prepared membrane-polysome fractions whose products were enriched in several secreted or incorporated proteins, while containing very little cytoplasmic protein. Recently, Hall and Schwartz (1982) proposed that the ribosome was simultaneously pulled towards the membrane after the initial contact of the hydrophilic portion of signal sequence with the membrane receptor, and that the hydrophobic region of the signal

sequence begins melting into the hydrophobic environment of the membrane.

The chemical nature of the pore or tunnel through which the growing polypeptide emerges is also a matter of conjecture. Blobel and his collaborators clearly favour a tunnel formed through the assembly of membrane proteins, envisaging that binding of signal peptide and ribosome somehow favours interaction among membrane proteins, which leads to the formation of a hole (Blobel and Dobberstein, 1975a,b). An alternative view, on purely theoretical grounds, has been presented by Von Heijne and Blomberg (1979). Based on thermodynamic considerations, they calculated that it is energetically feasible for proteins to go directly through the bilayer without a proteinaceous pore. For this direct transfer to be accomplished, it was hypothesized that energy for the process is supplied by interaction between ribosomes and a membrane protein. Experimental results obtained by Wickner (1979) favoured this model. In 1981, Engelman and Steitz proposed an even simpler theoretical scheme in which proteins pass directly through the membrane without the participation of ribosome or membrane proteins. However, recent biochemical and genetic data point to an important role both for ribosomes and for membrane components in mediating the export of many E. coli proteins (Michaelis and Beckwith, 1982).

e. Signal Sequence Processing

The signal peptide is cleaved prior to polypeptide chain completion in a cotranslational reaction taking place on the growing polypeptide chain (Blobel and Dobberstein, 1975a,b). This signal peptidase, as proposed by Blobel and Dobberstein (1975a,b), is an integral membrane protein, closely associated with the pore in the membrane or forming an integral part of the pore, and their premise was based on the fact that cleavage by signal

peptidase is intimately linked to chain transit and occurs on nascent but not on completed chains. Moreover, Palmiter et al. (1977) estimated that the cleavage of prelysozyme to lysozyme occurs at a chain length of only about 60 residues; this would indicate that binding of the signal peptide and the ribosome to the membrane, formation of the pore, and cleavage of the signal peptide all take place very early in the synthesis of a secretory polypeptide. However, in an elegant study, Josefsson and Randall (1981a,b) have shown that some proteins are processed both co-translationally and post-translationally in vivo. For all these proteins, removal of the signal peptide is a relatively late event occurring after these polypeptides have been elongated to at least 80% of their final length, revealing a great complexity in the export process.

The precise cellular location of signal peptidase is not clear. Genetic studies reviewed by Silhavy et al. (1983) demonstrate that the processing activity is located outside the cytoplasmic membrane. Signal peptidase has been solubilized from the membrane by detergent treatment (Jackson and Blobel, 1977; Kreil et al., 1980) and then reconstituted into vesicles. Some inhibitors of protease were found to have no effect on the enzyme (Strauss et al., 1980). The detection of signal peptides after their cleavage from the polypeptide chain has been attempted in several eukaryotic systems, but no clear results were obtained (Jackson and Blobel, 1977; Patzelt et al., 1978; Habener et al., 1979), suggesting that cleaved signal peptides must be very short lived in vivo and in vitro and must be rapidly hydrolysed. This rapid degradation could be important, since the hydrophobic nature of signal peptides might otherwise have an adverse influence on the properties of the membrane. However, Chang et al (1978) detected not only the cleavage of precoat protein, but also varying amounts of the cleaved signal peptide. Such results have not

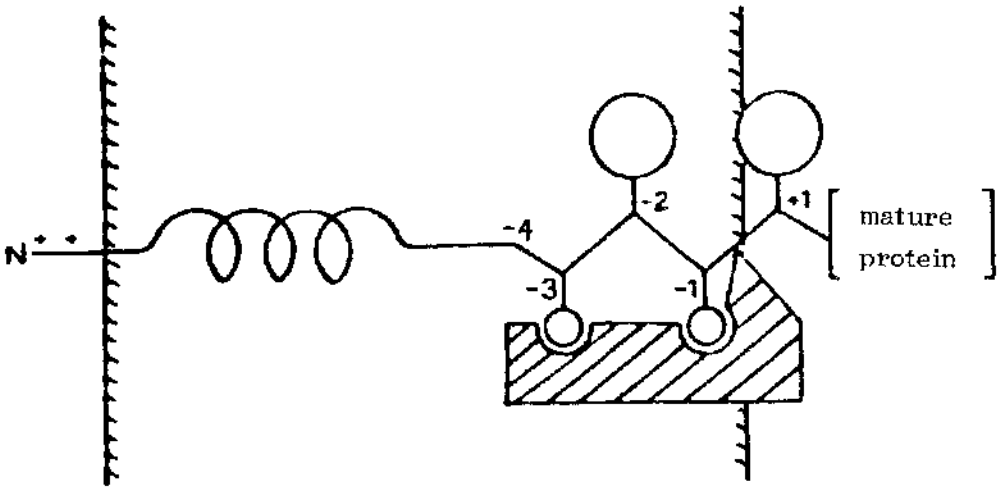
yet been achieved for any eukaryotic system. Possibly, further hydrolysis of bacterial signal peptides by "signal peptide hydrolase" proceeds at a slower rate. Bennett et al (1980) suggested that bacterial factors, which stimulate neutrophils during inflammatory response, may be derived from signal peptides released during synthesis of extracytoplasmic polypeptides. As they suggested, signal peptides would be excellent candidates for the natural bacterial chemotactic factors as they contain predominantly hydrophobic amino acids with an N-terminal f-Met. The released peptides would form a heterogenous population due to further proteolysis.

Zwizinski and Wickner (1980) reported a 6000-fold purification of E. coli signal peptidase. The endopeptidase also processes the precursor forms of several other exported proteins in vitro (Wolfe et al., 1982; Zwizinski et al., 1981). Recent structural studies have shown that this protein spans the membrane with a small N-terminal domain exposed on the cytoplasmic surface and a large C-terminal domain exposed to the periplasm (Wolfe et al., 1983). Studies of the biogenesis of this signal peptidase in vivo revealed that the peptidase is made without a cleaved signal peptide (Wolfe and Wickner, 1984). Based on a sample of 78 eukaryotic signal sequences, Von Heijne (1983) suggested that the region -5 to -1 of the signal sequence conferred processing specificity, and the proposed signal-sequence-protease structure is shown in Fig. 6.

f. Post-translational Modifications

Most eukaryotic proteins exported from the cytoplasm through the Palade pathway (Jamieson and Palade, 1967a,b) are glycoproteins (Silhavy et al., 1983) and they are usually modified post-translocationally by acylation (Schlesinger, 1984) of some amino acid residues with palmitic (and possibly other fatty) acid(s). Similar post-translational

Figure 6. A Schematic Representation of the Proposed Signal Sequence-Protease Complex*



* Reproduced with permission of G.Von Heijne.
Eur. J. Biochem. 133 (1983) p20.
The signal sequence spans the membrane as a "helix + sheet" structure. The small, neutral residues in position -1 and -3 fit into a pocket in the protease, thereby defining the cleavage site between position -1 and +1.

modifications have also been reported in prokaryotic system. Perhaps the most extensively modified protein in E. coli is the outer membrane lipoprotein (Inouye and Halegoua, 1979). To the N-terminal cysteine residue ,a diglyceride is attached by a thioether linkage and a fatty acid is attached by an amide linkage. The C-terminal lysine residue is covalently linked by the ε-amino group to peptidoglycan to form the bound form of the protein. E. coli also produces several other proteins localized in the inner and outer membrane that are modified in a similar manner (Ichihara et al., 1981).

Amongst gram-positive organism, the penicillinases of B. licheniformis, B. cereus and S. aureus are related in structure and cellular localization by retaining a substantial proportion as hydrophobic membrane-bound forms. Recently, Nielsen et al (1981) showed that for the membrane-bound form of B. licheniformis penicillinase, attachment was achieved through a glyceride thioether modification identical to that found in several gram-negative outer membrane proteins. That the membrane penicillinases of S. aureus and B. cereus also possess such modification was also reported by Nielsen and Lampen (1982). The complete event of such modification has been proposed by Chang et al. (1982). By comparing the modification-susceptible signal sequences of gram-positive penicillinases and of gram-negative outer membrane proteins with those of non-modified gram-negative penicillinases, Nielsen and Lampen (1982) described a possible structure requirement within the signal sequence for the addition of the glyceride modification.

OBJECT OF RESEARCH

Although the physicochemical properties of both α-lysin and δ-lysin have been thoroughly investigated, the modes of secretion have received relatively little attention. Many secretory and membrane proteins are initially synthesized as precursor molecules in which an extra hydrophobic peptide segment precedes the N-terminus of the mature protein. The primary objective of this research was to investigate the export of these lysins.

The specific objectives of this investigation were :

1. to investigate the relationship between δ-lysin production and growth phase in the superproducing strain of *S. aureus* (strain NCTC 10345) and the metabolism of δ-lysin mRNA *in vivo*.
2. to obtain *in vitro* synthesized δ-lysin by translation of total cellular RNA of *S. aureus* NCTC 10345 in the *E. coli* S-30 extract and compare it with the native δ-lysin in SDS-PAGE.
3. to study the effect of a membrane perturbing agent, phenethyl alcohol (PEA) on the production of exoproteins of *S. aureus* Wood 46.
4. to detect any precursor of α-lysin in the membrane of PEA-treated *S. aureus* Wood 46.

MATERIALS AND METHODS

A. ORGANISMS

Staphylococcus aureus strain NCTC 7121 (Wood 46) was used in work which involved α-lysin and S. aureus strain NCTC 10345 was used for work with δ-lysin.

B. MEDIA

A yeast extract enriched medium modified from that of Bernheimer and Schwartz (1963), and known as B.S. Medium (See Appendix I), was used throughout for the growth and lysin production of staphylococci.

C. MAINTENANCE OF CULTURES

The organisms, which were originally taken from freeze-dried ampoules, were reconstituted in sterile saline before plating on to blood agar. Cultures were maintained by passage on freshly prepared nutrient agar (Oxoid Ltd., London, U.K.) slopes at monthly intervals with routine gram staining to confirm culture purity.

D. CULTURAL CONDITIONS

It was essential to subculture both strains on 10% (v/v) horse blood agar (Oxoid Blood Agar Base No. 2, Oxoid Ltd., London, U.K.) plates before cultivation in broth. Plates were inoculated from a loopful of stock culture, and incubated overnight at 37 °C. After incubation, 50ml of B.S. Medium in a 250 ml flanged Erlenmeyer flask was inoculated as a starter culture from an isolated colony which showed good haemolytic activity. After incubation overnight at 37 °C on an orbital shaker operating at 150 cycles min^{-1}, the starter culture was added as a 5% (v/v) inoculum to aliquots of pre-warmed B.S. Medium and incubation was continued at 37 °C.

E. MEASUREMENT OF BACTERIAL GROWTH

Growth was estimated by measuring the A_{650nm} of samples of culture withdrawn at 1 h intervals; where A_{650nm} exceeded 0.40 the culture was diluted with B.S. Medium to bring the absorption value to within the range of 0.00 - 0.40. Absorption values were measured on a MSE Spectro-Plus spectrophotometer. Uninoculated B.S. Medium served as a blank.

F. BIOCHEMICAL ASSAYS

a. Assays of Haemolytic Activity

1. Haemolytic Assay Buffers

α-lysin : PBS (Dulbecco A, Oxoid Ltd., London, U.K.) pH 7.3 (Appendix II).

δ-lysin : CDS containing 12.5% (v/v) DMSO (see Appendix II).

2. Standard Erythrocyte Suspensions

Rabbit or cod erythrocytes were washed in the appropriate buffer (PBS and 12.5% DMSO in CDS respectively) 4 times and the packed cell volume determined by centrifugation in graduated conical centrifuge tubes (MSE Scientific Instruments, Crawley, U.K.) at 540 g for 10 min at room temperature (MSE Super Minor). From the packed cell volume the erythrocytes were resuspended to a final concentration of 1% (v/v) in the appropriate buffer.

3. Doubling Dilution Titration

The haemolytic titres of lysin samples were determined by making serial doubling dilutions of lysin in 0.1 ml amounts of buffer in wells of WHO haemagglutination trays, and addition of 0.1 ml of standard erythrocyte suspensions to each well. Wells without lysin served as controls. The trays were incubated for 30 min at 37 °C (rabbit blood) or at room

temperature (cod blood). The dilution of lysin give 50% haemolysis was estimated visually and this amount of lysin was defined as constituting one haemolytic unit (HU_{50}).

b. Protein Estimation

Protein estimations were carried out by the method of Bradford (1976) with BSA (Fraction V, Sigma London Chemical Co. Ltd., Poole, Dorset.U.K.) as a standard. The protein content in the total cellular RNA extract was monitored by measuring A_{280nm} using a Pye Unicam SP 8-100 ultraviolet spectrophotometer. Distilled water served as a blank.

c. Assay for Staphylococcal Extracellular Protease

The method used was modified from that of Charney and Tomarelli (1947) and Tomarelli et al. (1949). Azocasein (Sigma) was dissolved in PBS at a concentration of 25 mg ml^{-1}. The standard protease used for reference was subtilisin (EC 3.2.21.14, Type VII from Bacillus amyloliquifaciens, Sigma) which was diluted in PBS over the range 5-25 $\mu g\ ml^{-1}$. To 0.3 ml of substrate (azocasein) was added 0.3 ml standard protease or test samples. The assay mixtures were incubated at 37°C for 30 min and the reaction was then stopped by addition of 2.4 ml chilled 5% (w/v) TCA. The unhydrolysed substrate was collected by centrifugation (860 g, 10 min at room temperature) and 2 ml of the supernatant fluid was removed and added to 2 ml 0.5N sodium hydroxide. The A_{440nm} was measured against a PBS blank using an MSE Spectro-Plus spectrophotometer. A standard curve was drawn of A_{440nm} against standard protease concentration, from which the protease content of the samples was estimated.

d. RNA Estimation

RNA estimations were carried out by the method of Albaum and Umbreit

(1947) as modified by Ogur and Rosen (1950). Samples were adjusted to 3 ml with distilled water, equal volumes of $FeCl_3$ reagent (0.1% (w/v) $FeCl_3$ in conc. HCl) and orcinol reagent (10% (v/v) orcinol in 95% (v/v) ethanol) were added and the samples heated in a boiling water bath for 50 min. The standard RNA used for reference was yeast RNA (Type III from bakers yeast, Sigma) which was diluted in distilled water over the range 5-200 $\mu g\ ml^{-1}$. The A_{670nm} was read against a distilled water blank using an MSE Spectro-Plus spectrophotometer. A standard curve was contructed of A_{670nm} against reference RNA, from which the RNA in the samples was estimated. The RNA content in the total cellular RNA extract was monitored by measuring A_{260nm} using a Pye Unicam SP 8-100 ultraviolet spectrophotometer, in which distilled water served as a blank.

G. PREPARATION OF PURIFIED LYSINS

a. α-lysin

α-lysin, purified according to a modification of the method of Cassidy and Harshman (1976a) from a 15 h culture supernate of S. aureus Wood 46, was a gift from Dr.J.H.Freer.

b. δ-lysin

This was based on the method of Kreger et al. (1971). The 7 h culture supernates of S. aureus NCTC 10345 were stirred for 48 h with hydroxylapatite (200 g per 3000 ml) at 4 °C and unadsorbed material removed after centrifugation (300 g for 10 min). The lysin/hydroxylapatite mixture was washed 5 times with 400 ml of 0.01 M phosphate buffer pH 6.8 and then washed 5 times with 500 ml of 0.4 M phosphate buffer pH 6.8. The mixture was stirred for 30 min each time and then centrifuged at 300 g for 10 min to separate hydroxylapatite from the supernatant fluid. δ -

lysin was recovered from the hydroxylapatite by washing for 30 min with 200 ml of 1 M phosphate buffer pH 7.4. Five such washes were carried out after which the supernates (containing the eluted δ-lysin) were bulked and centrifuged (12,000 g for 10 min) to remove traces of hydroxylapatite. Following overnight dialysis against running water, lysin was recovered from the dilute solution by lyophilisation. The freeze-dried preparation was redissolved in approx. 250 ml distilled water and dialysed for a further 72 h against distilled water with frequent changes of the water. At this stage, any insoluble material was removed by centrifugation at 47,000 g for 20 min. The supernates, containing purified soluble δ-lysin, were lyophilized.

H. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

a. Preparation and Electrophoresis of Polyacrylamide Gels

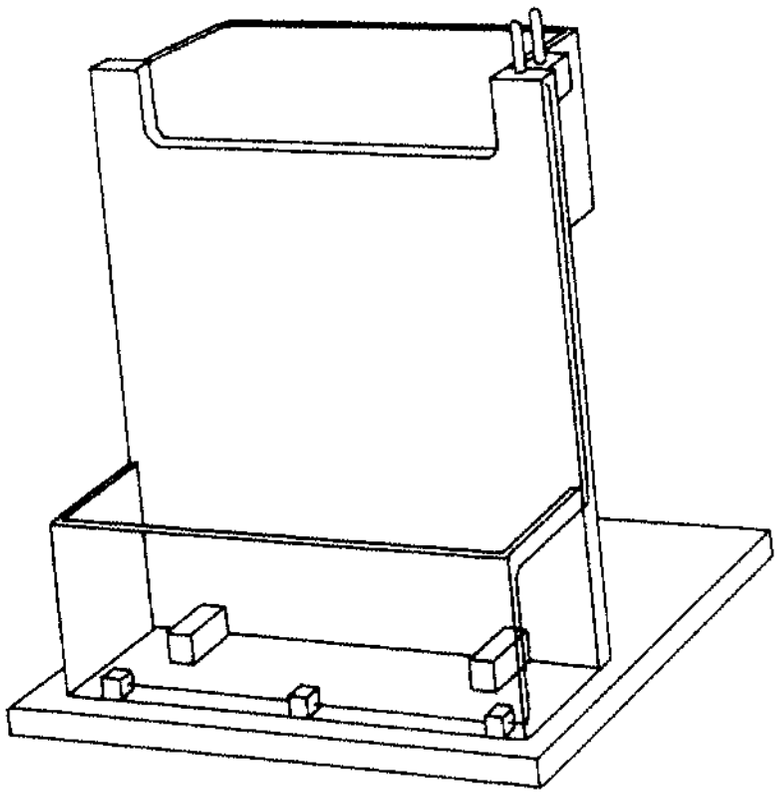
1. Preparation of Slab Gels

The method used was based on those described by Laemmli (1970) and Ames (1974) with a vertical slab gel tank which was constructed in the laboratory workshop (Fig. 7) (Studier,1973). The list of stock solutions and the recipe for gel preparation are shown in Appendix III. The gel was formed between two glass plates (17 cm x 19 cm x 0.3 cm) with a spacer (0.15 cm thick) running down each vertical side of the sandwich. Separating (lower) and stacking (upper) gels contained 15% (w/v) and 4.5% (w/v) acrylamide respectively.

2. Sample Preparation

Purified α-lysin and δ-lysin were adjusted to 0.5 - 1.0 mg ml^{-1} protein, as determined by the method of Bradford (1976), whereas culture supernates, reaction mixtures of cell-free translation system and SDS extracts of

Figure 7. A Studier-Type Slab Gel Apparatus*



* Reproduced with permissiom of B.D.Hames and D.Rickwood. In Gel Electroresis of Proteins : a Practical Approach. (1981) p22. IRL Press, London.

membrane fractions were used neat. Each sample was added to an equal volume of solubilising buffer (Appendix III) and was heated at 100 °C for 3 min prior to the addition of 30 µl of sample to the gel.

3.Electrophoresis

Electrophoresis was performed in a cold room at a constant current of 35 mA per gel until the tracking dye has reached the bottom of the gel. Power was provided by a Vokam SAE 2761 power pack (Shandon Southern Instruments Ltd., Camberley, U.K.).

b.Analysis of Gels Following Electrophoresis

1.Protein Staining

Staining and destaining of the gels was done by the method of Weber and Osborn (1969). Coomassie blue R-250 (BDH Chemicals Ltd., Poole, Dorset, U.K.) at 0.1% (w/v) in water:methanol:glacial acetic acid (5:5:2, v/v/v) was used as the dye for protein staining. Gel slabs were stained overnight at room temperature and destained the following day. Destaining involved several changes of destaining solution (Appendix III). The gel was picked up on 3 MM filter paper and dried on a Savant slab gel dryer for 1 h. Best results were obtained if the gel was soaked overnight in 30% (v/v) methanol, 3% (v/v) glycerol solution prior to drying.

2.Molecular Weight Estimation

i.Principle

The method used was based on those of Ornstein (1964) and Weber and Osborn (1969). When denatured by heating in the presence of excess SDS and a thiol reagent (usually 2-mercaptoethanol or dithiothreitol), most polypeptides bind SDS in a constant weight ratio such that they have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity according to polypeptide size. Under these

conditions , a plot of log$_{10}$ polypeptide molecular weight versus relative mobility (R_f) reveals a straight-line relationship. The relative mobility refers to the mobility of the protein of interest measured with reference to a marker protein or to a tracking dye where :

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

The approach was therefore to electrophorese a set of marker polypeptide of known molecular weight and use the distance migrated by each to construct a standard curve from which the molecular weight of the sample polypeptides can be calculated based on their mobility under the same electrophoretic conditions.

iiMolecular Weight Markers

Whenever analytical SDS-PAGE was used, a mixture of polypeptides of known molecular weight was also included. The molecular weight marker set, Dalton Mark VI® (Sigma) was employed for non-radioactive gels. It contained a mixture of following six proteins :-

Lysozyme (M.W. 14,300)

β-Lactoglobulin (M.W. 18,400)

Trypsinogen, PMSF treated (M.W. 24,000)

Pepsin (M.W. 34,700)

Albumin, Egg (M.W. 45,000)

Albumin, Bovine (M.W. 66,000)

For radioactive gels. [^{14}C] methylated protein mixture (Amersham International Ltd., Buck, U.K.) was used. It contained a mixture of the following six proteins :-

[^{14}C] Methylated Lysozyme (M.W. 14,300)

[^{14}C] Methylated Carbonic Anhydrase (M.W. 30,000)

[^{14}C] Methylated Ovalbumin (M.W. 46,000)

[^{14}C] Methylated Bovine Serum Albumin (M.W. 69,000)

[^{14}C] Methylated Phosphorylase b (M.W. 92,500)

[^{14}C] Methylated Myosin (M.W. 200,000)

3.Detection of Radioactive Proteins

i.Fluorography

After separating proteins by electrophoresis in an acrylamide gel, the gel was placed in a polyethylene tray containing fixer solution (10% (w/v) TCA, 10% (v/v) glacial acetic acid, 30% (v/v) methanol) and gently agitated overnight. After fixing proteins in the gel, the gel was gently agitated for 1 h in three times the gel volume of EN^{3}HANCE™ (New England Nuclear, Southampton, U.K.). The fluor in the gel was then precipitated by gentle agitation in cold water for a further 1 h. When precipitation was complete, the gel was picked up on 3 MM filter paper and dried on a Savant slab gel dryer for 1 h. The gel was placed against Kodak X-OMAT AR film at -70°C until the desired visualization level was achieved after which the film developed according to the manufacturer's instructions.

ii.Gel Slicing and Counting

Slicing of the gel and scintillation counting of the solubilised fractions is the most widespread method of quantitation of labelled protein components following polyacrylamide gel electrophoresis. The amount of radioactive α-lysin separated after electrophoresis was therefore measured by counting the gel slice which contained radioactive α-lysin from the stained gel. The location of the radioactive α-lysin was determined by addition of non-radioactive purified α-lysin (which produced an approx. 1 mm wide band on a Coomassie blue stained gel) to the samples prior to electrophoresis and by running a track of purified α-lysin parallel to

samples on the slab gel. Each gel slice (1 mm) was swollen by soaking in 0.5 ml of 90% (v/v) NCS (Amersham International Ltd., Buck,U.K.) for 2 h at 65 °C, followed by addition of 5 ml OCS (Amersham International Ltd., Buck, U.K.). The radioactivity of the gel slice was counted in a Packard Tri-Carb liquid scintillation spectrometer.

4.Immunological Identification of Proteins

i.Protein Blotting onto Nitrocellulose Membranes

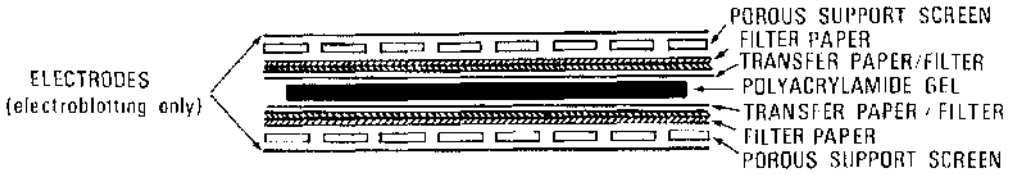
Electroblotting was carried out by the method of Towbin et al. (1979) in the Bio-Rad Trans-Blot transfer apparatus. The physical assembly used is shown diagrammatically in Fig. 8. The assembly was put into an electrophoretic transfer chamber containing 2.5 l of pre-cooled transfer buffer (Appendix II) with the Millipore HAHY 0.45 µm nitrocellulose membrane filter facing the cathode. Electrophoretic transfer was accomplished at 225 mA in 3 h using a Shandon 50V/1A power unit supply at 4 °C. For direct visualization of unlabelled proteins, the nitrocellulose sheet was stained for 5 min in a solution containing 0.2% (w/v) Coomassie blue R-250 (BDH Chemicals Ltd., Poole, U.K.), 40% (v/v) methanol, and 10% (v/v) glacial acetic acid. Rapid destaining was accomplished in 90% (v/v) methanol and 2% (v/v) glacial acetic acid.

ii.Labelling of Protein A with [^{3}H] NSP

This was based on the method of Muller (1980). [^{3}H] NSP reacts specifically with free amino groups and reacts in an analogous manner to the Bolton and Hunter reagent (Bolton and Hunter, 1973). It has the advantage of being a smaller molecule than the Bolton and Hunter reagent and hence causes less alteration to the protein structure. The labelled product has been found to have a biological activity comparable with that of the native protein.

(a)Preparation of Reagents

Figure 8. A Schematic Representative of the Protein Blotting Sandwich Used in Contact-Diffusion[+] and Electroblotting Experiments*



* Reproduced with permission of J.M.Walker and W.Gaastra. In Techniques in Molecular Biology. (1983) p52. Croom Helm, London.

+ In contact-diffusion blotting experiments protein migration is bidirectional and two transfer filters are required. Protein transfer is unidirectional in electroblotting experiments and a single transfer filter is used.

(1) PBS (Dulbecco A , Oxoid Ltd., London, U.K.) adjusted to pH 8 using sodium hydroxide.

(2) 0.05 M glycine in PBS pH 8.

(3) 0.1% (w/v) BSA (Fraction V, Sigma) in PBS pH 8.

(4) Protein A (P8143, Sigma) was reconstituted in PBS pH 8 (20 mg in 5 ml) dispensed in 0.1 ml (400 µg of Protein A) aliquots in 1.5 ml microfuge tube and stored at -20 °C.

(b)Column Packing and Equilibration

Sephadex G25 (Pharmacia Fine Chemicals, London, U.K.) was allowed to swell in PBS, pH 8 overnight at room temperature and then packed into a Bio-Rad Econo-Column (1.5 cm x 15 cm) to give a bed height of 6 cm. The packed column material was then equilibrated with 25 ml of 0.1% (w/v) BSA solution.

(c)Sample Preparation

(1) A 1 mCi ml^{-1} solution of [^{3}H] NSP (1.85 TBq $mmol^{-1}$,Amersham International Ltd.,Buck, U.K.) was dispersed into a 1.5 ml microfuge tube and toluene was removed by directing a gentle steam of nitrogen gas onto the surface of the solution. (evaporation of 1 ml toluene took 20 min at 20 °C).

(2) 0.1 ml of protein A solution was added to the microfuge tube containing [^{3}H] NSP. The tube which contained the protein A was rinsed with a further 0.4ml PBS , pH 8 and this was added to the microfuge tube containing [^{3}H] NSP.

(3) The microfuge tube containing [^{3}H]NSP and protein A was placed in an ice bath with occasional vortexing to mix the reactants.

(4) After 30 min, 40 µl of 0.05 M glycine solution was added to terminate the reaction and the tube was left for another 20 min with occasional vortex mixing.

(d)Sample Application and Elution

0.5 ml of tritiated protein A was applied to the Sephadex G25 column and eluted at flow rate of 10 ml h^{-1} at 4 °C. After sample application, elution was continued with 10 ml of 0.1% (w/v) BSA solution. 20 fractions of 0.5 ml fraction $^{-1}$ were collected and the radioactivity in each fraction was measured by adding 10 µl of each fraction to a scintillation vial containing 1 ml of water; 5 ml of Triton X-100 scintillation cocktail (Appendix V) was then added and the samples counted in a Packard Tri-Carb liquid scintillation spectrometer. The [^{3}H]-protein A fractions were pooled and stored at -20 °C.

iii.Immunological Detection of Protein on Nitrocellulose

Immediately following transfer, the nitrocellulose sheet was immersed in wash buffer (Appendix II) containing 3% (w/v) BSA (Fraction V, Sigma), and incubated at 40 °C for 45 min on a rocking platform. The sheet was transferred to a fresh 0.3% (w/v) BSA solution in wash buffer containing 0.5 ml of anti-α-lysin antiserum and put in the cold room (4 °C) for about 24 h. The nitrocellulose was then washed with rocking for 10 min in 200 ml of wash buffer, for 20 min in two changes of 200 ml each of wash buffer containing 0.05% (v/v) Nonidet P-40 (NP-40), and again in 200 ml of wash buffer overnight at room temperature. The nitrocellulose was then immersed in fresh 0.3% (w/v) BSA containing 200 µl of [^{3}H]-protein A (1×10^{9} cpm ml^{-1}). Binding of [^{3}H]-protein A was allowed to occur for 5 h with rocking at room temperature. The nitrocellulose sheet was removed and washed as described above. It was then briefly blotted with filter paper sheets and left to dry in a laminar flow cabinet for 30 min. The sheet was exposed to LKB Ultrofilm ^{3}H for 2 weeks at -70 °C and the film developed according to the manufacturer's instructions.

I. PREPARATION OF SDS EXTRACTS OF MEMBRANES AND WHOLE CELLS

a. SDS Extract of Staphylococcal Cytoplasmic Membranes

Cytoplasmic membranes were isolated by a modified method based on the procedure of Theodore et al. (1971). S. aureus Wood 46 was grown to late-exponential phase at 37°C and cells were collected by centrifugation at 4°C at 16.000 g for 10 min, washed twice in hypotonic buffer (0.05 M Tris-HCl, pH 7.5, 0.015 M $MgSO_4$), and suspended in hypertonic buffer (0.05 M Tris-HCl, pH 7.5, 0.015 M $MgSO_4$, 3.45 M NaCl). Routinely, 1.5 g (wet weight) of exponential-phase cells was suspended in 1.5 ml of hypertonic buffer and allowed to equilibrate on ice for 20 min. Then, 0.1 ml of lysostaphin solution (300 µg of lysostaphin (Sigma) in 1 ml of hypotonic buffer) was added and left overnight at 4°C. On the following morning, an additional 0.1 ml of lysostaphin solution and 0.1 ml of DNase solution (300 µg of DNase-I (EC 3.1.4.5, Type I from Bovine pancreas, Sigma) in 1 ml of hypotonic buffer) were added. The mixture was incubated at 37°C for 15 min and the osmotically sensitized cells were centrifuged at 10,000 g for 30 min at 4°C. The pellet was suspended with vortex blending in 10 ml of hypotonic buffer, and then stirred on ice for 20 min. 0.1 ml of DNase-I solution and 0.1 ml of RNase (300 µg of RNase (EC 3.1.4.22, Type III-A from Bovine pancreas, Sigma)) were added, and the mixture was shaken gently in a 37°C water bath for 10 min. The mixture was centrifuged at 2,000 g for 10 min, and the supernate containing the membrane fraction was retained and subjected to a further, similar centrifugation step. All subsequent steps were conducted at 4°C, and the membrane suspensions were kept on ice during manipulations. The supernates were collected by centrifugation at 35,000 g for 40 min, the membrane precipitate was washed five times with hypotonic buffer, and resuspended in 0.5 ml of

solubilisation buffer (0.05 M Tris-HCl, pH 7.5, and 2% (v/v) SDS) to solubilise the membrane proteins. The remaining insoluble debris was removed by centrifugation and the supernate which contained solubilised membrane proteins was stored in small fractions at -20 °C.

b. SDS Extract of Staphylococcal Whole Cells

Samples of S. aureus Wood 46 culture (1 ml) were withdrawn and cells were collected by centrifugation in an MSE microfuge for 15 min, washed twice in hypotonic buffer (0.05 M Tris-HCl, pH 7.5, 0.015 M $MgSO_4$), the cell pellets resuspended in 0.5 ml of 10% (v/v) TCA and left overnight. Precipitates were collected by centrifugation for 15 min in an MSE microfuge and washed twice with ethanol-ether (1:1, v/v) to remove residual TCA. After washing with ethanol-ether, TCA precipitates were dissolved in 0.1 ml of solubilising buffer (Appendix III) and heated at 100 °C for 3 min followed by brief centrifugation to remove insoluble debris prior to gel electrophoresis.

J. EXTRACTION OF TOTAL CELLULAR RNA

Prewarmed B.S. Medium was inoculated with 5% (v/v) of a starter culture of S. aureus NCTC 10345. Cells were grown to late-exponential phase and growth was terminated by rapidly cooling to 0 °C in an acetone/CO_2 bath. Cells were collected by centrifugation at 4°C at 16,000 g for 10 min and washed at 0 °C with standard buffer (0.03 M Tris-HCl, pH 7.8, 0.04 M $(CH_3COO)_2Mg$, 0.04 M NH_4Cl, 0.006 M 2-mercaptoethanol, and 0.002 M EDTA). After resuspension in standard buffer containing 0.02 M EGTA and 1 mg ml^{-1} of heparin, cells were disrupted in a pre-chilled X-press (LKB Instruments Ltd., South Croydon, Surrey, U.K.) at 1300 kg cm^{-1}. Electrophoretically pure DNase (Miles Laboratories Ltd., Slough, U.K.) was added to a

concentration of 3 mg ml^{-1} and after 15 min, the cell debris was separated from the suspension by centrifugation at 12,000 *g* for 3 min. The supernatant fraction, containing most of the polysomes, was carefully aspirated with a Pasteur pipette and the pellet of cell debris, containing large amounts of trapped polysomes, was resuspended in buffer and centrifuged for 3 min at 12,000 *g* . The supernate was retained along with the crude polysomal fraction and RNA was obtained from the crude pooled polysomal fraction using chloroform-phenol-SDS as deproteinising agents based on the methods of Perry *et al.* (1972) and Palmiter (1974). SDS was first added to 5% (w/v), the mixture was shaken for 5 sec and an equal volume of phenol buffer (80% (v/v) phenol : 8 hydroxyquinoline : m-cresol; 15 : 0.05 : 7; v/w/v) then added. The mixture was again shaken vigorously at room temperature for 5 sec and shaken again 3 min later. Half the volume of chloroform was then added and the mixture was again shaken at 25 °C for 5 sec and centrifuged at 15,000 *g* for 2 min. The aqueous phase was collected and re-extracted with chloroform until the interphase disappeared. RNA was precipiated from the aqueous phase by addition of two volumes of 95% (v/v) ethanol and collected by centrifugation at 15,000 *g* for 10 min; trace amounts of ethanol were removed by lyophilisation. The RNA was redissolved in sterile deionised distilled water and stored in small fractions at -70 °C.

K. PREPARATION OF CELL-FREE EXTRACT

An S-30 extract was prepared from *E. coli* strain MRE 600 based on the method of Nirenberg and Matthaei (1961). Prewarmed L-medium (see Appendix I), was inoculated with 1% (v/v) of a fresh stationary phase culture of *E. coli* and growth was terminated rapidly at mid-logarithmic phase by rapid

cooling to 0 °C. The cells were harvested and washed twice with cold buffer containing 0.01 M Tris-HCl, pH 6.8, 0.06 M NH_4Cl, 0.01 M $(CH_3COO)_2Mg$, and 0.006 M 2-mercaptoethanol before disruption by EDTA-lysozyme treatment (see Appendix IV) based on the method of Godson and Sinsheimer (1967). After complete lysis, NH_4Cl and 2-mercaptoethanol were added to final concentrations of 0.06 M and 0.006 M respectively and, electrophoretically pure DNase (Miles Laboratories Ltd., Slough, U.K.) was added to a concentration of 3 mg ml^{-1}. After thorough mixing, the extract was clarified by centrifugation for 30 min at 30,000 g at 4 °C, dialysed against 500 volumes of buffer for 5 h and again clarified by centrifugation at 30,000 g for 20 min at 4 °C. Since an S-30 extract should never be frozen and thawed more than once, it was divided into small portions, quickly frozen in a CO_2/acetone mixture, and stored in liquid nitrogen.

Commercial dialysis tubing is normally quite dirty. It was therefore cleaned before use. This was accomplished by boiling it in water, in 0.1 M $NaHCO_3$, twice more in water, once in 1 mM EDTA (30 min each time), and finally washing it with water. It was stored in 1 mM EDTA before use.

L. CELL-FREE SYSTEM FOR PROTEIN SYNTHESIS

a. Preparation of Stock Reagents

Unless otherwise indicated the reagents were stable in solution for relatively long periods of time when stored frozen. The following solutions were prepared :

1. 2M Tris-HCl (adjusted to pH 7.8).
2. 3M NH_4Cl (no refrigeration required).
3. 1M 2-mercaptoethanol.
4. 5mM (each) 21 amino acids (Sigma) minus methionine. This was most

easily prepared from 0.1 M stock solutions of the amino acids, mixing 1 volume of each of the 20 plus 20 volumes of sterile deionised distilled H_2O.

5. 2mM Methionine.
6. [^{35}S] Methionine (48.1 TBq mmol^{-1}, Amersham International Ltd., Buck, U.K.).
7. 1 ml of "Energy" solution, viz a solution containing :- 10mM ATP-Tris (Sigma),50mM Phospho(enol)pyruvate (PEP) (Tri-(cyclohexylammonium) salt, Sigma), 0.5mM Guanosine 5' -triphosphate (GTP) (Tris salt, Type VI, Sigma), and 400 µg Pyruvate kinase (PK) (EC 2.7.1.40, Type III from rabbit muscle, Sigma).

b.Preparation of Reaction Mixture

Table 14 shows the composition of a 35-µl reaction mixture for incorporating amino acids with S-30 extract and crude RNA extract from S. aureus NCTC 10345. The volume of the reaction mixture varied with the experiments performed. A 35-µl reaction mixture was prepared by mixing the following reagents in a sterilized 1.5 ml polypropylene microfuge tube kept at 0 °C :

1. 20 µl preincubated S-30 extract (iS-30). As the S-30 extract is saturated with endogenous RNA, additional mRNA will only be translated to the extent that it competes with the endogenous mRNA. The S-30 extract was therefore incubated, just before its addition to the reaction mixture,for 35 min at 34 °C to deplete its content of endogenous RNA (see Fig. 16).
2. 5 µl "Translation cocktail". The "cocktail" was prepared by mixing : 5µl of 1M 2-mercaptoethanol, 5 µl of 21 amino acids minus methionine, 5µl of 2mM methionine, 20 µl of [^{35}S]methionine (48.1 TBq mmol^{-1}),

Table 14. Components of an Amino Acid-Incorporating Mixture Directed by Total Cellular RNA of S. aureus.

Component	Concentration*
Tris-HCl (pH 7.8)	38.2 mM
NH_4Cl	61.0 mM
$(CH_3COO)_2Mg$	5.7 mM
2-Mercaptoethanol	6.5 mM
ATP-Tris	0.65mM
GTP	0.03mM
PEP	3.25mM
PK	26µg ml^{-1}
[^{35}S] Methionine (1300 Curies $mmol^{-1}$)	300 µCi ml^{-1}
20 other amino acids	0.03 mM
Total cellular RNA	5µl
iS-30	20µl

*For 35-µl reaction mixture

50 µl of "Energy" solution, and 25 µl of concentrated salts solution (the concentration of both Mg^{2+} and NH_4^+ ions significantly affected the protein synthesizing ability of the mRNA. Therefore, the concentration of Mg^{2+} and NH_4^+ necessary for optimal activity was first established experimentally (see Figs. 17 and 18). The 1 ml solution of concentrated salts was then prepared by mixing : 0.28 ml of 3M NH_4Cl, 0.5ml of 2M Tris-HCl, and 0.22ml of sterile deionised distilled H_2O).

3. 5 µl Total cellular RNA; A_{260}/A_{280} = 2.04. Addition of increasing amount of mRNA to a fixed incubation mixture (iS-30 and "translation cocktail") results in increasing incorporation of the radioactive amino acid into protein until saturation is reached. To avoid wastage of mRNA, the optimal concentration was determined by plotting a dose response curve (see Fig. 19).

4. 5 µl Sterile deionised distilled H_2O or other additions whose influence on the reaction were to be determined.

A background control was prepared simultaneously by mixing 20 µl of iS-30, 5µl of "translation cocktail" and 10 µl of sterile deionised distilled H_2O.

c. Incubation

The tubes were kept in an ice bath to prevent degradation of labile components and to that ensure protein synthesis did not commence before all components were added. The contents of the tubes were then briefly vortex mixed and centrifuged in a MSE microfuge to ensure that the complete mixture was at the bottom of each tube. This step was important because small droplets of reaction solution might otherwise stay on the wall of the tube and never enter the reaction. Assay tubes were then placed in a 37 ^{o}C

water bath for 25 min.

d.Analysis of Translation Products

1.Measurement of Total Incorporation into Protein

The reaction was terminated by chilling the tubes in an ice-water bath and 5µl samples of the reaction mixture were withdrawn, spotted on to 2 cm^2 pieces of Whatman 3 MM paper and dropped into a beaker of 10% (w/v) TCA. The TCA solution was then brought to a rolling boil for 10 min in a fume hood to hydrolyse aminoacyl tRNA (Ranu and London, 1979), crushed ice was added and the TCA discarded. Papers were rinsed twice with water, ethanol and acetone, before air-drying and counting in a Packard Tri-Carb spectrometer with 5 ml of Triton X-100 scintillation cocktail (Appendix V).

2.Immunological Techniques

i.Immunoprecipitation

Immunoprecipitation was performed immediately after translation. The translation products tend to aggregate when frozen and thawed. The procedure was that described by Kessler (1975) as modified by Ivarie and Jones (1979). To 100µl samples containing 0.5% (v/v) NP-40 was added 10µl of heat-killed, formalin-fixed, heat-inactivated S. aureus Cowan Type 1 (SAC). After 5 min at room temperature, SAC was removed by centrifugation at 10,000 µg for 9 min. 10µl of anti-δ-lysin antiserum was added to the supernate and the mixture incubated for 20 min at 0 °C. 20µl of SAC was then added to bind the antigen/antibody complexes and after 15 min at room temperature SAC was collected and washed five times with PBS/NP-40 (Dulbecco PBS-A containing 1mM EDTA and 0.25% (v/v) NP-40). The SAC-antigen/antibody complexes were dissolved in SDS-sample buffer (O'Farrell, 1975), heated to 100 °C for 3 min, and SAC was removed by centrifugation (10,000 g for 5 min). The supernate was prepared for SDS-PAGE .

ii. Immunoabsorption

A glutaraldehyde-polymerised immunosorbent, prepared from anti-δ-lysin antiserum by the method of Avrameas and Ternynck (1969), was resuspended in a small volume of PBS/NP-40 and incubated with an equal volume of cell-free translation mixture for 1 h at room temperature. The supernate, containing unadsorbed proteins, was collected after centrifugation at 10,000 g for 5 min and was analysed by SDS-PAGE.

RESULTS

A. PRODUCTION OF δ-LYSIN BY S.AUREUS NCTC 10345

The relationship between δ-lysin production and growth phase was studied in shake flask cultures of 100 ml B.S.Medium of 37 °C. Aliquots (6 ml) were withdrawn at intervals and ammonium sulphate concentrates of the supernatants were analysed by SDS-PAGE and assayed for δ-lysin activity. The results (Fig. 9) showed that δ-lysin was first detected at the mid-exponential phase of growth, that production of δ-lysin appeared to obey exponential kinetics and production continued well into the stationary phase of growth. When supernatant proteins were separated by SDS-PAGE in a 15% gel, δ-lysin appeared as a very diffuse band just behind the dye front with an apparent molecular weight of less than 10 kdal (Fig. 10).

B. STUDIES IN VIVO OF S.AUREUS δ-LYSIN mRNA METABOLISM

a. Effect of Rifampicin on RNA Synthesis

This was studied in shaken cultures of 50 ml of B.S. Medium at 37 °C. 25 µCi of [5- ^{3}H] uridine solution (25 µCi [5- ^{3}H] uridine plus 1.25 mg unlabelled carrier per ml) and rifampicin (final concentration 200 µg ml^{-1}) were added simultaneously to an early stationary phase culture (5 h) of S. aureus. Fig 11A shows that the uptake of [^{3}H] uridine into the TCA-precipitable fraction in the presence of 200µg ml^{-1} rifampicin ceased within 5 min. RNA synthesis was inhibited very rapidly and in Fig. 11B, the time course of residual RNA synthesis shows that two types of RNA were found. The increase from t = 0 to t = 2 min represents RNA production by RNA polymerase molecules involved in chain elongation at the time of antibiotic block when further rounds of transcriptional initiation were inhibited. The proportion of stable to unstable RNA was determined by extrapolation to be about 50%.

Figure 9. Production of δ-Lysin by S. aureus NCTC 10345 in Relation to Growth of the Organism.

S. aureus NCTC 10345 was grown in B.S. Medium at 37°C in an orbital shaker operating at 150 cycles min^{-1} ; Growth was assessed by $A_{650\ nm}$ measurement (◇) ; The $(NH_4)_2SO_4$-concentrated supernatant was assayed for δ-lysin activity (▲).

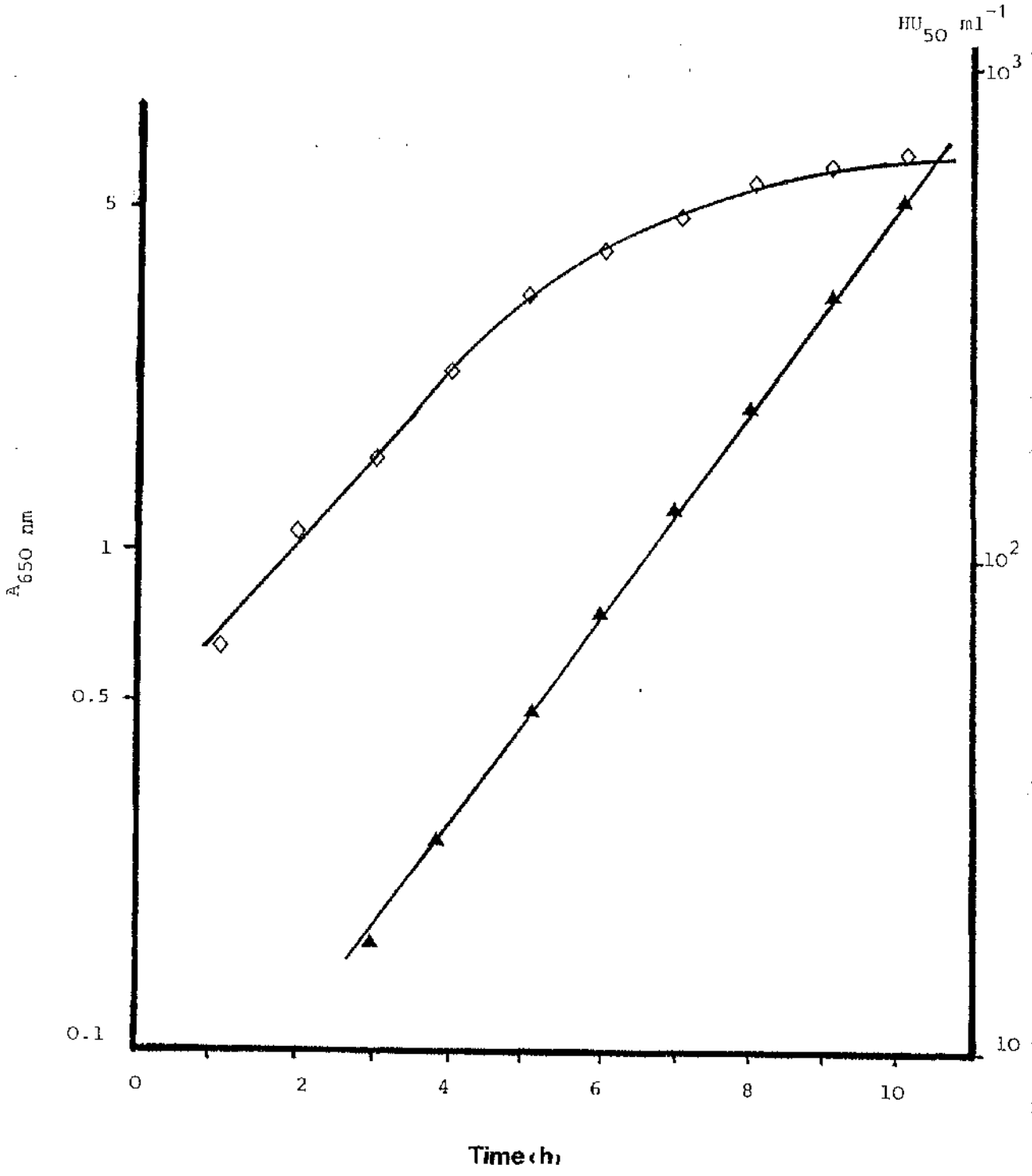
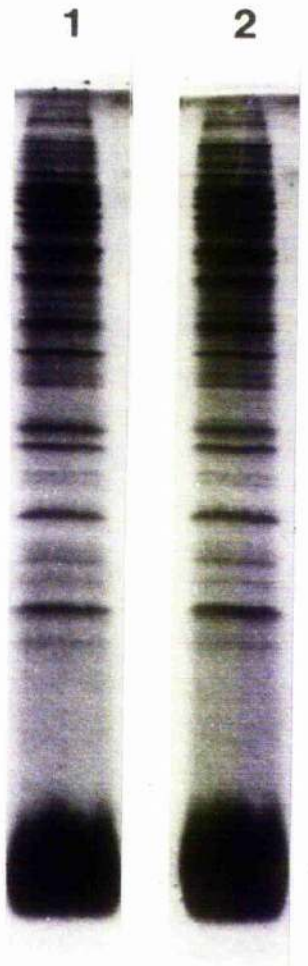

HU_{50} ml^{-1}
10^3
10^2
10
5
1
0.5
0.1
A_{650} nm
0
2
4
6
8
10
Time (h)

Figure 10. SDS-PAGE Analysis of $(NH_4)_2SO_4$-Concentrated Supernatants of S. aureus NCTC 10345.

Supernatants of S. aureus NCTC 10345 obtained at 5 h (lane 1) and 7 h (lane 2) were analysed on 15% gel. Proteins were visualized by Coomassie blue staining.

The lysin band was identified by comparison with purified δ-lysin.



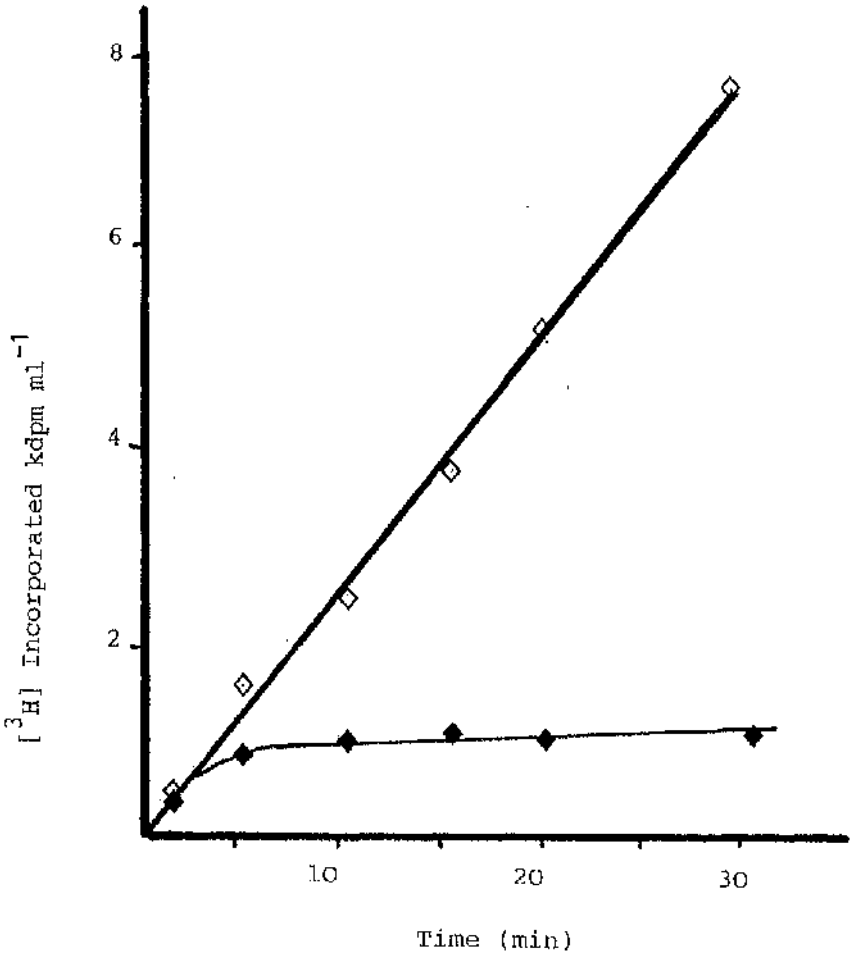
δ-LYSIN

Figure 11. The Effect of Rifampicin on RNA Synthesis in S.aureus NCTC 10345

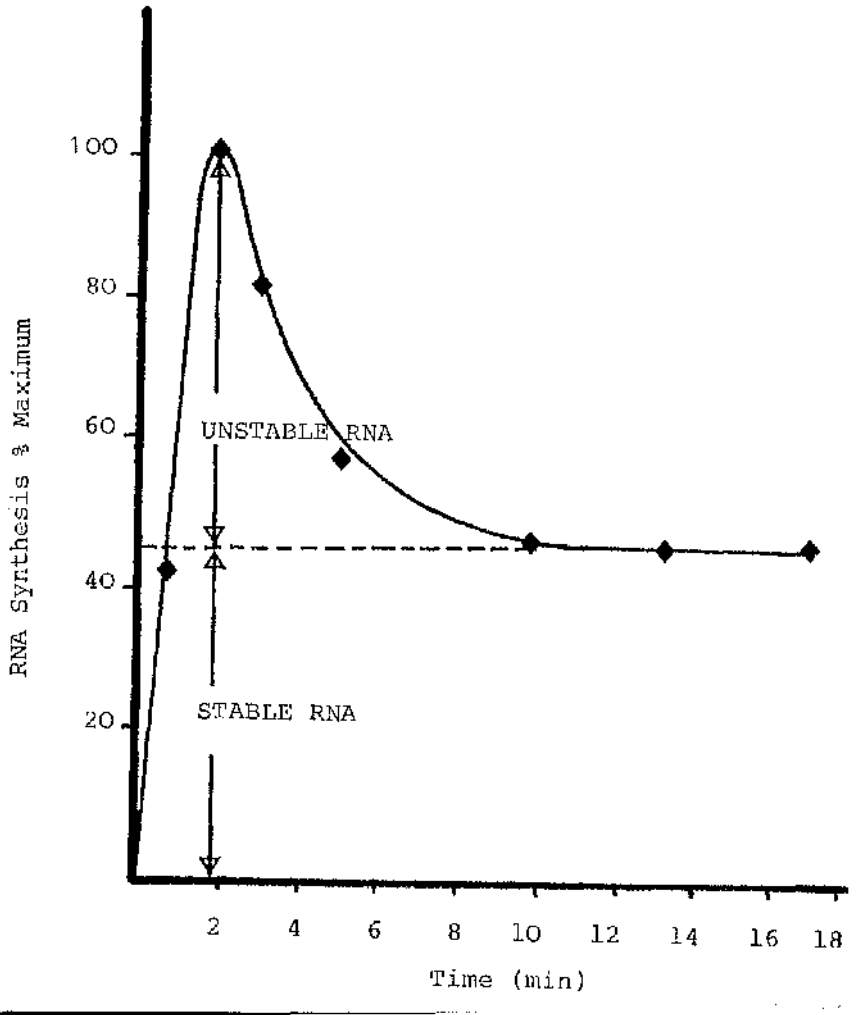
(A) The RNA synthesis of both control (◇) and rifampicin-inhibited (◆) cell cultures at various times after addition of rifampicin (200 μg ml^{-1} of culture) to the test culture of late logarithmic-early stationary growth phase were determined by measuring the radioactivity in TCA-precipitable material of culture supernatants.

(B) RNA synthesis % maximum against time; The % maximum RNA synthesis at time X after addition of rifampicin= Uptake of [^{3}H] uridine at time X divided by the maximum uptake of [^{3}H] uridine after addition of rifampicin and multiplied by 100.

(A)


8
6
4
2
[3H] Incorporated kdpm ml-1
10
20
30
Time (min)

(B)


100
80
60
40
20
RNA Synthesis % Maximum
UNSTABLE RNA
STABLE RNA
2
4
6
8
10
12
14
16
18
Time (min)

b. Effect of Rifampicin on Bacterial Growth

Whereas rifampicin rapidly inhibited RNA synthesis in early stationary phase cultures of S. aureus it would be expected to have a much slower effect on bacterial growth, and the effect of rifampicin on growth was studied in shake flask cultures of 150 ml of B.S. Medium of 37 °C. At the mid-logarithmic (2 1/2 h) and early stationary (5 h) phases of growth, 50 ml of culture was withdrawn, transferred to fresh flasks and rifampicin added (final concentration 200µg ml^{-1}). Growth was estimated by measuring the A_{650nm} of samples of culture and the results (Fig. 12) showed that at the mid-logarithmic phase of culture, growth of bacteria ceased within 1 h of the addition of rifampicin. Addition of rifampicin to the early stationary phase culture had less effect and growth of bacteria ceased after 2 h.

c. Functional Half-Life of δ-Lysin mRNA

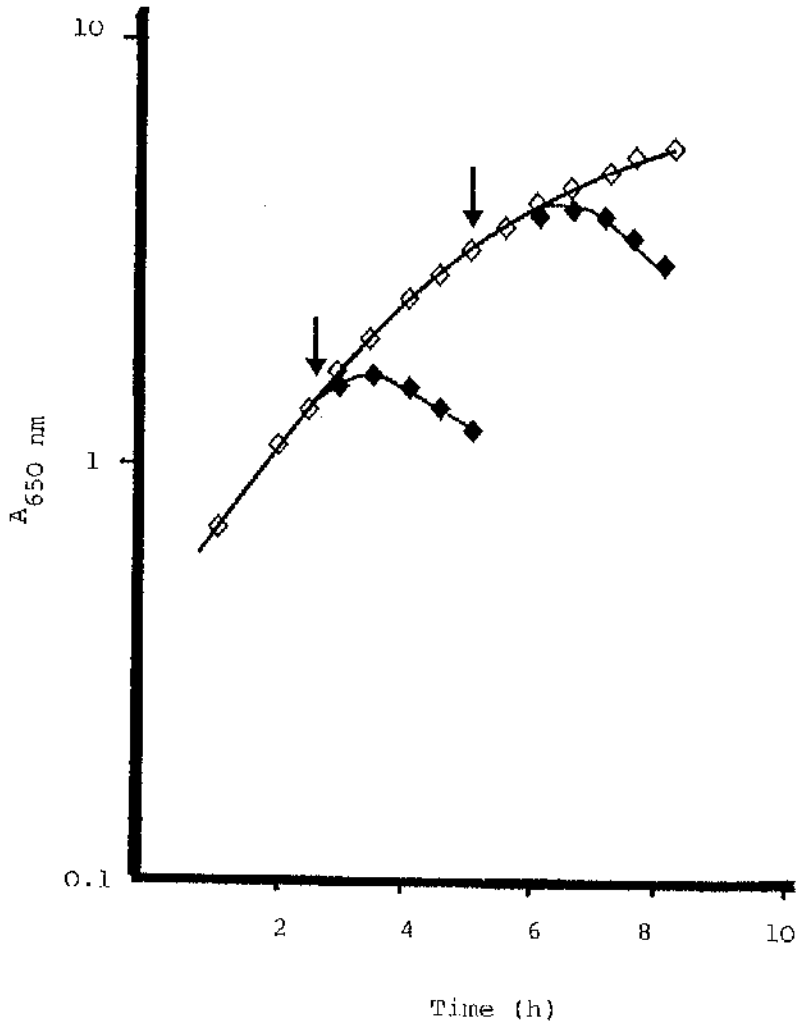
The stability in vivo of mRNA for δ-lysin was assessed by measuring the residual synthesis of δ-lysin after blockage of further RNA synthesis by addition of rifampicin. Delta-lysin was quantified by precipitation of radiolabelled lysin with rabbit anti-δ-lysin and staphylococcal protein A in the form of S. aureus Cowan Type 1 cells (SAC). This required titration of the anti-δ-lysin antiserum and the SAC for optimal recovery of δ-lysin.

1. Immunotitration of δ-Lysin with Anti-δ-Lysin Antiserum Using SAC Precipitation

To titrate the amount of anti-δ-lysin antiserum required to remove δ-lysin from solution quantitatively by the bacterial absorbent, varying amounts of anti-δ-lysin antiserum were reacted at room temperature for 5

Figure 12. The Effect of Rifampicin on Growth of S.aureus NCTC 10345

Rifampicin (◆) at 200 µg ml^{-1} of culture was added at mid-logarithmic phase (2 1/2 h) or at late logarithmic-early stationary phase (5 h), and growth of the culture was measured spectrophotometrically at 650nm (A_{650nm}). Control culture (◇). The arrow indicates the point at which rifampicin was added.


10
1
0.1
$A_{650\ nm}$
2
4
6
8
10
Time (h)

min with 100µl of $(NH_4)_2SO_4$ - concentrated supernatant fraction containing 128 HU_{50} δ-lysin after which time 10 µl of SAC was added. After removing SAC by centrifugation, the amount of δ-lysin activity remaining in the supernate was assayed. The data are shown in Fig. 13. It can be seen that at slight antibody excess 100 µl of anti-δ-lysin antiserum quantitatively bound 124 HU_{50} of δ-lysin to SAC (98% of the original amount). Increasing the volume of SAC (up to 20 µl) did not further enhance the efficiency of precipitation.

2. Assessment of δ-Lysin mRNA Functional Half-Life Using SAC Immunoprecipitation

The functional half-life of δ-lysin mRNA at early stationary phase culture was assessed by measuring the residual δ-lysin synthesis from remaining preformed mRNA after inhibition of RNA synthesis with rifampicin. The residual δ-lysin synthesis was measured by [^{35}S] methionine incorporation with SAC immunoprecipitation of the rifampicin-inhibited $(NH_4)_2SO_4$ - concentrated samples collected at intervals after the addition of rifampicin. In Fig. 14, the decline in the rate of incorporation of labelled amino acid into δ-lysin after 60 min incubation in a rifampicin-inhibited culture was attributed to the loss of functional activity in the δ-lysin mRNA pool. The plateau, taken as the total capacity of the inhibited culture for δ-lysin synthesis, was reached 60 min after rifampicin addition. A semi-logarithmic plot of the residual capacity of δ-lysin synthesis (the difference between the incorporation at time X and the total capacity for δ-lysin synthesis) against time after inhibition of transcriptional initiation produced a linear relationship (Fig. 15), the slope of which was taken as an assessment of the functional half-life of the δ-lysin mRNA pool. In early stationary phase cells of S.aureus NCTC 10345, the mRNA of δ-lysin was characterised by a remarkable longevity,

Figure 13. Immunotitration of δ-Lysin with Anti-δ-Lysin Antiserum and S. aureus Cowan Type 1 cells (SAC)

The amount of anti-δ-lysin antiserum required to remove quantitatively 128 HU_{50} ml^{-1} δ-lysin in 100µl of $(NH_4)_2SO_4$-concentrated S. aureus NCTC 10345 supernate fraction was determined by addition of antiserum and the bacterial absorbent (SAC). The amount of δ-lysin activity remaining in the supernate was assayed by titration of δ-lysin haemolytic activity.

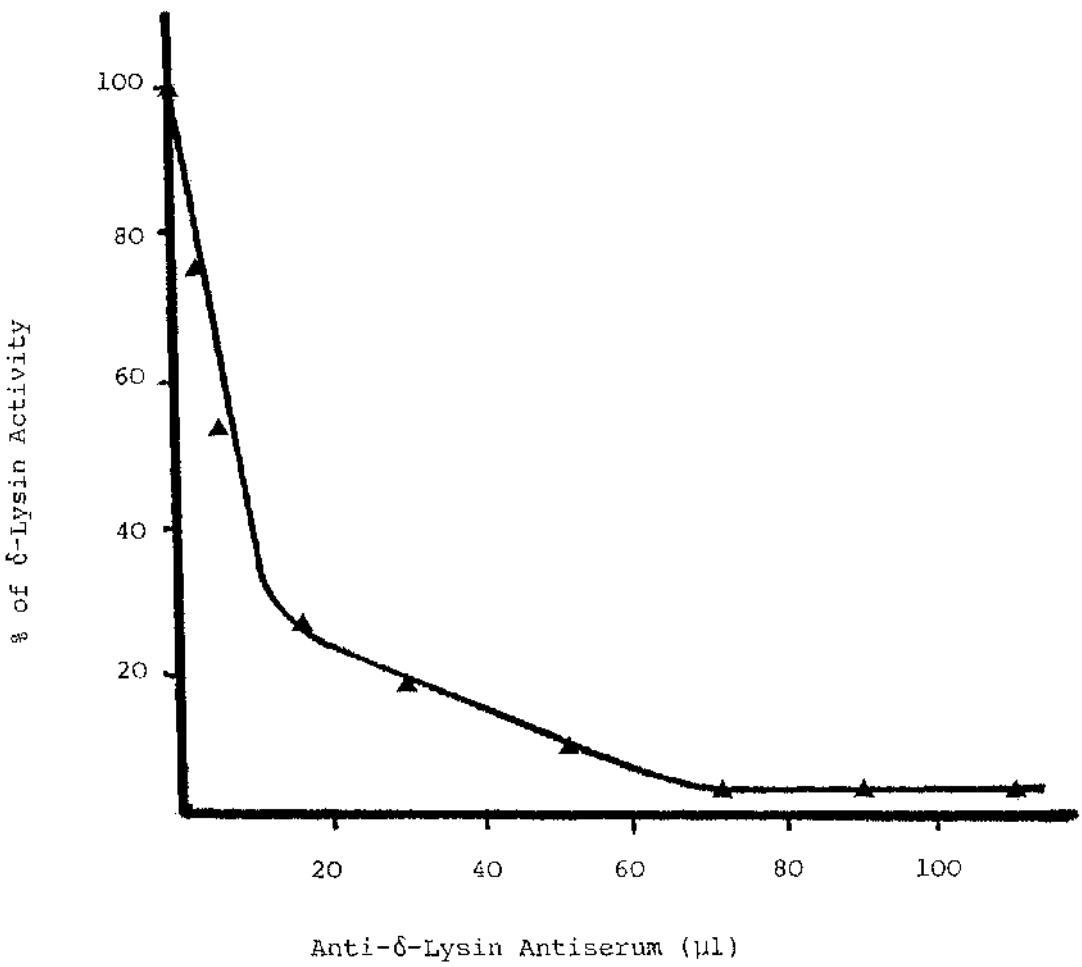

100
80
60
40
20
% of δ-Lysin Activity
20
40
60
80
100
Anti-δ-Lysin Antiserum (µl)

Figure 14 The Effect of Rifampicin on δ-Lysin Synthesis in S.aureus NCTC 10345

Rifampicin (200 μg ml^{-1} of culture) and 10 μCi of [^{35}S] methionine were added simultaneously to a late logarithmic-early stationary phase culture of S.aureus. The residual δ-lysin synthesis was measured by [^{35}S] methionine incorporation with SAC immuno-precipitation of the rifampicin-inhibited (NH_4)$_2SO_4$-concentrated samples collected at intervals after the addition of rifampicin.

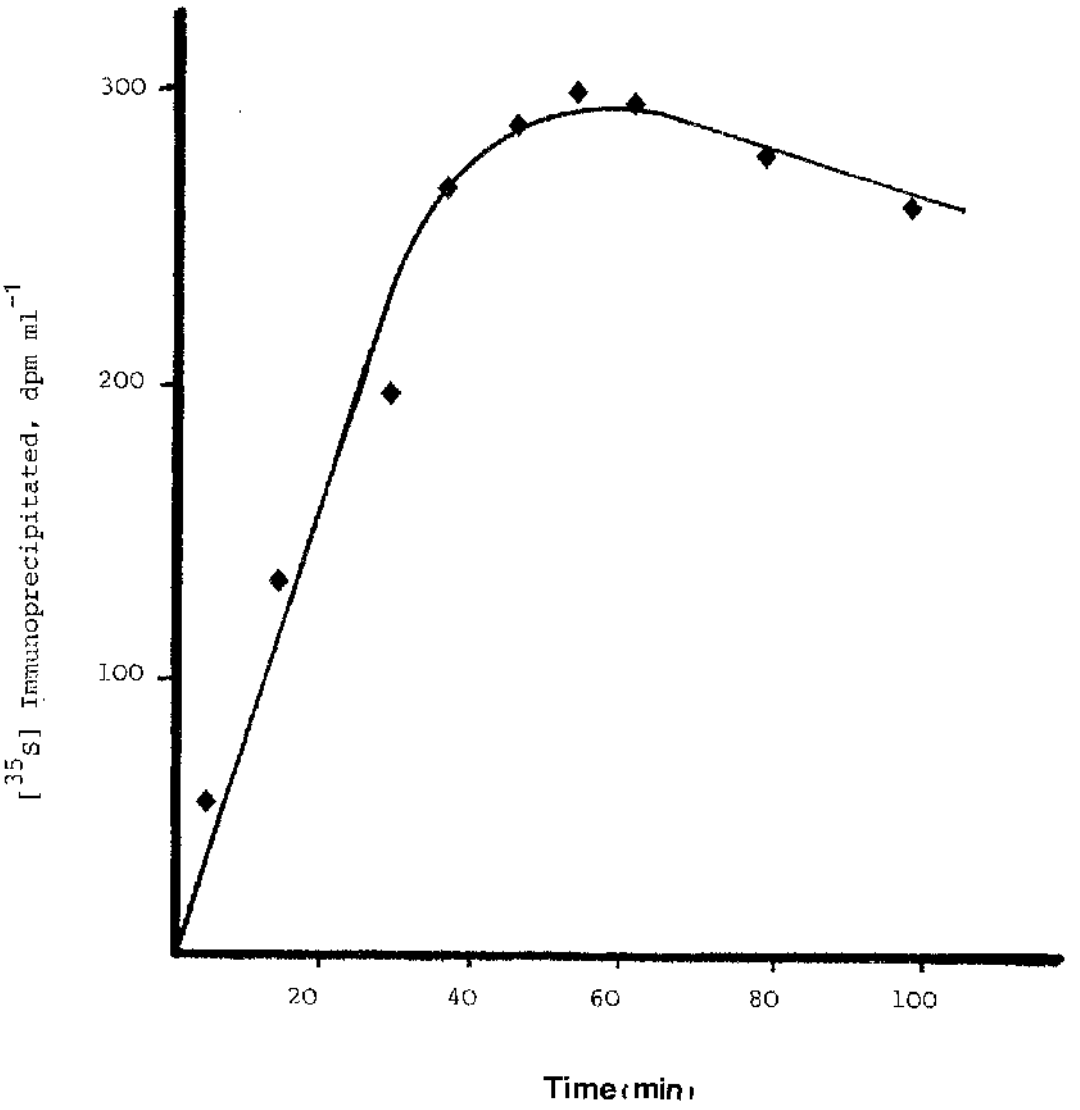
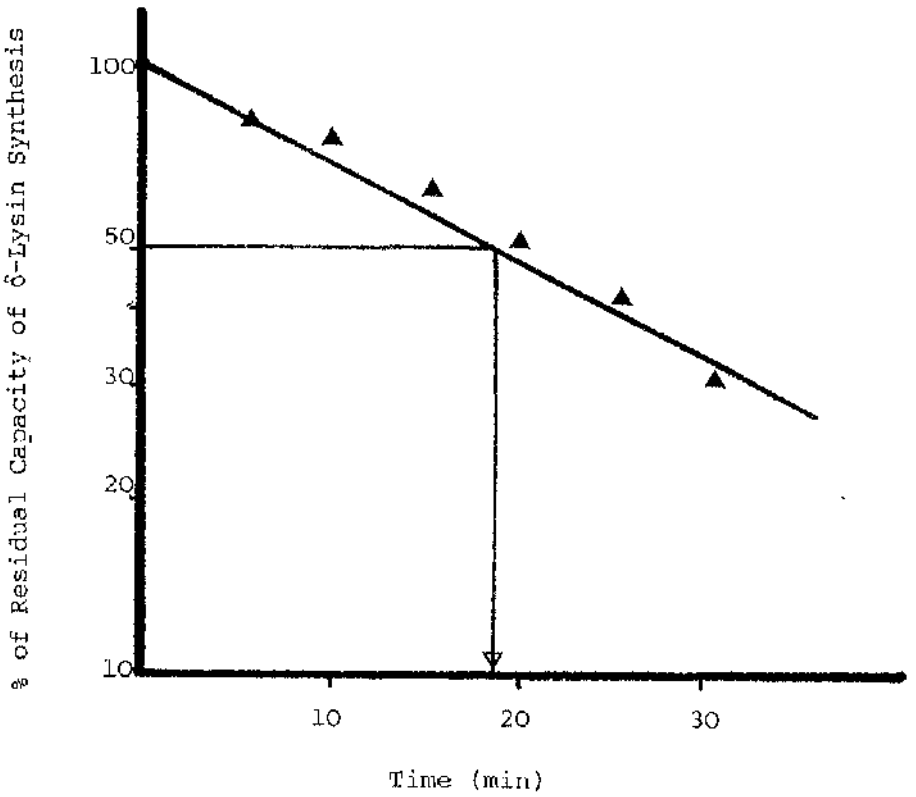

300
200
100
[35S] Immunoprecipitated, dpm ml-1
20
40
60
80
100
Time (min)

Figure 15. Semi-Logarithmic Plot of the Residual Capacity of δ-Lysin Synthesis Against Time

The % of the residual capacity of δ-lysin synthesis at time X after addition of rifampicin (200 μg ml^{-1} of culture) = The difference between the incorporation of time X and the total capacity for δ-lysin synthesis divided by the total capacity for δ-lysin synthesis multiplied by 100. The total capacity of the rifampicin-inhibited culture for δ-lysin synthesis was reached after 60 min of rifampicin addition (Fig.14).


% of Residual Capacity of δ-Lysin Synthesis
100
50
30
20
10
10
20
30
Time (min)

with a half-life of approximately 20 min and it was therefore decided to extract RNA from such cells for *in vitro* translation.

C. EXTRACTION OF TOTAL CELLULAR RNA FROM S.AUREUS NCTC 10345

Extraction of total cellular RNA from *S. aureus* was carried out with late exponential - early stationary phase cultures to maximize the amount of available δ-lysin mRNA. Cells were disrupted in a pre-chilled X-press in the presence of RNase inhibitors (heparin and EGTA) and protein contaminants were removed from the crude polysomal extract by chloroform-phenol-SDS treatment. Chloroform-phenol-SDS provided an effective deproteinization mixture removing almost 98% of protein with recovery of 96% of RNA (Table 15). The total cellular RNA preparation contained approximately 1.6% protein (expressed as a percentage of the total nucleic acid + protein) and the ratio of Absorption at 260 nm to 280 nm ($A_{260/280}$) was 2.04.

D. PREPARATION OF E. COLI S-30 EXTRACT

a. Characterization of S-30 Extract

The dialysed and clarified S-30 extract prepared from mid-logarithmic phase cultures of *E. coli* strain MRE 600 (as described in Methods section) had an amber-yellowish colour without apparent turbidity. It contained approximately 2.4 mg of ribosomes and 3 mg protein ml^{-1} extract. A small amount of protease (0.06% of the total protein) was also detected in the extract. Ribosome content of S-30 extract (mg ml^{-1}) = $A_{260nm} \times 0.04$

b. Studies on the Degradation of Endogenous RNA

As the S-30 extract is saturated with endogenous RNA (3.65 mg ml^{-1}),

Table 15. Estimation of RNA* and Protein+ Before and After Chloroform-Phenol-SDS Treatment

Sample	Protein Content ($\mu g\ ml^{-1}$)	RNA Content ($\mu g\ ml^{-1}$)
1. Before treatment	3550	3800
2. After treatment	60	3650
Amount of protein removed (%)	98	-
Amount of RNA recovered (%)	-	96

*RNA Estimation : The standard RNA used for reference was yeast RNA (Type III from bakers yeast)

+Protein Estimation : The standard protein used for reference was BSA (fraction V).

additional heterologous mRNA will only be translated to the extent that it competes with the endogenous mRNA. The activity of endogenous mRNA of E. coli MRE 600 which remained after incubation at 34°C for various times was measured by the degree of incorporation of [^{35}S] methionine at 37°C over a 25 min period. As shown in Fig. 16, preincubation of the S-30 extract for 15 min at 34°C reduced the endogenous mRNA activity by 70% and it was further reduced more slowly (approximately 20%) by further incubation.

E. IN VITRO PROTEIN SYNTHESIS DIRECTED BY STAPHYLOCOCCAL mRNA

a. Optimization of Ion Concentration

In vitro protein synthesis is very sensitive to cation concentration and the types of cation in the system. The concentrations of monovalent (NH_4^+) and divalent (Mg^{2+}) cations were therefore adjusted to the optimum concentrations for translation of the optimal amount of RNA extract of S. aureus.

1. Determination of Optimum Concentration of Mg^{2+}

The effect of Mg^{2+} concentration on translation efficiency was determined by measuring the incorporation of [^{35}S] methionine into protein at different Mg^{2+} concentrations. No extra NH_4^+ was added to the reaction mixtures and the RNA ($A_{260/280}$ = 2.04) concentration was 14% (v/v) of the reaction mixture. All incorporations were carried out at 37°C for 25 min and measured on 5 µl of reaction mixture. As shown in Fig. 17, optimal translation occurred with no further addition of Mg^{2+} ions. The final concentration of Mg^{2+} ion in the 35-µl reaction mixture was approximately 5.7 mM.

2. Determination of Optimum Concentration of NH_4^+

The effect of NH_4^+ concentration on translation was determined by

Figure 16. Degradation of Endogenous mRNA in an E. coli S-30 Extract by Incubation at 34°C.

Residual activity of endogenous RNA of E. coli MRE 600 in an S-30 extract after incubation at 34°C for various times was measured by the ability to incorporate [^{35}S] methionine at 37°C for 25 min.

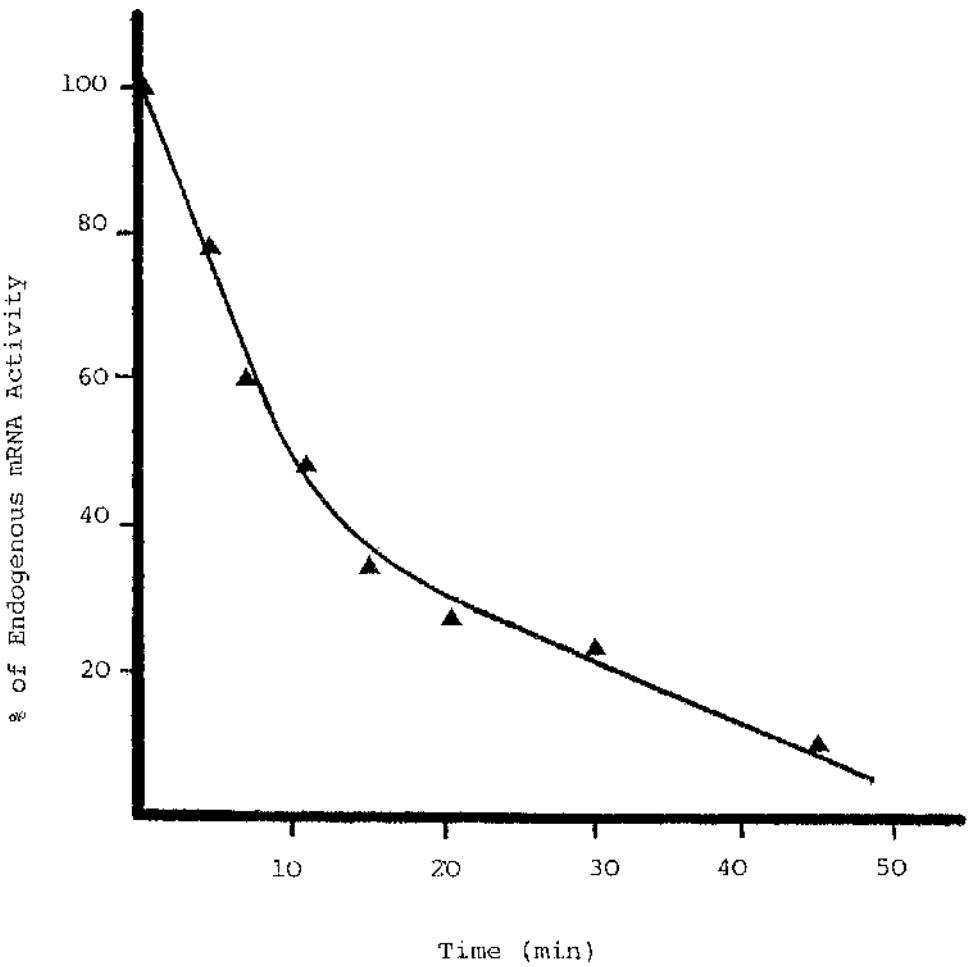
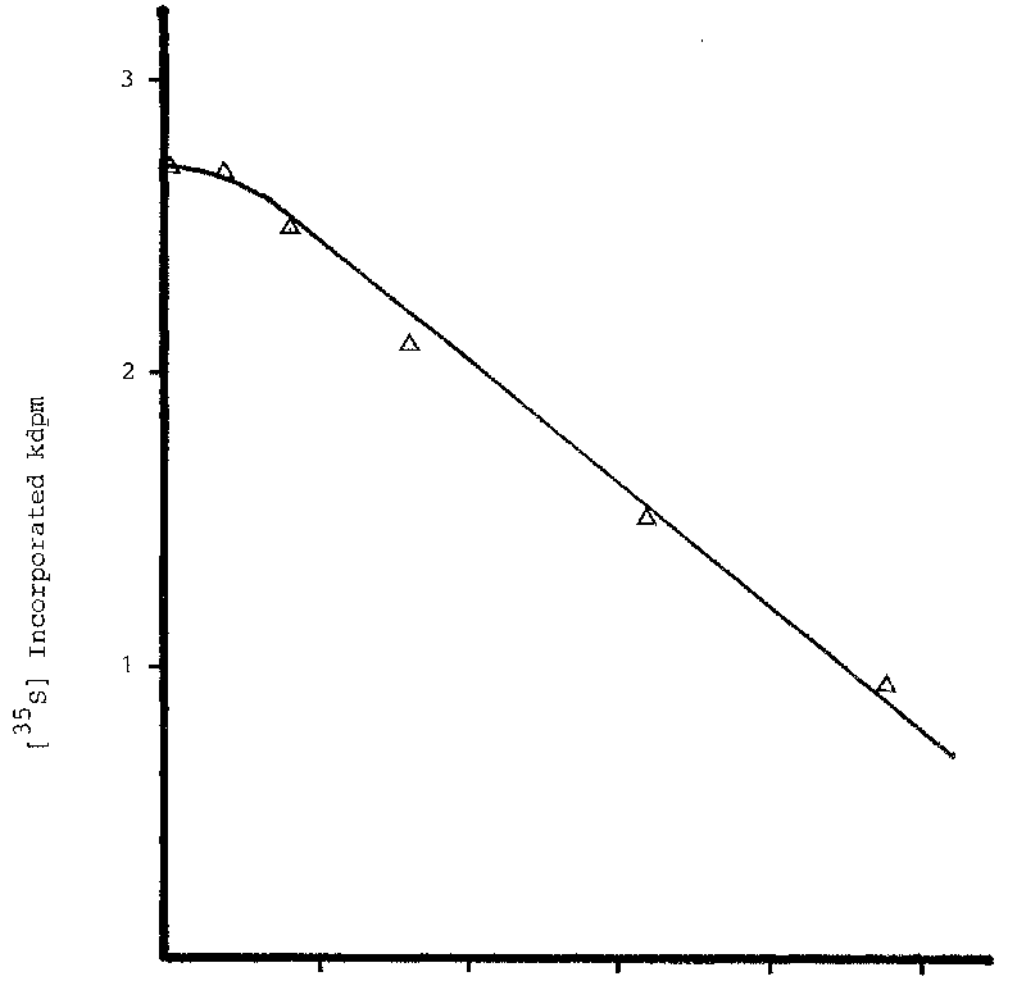

100
80
60
40
20
% of Endogenous mRNA Activity
10
20
30
40
50
Time (min)

Figure 17. The Effect of Mg^{2+} Concentration on In Vitro Translation of Staphylococcal mRNA.

Incorporation of [^{35}S] methionine into TCA-insoluble protein at different Mg^{2+} concentration was measured with no extra NH_4^+ added to the 35-µl reaction mixtures which contained 14% (v/v) staphylococcal RNA ($A_{260/280}$ = 2.04). All incorporations were carried out at 37°C for 25 min and measured on 5-µl of reaction mixture.


3
2
1
[35S] Incorporated Kdpm
Conc. added : 0 1 2 3 4 5
5.7 6.7 7.7 8.7 9.7 10.7
Total conc. in 35µl-reaction mixture :-
Mg2+ Concentration (mM)

measuring the incorporation of [^{35}S] methionine into protein for the S-30 translation system with a range of NH_4^+ concentrations with an optimum Mg^{2+} concentration. The RNA concentration was 14% (v/v) of the reaction mixture. All incorporations were carried out at 37°C for 25 min and measured on 5 µl of reaction mixture. Fig. 18 shows that the optimal NH_4^+ ion concentration was approximately 60 mM of which 27mM was added to the reaction mixture.

b. Determination of Optimum RNA Concentration

Addition of increasing amounts of mRNA to a reaction mixture will result in increasing incorporation of the radioactive amino acid until saturation is reached. To avoid wastage of mRNA or use of an excess of mRNA, which may actually inhibit protein synthesis, the optimal concentration was determined by incorporation of various concentrations of RNA in the translation mixture. The dose response curve (Fig. 19) showed that under the assay conditions used the optimal RNA concentration for cell-free protein synthesis in *E. coli* iS-30 extract was 14% (v/v) of *S. aureus* NCTC 10345 total cellular RNA ($A_{260/280}$ = 2.04) in 35-µl of reaction mixture.

c. Time Course of Protein Synthesis In Vitro

The time course of protein synthesis *in vitro* directed by mRNA of *S. aureus* in an *E. coli* iS-30 extract was studied by determining the [^{35}S] methionine incorporation into 5-µl volumes of reaction mixture (at 37°C) withdrawn at various times. Fig. 20 shows that the rate of amino acid incorporation was linear for 15 min. After this time, the rate decreased such that by 25 min, the reaction was completed.

d. Analysis of Protein Products of In Vitro Translation

Figure 18. The Effect of NH_4^+ Concentration on In Vitro Translation of Staphylococcal mRNA

Incorporation of [^{35}S] methionine into protein at different NH_4^+ concentration. No extra Mg^{2+} was added to the 35-µl reaction mixtures and staphylococcal RNA ($A_{260/280}$ = 2.04) was 14% (v/v) of the reaction mixture. All incorporations were carried out at 37°C for 25 min and measured on 5-µl of reaction mixture.

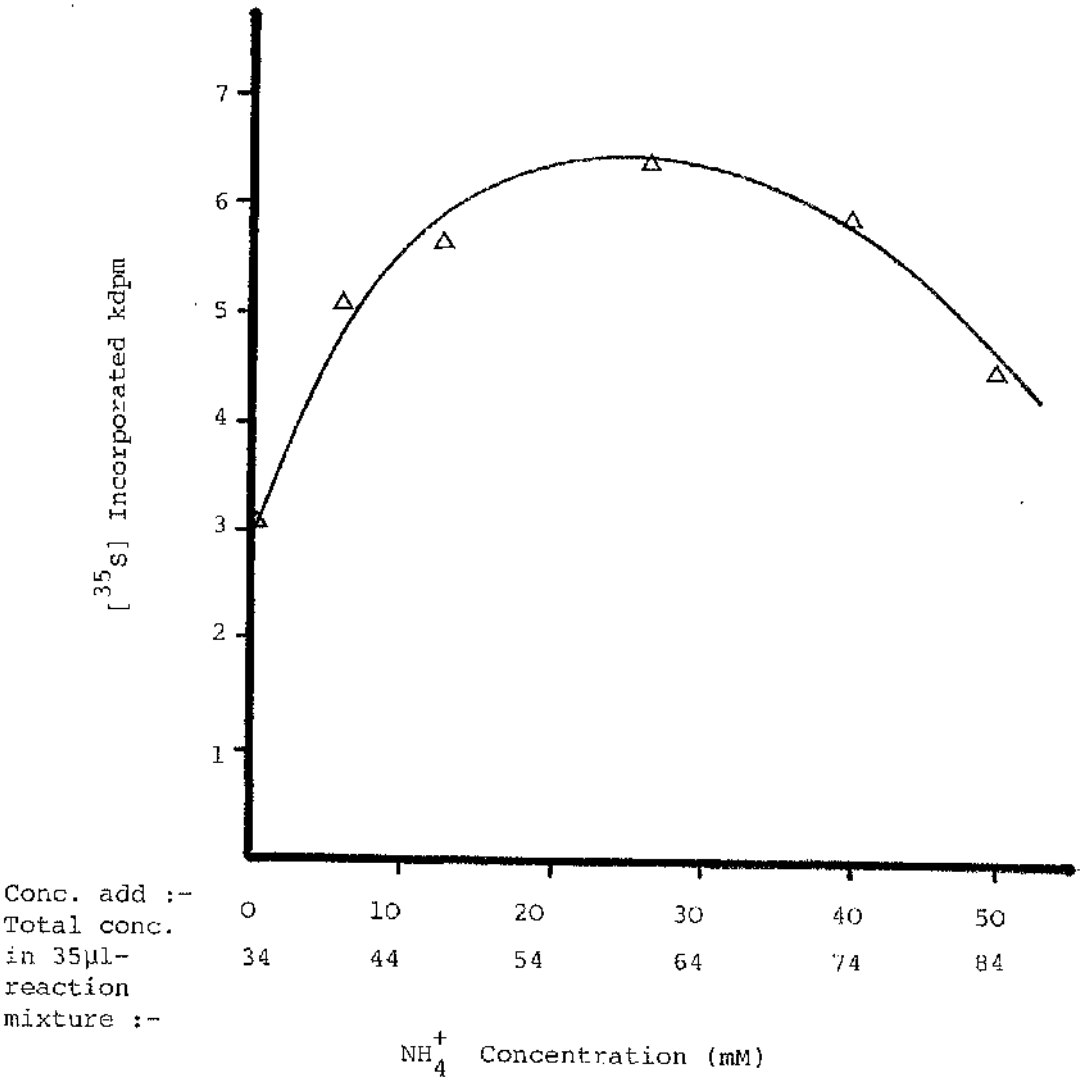

[35S] Incorporated kdpm
7
6
5
4
3
2
1
Conc. add :-
Total conc. in 35µl-reaction mixture :-
0
10
20
30
40
50
34
44
54
64
74
84
NH4+ Concentration (mM)

Figure 19. The Effect of RNA Concentration on In Vitro Translation of Staphylococcal mRNA

Incorporation of [^{35}S] methionine into protein directed by different concentrations of staphylococcal RNA ($A_{260/280}$ = 2.04) at optimal ion concentrations is shown. All incorporations were carried out at 37^{o}C for 25 min and measured on 5-µl of reaction mixture.

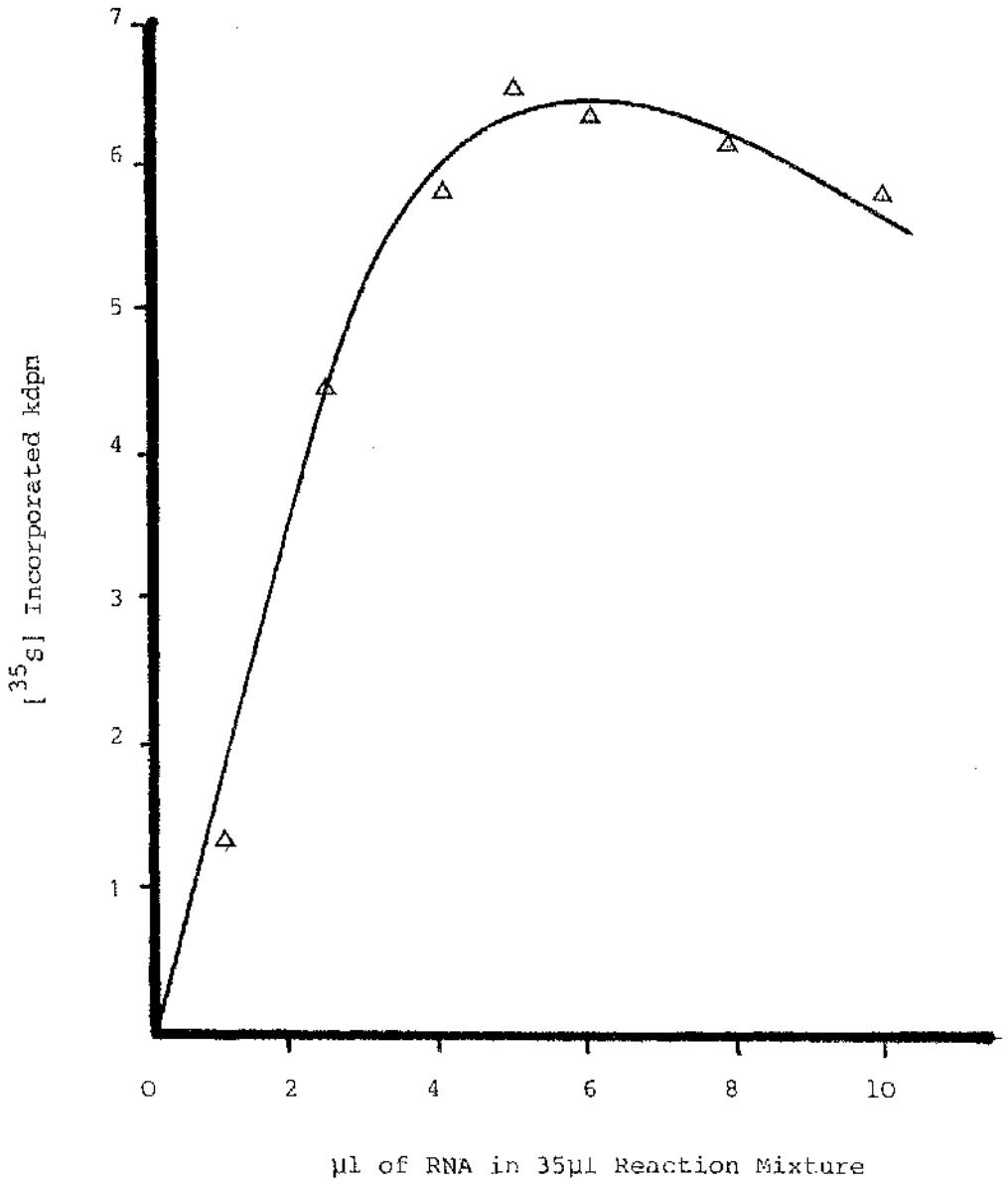
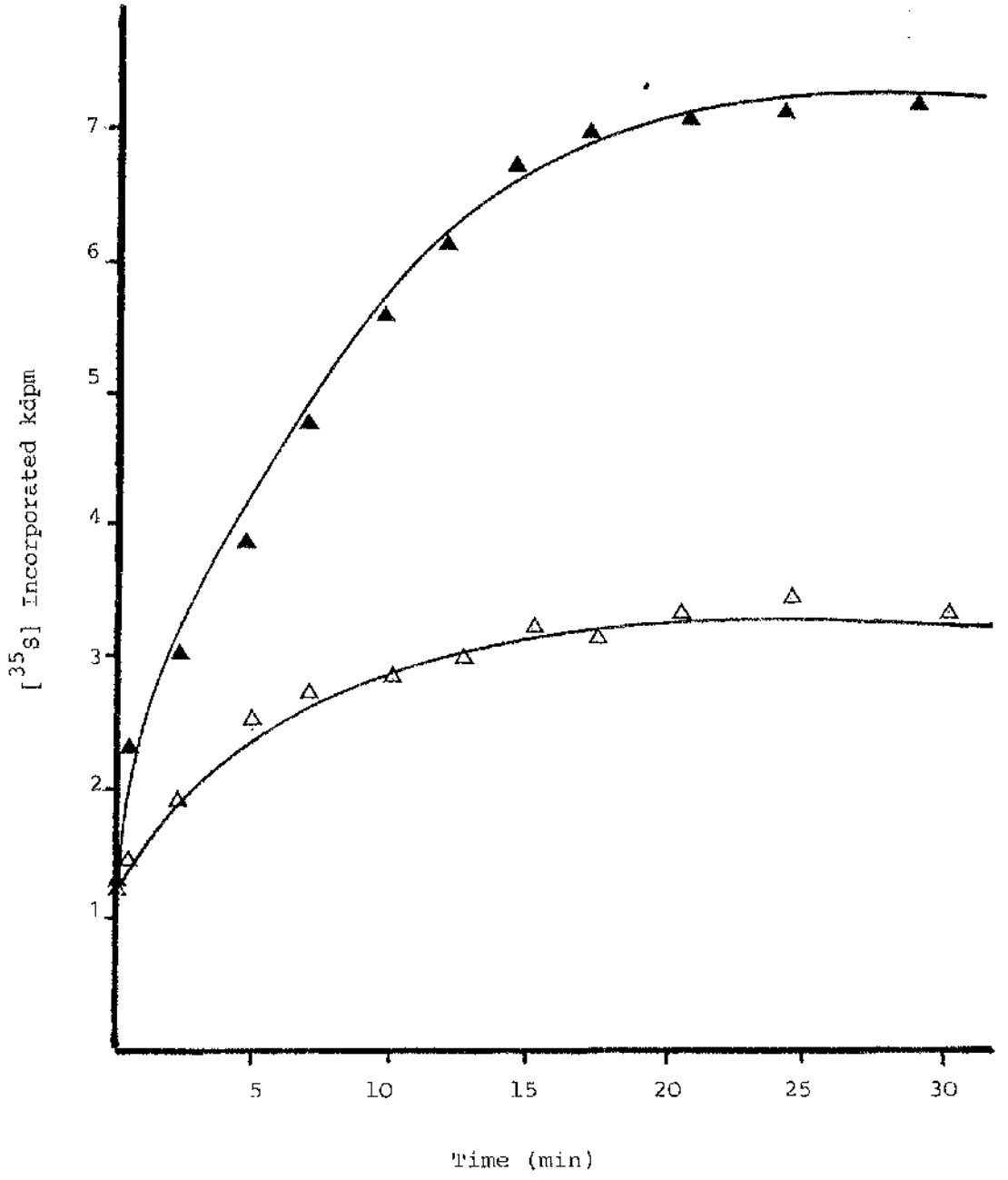

7
6
5
4
3
2
1
[35S] Incorporated kdpm
0
2
4
6
8
10
µl of RNA in 35µl Reaction Mixture

Figure 20. Time Course of In Vitro Protein Synthesis Directed by Total Cellular RNA of S. aureus NCTC 10345.

Protein synthesis directed by total cellular RNA of S. aureus NCTC 10345 (▲) and the residual endogenous RNA of E. coli MRE 600 (△) at 37°C, was measured as [^{35}S] methionine incorporated per 5-µl reaction mixture.


[35S] Incorporated kdpm
7
6
5
4
3
2
1
5
10
15
20
25
30
Time (min)

When total cellular RNA of S. aureus was added to E. coli iS-30 extract, the staphylococcal mRNA stimulated the incorporation of [^{35}S] methionine into hot-TCA-insoluble material to a level approximately 20 fold above the background (minus RNA) (Table 16). This protein synthesis was almost completely abolished by chloramphenicol (50 μg ml^{-1}), was dependent on an exogenous energy source (ATP, GTP, PEP) and was barely affected by the protease inhibitor, PMSF (1 mg ml^{-1}) or the RNase inhibitor, heparin (1 mg ml^{-1}). The products of cell-free synthesis were separated by SDS-PAGE ; several polypeptides were synthesized in the iS-30 extract, directed by mRNA of S. aureus, including a polypeptide of the same R_f value as native δ-lysin. This polypeptide was the only one to be immunoprecipitated by anti-δ-lysin antiserum (Fig. 21) and the only polypeptide to be removed from the mixture by an immunosorbent prepared from anti-δ-lysin antiserum (Fig. 22). In vitro synthesized δ-lysin also appeared as a very diffuse band similar to its native form when analysed on the SDS-PAGE. However,a putative precursor of δ-lysin was not detected in the initial translation product of the gene of δ-lysin translated in this cell-free protein synthesizing system.

Thus, δ-lysin does not seem to require an N-terminal signal sequence for its transmembrane translocation or to be a signal sequence for other secretory proteins. It could be of interest to know whether this is general for other membrane-active staphylococcal lysins (such as α-lysin) or only restricted to δ-lysin. The approach used for δ-lysin was applied to α-lysin of S.aureus Wood 46; this did not yield promising results as there was little incorporation of radiolabel stimulated by Wood 46 mRNA. Therefore, attempts were made to demonstrate a presursor to α-lysin directly in the membrane fractions of S.aureus Wood 46.

Table 16. Activity of E. coli iS-30 Extracts with Endogenous and Exogenous (Total Cellular RNA of S. aureus NCTC 10345)mRNA.

Reaction Mixture	[^{35}S] Incorporated (dpm $^{35}\mu l^{-1}$)
Complete* (preincubated+after storage)	37,522
Complete (preincubated+before storage)	30,000
Complete - Exogenous RNA	2,262
Complete - Exogenous Energy (ATP, GTP and PEP)	2,500
Complete + Chloramphenicol (50µg ml^{-1})	825
Complete + PMSF (1 mg ml^{-1})	37,658
Complete + Heparin (1 mg ml^{-1})	36,989

* Complete reaction mixture consists of 20µl iS-30, 5µl "translation cocktail", 5µl exogenous RNA, and 5µl sterilized deionized distilled H_2O.

+ S-30

Figure 21. SDS-PAGE Analysis of In Vitro Synthesized Proteins by Total Cellular RNA of S. aureus NCTC 10345

Lanes 1 and 4 are LKB ultrascan densitometer traces of lane 5 to 8 respectively. Lane ; 5, autoradiograph of S-30 background translation products; 6, autoradiograph of S-30 translation products from S. aureus NCTC 10345 RNA; 7, autoradiograph of S-30 translation products from S. aureus RNA immunoprecipitated with anti-δ-lysin antiserum; 8, 35µg of purified δ-lysin, Coomassie blue stained. The direction of migration is from right to left.

The arrow marks an artefact on the photographic negative.

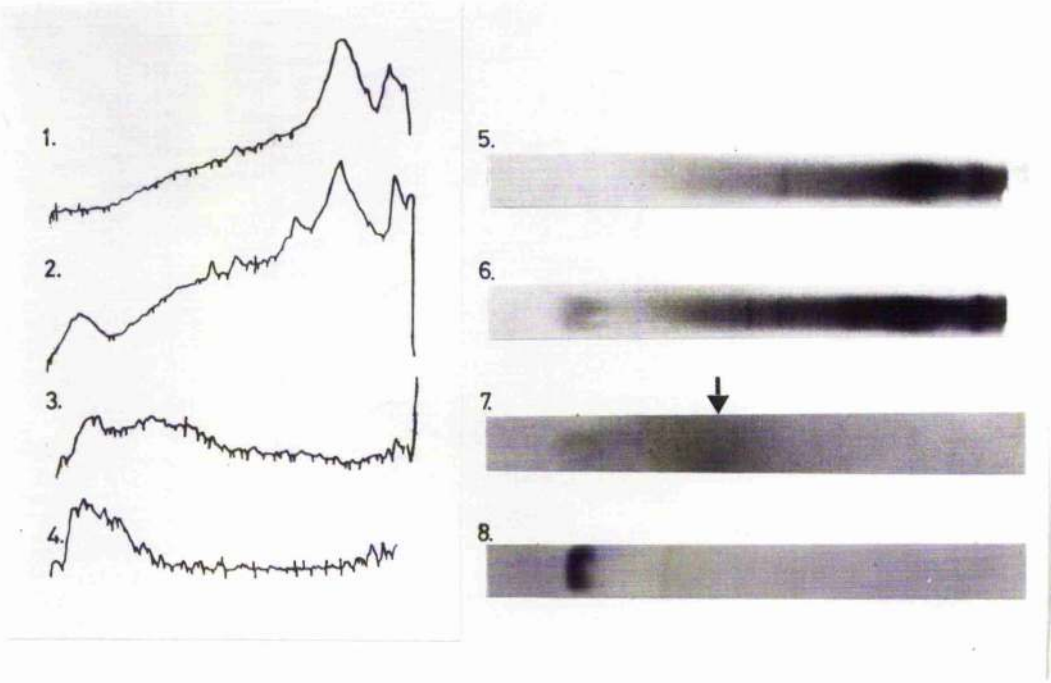
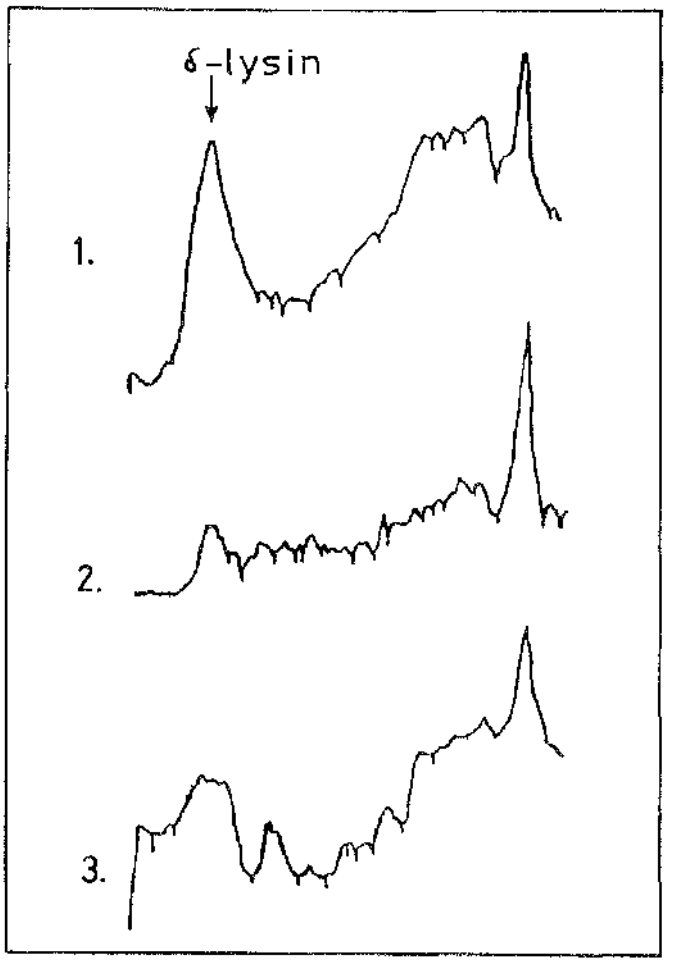

1.
2.
3.
4.
5.
6.
7.
8.

Figure 22. Densitometer Traces of Autoradiographs of the In Vitro Synthesized Protein Separated by SDS-PAGE.

Lanes ; 1, S-30 translation of S. aureus total cellular RNA ; 2, supernatant fraction of the above translation mixture after immunoabsorption with a polymer prepared from the immunoglobulin fraction of an anti-δ-lysin antiserum; 3, supernatant fraction of the above translation mixture after immunoabsorption (as a control) with a polymer prepared from an immunoglobulin fraction of an anti-ovalbumin antiserum. The direction of migration is from right to left.

The apparent reduction in the material to the right of the δ-lysin peak (lane 2) did not represent significant absorption of protein.


δ-lysin
1.
2.
3.

F. IMMUNOLOGICAL DETECTION OF α-LYSIN IN THE MEMBRANE OF S.AUREUS WOOD 46

Initially, attempts were made to demonstrate a precursor to α-lysin directly in the membrane fractions of S. aureus Wood 46. Membrane samples were isolated from both X-press-disrupted and lysostaphin-disrupted cells from late-logarithmic phase cultures of S. aureus. α-lysin and antigenically related proteins, in the membrane preparations and culture supernatants were demonstrated immunologically by fractionation of polypeptides by SDS-PAGE, electrotransfer to nitrocellulose paper and reaction with anti-α-lysin antiserum. Antibody-antigen reactions that took place on nitrocellulose paper were visualized using [^{3}H] -protein A and autoradiography (see Fig. 23A). Two types of band were visualized on the autoradiograph (Fig. 23A, lane 5 and 6); the 34 kdal-molecular weight bands corresponded to the purified α-lysin as determined by Coomassie blue staining of a parallel gel (Fig. 23B). The 27kdal-molecular weight bands were only seen with membrane samples where lysostaphin was employed to disrupt the cells (Fig. 23A, lane 5 and 6) and were not found in membrane samples where an X-press was used to disrupt the cells (Fig. 23A, lane 1 and 2). Membrane-associated α-lysin remained firmly bound in the membrane after washing the membrane preparations five times with 1M NaCl prior to fractionation in gels (Fig. 23A, lane 2 and 6). A larger precursor of α-lysin could not be detected in this case.

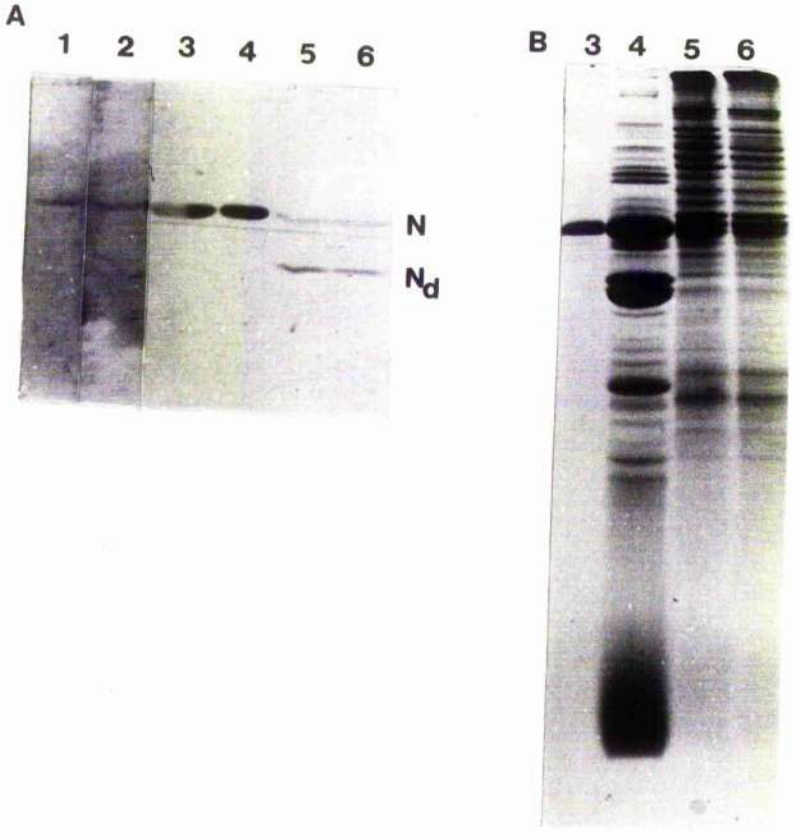
G. EFFECT OF PHENETHYL ALCOHOL (PEA) ON GROWTH OF S.AUREUS WOOD 46.

Failure to detect a precursor to α-lysin in the membrane indicated that the processing of α-lysin during the transmembrane translocation may be a very rapid process. To facilitate the detection of a precursor to α-lysin, phenethyl alcohol, a membrane perturbing agent, was employed to disturb the normal processing of the precursor. The effect of PEA on

Figure 23. Immunodetection of α-Lysin (Mature and Degraded Form) from Membranes of S. aureus Wood 46.

Immunodetection of α-lysin in membrane of S. aureus Wood 46. (A) Nitrocellulose blot analysis of membrane proteins from S. aureus Wood 46 at late-logarithmic phase of growth separated by SDS-PAGE and reacted with anti-α-lysin antiserum and [^{3}H] protein A. The positions of mature α-lysin (N) and its degraded form (Nd) are shown on the autoradiograph. Lanes 1 and 2, S. aureus membrane samples prepared from X-press-disrupted cells; Lane 3, purified α-lysin; Lane 4, culture supernatant obtained at late-logarithmic phase of growth; Lane 5 and 6, S. aureus membrane samples prepared from lysostaphin-disrupted cells. Membrane samples in Lanes 2 and 6 received 5 washes with 1M NaCl solution prior to separation on the gel.

(B) Coomassie blue stained of SDS-PAGE of Lane 3 to 6 of (A).


A
1
2
3
4
5
6
N
N_d
B
3
4
5
6

growth of a late-logarithmic phase culture of S.aureus Wood 46 was studied in shake flask cultures of 500 ml of B.S. Medium at 37°C. At late-logarithmic phase, the original culture was divided into 50 ml-cultures in 250 ml flanged Erlenmeyer flasks which contained different amounts of PEA. Growth was followed by measuring A_{650nm} of the cultures. The effect of graded concentrations of PEA on the final growth density (after 2 h of incubation at 37°C) of S. aureus is shown in Fig. 24 and growth of late-logarithmic phase culture of S. aureus was not affected by up to 0.3% (v/v) of PEA (1 mg ml^{-1}).

H. EFFECT OF PEA AT SUBINHIBITORY CONCENTRATION ON EXTRACELLULAR PROTEINS PRODUCTION BY S.AUREUS WOOD 46

The effect of PEA at maximum subinhibitory concentration (MSC) on the production of several extracellular proteins of S. aureus Wood 46 was studied in shake flask cultures of 50 ml of B.S. Medium at 37°C when PEA (0.3% (v/v)) was added to late-logarithmic phase cultures. Aliquots (1 ml) were withdrawn at intervals and the supernatant, after centrifugation in a MSE microfuge for 15 min, was assayed for α-lysin, δ-lysin and exoprotease production. Fig. 25 shows that, at the MSC of PEA, the production of α-lysin and exoprotease were preferentially suppressed with production of δ-lysin being unaffected. The treatment had no effect on biochemical activity of the proteins and no cryptic activity was released after disruption of the PEA-treated cells. The protein profiles on SDS-PAGE of both control and PEA-treated culture supernatant are shown in Fig. 26.

Figure 24. The Effect of Graded Concentration of PEA on Final Growth of S. aureus Wood 46.

Late-logarithmic phase cell cultures were incubated with graded concentrations of PEA at 37°C. After 2 h, final growth density was estimated by measuring A_{650nm}. The arrow indicates the initial cell density of the late-logarithmic cultures of S. aureus Wood 46.

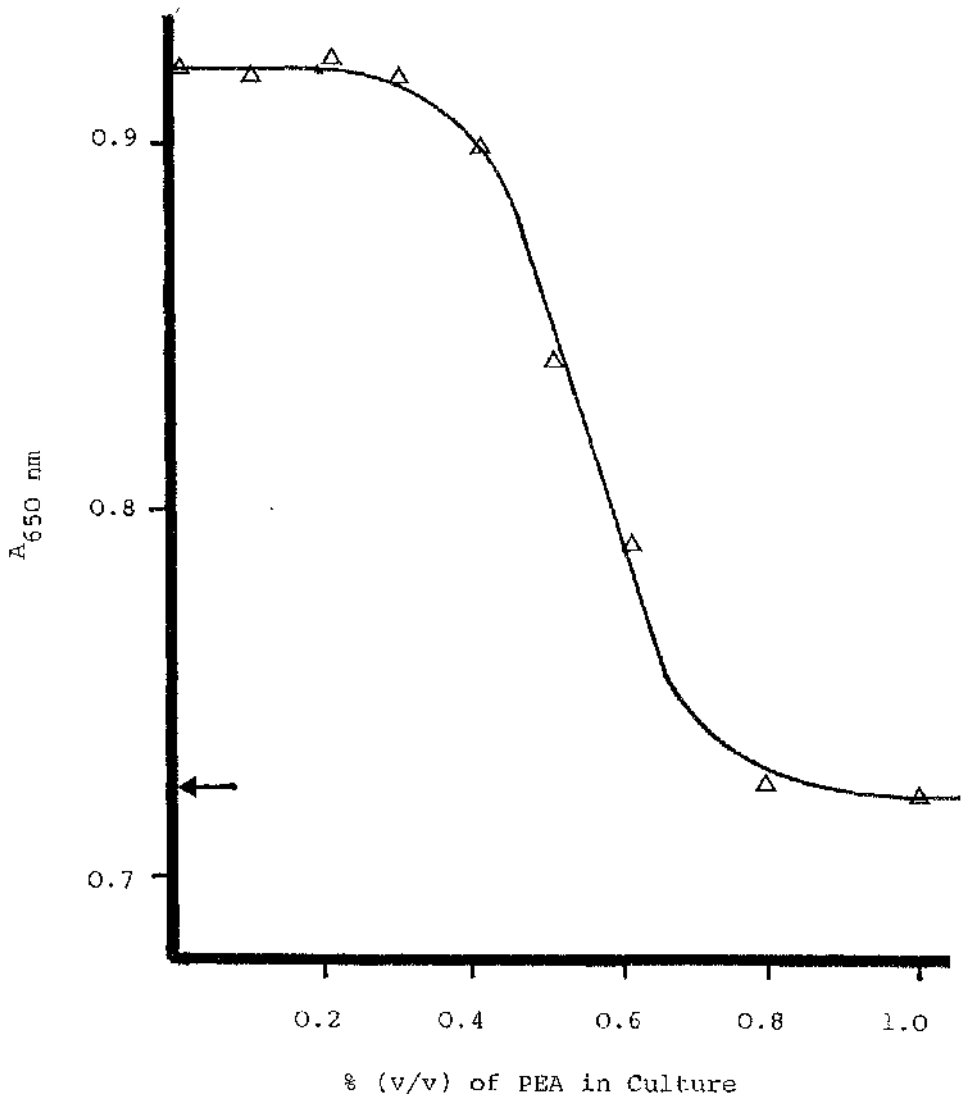

0.9
0.8
0.7
$A_{650\ nm}$
0.2
0.4
0.6
0.8
1.0
% (v/v) of PEA in Culture

Figure 25. The Effect of PEA at Maximum Subinhibitory Concentration on Production of Staphylococcal α- and δ-Lysins and Exoprotease by S. aureus Wood 46.

Supernatants obtained from both control and PEA-treated cell cultures were assayed for (i) α-lysin (▲, control and △, PEA-treated), (ii) δ-lysin (◆, control and ◇, PEA-treated), and (iii) exoprotease* (■, control and □, PEA-treated). The growth of the cultures was estimated by measuring $A_{650\,nm}$ (●, control and ○, PEA-treated) and the arrow indicates the point at which PEA was added to the test culture (final concentration 0.3% (v/v).

* Protease activity is expressed as A_{440nm} (see Materials and Methods, pg. 56 - for details).

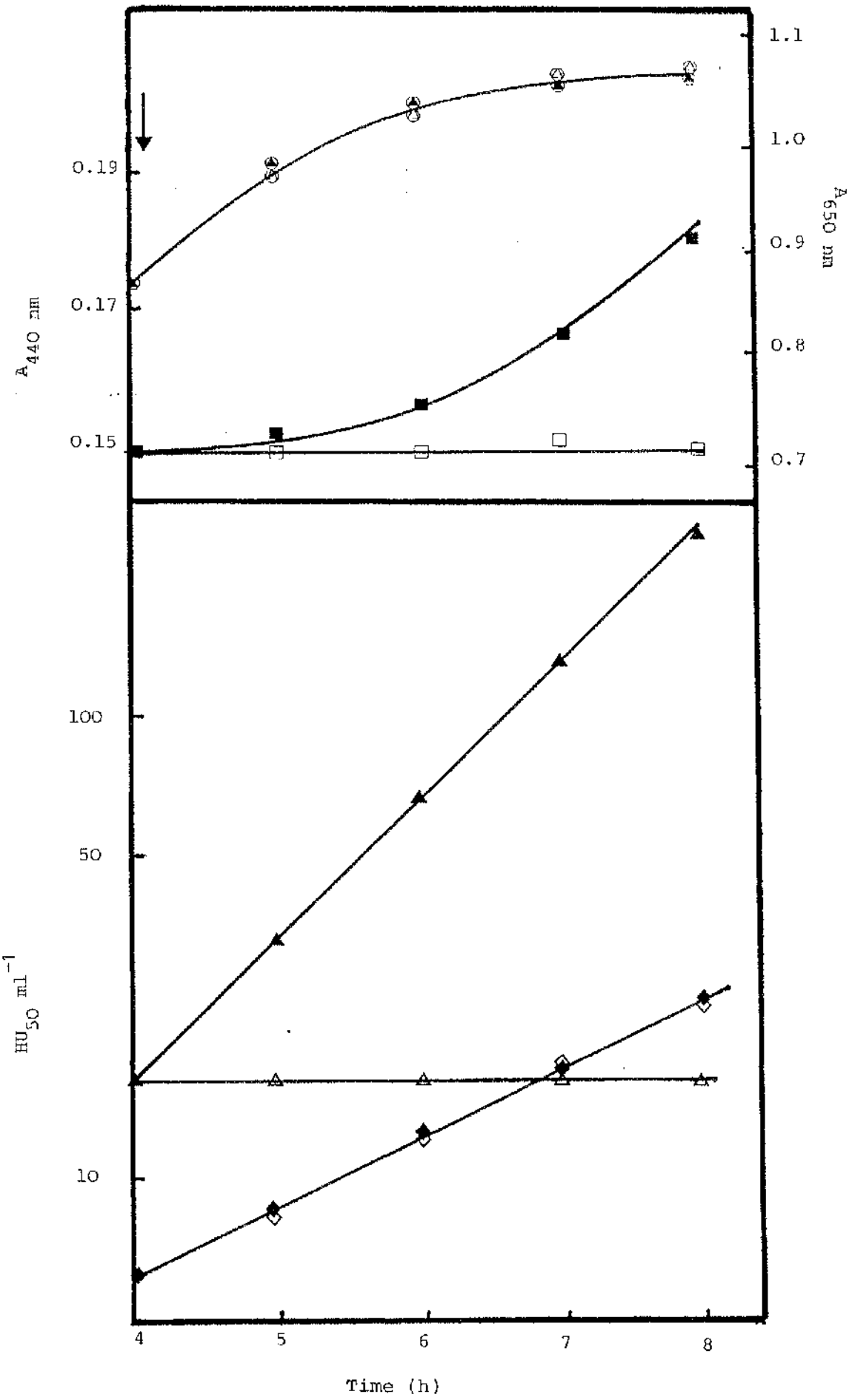
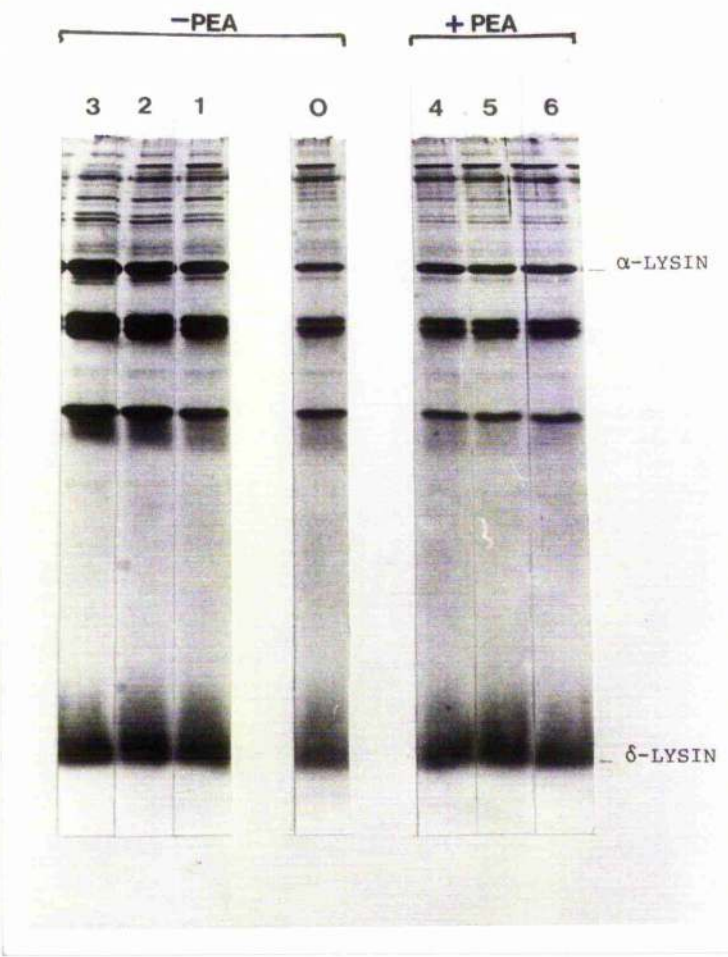

0.19
0.17
0.15
A_{440} nm
1.1
1.0
0.9
0.8
0.7
A_{650} nm
100
50
10
HU_{50} ml^{-1}
4
5
6
7
8
Time (h)

Figure 26. SDS-PAGE Analysis of Culture Supernatants of Control and PEA-treated Cell Cultures of S. aureus Wood 46.

Supernatants obtained from both control (Lane 1, 2 and 3) and PEA-treated (Lane 4, 5 and 6) cell cultures at 1, 2 and 3 h intervals after addition of PEA to the test culture (final concentration 0.3% (v/v)) were analysed on the 15% gel. Supernatant obtained just before the addition of PEA (Lane 0) was also analysed.


−PEA
+PEA
3 2 1 0 4 5 6
α-LYSIN
δ-LYSIN

I. EFFECT OF PEA AT SUBINHIBITORY CONCENTRATION ON TOTAL PROTEIN SYNTHESIS AND TOTAL EXOPROTEIN PRODUCTION OF S. AUREUS WOOD 46

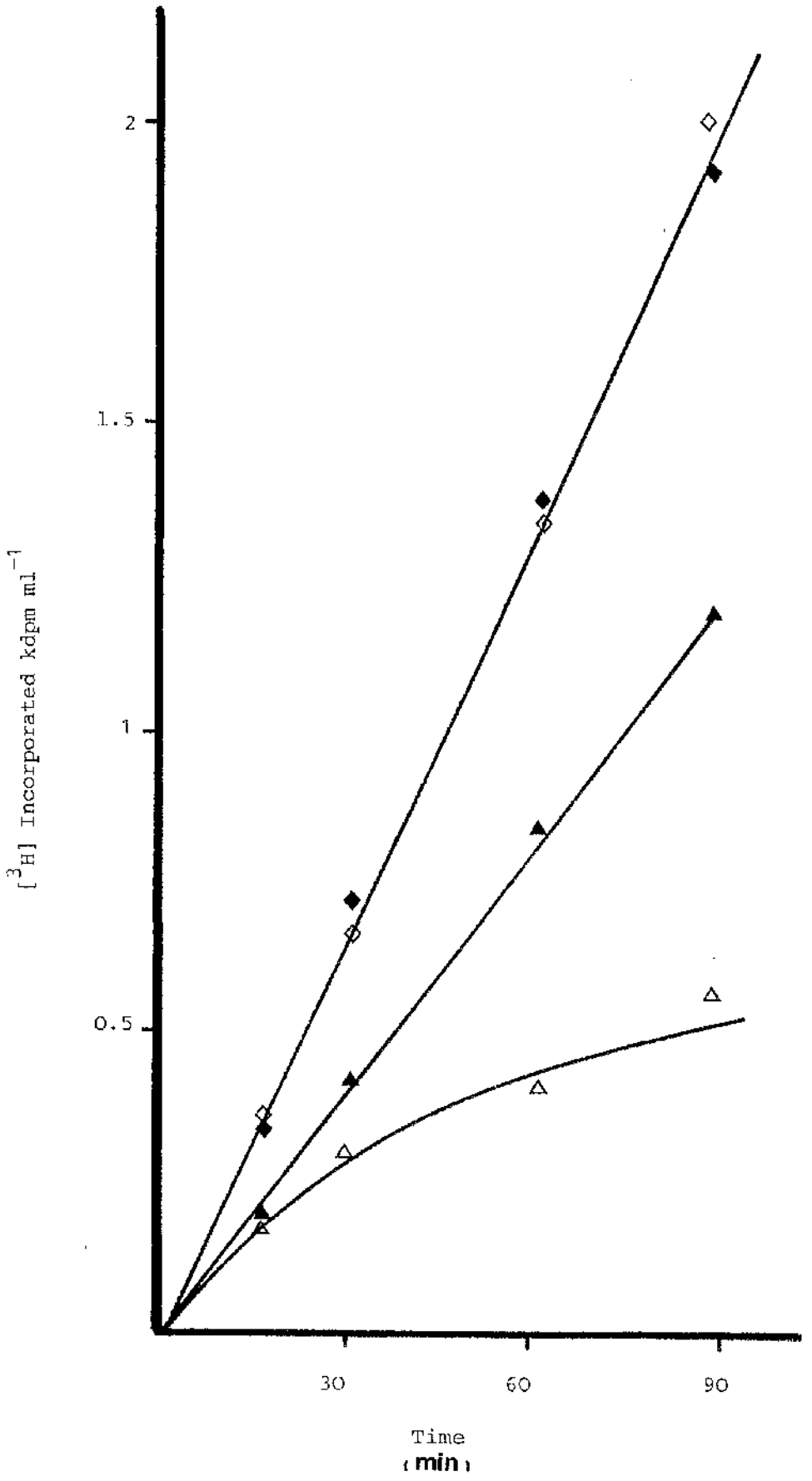
As PEA at maximum subinhibitory concentration, preferentially suppressed production of certain exoproteins (such as α-lysin and exoprotease), it would be of interest to know its effect on total protein synthesis and total exoprotein production. This was studied in shake flask cultures of 10 ml of B.S. Medium at 37°C; 16 μCi of [^{3}H]methionine and PEA (0.3% v/v final concentration) were added simultaneously to a late-logarithmic phase culture, samples of 1ml were withdrawn at various intervals and radioactivity in TCA-precipitable material of whole cell culture and culture supernatant were determined. Fig. 27 shows that the total protein synthesis, as measured by [^{3}H] methionine incorporation into proteins, was unaffected for at least 90 min after addition of PEA. However, exoprotein production, as measured by [^{3}H] methionine incorporation into exoproteins in the culture supernatant, were inhibited by nearly 50% after 90 min incubation. The results also imply that there is an increased protein level inside the PEA-treated cells.

J. EFFECT OF PEA AT SUBINHIBITORY CONCENTRATION ON THE LEVEL OF CELLULAR α-LYSIN IN S. AUREUS WOOD 46

The higher level of protein in the PEA-treated cells may be due to accumulation of exoproteins inside the cells and/or increased production of cellular protein after cessation of exoprotein synthesis. To differentiate between these possibilities, the effect of PEA at MSC on the level of cellular α-lysin was studied in shake flask cultures of 20 ml of B.S. Medium at 37°C in which 15μCi of [^{35}S]methionine was added to the mid-logarithmic cultures of S. aureus Wood 46 and after 2 h, PEA (0.3% (v/v)

Figure 27. The Effect of PEA at Maximum Subinhibitory Concentration on Total Protein Synthesis and Total Exoprotein Production of S. aureus Wood 46.

The total protein synthesis/exoprotein production of both control (◆/▲) and PEA-treated (◇/△) cell cultures at various times after addition of PEA to the test culture were determined by measuring the radioactivity in TCA-precipitable material of culture supernatants/supernatants.


2
1.5
1
0.5
[3H] Incorporated kdpm ml-1
30
60
90
Time
(min)

final concentration) was added to the test culture. At various intervals, samples of 1-ml were withdrawn, centrifuged for 15 min in MSE microfuge, the cells from each sample washed once with prewarmed B.S. Medium and resuspended in an equal volume (1 ml) of fresh prewarmed B.S. Medium containing chloramphenicol (100μg ml^{-1}) and unlabelled methionine (50 mg ml^{-1}). After 1 h at 37°C, cells were removed by centrifugation and each culture supernate was analysed by SDS-PAGE and the quantity of radiolabelled α-lysin in each sample was measured by excising the α-lysin band from the gel and scintillation counting of the solubilised fraction. A transient accumulation of cellular α-lysin occurred within 15 min of addition of PEA and the level of cellular α-lysin slowly decreased with further incubation (Fig. 28) probably due to cessation of α-lysin synthesis and proteolytic cleavage of existing cellular α-lysin.

K. REVERSAL OF INHIBITION BY PEA OF α-LYSIN PRODUCTION OF S.AUREUS WOOD 46

This was studied in shake flask cultures of 20 ml of B.S. Medium at 37°C in which PEA (0.3% (v/v) final concentration) was added to a late-logarithmic phase culture. After 1 h of incubation, both the control and PEA-treated cells were harvested by centrifugation and washed with prewarmed B.S. Medium. They were then transferred to 20 ml fresh B.S. Medium devoid of PEA and growth was allowed to continue at 37°C. Supernatant fractions obtained at various intervals were assayed for α-lysin and analysed by SDS-PAGE. Fig. 29 shows that when PEA was removed from the medium, production of active α-lysin was resumed to normal levels, showing that the drug reversibly inhibited α-lysin production and that the capacity for α-lysin production was not affected.

Figure 28. The Effect of PEA at Maximum Subinhibitory Concentration on the Level of Cellular α-Lysin in S. aureus Wood 46.

S. aureus was grown for 2 h in medium containing [^{35}S] methionine before addition of PEA to test culture. The amounts of cellular α-lysin in control (◆) and PEA-treated (◇) cells at various times after addition of PEA to the test culture were determined after release of radiolabelled cellular α-lysin into fresh medium (See Materials and Methods, section H-b-3-ii).

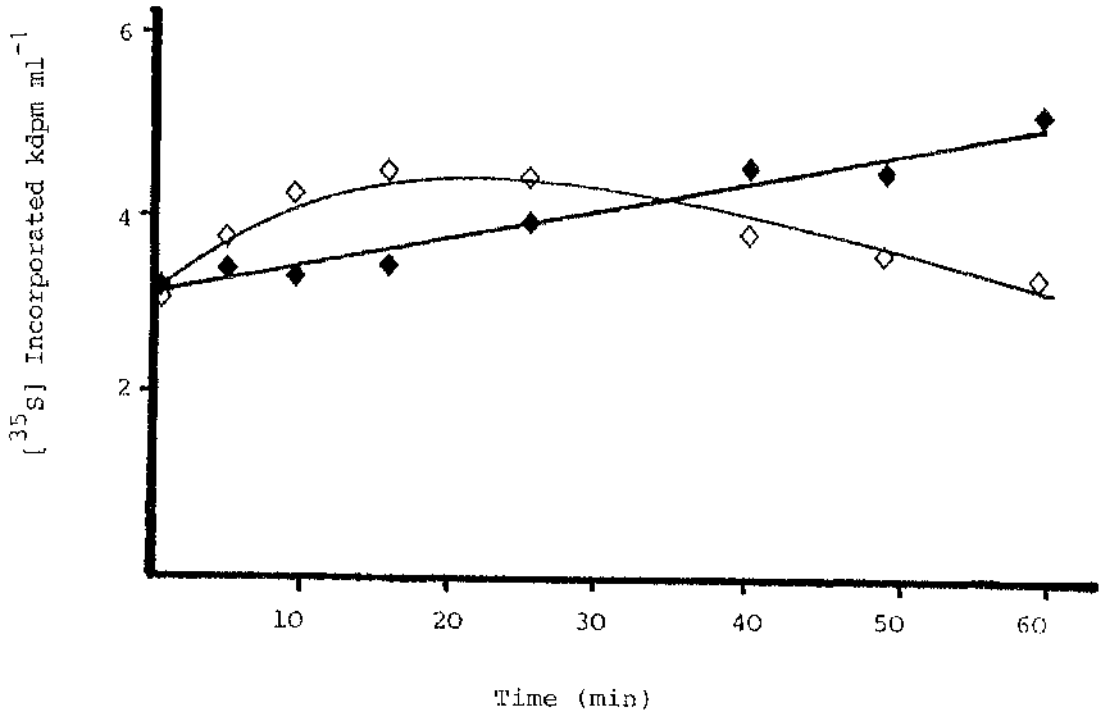
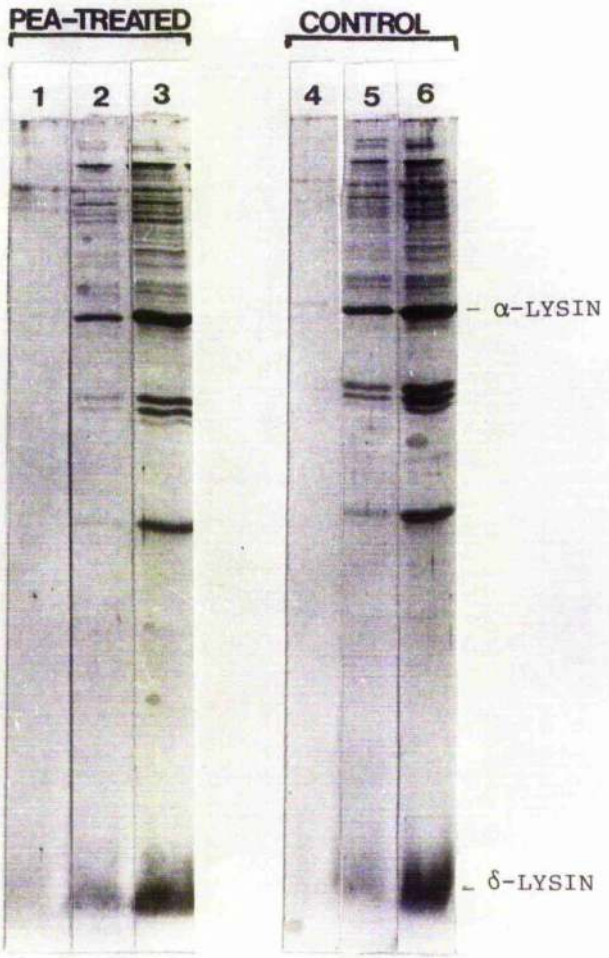

[35S] Incorporated kdpm ml-1
6
4
2
10
20
30
40
50
60
Time (min)

Figure 29. SDS-PAGE Analysis of Supernatants of Control and PEA-treated Cells Growth in Fresh Medium Devoid of PEA.

Supernatants were obtained at 0, 1 and 2 h (Lane 4 to 6, respectively) from control cells and (Lane 1 to 3, respectively) from PEA-treated cells, after resuspending in fresh medium devoid of PEA at 37°C.


PEA-TREATED
CONTROL
1
2
3
4
5
6
α-LYSIN
δ-LYSIN

L. DETECTION OF α-LYSIN PRECURSOR IN PEA-TREATED SYSTEM

Membrane fractions were prepared from late-logarithmic phase cultures of S. aureus Wood 46 treated with 0.3% (v/v) PEA for 20 min, and SDS-extracted membrane proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose paper. Antibody-antigen reactions that took place on nitrocellulose paper following treatment with anti-α-lysin antiserum were visualized using [^{3}H]-protein A and autoradiography (Fig. 30). Two proteins were visualized on the autoradiograph (Fig. 30, lane 3). The larger protein (P) had a molecular weight of 37 kdal, whereas the smaller protein (Nd) had a molecular weight of 27 kdal (Fig. 31); No mature lysin (N) was detected. However, when samples of SDS extract of PEA-treated whole cells were analysed, two proteins were visualized on the autoradiograph (Fig. 32), the larger protein (P) and the mature α-lysin (N) being detected. The 27 kdal-protein (Nd) was not detected in this preparation. Fig. 32 also shows the accumulation of α-lysin precursor with time in the presence of PEA, whereas levels of mature α-lysin did not substantially increase but decreased when cells were treated for 45 min.

Figure 30. Immunodetection of α-Lysin (Mature, Precursor and Degraded form) from Membrane of PEA-treated S. aureus Wood 46.

Nitrocellulose blot analysis of membrane proteins from PEA-treated S. aureus Wood 46 separated by SDS-PAGE. The positions of mature α-lysin (N), its precursor (P) and the degradation product of mature lysin (Nd) as immunodetected with anti-α-lysin antiserum, [^{3}H]-protein A and autoradiography are marked on the autoradiograph. Lane 1, [^{14}C] methylated protein molecular weight markers; Lane 2, purified mature α-lysin; and Lane 3, membrane proteins of PEA-treated S. aureus Wood 46.

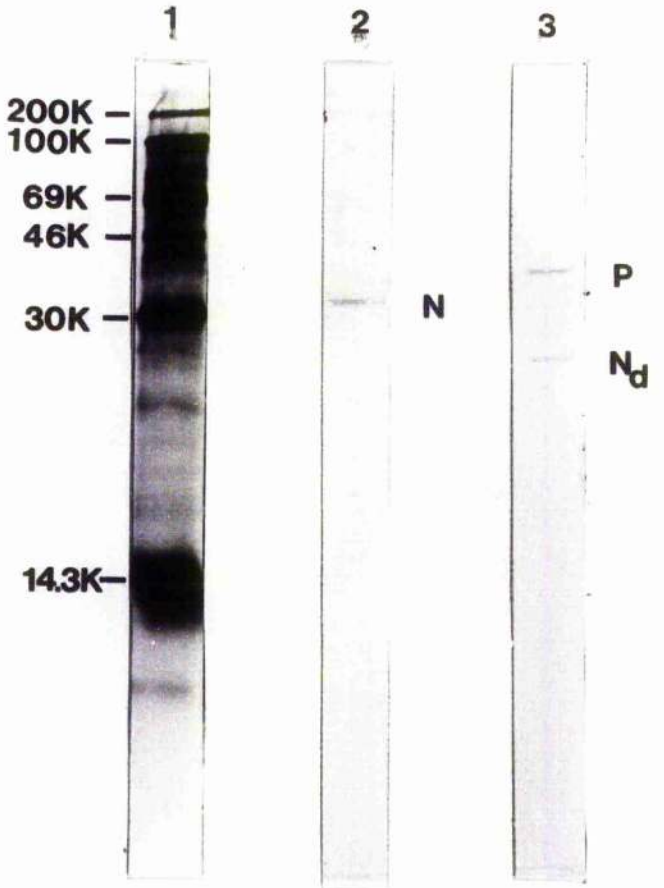

1
2
3
200K
100K
69K
46K
30K
14.3K
N
P
N_d

Figure 31. Plot of Relative Mobility of Standard Protein Markers Against Logarithm of their Molecular Weight.

Molecular weight plot of proteins from autoradiograph shown in Fig. 30. From left to right, the (▲) indicate the following markers; [^{14}C] methylated lysozyme (14.3 kdal), [^{14}C] methylated carbonic anhydrase (30 kdal) and [^{14}C] methylated ovalbumin (46 kdal). The position of precursor of α-lysin (□), mature α-lysin (■) and degraded form of α-lysin (▣) were also indicated.

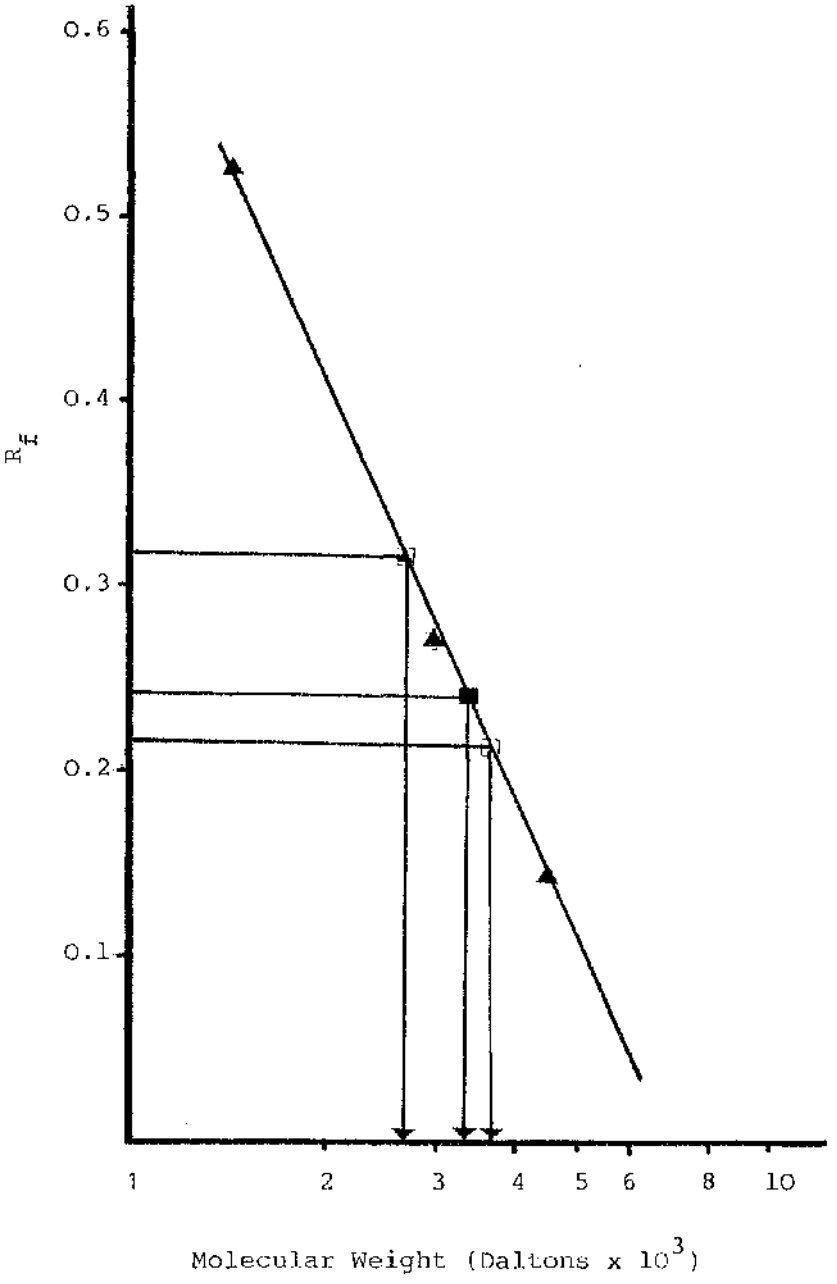
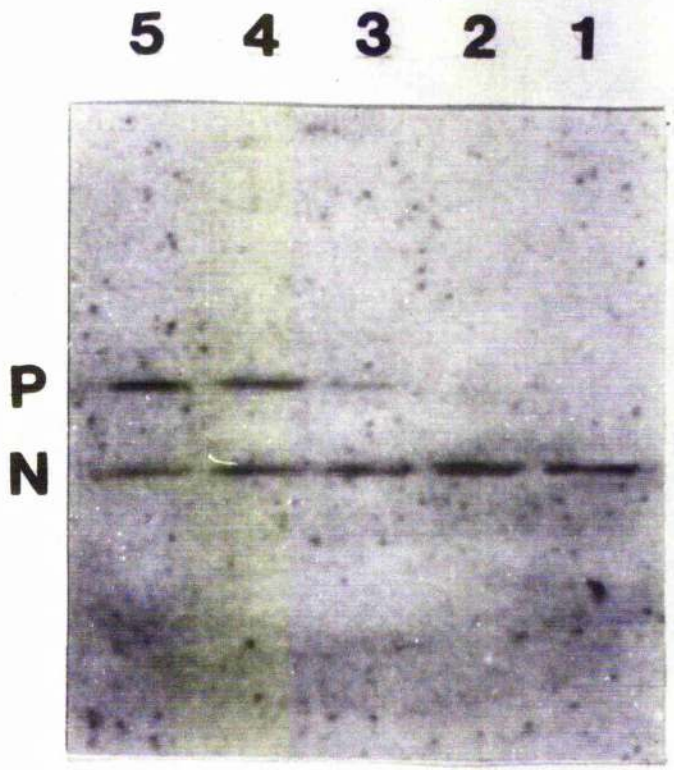

0.6
0.5
0.4
0.3
0.2
0.1
R_f
1
2
3
4
5
6
8
10
Molecular Weight (Daltons x 10^3)

Figure 32. Immunodetection of Mature α-Lysin and Precursor α-Lysin from PEA-treated S. aureus Wood 46 Whole Cells.

Nitrocellulose blot analysis of proteins from PEA-treated staphylococcal whole cells separated by SDS-PAGE. The positions of mature α-lysin (N) and its precursor (P) as immunodetected with anti-α-lysin antiserum, [^{3}H]-protein A and autoradiography are marked on the autoradiograph. Samples were taken at 0, 15, 30 and 45 min (Lanes 2 to 5, respectively) after the addition of PEA. Lane 1., purified α-lysin.


5
4
3
2
1
P
N

DISCUSSION

A. STUDIES IN VIVO OF δ-LYSIN mRNA METABOLISM

a. Effect of Rifampicin on RNA Synthesis

Rifampicin, a potent inhibitor of bacterial DNA-dependent RNA polymerase (Wehrli et al., 1968) rapidly blocked the incorporation of [^{3}H] uridine into staphylococcal RNA. As rifampicin inhibits only further rounds of transcriptional initiation and does not affect the synthesis of those mRNA chains already begun (Sippel and Hartmann, 1968) all RNAs initiated are synthesized to completion. This residual RNA synthesis (2 min) represents RNA production by RNA polymerase molecules involved in chain elongation at the time of the antibiotic block. This delay is a typical rifampicin-specific effect and has been described by other workers with other experimental systems (Schlessinger et al., 1977; Kennell and Talkad, 1976; Pedersen and Reeh, 1978; Kaluza and Hennecke, 1981). The length of the delay is reported to be influenced by the concentration of rifampicin (Reid and Speyer, 1970).

One noticeable feature of the curve of residual RNA synthesis after addition of rifampicin (Fig. 11) is the appearance of label in an unstable as well as stable RNA fraction. The RNA population within the bacterium can be divided into two major groups with respect to in vivo decay rate: stable and unstable RNA. The former is fairly permanent once synthesized and accounts for around 50% of total RNA synthesized at late logarithmic-early stationary phase of S. aureus. Similar observations were made by Kennell (1968) who suggested that the RNA synthesized in E. coli is about half messenger and half stable. This classification is useful for a functional approach to cellular RNA, as stable RNA consists mainly of rRNA and tRNA species, whereas unstable RNA is mRNA. The latter represents only around 3% of the total RNA mass because it is unstable. Such

differences in lability may arise because the stable RNA is either present as a ribonucleoprotein complex or has pronounced secondary structure, properties that shield the 5'-terminus or susceptible internal targets from attack by endogenous ribonucleases (see below).

b. Effect of Rifampicin on Bacterial Growth

The same concentration of rifampicin required a considerably longer period of time to cease cell growth at the late logarithmic-early stationary phase than the mid-exponential phase culture (Fig. 12). It is possible that during the onset of the stationary phase, due to lack of exogenous carbon substrate or utilizable nitrogen source, there is no net synthesis of RNA and protein and new protein is made from existing materials (hydrolysis of inessential RNA and proteins). Thus, inhibition of RNA synthesis at this stage will take a considerable time to affect bacterial growth in comparison to cells in the logarithmic phase of growth where cell growth primarily depends upon the active synthesis of the protein forming system (mainly rRNA and tRNA; Nierlich, 1978). Such an explanation is also supported by the following points : (1) in the transition to a lower growth rate the decrease in the rate of synthesis is immediate and marked for RNA but more gradual for protein and for DNA (Neidhardt and Magasanik, 1960) (2) in an "upshift" and "downshift" experiment, it has been suggested that cells growing at the faster rate are richer in RNA, therefore during the shift-up, small cells which are relatively poor in RNA acquire the character of fast-growing cells. The rate of RNA synthesis accelerates almost immediately, and is followed by an increase in the rate of protein synthesis and, later, by an increase in the rate of DNA synthesis. Conversely, after shift-down, cell division and DNA synthesis continue for some time, extensive turnover of RNA takes place

and net protein and RNA synthesis are only resumed gradually (Kjeldgaard, 1967). (3) rifampicin enters bacteria in a purely passive manner and logarithmic phase cells of E. coli bind twice the amount of rifampicin as stationary phase cells solely because the amount of DNA-dependent RNA polymerase in a cell varies during the growth cycle (White and Lancini, 1971).

c.Stability of Staphylococcal δ-Lysin mRNA

The stability of specific mRNA is generally expressed either by measuring the degradation of pulse-labelled RNA in the presence of an inhibitor of RNA synthesis (chemical half-life or decay rate) or by observing the time required for protein synthesis to cease after administering the inhibitor of RNA synthesis (functional half-life). Functional inactivation and chemical degradation both occur at exponential rates and the rates are markedly different from each other (Schwartz et al., 1970). Little or no information is available in the literature regarding the stability of mRNA in S. aureus. The fact that the half-life for chemical decay of total mRNA of S. aureus agrees with that for E. coli (Kennell, 1968) suggests that the bulk, if not all of mRNA species in bacteria have similar chemical half-lives. However, by measuring the residual δ-lysin synthesis in rifampicin-inhibited cultures, the functional half-life of δ-lysin mRNA in late logarithmic-early stationary phase was found to be around 20 min. Usually, in bacterial systems, mRNA half-lives of the order of 1 to 3 min have been found (Blundell et al., 1972 ; Leive and Kollin, 1967 ; Leive, 1965 ; Schwartz et al.,1970) but more recent studies have indicated much longer half-lives of up to 20 min for some mRNA species (Csanyi et al., 1971; Kaluza and Hennecke, 1981; Pedersen and Reeh, 1978; Hirashima and Inouye, 1973).

The stability of each mRNA is unique and independent of its size, function or level of synthesis (Blundell et al., 1972; Pedersen and Reeh, 1978). The stability of mRNA may be viewed as the resultant between degradation processes in the exo- and endonucleases and the protection given to mRNA by ribosomes (Smith, 1979). To account for the observation that mRNAs have different half-lives, a number of explanations have been proposed by Pedersen and Reeh (1978) and these include (1) secondary mRNA structure, (2) restricted accessibility of ribonucleases to particular classes of polysomes by reason of cellular location, (3) the number of ribosomes per unit of mRNA.

The functional inactivation of δ-lysin mRNA appear to obey exponential kinetics in agreement with the findings of Schwartz et al. (1970) and Apirion (1973). Exponential inactivation implies random inactivation for mRNA molecules irrespective of age, rather than a fixed life span or fixed number of cycles of utilization.

B. IN VITRO SYNTHESIS OF STAPHYLOCOCCAL δ-LYSIN

a. RNA Extraction

Protein synthesis in S. aureus is controlled such that extensive synthesis of structural proteins and constitutive enzymes occurs during the exponential phase of growth, but the numerous exoproteins, including δ-lysin, are secreted at a high rate after the end of exponential growth (Abbas-Ali and Coleman, 1977 a,b). The mechanisms regulating exoprotein synthesis in S. aureus are not known but could include competition at the level of transcription resulting in the accumulation of exoprotein mRNA (Coleman et al., 1975). Therefore, extraction of total cellular RNA from S. aureus was done with late exponential-early stationary phase

cultures to maximize the amount of available δ-lysin mRNA. Another advantage of using late exponential-early stationary phase cultures is that a greater functional stability of the mRNA pool may occur at lower growth rates (Smith, 1979).

Denaturation and breakdown of RNA is most likely to occur during the initial stages of extraction when precise control of the ionic composition and strength of the extract is impossible, and before contaminating nucleases have been removed. Enzymic degradation was minimized by performing all operations between 0 C-4 C, and by the use of RNase inhibitors, namely heparin and EGTA. Heparin, a sulphated polysaccharide which inhibits the activity of RNase by competitive binding (Zollner and Kellig, 1953) is reported to decrease RNase activity by more than 95% at 37 C (Palmiter et al., 1970; Palmiter, 1974). On the other hand, EGTA is a chelator of divalent metal cations such as Ca^{2+}, Cu^{2+} and Zn^{2+} (Marhol and Cheng, 1970), which may act as cofactor for RNase (Hiramaru et al., 1969) but to prevent possible chelation of Mg^{2+} by EGTA, extraction of RNA was done in the presence of excess Mg^{2+} ions as suggested by Kaufer and coworkers (1981). The chloroform-phenol-SDS mixture was an effective deproteinizing reagent, removing 98% of protein with recovery of 96% of RNA. SDS was included in the extraction medium as a dissociating agent to free RNA from protein and lipoprotein complexes and it also partially inhibits RNase action (Noll and Stutz, 1968). Unlike SDS, phenol is a very efficient protein denaturant (Kirby, 1956) and was employed together with chloroform as deproteinizing agent. Chloroform has several advantages over phenol; these include (1) rendering the organic phase sufficiently dense that it always remains at the bottom, thereby allowing more rapid separation of the aqueous and organic phases as well as permitting easy removal of the aqueous phase, (2) phenol alone retains 10-15% of the

aqueous phase at each extraction; chloroform prevents this water retention and thus improves the RNA yield, and (3) mRNA, especially poly(A)-containing mRNA, preferentially fractionates into the phenol phase under certain conditions but this is prevented by chloroform (Perry et al., 1972). Addition of 8-hydroxyquinoline to the phenol provides several advantages. It prevents oxidation of phenol, partially inhibits ribonuclease activity and chelates metal ions involved in binding RNA to proteins (Kirby, 1962). Also, addition of 10% m-cresol not only improves the ability of the mixture to deproteinize extract but also lower the freezing point of the phenol-8-hydroxyquinoline mixture (Kirby, 1965).

The ratio of A_{260} to A_{280} of the extracted total cellular RNA of S. aureus was within the acceptable range (1.9 to 2.1) suggested by Goldstein (1971) for the clean RNA extract. Lower ratios indicate protein contamination and higher ratios may indicate the presence of phenol.

b. In Vitro System of Protein Synthesis

Many functional protein-synthesising systems have been prepared from extracts of a variety of animals, plants and microorganisms (Shafritz, 1977; Legault-Demare and Chambliss, 1974; Hofbauer et al., 1982). These extracts vary widely in their degree of fractionation of protein synthesis components, their level of background amino acid incorporation (endogenous messenger activity) and their responsiveness to individual mRNA species. The E. coli S-30 extract, originally reported by Nirenberg and Matthaei (1961) is one of the earliest and most widely used systems for cell-free translation of prokaryotic exogenous mRNA. The fidelity of translation achieved by this system directed with exogenous RNA is exemplified by the synthesis of active enzymes (Shafritz, 1977) and by early termination at a nonsense mutation and suppression with appropriate

genetic suppressors (Webster et al., 1967; Engelhardt et al., 1967; Lodish and Roberton, 1969).

Since the S-30 extract is saturated with endogenous RNA, additional exogenous mRNA will only be translated to the extent that it competes with the endogenous mRNA. The S-30 extract was therefore preincubated to deplete its content of endogenous messenger; this was done before the dialysis step in the original procedure of Nirenberg and Matthaei (1961) during the preparation of E. coli S-30 extract but in our laboratory, postponing preincubation until just before use (Capecchi, 1966; Webster et al., 1967), resulted in increased activity (Table 16).

Protein synthesis directed by both residual endogenous and exogenous RNA was completely sensitive to chloramphenicol and only little incorporation occurred when exogenous energy was omitted. This is consistent with incorporation being due to ribosomal action and largely dependent on exogenous supplies of energy. Addition of both a protease inhibitor (PMSF) and an RNase inhibitor (heparin) to the translation system did not stimulate the incorporation (Table 16) showing that no significant amount of protease and RNase contamination.

c. In Vitro Synthesized δ-Lysin

Translation of mRNA species for eukaryotic secretory proteins in cell-free systems in the absence of microsomal membranes resulted in the synthesis of larger forms still containing the signal peptide (Inouye and Halegoua, 1980). A similar approach has demonstrated in vitro synthesis of proteins larger than their mature size for Escherichia coli (Michaelis and Beckwith, 1982), Bacillus licheniformis (Chang et al., 1982) and Corynebacterium diphtheriae (Smith, 1980). In vivo, most of the precursor proteins are rapidly processed to their final size and it is

therefore very difficult to detect the unprocessed molecules existing in the cell at any given time. In general, *in vitro* translation systems do not contain the necessary enzyme(s) for processing, as most are membrane-bound, and the precursor forms are therefore stable and detectable. *In vitro* synthesized δ-lysin seems not to be larger than the mature, secreted form by analysis on the SDS-PAGE. It is unlikely that the cell-free system processed the lysin as such processing has never been reported in *E. coli* S-30 extract.

C. TRANSMEMBRANE TRANSPORTATION OF δ-LYSIN

Many secretory and membrane proteins are initially synthesized as precursor molecules in which an extra hydrophobic peptide segment precedes the N-terminus of the mature protein. The finding that δ-lysin seems not to have a transient, hydrophobic signal sequence raises important questions about the mechanism of δ-lysin secretion. However, in some instances, proteins may be synthesized and secreted without cleavage of hydrophobic signal peptide (Wickner, 1980; Michaelis and Beckwith, 1982). Ovalbumin, the major secretory protein of the hen oviduct, is the only well-characterized secretory protein to be synthesized and secreted without a transient signal sequence (Palmiter *et al.*, 1978). It was later suggested that the signal sequence is located within the molecule (Lingappa *et al.*, 1979), and more recent studies have shown that the functional signal for membrane translocation of ovalbumin lies between residue 25 and 45 and becomes accessible when the nascent chain is 50 to 60 residues long. This hydrophobic sequence folds back on the preceding residues to form an amphipathic hairpin structure which is the signal recognized by the membrane (Meek *et al.*, 1982). This discovery also indicated that

proteolytic processing is not necessary for protein translocation, agreeing with the observation of Lin et al., (1978) who found that mutational change leading to the replacement of a glycine residue by an aspartic acid residue in the signal sequence of bacterial prelipoprotein prevented proteolytic cleavage of the signal, yet the precursor form was secreted. In E. coli, colicin E1, colicin E3 and colicin E3 immunity protein are reportedly secreted in the form in which they are synthesized. They appear to leave the cells where they are made long after their synthesis by a non-specific mechanism which results in increased permeability or actual lysis of the producing cells (Jakes and Model, 1979). However, more recent studies indicate that colicin E1 is exported with the aid of a product of the downstream gene and that the COOH-terminal portion, perhaps functioning as an internal signal-like sequence, is necessary for the export (Yamada and Nakazawa, 1984).

Turning to δ-lysin, from the published amino acid sequence (Fitton et al., 1980;1984), there appears to be no long sequence of hydrophobic residues in one region of the molecule. As such, it is unlikely that δ-lysin possess an internal sequence or itself act as a signal sequence for other secreted proteins. We have also no evidence that δ-lysin leaves the cells long after their synthesis by a non-specific mechanism as does the colicin of E. coli because δ-lysin was never detected in significant amounts in the cytoplasm at any stage of bacterial growth. However, δ-lysin contains 14 hydrophobic residues and a high percentage of non-ionizable side-chain amino acid. The periodic distribution of the charged residues in δ-lysin suggests that it may adopt an α-helical structure, resulting in a laterally amphipathic rod with separate hydrophobic and hydrophilic faces (Freer and Birkbeck, 1982). This strongly surface-active amphiphilic molecule may represent a new class of secretory proteins

in the prokaryotic system that initiates translocation directly without the need for a cleavable N-terminal signal sequence in molecule. However, the precise mechanism of translocation of δ-lysin is not clear. δ-lysin is most likely synthesized by the membrane-bound polysomes and translocated immediately after or during its synthesis to prevent aggregation of such hydrophobic molecules inside the cells. Purified δ-lysin has been shown to be soluble in both organic solvents and aqueous solution (Heatley, 1971).

Although monomeric δ-lysin has not been observed in aqueous solution, it is likely that this is the active form in the membrane or in solvents of low dielectric constant as has been suggested by Fitton et al., (1981). Highly purified δ-lysin has also been shown to insert into a lipid monolayer with a concomitant increase in the surface pressure (Bhakoo et al., 1982) and is cytolytic towards a wide variety of membrane, including bacterial protoplasts (Kreger et al., 1971); these data suggest a detergent-like peptide with the capacity to assume alternative conformations and upon meeting a cell membrane or liposome, the newly synthesized δ-lysin may be triggered to insert into the lipid bilayer without "topographic catalysis" (Wickner, 1980) and thus be conducted directly through the bilayer.

D. EFFECT OF PEA ON THE PRODUCTION OF STAPHYLOCOCCAL α-LYSIN

The view that the cytoplasmic membrane has a specific role in the production of bacterial exoproteins and therefore is expected to be susceptible to alterations in membrane structure (membrane composition or physical state of membrane) was supported by works on staphylococcal exterotoxin A (Berkeley et al., 1978), exterotoxin B and C (Altenbern,

1977), α-lysin (Yoshikawa et al., 1974; Fishman et al., 1980; Saleh and Freer, 1984), outer membrane proteins (Halegoua and Inouye, 1979; DiRienzo and Inouye, 1979) and periplasmic proteins (Pages et al., 1978; Lazdunski et al., 1979; Pages and Lazdunski, 1981; Kimura and Izui, 1976) of E. coli, and exotoxin A in P. aeruginosa (Nory et al., 1983).

Phenethyl alcohol (PEA), a membrane perturbing agent (Papahadjopoulos, 1972; Eliasz et al., 1976), at maximum subinhibitory concentration selectively suppressed production of α-lysin and exoproteases of late-logarithmic phase cultures of S. aureus Wood 46 but production of δ-lysin was not affected. Such a differential effect in the presence of PEA was also observed in the synthesis and assembly of major outer membrane proteins and periplasmic proteins of E. coli (Halegoua and Inouye, 1979; Pages and Lazdunski, 1981). The possible explanations might be that (1) the sites of processing or the enzymes of processing, of α-lysin and exoprotease are different from that of δ-lysin; (2) different levels of membrane potential are needed for a given protein to be effectively processed and secreted (Randick et al., 1983), and (3) α-lysin and δ-lysin are secreted by entirely different process as δ-lysin does not seem to required a signal sequence (see above).

The mechanism by which PEA exerts its inhibitory effect has not been established, but it has been suggested that the primary action of PEA is on the cell membrane resulting in reversible breakdown of the permeability barrier of the cell (Silver and Wendt, 1967). Absorption of PEA may alter the conformation of the membrane, thereby affecting its structure integrity with the membrane returning to its original configuration when PEA is removed. More recently, it has been shown that the inhibition of phospholipid synthesis by this compound is not a secondary effect leading

to the perturbation of the above cellular processes (Nunn and Tropp, 1972). Results of the incorporation experiments of amino acid precursor into total proteins suggest that PEA at MSC did not inhibit de novo protein synthesis. However, the reduction of exoprotein production and transient accumulation of cellular α-lysin suggest that PEA exerts its effect on the physical state of membrane lipids by its ability to perturb membrane structure and alter the fluidity (Halegoua and Inouye, 1979) and membrane potential (Landick et al, 1983). This might subsequently influence one or several events required for the production of certain exoproteins, including the direct interaction between membrane-bound polysomes or the mechanism of translocation of the protein across the cytoplasmic membrane. Such a conclusion is also supported by the finding that production of active α-lysin was resumed to normal levels after removal of PEA.

E.DETECTION OF α-LYSIN PRECURSOR

An earlier study showed cell-associated α-lysin in S. aureus Wood 46 by use of ferritin-labelled antibody (Coulter and Mukherjee, 1971). Although this analysis yielded information on antigenic similarity, it failed to distinguish a true membrane-bound protein from contaminating extracellular α-lysin.

When membranes of late-logarithmic phase cultures of S. aureus Wood 46 were analysed by SDS-PAGE and nitrocellulose blotting, no larger precursor to α-lysin was detected, an observation supported by those of Tweten et al., (1983). More interestingly, the appearance of the 27 kdal component of α-lysin (Nd) in membrane samples but not in culture supernatant or membrane samples where an X-press was used to disrupt the cells, indicated that this component might be a product of in vitro

degradation (N ⟶ Nd) of membrane-bound α-lysin (N) due to lysostaphin treatment or contamination of lysostaphin with protease. Mature α-lysin was detected in the SDS extract of whole cell but not in the SDS extract of the membrane suggesting that in vitro degradation (N⟶Nd) only occurred during lysostaphin treatment. Dalen (1976) reported that α-lysin from Strain Wood 46 consisted of 27.5 kdal and 39 kdal-molecular-weight components that were antigenically identical and suggested that autodigestion, resulting from inherent proteolytic activity with α-lysin was responsible for formation of the 27.5 kdal component. However, the observed changes could also have resulted from interaction of the lysin with extraneous proteases.

Alteration of membrane fluidity by agents such as PEA (Halegoua and Inouye, 1979) or procaine (Lazdunski et al., 1979), ethanol (Lory et al., 1983) or growth of a fatty acid auxotroph in the presence of cliadate (DiRienzo and Inouye, 1979; Pages et al., 1981) have been used to inhibit the processing of precursors of exported proteins and facilitate the detection of precursors. A larger precursor to α-lysin was detected in the SDS extracts of both membrane and whole cell prepared from PEA-treated late-logarithmic phase culture of S. aureus Wood 46. The molecular weight of the precursor was approximately 37 kdal, whereas that of mature α-lysin is 34 kdal. This corresponds to a molecular weight difference of about 3,000 or a peptide elongation of about 20 to 30 amino acids. Our results are considerably different to those of Tweten et al., (1983) who detected two larger precursors of α-lysin with peptide extension of 1,850 and 1,100 respectively in the cell membrane of dinitrophenol-inhibited S. aureus S6. Such variations in molecular weight might be due to strain differences but our results are supported by work on DNA sequencing which shows the N-terminal region of mature α-lysin

to be preceded by a 26 amino acids signal sequence (Gray and Kehoe,1984).

The mechanism by which PEA inhibits the cleavage of precursor form is unknown. Several hypotheses can be considered : (1) the signal peptidase might be directly inhibited by PEA, (2) PEA, a membrane perturbing agent, might alter the electrochemical gradients across the cytoplasmic membrane. Studies on E. coli have led to the suggestion that electrochemical potentials across the membrane may be an important factor in protein secretion (Enequist et al., 1981; Daniels et al., 1981; Date et al., 1980). The principal role of the membrane potential is to orient the precursor within the membrane so that it will be accessible to the signal peptidase, (3) PEA, by addition to its own bulk to the lipid molecules of the membrane, drastically increased the membrane fluidity (Halegoua and Inouye, 1979). Fluidity of the membrane is required for processing of the precursor forms of exported protein but the detailed mechanism by which this occurs is not clear (Pages et al., 1978; DiRienzo and Inouye, 1979). This limited alteration is probably small enough to avoid inhibitions of membrane-associated respiratory functions, but sufficient to prevent the processing of precursors to occur.

F.PROTEIN TRANSPORTATION OF S. AUREUS : GENERAL DISCUSSION AND FURTHER RESEARCH

S.aureus secretes several extracellular proteins, many of which have been characterized in terms of their physicochemical properties and biological effects (Rogolsky, 1979; Freer and Arbuthnott, 1983). The study of the transmembrane transport of these extracrellular protein has been largely neglected and only recently has work on the mode of secretion of staphylococcal penicillinase (Nielsen and Lampen, 1982 a,b), enterotoxin B

(Altenbern, 1977, 1978; Tweten and Iandolo, 1981, 1983), α-lysin (Saleh and Freer 1984; Tweten et al., 1983) and δ-lysin (Lee and Birkbeck, 1984) been reported. The information obtained is far from complete and indicates that different modes of secretion may be involved for different proteins of S. aureus. Secretion of δ-lysin is different from that of penicillinase (McLaughlin et al., 1981), enterotoxin B (Tweten and Iandolo, 1981) and α-lysin (Tweten et al., 1983) as a putative precursor of δ-lysin was not detected in the initial translation product of the gene of δ-lysin in the cell-free protein synthesizing system reported here. However, failure to detect a precursor form for δ-lysin in this system does not constitute full proof that it is not made in precursor form, as there is the possibility of co-migration of mature and precursor proteins in the electrophoresis system; also in vitro processing of the precursor protein cannot be totally excluded. Considerable work therefore remains to be done before the nature of δ-lysin secretion can be conclusively elucidated. One possible approach would be to study in detail the structure of the genetic determinant which encodes for δ-lysin by employing recombinant DNA techniques to construct a hybrid plasmid expressing δ-lysin. The DNA sequence of such a cloned δ-lysin gene could be used to infer indirectly the initial product of translation of δ-lysin gene to avoid the possibility of co-migration and in vitro processing in the cell-free protein synthesizing system. Systems such as plasmid-containing mini-cells or phage infection of uv-irradiated cells can also be constructed to reveal the initial product of translation (see review by Silhavy et al., 1983). Additional information can also be obtained by introducing the cloned lysin determinant into different bacteria, such as E. coli or B. subtilis to reveal the localization of protein in different systems. A similar approach to the suggested for δ-lysin has begun for α-lysin of S. aureus

(Kehoe et al., 1983; Fairweather et al., 1983; Gray and Kehoe, 1984).

Earlier studies on the secretion of staphylococcal enterotoxin B and α-lysin using cerulenin and protease inhibitors (Saleh and Freer, 1984; Altenbern, 1977, 1978) suggested that they were transported by a lipid intermediate/proteolytic-releasing mechanism similar to that of penicillinase (Nielsen and Lampen, 1982a). The membrane-bound penicillinase of S. aureus carries the same glyceride thioether modification as B. licheniformis membrane penicillinase (Nielsen and Lampen, 1982b) and by comparing the modification-susceptible signal sequence of gram-positive penicillinase and of gram-negative outer membrane proteins with those of nonmodified gram-negative penicillinases, Nielsen and Lampen (1982a) proposed that a conserved sequence of Leu-Ala-Gly-Cys-X-Ser-Asn (where X designates a neutral or non-polar residue) in the signal sequence is required both for the modification to occur and for correct signal peptidase removal of segment preceding the glyceride thioether. More recently, Bocquet-Pages et al., (1981) showed that cerulenin inhibits the synthesis or assembly of the E. coli outer membrane proteins OmpA, OmpC and OmpF (none of which is a lipoprotein). Thus, the inhibiting activity of cerulenin could be indirect and does not necessarily indicate the presence of a lipid intermediate in the transport of enterotoxin B and α-lysin. This view is supported by examination of the amino acid sequence of the signal peptide of α-lysin (Gray and Kehoe, 1984) which does not possess the proposed conserved sequence of Nielsen and Lampen (1982a). As an amino acid sequence of the signal peptide of enterotoxin B is not available, predictions of the involvement of glyceride thioether modification during its secretion becomes impossible. However evidence could be obtained by studying the effects of the peptide

antibiotic globomycin on their production and by *in vivo* radioisotopic labelling with palmitate or glycerol, similar to work of Nielsen *et al.* (1981) on penicillinase of *B. licheniformis*.

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