EFFECT OF CERULENIN ON THE PRODUCTION OF ALPHA-LYSIN BY *STAPHYLOCOCCUS AUREUS* Wood 46

By

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To my mother

and the memory of my father.

PREFACE

This thesis is the original work of the author.

Faraydoon A. Kader Saleh

Since the writing of this thesis part of the results have been accepted for publication under the title of:

"INHIBITION OF SECRETION OF STAPHYLOCOCCAL ALPHA TOXIN BY CERULENIN"

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"Everyone is free to declare his judgement, in things which have not been fully discovered"

Antonie van Leeuwenhoek (1685)

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SUMMARY

This investigation was started with the intention of determining whether the secretion of haemolysins by Staphylococcus aureus took place in accordance with the signal hypothesis of Blobel and Dobberstein (1975 a,b).

Before embarking on the main object of the investigation, various studies were made on the production and purification of the α-haemolysin of S.aureus Wood 46. The production of α-, β- and δ-haemolysins and protease(s) by S.aureus Wood 46 was biphasic, in agreement with the published data (Duncan and Cho, 1971; McNiven and Arbuthnott, 1972).

Purified α-lysin prepared by ammonium sulphate precipitation, chromatography on controlled-pore glass and electrofocusing had a pI of 8.5, a specific haemolytic activity of 1.63×10^5 HU_{50} mg^{-1}, and gave a single line in immunodiffusion tests against anti-serum to culture supernate of Wood 46. The molecular weight was estimated as 34,000 dalton, by sodium dodecyl sulphate polyacrylamide gel electrophoresis. These properties coincide with those of α-lysin prepared by other workers (Freer and Arbuthnott, 1983).

Bands of 27,000 and 18,000 daltons which appeared in preserved preparations of purified α-lysin, shared several peptides with 34,000 dalton α-lysin band when peptide mapping was performed confirming that these bands were derivatives of purified 34,000 dalton lysin (Dalen, 1976a).

Since the signal peptide hypothesis requires proteolysis of the pro-protein in order to permit secretion across the cytoplasmic membrane, various protease inhibitors were tested for their ability to inhibit secretion of the α-, β- and δ-lysins of S.aureus Wood 46. Phenylmethylsulfonyl fluoride (PMSF), quinacrine and O-phenanthroline at sub growth inhibitory concentrations did not reduce levels of α-lysin or other extracellular proteins of Wood 46.

Attention was next turned to two agents reported to affect membrane lipids, procaine and cerulenin. The former had no effect on the secretion of α-lysin but the latter had a major effect on production of the three lysins and protease(s) at concentrations which only marginally affected bacterial growth. The protein most sensitive to inhibition was α-lysin where the extracellular level was reduced by 98%.

Cerulenin caused a 50% reduction in total extractable cell lipids. Lipid synthesis was reduced by 50% as measured by the rate of incorporation of [^{14}C]-acetate into chloroform/methanol extracts, protein synthesis measured by the rate of incorporation of [^{3}H]-phenylalanine into trichloroacetic acid (TCA) precipitated material was not affected. Cerulenin also caused significant changes in the fatty acid profile of Wood 46. Exogenous oleate partially restored α-lysin production in cerulenin-inhibited cells.

It appeared from these observations that secretion of the haemolysins and the protease(s) was dependent or at least closely associated with synthesis of membrane lipids.

A reduction in total membrane and increased protein: lipid ratio was observed in cerulenin-inhibited cells. Alpha-lysin of molecular weight 34,000 dalton was present in control cell membranes but absent from cerulenin-inhibited cell membranes. No α-lysin precursors (heavy forms) were detected when one dimensional peptide mapping was performed on peptides isolated from membranes of control and cerulenin-inhibited cells. The 34,000 dalton band in control membranes gave similar maps to authentic 34,000 dalton α-lysin. A single precipitin line was formed in double diffusion tests in agar when purified α-lysin, culture supernate or sonicated membranes of control cells were tested against antiserum raised with purified α-lysin. No precipitin line was formed when culture supernate or membranes from cerulenin-inhibited cells were tested against this antiserum. No evidence was obtained for precursor-forms of α-lysin in control or cerulenin-inhibited cells.

Finally it was noted that cerulenin inhibition increased the sensitivity of S.aureus cells to lysostaphin, but decreased their autolytic activity. These effects may be explained in terms of the known inhibitory effects of cerulenin on lipoteichoic acid synthesis in other organisms (Carson and Daneo-Moore, 1978).

INTRODUCTION

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
AMP	adenosine monophosphate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Bis	N,N'methylene bis acrylamide
BLP	Braun's lipoprotein
Bq	becquerel (1 Becquerel = 1 nuclear transformation second^{-1} = 2.7 x 10^{-11} Curies)
BSA	bovine serum albumin
BS medium	Bernheimer&Schwartz Medium
CCCP	Carbonylcyanide-Chlorophenylhydrazone
CDS	citrate dextrose saline
CL	cardiolipin
CP	coat protein
CPG	control pore glass
cpm	count per minute
Cys	cysteine
DEAE	diethylaminoethyl
dpm	disintegration per minute
EDTA	ethylenediaminotetraacetic acid
EM	electron microscopy
ER	endoplasmic reticulum
g	gram
'g'	gravimetrical field - unit of centrifugation
GBq	gigabecquerel = 10^{9} Bq.
GLC	gas liquid chromatography
HMG - CoA	3-hydroxy-3-methylglutaryl-Co-enzyme A

HU	haemolytic unit
IEF	isoelectric focusing
IMP	integral membrane protein
IU	international unit
KBq	Kilobecquerel = 10^3 Bq.
K	Kilo = 10^3
Kg	Kilogram
λ receptor	an E.coli outer membrane protein, a receptor for the lambda bacteriophage
Lamb gene	the gene for lambda bacteriophage receptor protein in E.coli
LPG	lysylphosphatidylglycerol
LTA	lipoteichoic acid
µg	microgram
mg	milligram
mmol	millimole
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NCTC	National Collection of Type Cultures
OD	optical density
Omp	outer membrane protein
PA	phosphatidic acid
PABZ	para-aminobenzamidine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered Saline
PE	phosphatidylethanolamine
PEA	phenethyl alcohol
PG	phosphatidylglycerol
pI	isoelectric point

PMSF	phenylmethylsulfonyl fluoride
POPOP	1,4,bis-2-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
PSEB	precursor of staphylococcal enterotoxin B
r.b.c.	red blood cells (erythrocytes)
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
R_t	retention time
S	Svedberg unit
SDS	sodium dodecyl sulphate
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
SER	smooth endoplasmic reticulum
SFA	saturated fatty acid
SP	signal peptidase
SPB	saline phosphate buffer
SRP	signal recognising particle
TA	teichoic acid
TBq	terabecquerel = 10^{12} Bq
TBS	tris buffered saline
TEMED	N, N, N', N', tetramethylethylenediamine
TLC	thin layer chromatography
TLCK	N-tosyl-L-lysyl chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
Tris	tris (hydroxymethyl) amino methane
WHO	World Health Organisation

INTRODUCTION

I. HISTORICAL PERSPECTIVE

I.1. Staphylococcal Infections

Robert Koch in 1878 was first to describe the occurrence of staphylococci in human pus and two years later Louis Pasteur (1880) successfully cultivated the organisms in liquid medium. In 1881 Ogston showed that the organism was pathogenic for both mice and guinea pigs.

Staphylococcus aureus occurs as a harmless commensal organism in the nose and on the skin of many healthy people, but can cause a diverse array of both superficial and systematic infections. Superficial infections include boils, abscesses, impetigo, carbuncles, toxic epidermal necrolysis, pharyngitis, sinusitis, conjunctivitis, primary pneumonia, enterocolitis, cervicitis and pelvic abscesses in the vagina. It is also the cause of many deep seated or systematic infections such as bacteremia, osteomoyelitis of bones or joints, muscle abscesses, secondary pneumonia, endocarditis, myocarditis and pericarditis and abscesses and cerebritis in the central nervous system. S. aureus has also been implicated in abdominal visceral abscesses in the pancreas, spleen and liver (Shulman and Nahmias, 1972).

I.2 Extracellular Products of Staphylococcus aureus

In 1872 Klebs suggested that micrococci produce diseases by means of diffusible substances, and he

postulated a relationship between pathogenicity and toxin production in S. aureus. De Christams (1888) supported this hypothesis by demonstrating that the filtrates of staphylococcal cultures, and various chemical fractions of them, produced an inflammatory reaction in animals. Using analytical starch gel electrophoresis, Bernheimer and Schwartz (1961) described 10-14 different proteins in culture filtrates of S. aureus Wood 46. Isoelectric focusing studies in polyacrylamide gels have shown that some strains of S. aureus produce more than 25 bands on the gel while others give only 4-10 bands (Wadström, Thelestam and Möllby, 1974).

Numerous more detailed reviews on the extracellular products of S. aureus are available (Bernheimer, 1965, 1968, 1970; Gladstone, 1966; Jeljaszewicz, 1967, 1972; Bergdoll, 1967, 1970, 1972; Arbuthnott, 1970; Wiseman, 1970, 1975; Woodin, 1970, 1972 a, b; Rogolsky, 1979; Freer and Arbuthnott, 1983).

Many of the extracellular products of S. aureus are damaging to the tissues of man and animals; these include enzymes capable of degrading connective tissues (hyaluronidase), membrane and serum components (phospholipase and lipases), nucleic acids (deoxyribonuclease), proteins (proteases), esterases (sugar phosphate and cholesteryl esters) plasma clotting agent (coagulase) and clot dissolution agent (staphylokinase). There are also exotoxins, including the membrane-damaging agents alpha

(α), beta (β), gamma (γ) and delta (δ) toxins, Panton-Valentine leucocidin active against human and rabbit leucocytes, epidermolytic toxins A and B (exofoliatin A, and B) which induce cell separation in the stratum granulosum of the epidermis resulting in a variety of skin lesions, enterotoxins A, B, C_1, C_2, D and E responsible for food poisoning and pyrogenic toxins A, B and C causing staphylococcal scarlet fever.

Recently, pyrogenic exotoxin C and enterotoxin F have been implicated in the syndrome known as staphylococcal toxic shock (Bergdoll et al., 1981). The variety and number of such exoproteins, many of which are produced by a single strain, provide the organisms with a great variety of pathogenic activities.

II. STAPHYLOCOCCAL TOXINS OR LYSINS

Van de Velde in 1894 was the first to observe the haemolysis of rabbit erythrocytes by cultures of S. aureus. Later, in 1900, Kraus and Clairmount confirmed this observation.

In 1928, in the Australian town of Bundaberg, 12 out of 21 children who were inoculated with a diphtheria toxin-antitoxin preparation, died. A bacteriological investigation later concluded that the deaths were due to an overwhelming toxaemia arising from contamination of the vaccine by pyrogenic staphylococci.

This accident created increased awareness of the toxic properties of staphylococci and focused serious attention on the study of the organism. S.aureus produces four distinct haemolysins, designated α, β, γ and δ, in addition to a large number of extracellular enzymes both in vivo and in vitro.

II.1 Discovery

A lysin which did not lyse goat erythrocytes at 37°C, but caused rapid lysis on cooling to 0°C, was described by Walbum in 1927 as a hot-cold haemolysin. This was subsequently reinvestigated by Bigger, Boland and O'Meara (1927) and found to be active against sheep erythrocytes. Glenny and Stevens (1935) showed that the haemolysin was immunologically distinct from the haemolysin which lysed rabbit erythrocytes and the rabbit haemolysin was then termed alpha (α) and the sheep haemolysin beta (β).

In 1936 Morgan and Graydon described two serologically distinct rabbit haemolysins (α1 and α2), while Smith and Price (1938) named their second rabbit haemolysin gamma (γ). Plommet and colleagues later published details for the production and purification of γ-lysin from strain Smith 5R (Plommet and Bouillanne, 1966; Guyonnet, Plommet and Bouillanne, 1968; Guyonnet and Plommet, 1970). Some strains of S.aureus grown on sheep blood agar produced a haemolytic agent which was not neutralised by either α- or β- antitoxins. This was designated delta (δ) haemolysin by Williams and Harper (1947).

II.2. Nomenclature

The haemolysins of S. aureus were differentiated on the basis of their lytic activities for different species of erythrocytes, hence the term haemolysin. Later it became evident that these haemolysins were active against the membranes of cells other than erythrocytes and "cytolytic toxins" or cytolysins was proposed as an appropriate term by Bernheimer (1974) in order to define the haemolysins as bacterial products capable of causing destruction of cells other than red blood cells in vitro.

Low concentrations of cytolytic toxins induce permeability changes in the membranes of intact susceptible cells without necessarily causing lysis (Thelestam and Möllby, 1975 a,b), and therefore, the term "membrane damaging toxins" was suggested by McCartney and Arbuthnott (1978), to be more appropriate term than cytolytic toxins.

II.3 Staphylococcal Delta-Lysin

Delta-lysin is produced by most pathogenic strains of staphylococci (Elek, 1959; Jeljaszewicz, 1972; Gemmell, Thelestam and Wadström, 1976). It has a broad haemolytic spectrum (Elek, 1959), but is most commonly assayed using human or horse erythrocytes. However, fish erythrocytes are the most sensitive (Birkbeck, Chao and Arbuthnott, 1974). The lysin is usually purified by its pronounced adsorption to the inorganic matrices of alumna (Kantor,

Temples and Shaw, 1972) or hydroxylapatite (Kreger et al., 1971). However, a very high purity lysin was produced by introducing an additional isoelectric focusing step (Chao and Birkbeck, 1978).

The reported molecular weight (M_r) of δ-lysin ranges between 3000 to 7.4 x 10^8 (Table 1). This variation could reflect various degrees of aggregation of the lysin. It has been shown that δ-lysin is present as a tetramer in aqueous solutions at extremes of pH (Fitton, 1981). The pI values of this lysin obtained by different groups are not in agreement (Table 1). Amino acid analyses of δ-lysin show the absence of arginine, histidine, cysteine, proline and tyrosine (Yoshida, 1963; Kreger et al., 1971; Heatley, 1971; Turner, 1978; Fitton, Dell and Shaw, 1980).

A structural analysis of δ-lysin by Fitton (1981) suggested that it possesses two helical domains joined by a flexible hinge region. More recently Freer and Birkbeck (1982) proposed an α-helical rod-like configuration for the δ-lysin molecule with separate hydrophilic and hydrophobic faces.

Delta-lysin possesses some unusual features such as heat stability (Kantor, Temples and Shaw, 1972), high surface activity and solubility in chloroform: methanol (Freer and Arbuthnott, 1976). It has been reported that δ-lysin increases the cyclic AMP in guinea pig small intestine (Kapral et al., 1976; O'Brien and

Table 1. Some characteristics of purified staphylococcal δ-lysin. (After Freer and Arbuthnott, 1983).

	Strain of Organism	Molecular Weight (Dalton)	Sedimentation Coefficient ($S_{20,w}$)	Isoelectric Point (pI)	Specific Haemolytic Activity (units mg protein^{-1} or N)
Jackson & Little (1958)	1363, 2426 2428, 2429	[a]	-	-	3,200
Yoshida (1963)	Foggie	68 - 150,000	6.1	-	120 - 400
Kayser & Raynaud (1965)	-	12,000	1.4 ; 5.5	-	-
Hallander (1968)	-	> 200,000	-	-	-
Caird & Wiseman (1970)	E-delta	> 200,000	2.8 ; 9.8	-	12,000
Maheswaran & Lindorfer (1970)	-	-	-	(i) 3.32 (ii) 3.75 (iii) 8.45	-
Möllby & Wadström (1970)	-	-	-	9.6	-
Heatley (1971)	186 x[b]	-	4.9	-	250 - 300
Kapral & Miller (1971)	PG114	-	-	-	400[c] ; 200[d]
Kreger et al. (1971)	Wood 46 M[e]	-	4.9 ; 11.9	(i) 9.5 (ii) 5.0	400[c] ; 200[d]
Kantor, Temples & Shaw (1972)	Wood 46M[e]	103,000	6.04	(i) 4.65 (ii) 6.7 (iii) 9.0	75
Turner (1978)	CN 4108 (Newman D2)	82,000	-	4.5 ; 9.5	150 - 200
	CN 7450	< 10,000	-	7.8	200
Fitton, Dell & Shaw (1980)	168X	2,977	-	-	

Notes: a = not reported
b = derived from Newman by subculture
c = insoluble δ-lysin
d = soluble δ-lysin
e = derived from Wood 46 by UV irradiation.

Kapral, 1976). However, a subsequent study showed that the action of δ-lysin differed fundamentally from the primary effect of cholera toxin; the increase in ion flux was thought to arise by a direct stimulatory effect of δ- lysin on the membrane ion pump (Freer and Arbuthnott, 1983).

II.4 Staphylococcal Gamma-Lysin

Gamma-lysin was first produced and partially purified by Plommet and colleagues using S.aureus strain Smith 5R. The purification involved a combination of hydroxyl-apatite chromatography and membrane ultrafiltration (Plommet and Bouillanne, 1966; Guyonnet, Plommet and Bouillanne, 1968; Guyonnet and Plommet, 1970; Bezard and Plommet, 1973). The former group recovered two distinct toxin components from a hydroxylapatite column, γ1 and γ2, by elution with increasing ionic strength. A molecular weight of 29,000 dalton and a pI of 9.8 for component I and a molecular weight of 26,000 dalton and a pI of 9.9 for component II was later reported (Taylor and Bernheimer, 1974).

Freer and Arbuthnott (1983) summarised some important properties of γ-lysin:

1. It is inactivated by agar and other sulfonated polymers.
2. Haemolytic activity is inhibited by cholesterol and cholesteryl esters.
3. It is inactivated by heating at 60°C for 10 min.
4. Both components are required for toxic and haemolytic activity.

5. It preferentially lyses rabbit erythrocytes.
6. It is inhibited by a variety of phospholipids and fatty acids.
7. It is antigenic.

It has been suggested that γ-lysin has phospholipase activity (Fackrell and Wiseman, 1976). However, others suggested that there were similarities in the type of membrane lesions induced by α-lysin and γ-lysin (Szmigielski et al., 1975).

II.5 Staphylococcal Beta-Lysin

Beta-lysin is produced at the maximal rate at neutral pH during the early exponential phase of growth (Wiseman, 1970) and reaches maximum concentration at the end of exponential growth (Low and Freer, 1977b).

Activation of β-lysin occurs after ion-exchange chromatography (Haque and Baldwin, 1969; Wadström and Möllby, 1971a; Low and Freer, 1977a), probably due to the removal of an inhibitor. Table 2 summarises the strains used and the purification procedures of earlier studies, while Table 3 summarises the properties of β-lysin.

The phospholipase C activity of β-lysin was first observed by demonstrating the release of water soluble phosphorus from both sheep and rabbit cell stromata. It was further shown that the lysin is an enzyme with

Table 2. Purification of Staphylococcal β-lysin. (After Freer and Arbuthnott, 1983)

Reference	Strain	Procedures[a]	Specific Activity (HU mg protein^{-1}) (haemolytic activity mg protein^{-1}
Robinson, Thatcher & Gagnon (1958)	L16	A,C,A,D	
Jackson (1963)	J32A	A,A,C	
Chesbro et al. (1965)	UNH-DOnita	C	6×10^4
Doery et al. (1965)	1061 - 17	A,A,C	3.7×10^4
Wiseman (1965)	R-1, 252F	C,A	1.2×10^6
Maheswaran, Smith & Lindorfer (1967)	J19	A,B,C,C	6.8×10^4
Wiseman & Caird (1967)	R-1, 252F	C,A,C	
Gow & Robinson (1969)	MB 534	A,B,C,D[b]	5.2×10^8
Haque & Baldwin (1969)	681	A,C	6.6×10^6
Wadström & Möllby (1971a)	R-1	C,E,B	$10^7 - 10^8$ (2 IU mg^{-1})[c]
Colley et al. (1973)		A,B	(1 IU 20 µl^{-1})
Bernheimer, Avigad & Kim (1964)	G-128, R-1, 234	A,B,E	3.7×10^5 (140 IU mg^{-1})
Zwaal et al. (1975)	269 HH	A,B,C,C	(1900 IU mg^{-1})
Low & Freer (1977a)	G128	A,B,C,E	6.3×10^7 (312 IU mg^{-1})

A, precipitation; B, gel filtration; C, ion exchange chromatography; D, Electrophoresis (starch block); E, isoelectric focusing.

b, Electrophoresis (sucrose gradient)

c, Figures in brackets indicates specific activity of sphingomyelinase in international units (IU mg^{-1})

Table 3. Properties of purified staphylococcal β-lysin. (After Freer and Arbuthnott, 1983)

Reference	Strain	Molecular Weight (dalton)	Sedimentation Coefficient ($S_{20,w}$)	Isoelectric Point (pI)	Specific Haemolytic activity (HU mg protein^{-1})
Robinson, Thatcher & Gagnon (1958)	L16	a	-	-	-
Jackson (1963)	J32A	-	-	-	-
Chesbro et al. (1965)	UNH-Donita	59,000^{u}	-	8.6 - 8.9	6×10^{4}
Doery et al. (1965)	1061 - 17	-	-	-	3.7×10^{4}
Wiseman (1965)	R1 - 252F	-	-	-	1.2×10^{4}
Maheswaran, Smith & Lindorfer (1967)	J19	-	-	9.5	6.8×10^{4}
Wiseman & Caird (1967)	R1 - 252F	-	-	-	-
Gow & Robinson (1969)	MB 534	-	1.7	-	5.2×10^{8}
Haque & Baldwin (1969)	681	-	-	-	6.6×10^{6}
Wadström & Möllby (1971a)	R-1	38,000^{f}:33,000^{f}	-	9.4	$10^{7} - 10^{8}$ (2IUmg^{-}
Chesbro & Kucic (1971)	UNH - 15	15,500$^{u.f.}$	-	-	-
	243 - B1	15,500^{u}:13,800^{f}	-	-	-
	243 - B2	13,600^{u}:11,000^{f}	-	-	-
Bernheimer, Avigad & Kim (1974)	G-128;R-1;234	30,000^{f};29,000^{f}	-	-	-
		29,000^{e}	-	9.0	4.7×10^{5}(140 IU mg^{-}
Zwaal et al. (1975)	269HH	-	-	-	(1900 IU mg^{-1})
Fackrell & Wiseman (1976)	-	26,000^{f};16,00^{a}	1.8	9.5	-
Low & Freer (1977a)	G128	33,000^{e};32,500^{f}	3.1	9.3 - 9.7	6.2×10^{7}(312 IU mg^{-}

a - , not reported

M_r determined by: u, ultracentrifugation; f, gel filtration; e, sodium dodecyl sulphate polyacrylamide gel electrophoresis; a, amino acid analysis. Figures in brackets refer to specific activity of sphingomyelinase in international units mg^{-1}.

a substrate range limited to sphingomyelin and phosphatidylcholine (Doery et al., 1963), the degradation sequence was then suggested:

$$\text{sphingomyelin} + \text{water} \xrightarrow[Mg\ 2+]{\beta\text{-lysin}} \text{N-acylsphingosine} + \text{phosphorylcholine}$$

A close correlation between sphingomyelin content of the erythrocyte membrane and lysis by β-lysin has been reported (Smith and Price, 1938; Marks and Vaughan, 1950; Jackson and Mayman, 1958). This was supported by the fact that sheep, ox and goat erythrocytes which are sensitive to β-lysin contained 40% more sphingomyelin than rabbit erythrocytes (Wadström and Möllby, 1971b; Bernheimer, Avigad and Kim, 1974).

One of the most unusual features of β-lysin is the phenomenon of 'hot-cold' haemolysis. Incubation at 37°C of sensitive erythrocytes with small quantities of β-lysin in the presence of Mg^{2+} results in little or no lysis, but if the treated erythrocytes are then chilled to below 10°C, rapid lysis follows. As an explanation of the 'hot-cold' effect, Low and Freer (1977b), suggested that at temperatures about 20°C, the cohesive forces of the intact erythrocyte membrane are sufficient to hold the hydrolysis product of β-lysin, ceramide, in position in the membranes, with Mg^{2+} ions possibly preventing collapse of the weakened bilayer. On cooling, a phase separation occurs with condensation of ceramide

into large pools and the collapse of the bilayer follows rapidly.

In contrast to the well defined activity of ß-lysin on biological membranes, its toxicity for laboratory animals is still in doubt. The role of ß-lysin in the pathogenesis of staphylococcal disease is poorly understood. There is no convincing evidence that ß-lysin alone plays an important role in staphylococcal pathogenicity (Adlam et al., 1977). However, ß-lysin may play a significant part in the potentiation of the effects of other staphylococcal toxins produced concurrently (Freer and Arbuthnott, 1983).

II.6 Staphylococcal Alpha-Lysin

A. Description

Of all the staphylococcal haemolysins, α-lysin has been the most fully characterised. It is produced by the majority of coagulase-positive staphylococci, and assayed most frequently by its high lytic activity towards rabbit erythrocytes. The proposal by Burnet (1929) that a single substance was responsible for the haemolytic, dermonecrotising and lethal activities has since been shown to be a function of the α-lysin.

Mangalo, Pillet and Raynaud (1954), reported that α-lysin is liberated into the culture medium during the exponential phase of growth, after a lag phase of

approximately 3 hours. Gladstone and Glencross (1959) reported that the course of α-lysin production lagged slightly behind growth, reaching a maximum around 6-8 hours of growth and then declining sharply. Others, however, concluded that the lysin is produced only during active cell division, and the amount remains constant during stationary phase (Kapral, 1965, 1966; Kapral, Keogh and Taubler, 1965). Evidence of a link between lysogeny and toxigenicity in S. aureus came from studies of Blair and Carr (1961), who showed that toxigenicity could be conferred on non-toxigenic strains by lysogenisation with phages from toxigenic strains. Witte (1976) reported that α-lysin production in some strains of S. aureus is plasmid-controlled. However, both Rogolsky (1979) and Brown and Patte (1980) suggested that a transposon location for the α-lysin gene would go some way towards explaining the anomalous properties noted in relation to α-lysin activity.

B. Purification

Proteolysis or denaturation were the main factors responsible for the loss of α-lysin activity during purification procedures, particularly at low ionic strengths (Lominski, Arbuthnott and Spence, 1963; Coulter, 1966). High surface-activity of α-lysin (Bucklewe and Colacicco, 1971) may lead to loss of

haemolytic activity by polymer formation which is enhanced at liquid-air interfaces (Arbuthnott, Freer and Bernheimer, 1967; Colacicco and Buckelew, 1971) and during adsorption to glass surfaces. There are several excellent reviews which discuss the development of the earlier purification procedures of α-lysin (Arbuthnott, 1970; Wiseman, 1975; Rogolsky, 1979).

Reducing the complexity of the procedures and improving the lysin yield, was the feature of most of the recent purification methods (Cassidy and Harshman, 1976a, Dalen, 1976b). Cassidy and Harshman (1976a) introduced a relatively simple purification method involving chromatography of culture supernates on controlled-pore glass followed by ion-exchange chromatography on DEAE-Sephadex.

C. Physicochemical properties of alpha-lysin

1. Molecular weight and pI values

Purified α-lysin is a protein (Arbuthnott, 1970), and the claim that it contains carbohydrate (Goshi, Cluff and Norman, 1963) has not been confirmed. Some characteristics of α-lysin are shown in Table 4. The reported values for molecular weight varies from 10,000 to 45,000 daltons. This difference may arise from the different purification methods employed. When assessed by four different methods, the molecular weight determinations for a single preparation of α-lysin fell within the range

Table 4. Some characteristics of purified α-lysin. (After Freer and Arbuthnott, 1983)

Reference	Strain of Organism	Molecular Weight (dalton)	N-terminal amino acid	Sedimentation Coefficient (S_{20}, w)	C-terminal amino acid	Isoelectric Point (PI)	Specific Haemolytic Activity (Haemolytic units mg^{-1} protein
Madoff & Weinstein (1962)	Wood 46	[a]		–		–	4.2×10^{4}
Kumar et al.(1962)	Wood 46	$1.0\text{–}1.5 \times 10^{4}$		1.45		–	–
Bernheimer & Schwartz(1963)	Wood 46	4.4×10^{4}		3.0[b]; 12.0[c]		–	1.9×10^{4}
Goshi,Cluff & Norman (1963)	–	–		–		–	8×10^{6}
Lominski,Arbuthnott & Spence (1963)	Wood 46	–		3.1		–	1.2×10^{5}
Jackson (1963)	Wood 46 209-60	–		–		–	–
Robinson & Thatcher(1963)	–	–		–		–	–
Cooper, Madoff & Weinstein (1966)	Wood 46	–		2.8		–	1.2×10^{6}
Coulter (1966)	Wood 46	2.1×10^{9}	Histidine, Arginine	2.8		–	10^{4}
Arbuthnott,Freer & Bernheimer (1967)	Wood 46	–		3.0		–	2×10^{6}
Wadström (1968)	Wood 46 V8,M18	–		–		8.5[b](alpha 1)	–
Wiseman & Caird (1970)	Wood 46	–	Histidine	–		–	–
Forlani,Bernhheimer & Chiancone (1971)	(Wood 46?)	3.3×10^{4}		2.8		–	–
McNiven, Owen & Arbuthnott (1972)	Wood 46	3.6×10^{4}		–		8.55[b](Alpha A)	–

Table 4 Continued. Some characteristics of purified α-lysin. (After Freer and Arbuthnott, 1983)

Reference	Strain of Organism	Molecular Weight (dalton)	N-terminal amino acid	Sedimentation Coefficient (S_{20}, w)	C-terminal amino acid	Isoelectric Point(pI)	Specific Haemolytic Activity (Haemolytic units mg^{-1} protein
Fackrell (1973)	Wood 46	-		-		-	1.3×10^5
Six & Harshman (1973 a, b)	Wood 46	2.8×10^4	Alanine (A+B)	3.0(A) 3.0(B)	Lys (A + B)	7.2(A) 8.4(B)	-
Good & Baldwin (1973)	Wood 46	-		3.0[b]; 10.5		8.65[b]; 5.8	1.8×10^4
Good & Baldwin (1974)	57,2097, 3558,3565	-		3.0[b]; 10.5		8.65 ± 0.15	-
Watanabe & Kato (1974)	Wood 46	3.6×10^4		-		7.98 ± 0.05	-
Dalen (1975, 1976b)	Wood 46	3.9×10^4		-		8.6[b]; 7.4	-
Fackrell & Wiseman (1976)	Wood 46	4.5×10^4	Histidine	1.4[d]		8.5	-
Cassidy & Harshman (1976a)	Wood 46	-		-		-	2.8×10^4

Notes: a, not reported; b, major compound; c, inactive aggregate; d, change to 2.8 on standing.

2.6 to 3.1 x 10^4 daltons (Six and Harshman, 1973b). Most workers have reported pI values of 8.4 to 8.8 for the main form of α-lysin (Wadström, 1968; McNiven and Arbuthnott, 1972; Six and Harshman, 1973b; Good and Baldwin, 1974; Watanabe and Kato, 1974; Dalen, 1975, 1976b). Minor components with different pI values (see Table 4) have also been reported, but these generally accounted for a small proportion of total toxic protein. It is possible that some of the so-called 'multiple forms' of the lysin arise during the purification procedures by proteolysis (Freer and Arbuthnott, 1983).

2. Charge heterogeneity

Several methods have revealed that α-lysin can exist in multiple forms, with respect to both molecular weight and electrophoretic mobility.

Ultracentrifugation studies of purified α-lysin indicate a main component of approximately 3S, with a minor heavy component variously reported as 10.5S (Good and Baldwin, 1973, 1974), 11.5 - 12.5S (Bernheimer and Schwartz, 1963; Arbuthnott, Freer and Bernheimer, 1967) and 16S (Lominski, Arbuthnott and Spence, 1963). These heavy forms reported may represent aggregates of the 3S lysin, since treatment of the active 3S lysin by brief heating at 60°C caused aggregation to give soluble inactive 12S lysin (Arbuthnott, Freer and Bernheimer, 1967).

Arbuthnott, Freer and Bernheimer (1967) first reported that treatment of 12S lysin with 8 M urea could dissociate it with regeneration of biological activity, while Dalen (1976a) extended this observation to show that dialysis to remove the urea gave the 12S lysin spontaneously. This provides circumstantial evidence that α-12S is an aggregated form of 3S α-lysin. Dalen (1975, 1976 a,b) in his studies of S. aureus α-lysin isolated a protein of M_r 27,500 (pI 8.4) and two basic proteins of M_r 18,000 (pI 9.5) and 12,000 (pI 11.5) as well as a component of M_r 39,000 (pI 8.6 and 7.4) from supernates of S. aureus strain Wood 46. The 27,000 dalton protein was antigenically related to the 39,000 dalton protein (which had the classical properties of α-lysin), although it was non-haemolytic and present in at least two charged forms (Dalen, 1976b). He suggested that either the 39,000 M_r α-lysin was a dimer of partially degraded 27,000 M_r protein or that the 27,000 M_r protein was a degradation product of 39,000 M_r α-lysin, and as such, a number of charged forms might be expected (Table 4).

3. Amino acid composition of alpha-lysin

Bernheimer and Schwartz (1963) reported that α-lysin contained most of the amino acids commonly found in globular proteins which varied in amount from 4 residues

for histidine to 44 residues for aspartic acid, and that there was a complete absence of half-cystine. There is an overall similarity in the results of analyses from different laboratories, in that half-cystine is absent and relatively large amounts of lysine, aspartic acid and glutamic acid are present (see Table 5).

The progress in sequencing the lysin is summarised in Fig. 1, the first 10 residues from the amino terminus are identical in the toxin of Six and Harshman (1973b) and Kato and Watanabe (1980) as is the C-terminal residue (lysine).

D. Mode of action of alpha-lysin

The mode of action of α-lysin is still disputed, nevertheless, the primary site of action is generally accepted as being the plasma membrane. The study of α-lysin -membrane interactions has involved three systems 1) whole cells 2) isolated membranes and 3) artificial membranes.

Many of the previous investigations have been focused on three main aspects of α-lysin - membrane interactions. These are a) the possible enzymic activity b) the importance of hydrophobic or lipid-specific interactions and c) the possible existence of specific high affinity lysin receptors.

Table 5. Amino acid analyses of staphylococcal α-lysin. (After Freer and Arbuthnott, 1983)

	Bernheimer & Schwartz* (1963)	Coulter* (1966)	Fackrell & Wiseman (1976)	Six & Harshman+ (1973b)		Watanabe & Kato+ (1974)	Kato & Watanabe (1980)
				A	B		
Tryptophan				4	4	4.2	
Aspartate	44	52	20(40)	40	43	48	43
Methionine	10	5	2(4)	6	6	5.5	8
Threonine	23	26	10(20)	22	23	24.3	24
Serine	22	21	10(20)	19	19	18	21
Glutamate	21	23	16(32)	19	20	20.1	20
Proline	7	10	14(28)	8	9	5.3	10
Glycine	23	25	28(56)	20	24	22.5	36
Alanine	12	14	12(24)	11	11	12.1	17
Valine	12	17	8(16)	13	14	16.2	19
Isoleucine	13	16	6(12)	13	14	15	16
Leucine	15	15	8(16)	13	14	14.5	16
Tyrosine	9	10	6(12)	9	10	12.5	7
Phenylalanine	10	10	4(8)	8	8	9.3	9
Lysine	23	27	12(24)	21	23	25.5	31
Histidine	4	4	4(8)	4	4	4	8
Arginine	10	8	6(12)	8	8	9.5	8
No residues	258	283	166(332)	239	254	267	297
Calculated M_r of single peptide of above composition	28,500	31,000	17,300(34,600)	26,500	28,200	30,000	31,000

* Number of residues calculated for a peptide of M_r 44,000 (Bernheimer and Schwartz) and 31,000 (Coulter)

\+ Figures for each residue calculated assuming 4 residues His per molecule. Figures under Fackrell and Wiseman were derived from those originally published by assigning 4 or 8 residues to His.

Figure 1. Partial amino acid sequence of staphylococcal alpha-lysin (After Kato and Watanabe, 1980).

NH_2-Ala-ASp-Ser-Asp-Leu/Ile-Asn-Ile-Lys-Pro-Gly------Gly-Arg----Ile-Leu-His-Val-Arg-Ala-Val-Val---Thr-Tyr-Val-Lys-COOH

1 2 3 4 5 6 7 8 9 10.....121 122....149 150 151 152 153 154 155 156...291 292 293 294

1 ..... light chain ..... 122/ /149 .....heavy chain 294

M_r = 14,000 unstable non toxic

M_r; = 17,000 Lethal, non-haemolytic non-dermonecrotic

123 to 148 = 26 residue short chain(s) released in trypsin-cleavage region

1. Enzymic mode of action

Wiseman and colleagues (Wiseman and Caird, 1970, 1972; Wiseman, Caird and Packrell, 1975) reported a correlation between the susceptibility of erythrocytes from different species to lysis by α-lysin and the degree of natural proteolytic activity possessed by the erythrocyte membrane. They proposed that α-lysin is secreted as an inactive zymogen and then activated by erythrocyte membrane protease to become a protease itself. However, Freer, Arbuthnott and Billcliffe (1973) failed to reveal any difference in polypeptide pattern, or reduction in sedimentable protein in α-lysin-treated erythrocyte membranes, while the protease inhibitor, phenylmethylsulfonyl fluoride also failed to inhibit erythrocyte lysis by α-lysin. Dalen (1976a) reported proteolytic activity associated with purified α-lysin, and suggested autodigestion may explain the observed changes and heterogeneity in molecular weight of the lysin (see physicochemical properties).

2. Hydrophobic interaction of alpha-lysin (surface activity)

The first indication of the possible importance of membrane lipids in α-lysin-membrane interactions came from Weissmann, Sessa and Bernheimer (1966), who found that α-lysin released previously sequestered marker molecules from liposomes containing lecithin, cholesterol

and either dicetyl phosphate or stearylamine. Freer, Arbuthnott and Bernheimer (1968) demonstrated a relationship between the effect of α-lysin on artificial membranes and its action on natural membranes, in both cases the inactivation of the lysin was accompanied by the appearance of ring like structures, having similar dimensions to the α12S. These ring-like structures, which have also been found when osmotically prepared erythrocyte ghosts were treated with relatively large amounts of α-lysin (Freer, Arbuthnott and Bernheimer, 1968; Bernheimer et al., 1972), or when α-lysin was incubated with lipid dispersions, are morphologically identical to the naturally occurring 12S α-lysin (Arbuthnott, Freer and Billcliffe, 1973).

The surface activity of α-lysin has been investigated by measuring the penetration rate of the lysin into mixed lipid monolayers (Buckelew and Colacicco, 1971; Colacicco and Buckelew, 1971),and by studying the ability of lipid dispersions to induce polymerisation of α-lysin (Arbuthnott, Freer and Billcliffe, 1973). None of these investigations suggested a marked specificity of α-lysin for any individual lipids. Recently, Bhakdi, Füssle and Tranum-Jensen (1981) reported that through contact with deoxycholate detergent micelles the native α-lysin molecule can be induced to self-associate to form amphiphilic, ring structured hexamers that are indistinguishable from

the cytolytic membrane complexes. Thus, the presence of an appropriate physicochemical environment appears to be the only condition necessary for inducing the hydrophilic-amphiphilic transition of α-lysin.

Bernheimer et al., (1972) in a freeze-etching study of the interaction of α-lysin with platelets, suggested that the lysin can penetrate the hydrophobic region of the membrane, while Freer, Arbuthnott and Billcliffe (1973) in another study with erythrocyte ghosts showed a marked change in the hydrophobic fracture plane of the membrane attributed to disruption of the normal fracture plane by the penetration of toxic protein into the hydrophobic domain. Recently, Füssle et al., (1981) reported that α-lysin oligomerises at the membrane surface to form amphiphilic, ring shaped protein complexes that penetrate into or through the target lipid bilayer generating transmembrane channels. Their results confirmed the earlier observations and conclusions of Freer, Arbuthnott and Billcliffe (1973) and Arbuthnott, Freer and Billcliffe (1973), that the primary target of toxin attack is the lipid bilayer.

3. Specific receptors

Among mammalian erythrocytes, those of the rabbit are the most sensitive to lysis by α-lysin; therefore the haemloytic assay with rabbit erythrocytes is the most

convenient and sensitive way to detect biologically active α-lysin (Arbuthnott, 1970).

In an attempt to understand the basis for the high sensitivity of rabbit erythrocytes to α-lysin, many studies have been performed. Cassidy and Harshman (1973) found that the capacity of erythrocytes from different species to bind radio iodinated α-lysin correlated with the known haemolytic sensitivity of the erythrocytes. They further suggested (1976 a, b, c) that binding and haemolysis were separate events and that there were high specificity lysin receptors on rabbit erythrocyte membranes. It has since been reported (Kato et al, 1975 a,b; Cassidy and Harshman, 1976 b) that digestion of rabbit erythrocytes by pronase leads to a reduction in both labelled lysin binding capacity and haemolytic sensitivity of the erythrocytes. Cassidy and Harshman (1979) found that binding of labelled lysin was non-specific to either rabbit or horse erythrocytes and that the binding was not affected by the presence of concavalin A. Recently, Maharaj and Fackrell (1980) presented evidence which suggested that erythrocyte glycoprotein band 3 was the specific receptor for α-lysin.

Although the precise mode of action of α-lysin is still unknown there are several areas of general agreement regarding its mode of action on natural and artificial membranes which have been summarised by Freer and Arbuthnott (1983) as follows:

1) Binding of the toxin to membranes is a separate event from the formation of functional lesions.

2) Cytolysis can be delayed by hypertonic buffer, by low temperature or by low levels of Zn^{2+}.

3) The toxin has pronounced surface activity, forms thick solid films at an air/water interface, penetrates mixed lipid monolayers and releases sequestered markers from liposomes.

4) At high concentrations (10 - 100 $\mu g\ ml^{-1}$) the toxin forms hexameric ring structures on erythrocyte ghosts or liposomes.

5) The specificity of the toxin for certain membranes does not solely depend on lipid composition of the membrane.

E. Regulation and export and alpha-lysin

In spite of the α-lysin being one of the major extracellular proteins of S. aureus, only in relatively recent years have attempts been made to investigate the factors which control its biosynthesis and release from the cell.

Duncan and Cho (1971) found that α-lysin is produced in a biphasic manner in liquid batch media, and that low levels of the lysin are produced during exponential growth with a marked increase occurring just prior to the onset of a slower rate of growth prior to stationary phase. The data of McNiven and Arbuthnott (1972) supported the

previous finding and they also found that α-lysin was released rapidly after synthesis, without intracellular accumulation. Coleman and Abbas-Ali (1977) confirmed the finding of Duncan and Cho (1971) showing that α-lysin is produced in a biphasic manner, most of the toxin appearing in culture supernate fluid during the post-exponential phase of growth, with no evidence that the lysin accumulated in any appreciable quantities in the cytoplasm prior to its release (McNiven and Arbuthnott, 1972). This is entirely compatible with current ideas on synthesis and release of exported proteins (Davis and Tai, 1980) which may involve concurrent synthesis on membrane bound polysomes and export through the membrane.

The first evidence for the involvement of the plasma membrane as a key control site in the export of exoproteins came from Yoshikawa et al., (1974), who confirmed the earlier observations of Voureka (1952) on pleiotropic alterations to hyper production of a range of extracellular proteins in nitrosoguanidine-induced mutants of S. aureus. Yoshikawa's group also found that pleiotropic effects were associated with loss of yellow pigment which is largely lipid-soluble carotenoid, and this suggests that alteration occurs in the membrane lipid fraction.

Dalen (1973 a, b, c) suggested that α-lysin is formed in response to a specific inducer histidine, but Abbas-Ali and Coleman (1977 a, b) found that the mechanism of

α-lysin production was similar to the model for exoprotein regulation proposed by Coleman, Brown and Stormonth (1975). In this model, the increased differential rate of α-lysin synthesis after exponential growth results from competition at the transcriptional level between cell protein and extracellular protein synthesising machinery. Two separate effects are thought to be involved, the first being an increase in available RNA polymerase on switching off of ribosomal RNA synthesis, and the second an increase in substrate concentration due to ribosomal RNA turnover. Coleman (1981) showed that the differential rates of total extracellular protein produced by Wood 46 and a low α-lysin producing variant were identical, whereas the contribution of α-lysin to the extracellular proteins was very different in each case. These results are consistent with pleiotropic alteration of multiple functions reported by Yoshikawa et al., (1974) who suggested a common regulatory mechanism for the synthesis and release of exoproteins.

III. STAPHYLOCOCCAL PROTEOLYTIC ENZYMES

It has been suggested that the proteolytic enzymes may serve a nutritional role by cleaving proteins to peptides and amino acids which can be transported into the bacterium (Arvidson, Holmes and Lindholm, 1972).

Drapeau, Bouilly and Houmard (1972) purified a

protease from the culture filtrates of S.aureus strain V8, by a combination of procedures, including precipitation with ammonium sulphate and acetone, chromatography on DEAE-cellulose, and preparative electrophoresis on polyacrylamide gels. A sedimentaion coefficient (S_{20},w) of 2.9S and a molecular weight of 11,400 were respectively found by sedimentation equilibrium and sodium dodecyl sulphate polyacrylamide gel electrophoresis. The amino acid composition indicates 115 residues, with the absence of sulfhydryl groups (Drapeau, Bouilly and Houmard, 1972).

At least three different extracellular proteolytic enzymes are produced by S.aureus, named protease I, protease II, and protease III (Arvidson, Holmes and Lindholm, 1972, 1973; Björklind and Jörnval, 1974; Houmard and Drapeau, 1972). Björklind and Jörnval (1974) in a study of substrate specificity of the three extracellular proteolytic enzymes from S.aureus, using carboxymethylated derivatives of yeast and horse liver alcohol dehydrogenases as substrates, showed that protease I has the most selective specificity, cleaving mainly at the carboxy-terminal side of glutamic acid, protease II has the broadest specificity and most amino acids were found both at the carboxy- and amino-terminal side of peptide bonds cleaved. It was also suggested that this type of specificity together with other characteristics of protease II, such as low molecular weight and activation by reducing agents and inactivation by heavy metals are

all properties reminiscent of the papain type of protease. Finally, protease III has a proteolytic specificity for the amino-terminal side of hydrophobic residues. The substrate specificity of protease III, as well as molecular weight and sensitivity to EDTA are all properties typical of a thermolysin type of proteinase (Björklind and Jörnval, 1974).

Drapeau (1978) studied an extracellular protease produced by S. aureus and showed that it was a serine protease with a molecular weight of 27,000 and contained a 43 residue C-terminal peptide composed almost exclusively of aspartic acid, asparagine, and proline. He suggested that this C-terminal peptide may present a structural component involved in the transport of the enzyme through the cytoplasmic membrane.

IV. PROTEIN SECRETION ACROSS BIOLOGICAL MEMBRANES

All living cells, secrete proteins across their membranes either directly into the external environment, or into vesicles or organelles.

In recent years, considerable attention has been focused on the study of the mechanism of protein secretion in both eukaryotes and prokaryotes.

IV.1. Protein Secretion Across Eukaryotic Membranes

A. Existence of membrane bound polysomes

The first attempt to study the mechanism of protein secretion across membranes was made by Palade in 1955 who found that animal cells contain ribosomes bound to the membrane of the endoplasmic reticulum (ER) as well as ribosomes free in the cytoplasm. Two decades later it was found that various secretory proteins are synthesised on membrane bound ribosomes, and various cytoplasmic proteins are synthesised on the free or unbound ribosomes (Rolleston, 1974; Palade, 1975). This ribosomal distribution together with the observation that puromycin released incomplete chains from the ribosomes of microsomes into the interior of microsomes (Redman and Sabatini, 1966), led to the suggestion that secreted proteins might cross membranes as growing chains (Sabatini and Blobel, 1970).

Sabatini and Blobel (1970) showed that microsomes

protect the nascent secretory chains of exportable proteins, but not of cytoplasmic proteins from proteolytic degradation. This support the previous ideas that growing polypeptide chains are translocated through the membrane of the endoplasmic reticulum during protein synthesis.

1. The signal hypothesis

Sabatini, Tashiro and Palade (1966) observed the close association of the large ribosomal subunit with the endoplasmic reticulum and close association of the nascent polypeptide chain with the endoplasmic reticulum membrane was observed by Redman and Sabatini (1966) and by Sabatini and Blobel (1970). These early observations indicated the role of ribosome-membrane interaction in the vectorial discharge of proteins into the cisternae of the endoplasmic reticulum, and from the suggestion that there was selective translation of specific mRNA on either free or membrane bound ribosomes (Takagi and Ogata, 1968; Hicks, Drysdale and Munro, 1969; Redman, 1969), Blobel and Sabatini (1971) postulated that the information as to whether a particular mRNA is to be translated by free or membrane-bound ribosomes lies in the mRNA itself. They further suggested that mRNAs are coding for a special sequence near the beginning of the polypeptide chain, that might be recognised by a membrane binding

factor presumably. The peptide chain would then be secreted while growing. Their last postulation (Blobel and Sabatini, 1971) was that all ribosomes are alike, and it is the mRNA that determines their location either free in the cytoplasm or on the membrane.

Milstein et al., (1972) in their classical studies on the translation of immunoglobulin light chain mRNA in cell-free systems, demonstrated the attachment of bound polysomes via the amino-terminal region of the growing polypeptide chain. They showed that a large precursor containing extra amino acids at the NH_2 - terminus was synthesised in the reticulocyte lysate but not in the presence of microsomal membranes. The extra sequence, with a molecular weight of 3,000 dalton was thought to provide a signal for attaching the growing chain of secretory protein to the membrane. They further suggested that only during protein synthesis can the homologous membrane-fragments convert the precursor protein to the ultimate product; after completion of the precursor the cleavage site evidently becomes inaccessible to the enzyme. Mach, Faust and Vassali (1973) confirmed the previous findings (Blobel and Sabatine, 1971; Milstein et al., 1972) and Schechter (1973) presented the first amino acid sequence for the immunoglobulin light chain. Further support for these findings came from Palade (1975) who observed that in animal cells there is a parallel

between the abundance of ribosomes bound to the endoplasmic reticulum and secretion of extracellular proteins. Working with immunoglobulin light chain mRNA, Blobel and Dobberstein (1975a) found that translation in the presence of membranes yielded the authentic product which was located inside the microsomal vesicles, whereas in the absence of membranes, the precursor was synthesised and upon addition of membranes after completion of the polypeptide chain, chain processing of the preprotein and translation do not take place.

In an assessment of current information on protein biosynthesis, Blobel and Dobberstein (1975 a,b) proposed the signal hypothesis for protein synthesis and secretion (Fig. 2). This hypothesis hinged on the following proposals:

(1) mRNAs coding for all proteins destined to be translocated across the ER membrane contain at their 5' end (following the initiation codon) a series of codons whose translation product is termed the signal sequence.

(2) All protein synthesis is begun on an initially free (unattached) ribosome whose subunits are derived from the common cytoplasmic pool.

(3) As the signal sequence of the growing chain emerges from the ribosome it interacts with specific integral membrane proteins to catalyse the formation of a functional ribosome - membrane junction in association with a newly assembled proteinaceous channel across the membrane.

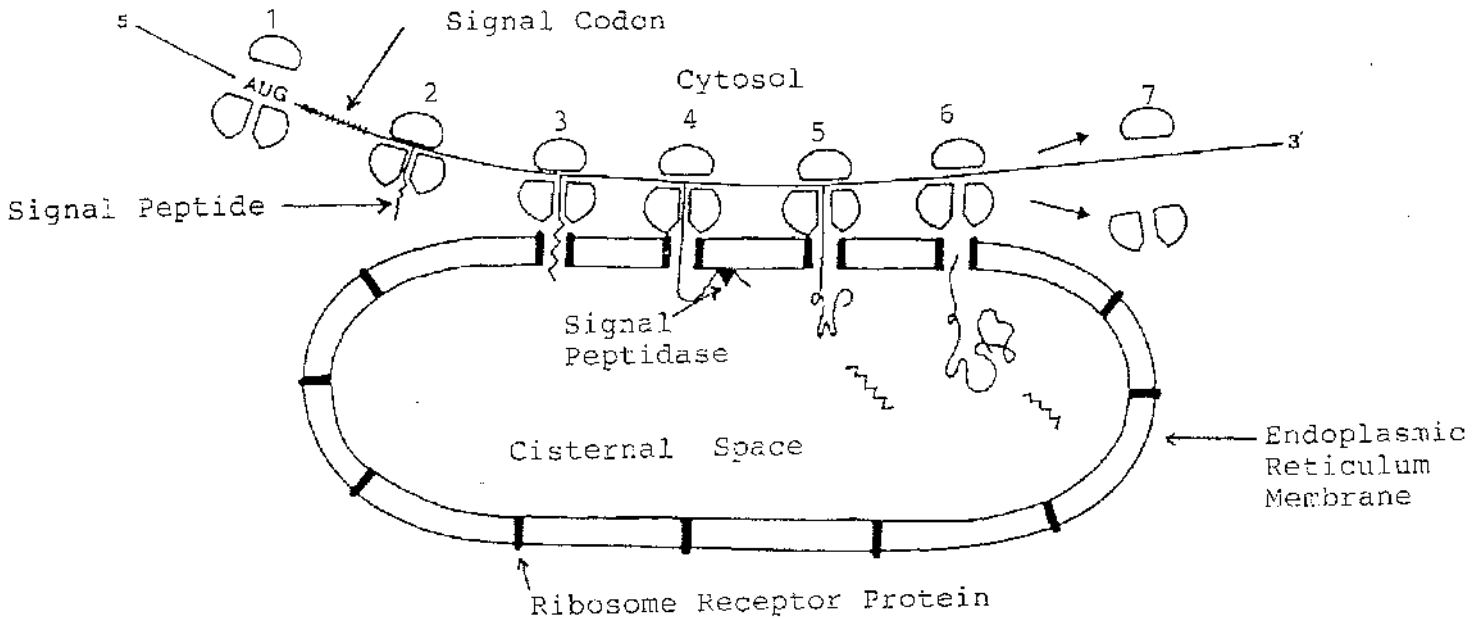


Figure 2. Schematic illustration of the signal hypothesis for the synthesis and segregation of secretory proteins.

(After Lingappa and Blobel, 1980)

(4) The growing chain, now on a membrane-bound polysome, passes through the channel and enters the cisternal space of the ER.

(5) The signal peptide is cleaved from the growing chain by a protease, termed signal peptidase, localised on the cisternal face of the ER membrane.

(6) Upon chain completion the ribosome detaches from the membrane; its subunits reenter the free cytoplasmic pool; the channel in the membrane is disassembled; and the completed chain consigned exclusively within the ER cisternae.

(7) The location and fate of the signal sequences might be subject to evolutionary variation. In some cases (physiological or pathological), the signal might not be cleaved off. In other cases, recombination might localise the signals to regions of the chain other than the amino-terminus. A schematic illustration summarising the signal hypothesis for the synthesis and segregation of secretory proteins, is given in Fig. 2 (After Lingappa and Blobel, 1980). This type of protein secretion is called co-translational secretion i.e. secretion while translating.

A somewhat different model from the signal hypothesis was presented by Wickner (1979). This model, termed "The membrane trigger hypothesis", emphasised the importance of induced folding of a polypeptide into a conformation

highly favourable for its insertion into a membrane. The signal sequence would alter the folding pathway, whether during or after protein synthesis and the driving force for the insertion of the protein being of self-assembly type. Von Heijne and Blomberg (1979) and Von Heijne (1980 a, b) postulated a "direct transfer model", in which the physicochemical parameters for a transmembrane transport of a nascent chain through the lipophilic core of the membrane is evaluated. In this model the nascent chain is extruded directly through the lipophilic portion of the membrane, the necessary driving force being provided by the direct, salt-labile membrane-ribosome interaction. Based on the specific three dimensional polypeptide structure and energetic considerations, Engleman and Steitz (1981), proposed that both the insertion of a protein into and the secretion of a protein through a membrane are initiated by the formation of a helical hairpin structure, which spontaneously partitions from the aqueous cytoplasm into the hydrophobic interior of a lipid bilayer (The helical hairpin hypothesis).

2. The signal peptides

Following the discovery of the signal sequence of an immunoglobulin light chain and the formulation of the signal hypothesis (Blobel and Dobberstein, 1975 a, b), extra pieces at the NH_2-termini of prehormones, precursors

of pancreatic proteins and several other proteins, have been found (Chan, Keim and Steiner, 1976).

In a series of more refined studies, Blobel and co-workers (1979), sequenced a number of eukaryotic proteins. They showed that the signal sequences were subject to considerable variation both in length and in primary structure across the animal kingdom. The size of the signal may vary from 15-29 amino acids but the main feature of a central cluster of hydrophobic amino acids with charged or hydrophilic residues at either end of the signal is conserved. The residues found in the penultimate C-terminal position of the signal are one of glycine, alanine, serine, cysteine or threonine i.e. amino acids with short side - chains (Blobel et al., 1979). Based on results of in vitro translation, Palmiter, Gagnon and Walsh (1978) showed that ovalbumin was not synthesised as a larger presecretory protein but clearly contains an uncleavable internal signal sequence. This raised the interesting question of how nascent ovalbumin is translocated across the membrane. Two possible mechanisms have been suggested by Lingappa and Blobel (1980),

(a) translation in a form of a loop with the signal at its leading edge, whereby both the left (amino-terminal) and right (carboxy-terminal) wings of the molecule are translocated simultaneously while the right wing of the molecule is being completed.

(b) co-translational translocation primarily of the right wing, followed by post-translational translocation of all or the remainder of the left wing of the molecule.

Lingappa, Lingappa and Blobel (1979) suggested that the internal signal of ovalbumin is located around residue 240 in a chain of 385 amino aicds, whereas Brael and Lodish (1982), suggested that the signal sequence is located prior to residue 150.

The detection of signal peptides liberated by cleavage from the polypeptide chain has been attempted, but no convincing results have been published (Jackson and Blobel, 1977; Patzelt et al., 1978; Habener et al., 1979). For this reason Kreil (1981) suggested that cleaved signal peptides must be very short lived in vivo and in vitro and rapidly hydrolysed. This rapid degradation could be essential, since the hydrophobic portions of signal peptides might otherwise have an adverse influence on the properties of the membrane. Analysis of 34 hydrophobic core-segments from known signal sequences (Von Heijne, 1982) with regard to the mean hydrophobicity at each position in the segment showed that these core-segments were not uniformly hydrophobic, but were more hydrophobic in the central region of the segment. In contrast, the hydrophobic residues in 12 membrane-spanning segments from transmembrane proteins were much more evenly distributed along the segment.

3. The signal peptidase

Blobel and Dobberstein (1975 a,b) suggested that signal peptidase is an integral membrane protein, closely associated with the pore in the membrane or forming an integral part of the pore. This suggestion was based on the fact that cleavage by signal peptidase is intimately linked to chain transit and occurs only on nascent but not completed chains. Signal peptidase, solubilised from the membrane by detergent treatment (Jackson and Blobel, 1977) retains its activity and specificity in the rough endoplasmic reticulum (RER), with little if any activity in the smooth endoplasmic reticulum (SER). Also, proteolytic enzymes added to intact vesicles did not inactivate the enzyme (Walter et al., 1979) suggesting that the signal peptidase activity is located on the cisternal side of the RER. Attempts to characterise the signal peptidase by protease inhibitors revealed that the enzyme bears some relationship to both trypsin and chymotrypsin (Gayda, Henderson and Markovitz, 1979; Strauss et al., 1979). Tabe, May and Elliott(1980) found that the release of α-amylase from rat pancreatic microsomes was inhibited by protease inhibitors which also inhibited signal peptidases of several secretory proteins. Examples of these protease inhibitors are; O-phenanthroline a metalloprotease inhibitor (Steiner, 1979), TLCK (N-tosyl-L-lysyl chloromethyl ketone) a trypsin inhibitor (Gayda,

Henderson and Markovitz, 1979). TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) a chymotrypsin inhibitor (Sussman, Tushniski and Bancroft, 1976) and PMSF (Phenylmethylsulfonyl fluoride) a serine protease inhibitor (Tabe, May and Elliott, 1980). Protease inhibitors which did not inhibit signal peptidase did not affect the production of α-amylase.

B. Translocation of integral membrane proteins

It was proposed in the signal hypothesis (Blobel and Dobberstein, 1975 a, b) that the initial events in the synthesis of certain membrane proteins could be identical to those of secretory proteins. Substantial support for this proposal came from *in vitro* translation experiments with two membrane proteins, the glycoprotein G of vesicular stomatitis virus (Katz *et al.*,1977; Rothman and Lodish, 1977; Lingappa *et al.*, 1978a) and the coat protein (CP) of bacteriophage f1 (Chang, Blobel and Model, 1978; Chang, Model and Blobel, 1979). These proteins compete for the same site of entry into the membrane even though their interaction with the membrane later becomes quite different (Blobel *et al.*, 1979). Blobel (1977 a,b) suggested that with membrane proteins, translocation is interrupted, so that the nascent chain becomes lodged in the membrane with a carboxy-terminus exposed to the cytoplasm. The information for this

interruption of translocation is thought to be contained in a portion of the nascent chain referred to as "stop transfer" sequence. The stop transfer sequence may reside in or near the membrane. Lingappa and co-workers (1978b) believe that the stop-transfer sequence is a common feature of numerous different transmembrane proteins and that it serves to position the protein in the membrane during translocation. Blobel and co-workers (1979) classified the integral membrane proteins (IMPs) into three groups: monotopic, bitopic and polytopic. Monotopic integral membrane proteins are unilateral in nature, i.e. they contain a hydrophilic domain only on one side of the lipid bilayer. An example of a monotopic integral membrane protein is cytochrome b_5 (Strittmatter, Rogers and Spatz, 1972). Bitopic IMPs are bilateral in nature. They contain two hydrophilic domains, one on each side of the membrane, and one stop-transfer sequence. Bacteriophage f1 coat protein, vesicular stomatitis virus glycoprotein and glycophorin (Bretscher, 1971; Tomita and Marchesi, 1975) are examples of bitopic IMPs. Polytopic IMPs are also bilateral but their polytopic chain contains more than two hydrophilic domains and more than one stop-transfer sequence (Bonatti, Cancedda and Blobel, 1979).

C. Post-translational translocation

A large number of chloroplast or mitochondrial proteins are synthesised in the cytosol and have to cross

either one or two membrane(s) in order to reach the chloroplast stroma or mitochondrial matrix. The first evidence for an alternative mechanism to co-translational translocation came from studies of Dobberstein, Blobel and Chua (1977), on the biosynthesis of the small subunit of ribulose-1, 5-biphosphate carboxylase. They found that *in vitro* cell free translation gave a molecule larger by some 40-50 amino acid residues. Also, this protein was synthesised by free ribosomes and could be cleaved post-translationally yielding the authentic (mature) protein and a small peptide, and that cleavage was by a soluble enzyme. It has been suggested (Dobberstein, Blobel and Chua, 1977) that the previously mentioned additional sequence functions in a receptor-mediated transfer through the chloroplast envelope, with the receptor localised in the closely distinct portion of the chloroplast envelope where the two membranes are opposed to each other. Maccecchini *et al.*, (1979) found that all three subunits of yeast F_1 ATPase were synthesised as larger precursors, when their mRNAs were translated in a cell-free protein-synthesising system. They further showed that incubation of the precursors with intact mitochondria resulted not only in their translocation into mitochondria but also in their proteolytic processing to their mature counterparts. Thus, the previous group suggested that translocation of proteins

from the cytoplasm into the mitochondrial matrix also proceeds post-translationally. Furthermore, the mature forms of the subunits (α, β and γ) could not be translocated into mitochondria, clearly demonstrating that translocation from the cytoplasm into the mitochondrial matrix depends on sequence information which is present only in the larger precursors and absent in the mature forms (Maccecchini et al., 1979).

A highly speculative model for post-translational translocation was suggested by Blobel et al., (1979) (Fig. 3) "All proteins to be translocated across intracellular membranes post-translationally are synthesised on free ribosomes, not on membrane bound ribosomes. Information for transfer resides in a group-specific signal sequence that is addressed to a receptor which is restricted in its location to specific membrane (mitochondrial or chloroplast or peroxisomal membrane). Thus, all species within a class of proteins to be translocated are synthesised with class - specific, functionally identical signal sequences. Interaction of the signal with the receptor is proposed to result in the assembly (or opening) of a pore (or a gate) that permits passage of a single polypeptide chain at a time. Passage through a membrane is envisaged to be accompanied by an unfolding (during passage) and refolding (following passage) of the polypeptide chain. In all cases, translocation should be initiated only by one and the same end of the molecule.

Figure 3. The protein to be translocated contains a signal sequence, indicated here as a cross-barred region on the amino-terminal of the polypeptide chain. The protein is folded (indicated by dots) but the signal sequence is exposed and free to interact with the signal receptors (SR). This interaction is indicated to result in the aggregation of the signal receptors in the phase of the membrane to form a pore.

Although not indicated in this model, it is conceivable that the signal sequence for post-translational translocation posesses two distinct domains. Both of these domains could be analogous to the two separate sites which have been invoked for co-translational translocation: one, which could be the analogue of the translocation domain of the signal sequence of nascent presecretory proteins, and another one which could be the analogue of a site on the large ribosomal subunit (eg. a portion of a ribosomal protein). By further analogy to co-translational translocation, the two putative domains of the siganl sequence for post-translational translocation could be recognised by two distinct receptors, each of them represented by a specific membrane protein.

Translocation is indicated arbitrarily to proceed carboxyl-terminal first. Signal peptidase (SP) would cleave when translocation is complete or nearly complete. Cleavage could be coupled to disassembly of the pore by lateral diffusion of the receptors in the plane of the membrane. No preferences are expressed as to the location of the signal peptide.

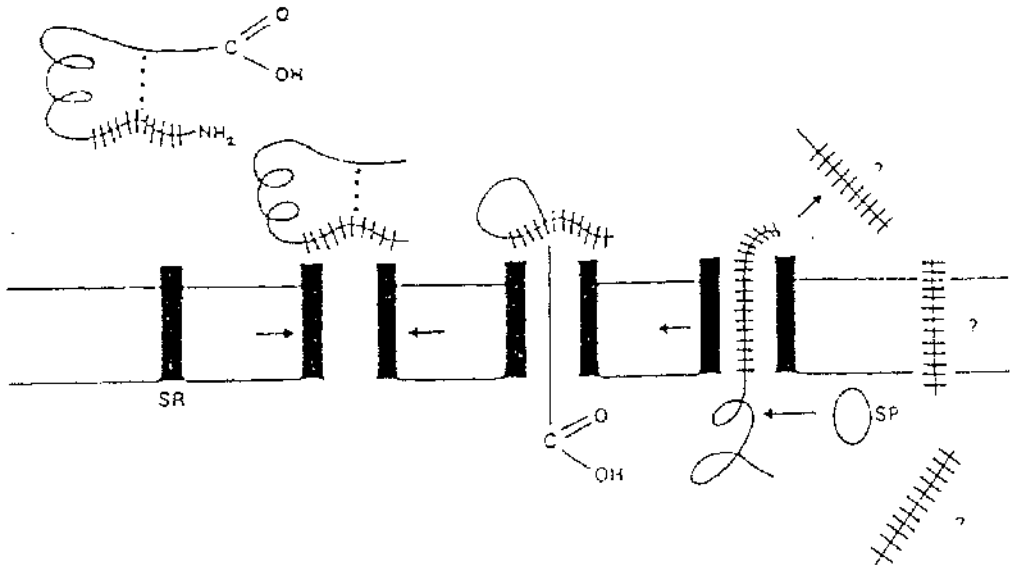


Figure 3. Hypothetical model for the post-translational translocation of a protein across a membrane.

(After Blobel et al., 1979)

Thus, unlike the case of co-translational translocation where both the signal sequence and the ribosome are postulated to provide the information for pore formation (or opening), in post-translational translocation this information is present only in the signal sequence. It is conceivable that post-translational translocation could be interrupted by a "stop-transfer" sequence functionally analogous to the one described for co-translational translocation. This would provide an important alternative mechanism for insertion of integral membrane proteins into the membranes. With mitochondrial or chloroplast proteins may be post-translationally translocated across only the outer membrane or both the outer and inner membranes. Two distinct signals and corresponding receptors may be involved. Both of the signals could be addressed to specific receptors in the outer membrane, one of the specific receptors could be part of a system that translocates only across the outer membrane. The other specific receptor could be part of a system that translocates across both membranes simultaneously. For the latter system one could invoke a pictorial analogy to gap junctions. Thus, a bitopic integral membrane protein (IMP) in the outer membrane could interact with a bitopic IMP in the inner membrane. Their assembly into a pore could be triggered by the interaction of the signal with a receptor domain which is located on the cytoplasmic side of the outer membrane".

D. The existence of specific protein assemblies within the lipid bilayer which facilitate translocation of proteins across membranes

The binding of ribosomes to the membrane of the endoplasmic reticulum through their large subunit is well established (Palade, 1975). As depicted in the signal hypothesis, ribosome binding would occur shortly after interaction of the membrane with the signal peptide (Blobel and Dobberstein, 1975a, b). The existence of specific protein assemblies within the lipid bilayer which are responsible for the initial binding of the signal peptide and for facilitating the motion of proteins across the membrane during protein synthesis was postulated by Blobel and co-workers (Blobel and Dobberstein, 1975a,b; Blobel et al., 1979; Blobel, 1980). Two proteins, ribophorins I and II which have been invoked as being responsible for ribosome binding are only present in the rough endoplasmic reticulum (Kreibich, Ulrich and Sabatini, 1978; Kreibich et al., 1978). It has been demonstrated that protein translocation is highly susceptible to protease or high salt washes (Warren and Dobberstein,1978). Prehn, Tsamaloukas and Rapoport (1980) have shown that preproinsulin, but not insulin, binds to the stripped membranes of the rough endoplasmic reticulum and that this binding can be abolished by pretreatment of membranes with protease.

In 1980 Walter and Blobel purified an 11S protein

complex (signal recognition protein, SRP) of 250K (M_r) (composed of six polypeptide chains) from dog pancreas microsomal membranes that was required for translocation of nascent secretory protein across the microsomal membrane. It specifically bound to and arrested the translation of the signal sequence of nascent secretory proteins, but not of cytoplasmic proteins on free ribosomes. This arrest was released upon SRP-mediated binding of the elongation-arrested ribosomes to the microsomal membrane, resulting in chain completion and translocation into the microsomal membrane (Walter and Blobel, 1981a,b; Walter, Ibrahimi and Blobel, 1981). Thus, Walter and Blobel (1981a) proposed that the initial events that lead to translocation and provide its specificity are not protein - lipid interaction (signal sequence- lipid bilayer) (Wickner, 1979; Engleman and Steitz, 1981), but protein-protein interactions (signal sequence plus ribosome - SRP). Meyer and Dobberstein (1980a, b) reported that a 60K protein can be released from salt-washed rough microsomes by elastase and high salt treatment and further suggested that this component was ER-specific, required for vectoral transfer of secretory proteins and derived from a membrane-bound protein of 72K molecular weight (Meyer, Louvard and Dobberstein, 1982). It appears that the 72K membrane protein is the molecule which relieves the SRP-induced block (Meyer, Kraus and Dobberstein, 1982), and that

translation can proceed only when the initiated ribosomal complex has made contact with the correct membrane, that is, the one containing the 72K protein. In accordance with its function, they henceforth referred to the 72K protein as the "docking protein".

A model consistent with all data reported previously (Meyer and Dobberstein, 1980a, b; Walter and Blobel, 1980, 1981a, b) and with data obtained by Meyer, Krause and Dobberstein (1982) is shown in Fig. 4. The initial events in protein translocation can be described as follows:

After 60-70 amino acids have been polymerised and the signal sequence has emerged from the large ribosomal subunit, further translation is interrupted by SRP. This block persists until contact is made with the 72K docking protein of which the 60K fragment represents the cytoplasmic domain. At this point translation, coupled with translocation proceeds (Meyer, Krause and Dobberstein, 1982). Recently, Walter and Blobel (1982) reported that the signal recognition protein contains a 7S RNA molecule; they suggested that this molecule is required for both structural and functional properties of signal recognition protein, hence they renamed the signal recognition protein as signal recognition particle.

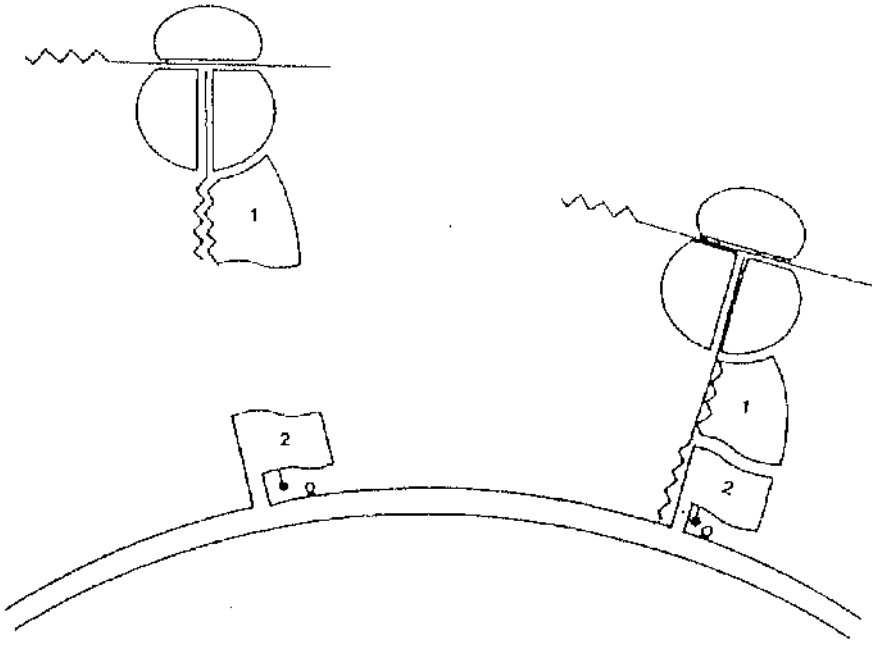


Figure 4. Sequence of events in ER-specific translocation of secretory proteins.

Initiation begins in the cytoplasm on free ribosomes. Translation is blocked by SRP (component 1) after 70-80 amino acids have been polymerised and the signal sequence emerges from the large ribosomal subunit (shown here in cross section). This arrest of translation persists until contact is made with the 'docking' protein (component 2) which is a 72K ER-specific membrane protein. Translation then resumes and translocation proceeds.

(After Meyer, Krause and Dobberstein, 1982).

IV.2. Protein Secretion Across Prokaryotic Membranes

The proposal that proteins that are secreted across a membrane are made on membrane-bound ribosomes was first confirmed by experiments in eukaryotic cells. A correlation between the amount of ribosomes bound to the endoplasmic reticulum and secretion of protein was first shown by Palade in 1975. It was later found that puromycin released incomplete chains to the interior of microsomes, suggesting that the secreted proteins pass through the membrane while the peptide chain is elongated (Redman and Sabatini, 1966). From these and other results it was proposed that membrane-bound ribosomes in animal cells are responsible for protein secretion across the membrane (see previous Section).

Recently, co-translational secretion in prokaryotes has been directly demonstrated through the study of protein secretion in bacteria. Moreover, the use of bacterial systems has allowed new approaches to the study of secretion, these being based on the accessibility of the relevant bacterial membrane surface and the isolation of bacterial mutants altered in secretion. However, in bacteria, the presence of a multi-layered rigid envelope (cell wall) presents a special problem for secretion of proteins into the medium. At the moment it is not known how the extracellular proteins are translocated through this layer. In addition the segregation of different proteins into the inner and the outer membrane of Gram-

negative bacteria presents a problem of membrane morphogenesis analogous to that of eukaryotic cells.

A. Protein secretion and localisation in Gram-negative bacteria

1. Existence of membrane-bound polysomes:

Cancedda and Schlesinger (1974) first separated polysomes of Escherichia coli into two classes, membrane-bound and free polysomes. They demonstrated that alkaline phosphatase, a periplasmic protein, was preferentially synthesised on the membrane-bound polysomes. Using sonic disruption and sucrose density gradient centrifugation, Randall and Hardy (1977) succeeded in isolating membrane-bound polysomes from E. coli. They found that the membrane-bound ribosomes synthesised two classes of proteins, one that remained membrane-bound and another that became soluble upon release from ribosomes. Smith et al., (1977) found that upon reacting the surface labelling agent [^{35}S]-acetyl methionyl methylphosphate sulphone (which labels amino groups) with E. coli spheroplasts which were synthesising proteins, the radioactivity was recovered in peptidyl-tRNA of the membrane-associated polysomes. A portion of the labelled product, after in vitro chain completion, was identified as alkaline phosphatase. This result provided the first evidence in a bacterial system that proteins are secreted across cytoplasmic membranes as growing chains or co-translationally.

In 1980, Emr, Hall and Silhavy stated that the molecular mechanism of cellular protein localisation is strikingly similar in both animal and bacterial cells. However, one difference between prokaryotic and eukaryotic systems appears to be the ribosome membrane interaction. A weaker ribosome-membrane attachment susceptible to puromycin alone was found in bacteria (Smith, Tai and Davis, 1979; Davis and Tai, 1980). Recent evidence indicated that the ribosomes are simultaneously attached to the membranes after the initial contact of the hydrophilic portion of the signal sequence with the membrane receptor, the hydrophobic portion of the signal sequence being melted into the hydrophobic environment of the membrane (Hall and Schwartz, 1982).

2. Precursor accumulation of secreted proteins

In a pioneering study exploring the characteristics of membrane and cytoplasmic protein synthesis in vivo, Hirashima, Childs and Inouye (1973) examined the effect of a number of antibiotic inhibitors of protein synthesis. The effect of five ribosome-directed antibiotics (Kasugamycin, tetracycline, chloramphenicol and puromycin) on the synthesis of E. coli cytoplasmic and specific envelope proteins were examined. In general, when the antibiotics were added to growing cultures labelled with radioactive amino acid, effects on the biosynthesis of

envelope proteins differed from that on biosynthesis of cytoplasmic proteins, indicating differences in the mode of synthesis. When labelled envelope proteins were examined further by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), different degrees of sensitivity to the antibiotics were found among the individual envelope proteins. The former group suggested that the differential effects of antibiotics may be due to compartmentalisation of polysomes for specific proteins or differences in the biosynthesis machinery for different proteins.

The effect of rifampicin, an inhibitor of RNA synthesis, on the synthesis of envelope and cytoplasmic proteins *in vivo* was examined. Increased resistance to rifampicin of *in vivo* envelope protein synthesis reflected an increased stability of their mRNA (Hirashim, Childs and Inouye, 1973). Hirashima, Wang and Inouye (1974) isolated biologically active lipoprotein mRNA, and found that the *in vivo* translation product was cross-reactive with antilipoprotein serum. Also, peptide mapping of the product revealed an identical carboxy-terminal structure to that of lipoprotein. Using toluene (a membrane perturbant) at concentration without effect on *E. coli* cell protein synthesis, a higher molecular weight form of lipoprotein (prolipoprotein) was accumulated in the cytoplasmic membrane (Halegoua *et al.*, 1976; Halegoua Sekizawa and Inouye, 1977). Halegoua, Sekizawa and

Inouye (1977) mixed the immunoprecipitated prolipoprotein from toluene-treated cells with cell free products directed by purified lipoprotein mRNA, the two products were found to comigrate on SDS-gel electrophoresis, suggesting that the two proteins are the same. Furthermore, they found that the new protein (prolipoprotein) had the same carboxy-terminal structure as the lipoprotein, as revealed by peptide mapping, however, the N-terminal residue was methionine instead of glycerylcysteine found in the case of lipoprotein. The new protein contained an amino-terminal peptide extension on the lipoprotein sequence containing 18-19 residues, predominantly hydrophobic in nature. The new form of lipoprotein, designated prolipoprotein, was believed to play a major role in biosynthesis an assembly of lipoprotein.

Halegoua and Inouye (1979a) found that phenethyl alcohol, (PEA), a membrane perturbant, at a concentration of 0.3% inhibited the synthesis of E.coli matrix protein (an outer membrane protein) with concomitant accumulation of promatrix protein. Under these conditions, unlike the case of toluene, the accumulation of promatrix protein was reversible, upon removal of PEA and regrowth of the cells in the culture, promatrix protein was processed and the resulting matrix protein was normally assembled into the outer membrane. From previous results, a direct precorsor-product relationship between the pre-protein and processed outer membrane protein was demonstrated

(Halegoua and Inouye, 1979b).

An important factor in the assembly processes of outer membrane proteins is the state of fluidity of the envelope membrane. It has been reported that a reduction in lipid fluidity of the E. coli plasma membrane resulted in inhibition of induction of active alkaline phosphatase, a periplasmic protein (Kimura and Izui, 1976). It was thought that inhibition of processing of proalkaline phosphatase, a precursor of alkaline phosphatase, resulted in the accumulation of the precursor in the envelope (Pages et al., 1978). In unsaturated fatty acid auxotrophs, fatty acid composition of the membrane lipid can be dictated by growing cells on specific unsaturated fatty acids and fluidity of the membrane can be controlled by altering the growth temperature (Halegoua and Inouye, 1979b).

Di Rienzo and Inouye (1979) used an unsaturated fatty acid auxotroph of E. coli grown on elaidate, to investigate the effect of lipid fluidity on the biosynthesis and assembly of three major outer membrane proteins (lipoprotein, Tol-G protein and matrix protein). Each of these proteins were synthesised and assembled in the outer membrane by different mechanisms on which the physical state of the membrane caused different effects: normal assembly of the lipoprotein, accumulation of a precursor of the Tol-G protein (proTol-G protein) and no production of matrix protein. They suggested that the outer membrane proteins are synthesised and assembled according to several

different mechanisms, on which the physical state of the membrane has various effects. Lazdunski, Baty and Pages (1979) found that treatment of E.coli with 0.55% procaine (which causes an increase in molecular packing of membrane lipid molecules) resulted in accumulation of precursors in addition to mature forms of two periplasmic proteins, alkaline phosphatase and glutamine-binding protein. The precursor form of alkaline phosphatase had a molecular weight increased by 2000 dalton over the mature form. They suggested that the inhibition of precursor processing was caused either by direct inhibition of signal peptidase, or, since the fluid state of membrane is required for precursor processing, the procaine induced decrease in fluidity caused processing inhibition.

All the previous results are consistent with predictions made by the signal hypthesis. The existence of protein precursors appears to be a general phenomenon for secreted proteins.

a. The loop hypothesis: From the amino acid sequence of prolipoprotein peptide extension Inouye et al. (1977) pointed out several unique features of the sequence believed to be important in the function of translocation of the protein across the cytoplasmic membrane. The features are:

1. The extended region is basic and positively charged at neutral pH because it contains two lysine but no

acidic amino acid residues.

2. The peptide extension contains three glycine residues, an amino acid that is not present in the lipoprotein sequence.

3. The extended peptide is much more hydrophobic than lipoprotein, since 60% of the residues in this region are hydrophobic in contrast to 38% in lipoprotein.

4. The distribution of the hydrophobic amino acids in the peptide extension is completely different from their periodic distribution in lipoprotein.

The amino acid sequence of β-lactamase (an E.coli periplasmic protein) was determined from the nucleotide sequence of the plasmid gene coding for the protein (Sutcliffe , 1978). Although β-lactamase is a periplasmic enzyme, the sequence of the pro β-lactamase peptide extension of 23 amino acid residues was remarkably similar to that of prolipoprotein. (Halegoua and Inouye, 1979b). Based on the general features of the peptide extension, a possible role in the translocation process has been suggested (Inouye et al., 1977; Di Rienzo, Nakamura and Inouye, 1978; Halegoua and Inouye, 1979b) (Fig. 5):

1. As the precursor protein is synthesised on the polysomes, the positively charged section I allows the initial attachment of the peptide extension and consequently the polysome to the negatively charged inner surface of the cytoplasmic membrane through ionic interactions. The E. coli membrane surface is negatively charged at

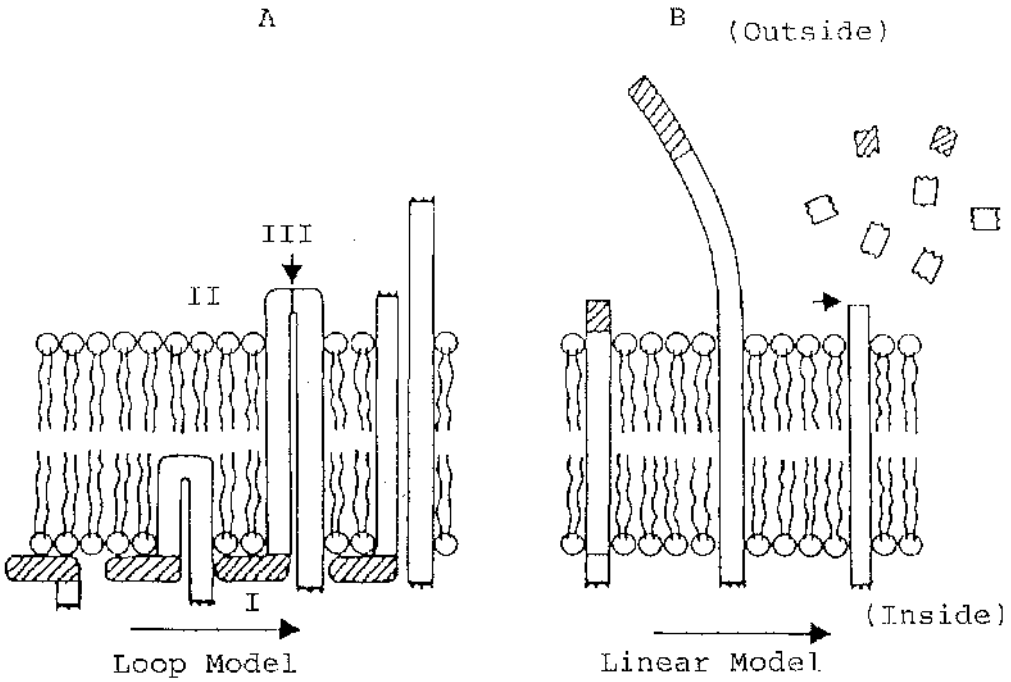


Figure 5. Loop Model and linear model for the translocation of secretory proteins across the membrane. (A) Loop model and (B) linear model. Solid portions represent the basic regions of the first several amino acid residues of the peptide extension (Section I). The following blank portions represent the hydrophobic regions of the peptide extension (Section II) and cleavage site (Section III) followed by portions of mature proteins. Small arrows show the cleavage sites of the precursor molecules.

(After Halegoua and Inouye, 1979b).

neutral pH as a result of the presence of phosphatidyl-glycerol.

2. The next hyrophobic section, section II, is then progressively inserted into the cytoplasmic membrane by hydrophobic interaction with the lipid bilayer.

3. As the peptide further elongates, the loop formed by section II is further extended into the lipid bilayer.

4. The processing or cleavage site of the peptide extension (section III) is eventually exposed to the outside surface of the cytoplasmic membrane.

5. The length of section II and III, about 53Å, is long enough to extend across the lipid bilayer to expose section III to the outside surface of the membrane where processing at the cleavage site may take place.

6. The role of proline or glycine residues in section II may be important in bending the peptide at the position to form a loop. This proposed mechanism of protein translocation across prokaryotic membranes was named as the loop mechanism or the "loop hypothesis".

The linear model (signal hypothesis) of Blobel and Dobberstein (1975a, b) does not take into account the specific characteristic of the peptide extension as described in loop hypothesis. It may not be difficult to distinguish between the two models experimentally, since in the linear model, the peptide extension is

transported to the outside, while it is left in the cytoplasmic membrane in the loop model (Halegoua and Inouye, 1979b).

In 1980, Davis and Tai reviewed several possible sources of energy for the extrusion of polypeptide after the initial spontaneous binding and insertion of nascent chains. They favoured a model which suggested that there is an active secretory machinery in the membrane. Such an active carrier would differ from the passive tunnel that has been postulated previously (Blobel and Dobberstein, 1975 a, b; Di Rienzo, Nakamura and Inouye, 1978; Halegoua and Inouye, 1979b). Daniels et al (1981) reported that secretion of leucine-specific binding protein and β-lactamase (E. coli perplasmic proteins) were inhibited by phenethyl alcohol and some other proton ionophores such as carbonylcyanide-chlorophenylhydrazone (cccp) and valinomycin which causes significant decrease in the membrane potential. They further reported that processing can be restored in cccp-treated cells and valinomycin-treated sphaeroplasts by dilution of the treated cells in fresh media. From their results they suggested that there is a role for membrane potential in the secretion of periplasmic protein. They further presented a model in which membrane potential plays a primary role in the proper orientation of the precursor signal sequence within the membrane, thus promoting processing and secretion.

b. The signal sequences: Precursors with N-terminal signal sequences have been demonstrated or, inferred from the corresponding DNA sequences, for several proteins that are secreted from bacteria (Inouye and Beckwith, 1977; Randall, Hardy and Josefsson, 1978; Sarvas et al., 1978; Sutcliffe, 1978). Randall, Hardy and Josefsson (1978) have shown that λ phage receptor (an outer membrane protein in E.coli) is synthesised preferentially as a larger precursor by ribosomes bound to the cytoplasmic membrane. Hedgepeth et al. (1980) demonstrated that removal of the NH_2-terminal sequence of the pre-λ receptor occurred during transport of the protein to the outer membrane. Emr, Hall and Silhavy (1980) identified a series of mutations within the signal peptide of the λ receptor where the precursor form accumulated in the cytoplasm. Sequence analyses revealed that most of these mutations were the result of single amino acid substitutions, usually the exchange of a hydrophobic residue for a charged one. Similar results were obtained for the maltose binding protein (Bedouelle et al.,1980). Most signal peptides also contain, besides the α-amino group, at least one more charged residue near the amino end, frequently an arginine or lysine (Emr, Hall and Silhavy, 1980).

Hall and Schwartz (1982) reviewed a unique mutation affecting the hydrophilic signal sequence of the λ receptor (Schwartz et al.,1981; Hall et al., 1982) and

suggested that the hydrophilic portion of the signal sequence mediates the initial interaction between a nascent secreted protein and the membrane. They also proposed the existence of a so called "stop translation sequence". 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 a membrane receptor.

By using the technique of gene fusion, it was not only clearly demonstrated that the central hydrophobic region of the signal peptides is essential and the introduction of a single amino acid change can block its ability to initiate export but also demonstrated that in some cases a single signal sequence is not sufficient for protein translocation (Moreno et al., 1980; Koshland and Botstein, 1980).

Knowledge of the signal sequences for secretion has been applied in an effort to increase production of insulin in recombinant bacteria. Talmadge, Kaufman and Gilbert (1980) constructed a variety of hybrid bacterial eukaryotic signal sequences by inserting the rat preproinsulin gene into the bacterial prepenicillinase gene. Among their hybrids were four constructions: rat proinsulin attached to the entire penicillinase signal sequence and rat preproinsulin fused to all of, to half of, or only to the first four amino acids of the bacterial

signal sequence. In all four cases, more than 90% of the rat insulin antigen appeared in the periplasmic space. They further showed (using immunoprecipitation and amino acid sequence determination of the radiolabelled products) that the bacteria correctly process both the bacterial and the eukaryotic signal sequences of these hybrid proteins, and that the cleavage of the eukaryotic signal by bacterial peptidase, in this case generates proinsulin. Using constructed plasmids in which most of the coding sequence for human preproinsulin was fused to portions of the prepeptide region of ampicillinase, Chan et al. (1981) succeeded in producing human proinsulin in E.coli. A perfect hybrid of the NH_2-terminal half of the leader sequence of ampicillinase (residues 23 to12) with the human preproinsulin peptide beginning at residue 13 was formed; the result was the synthesis and secretion of human proinsulin into the periplasmic space.

The signal peptides are removed by signal peptidase and attempts have been made to purify this enzyme. Zwizinski and Wickner (1980) reported a 600 fold purification of the signal peptidase responsible for the cleavage of precursor of M13 coat protein (an E.coli outer membrane protein). Tokunaga et al. (1982) reported a signal peptidase activity in E. coli cell envelope which processes prolipoprotein modified with glyceride. In order to find whether the signal peptidase for prolipoprotein is

distinct from the signal peptidase for M13 procoat protein, Tokunaga et al. (1982) used antibody against purified procoat protein signal peptidase to study the processing of proliprotein and M13 procoat protein in vitro. They found that the signal peptidase for modified prolipoprotein remained fully active in solubilised membrane preparations which had been treated with antibody against purified procoat protein signal peptidase, whereas the activity towards procoat protein was completely abolished by immunoadsorption. Furthermore, both unmodified and glyceride-modified prolipoprotein were not cleaved by the highly purified signal peptidase preparation. They concluded that prolipoprotein signal peptidase was distinct from the M13 procoat protein signal peptidase.

A comparison of the NH_2-terminal extensions found in bacterial proproteins with signal peptides of secretory polypeptides of eukaryotic cells reveals no principal differences. They are similar in length, distribution of polar and apolar amino acids and in the nature of the c-terminal residue (Kreil, 1981).

3. Post-translational secretion

Certain proteins are exported or incorporated in the form in which they are synthesised, that is without the need of precursor form. These include colicin E1 and E2 (Jackes and Model, 1979) and several products of

sex factors (Achtman et al., 1979). It is not known whether these proteins are synthesised on membrane bound or free ribosomes. It has been shown that ß-lactamase is sythesised initially in a precursor form, with an amino-terminal "signal" peptide (Sutcliffe, 1978; Ambler and Scott, 1978). This signal peptide is cleaved from the amino-terminus after the synthesis of ß-lactamase is complete (Koshland and Botstein, 1980; Josefsson and Randall, 1981). ß-Lactamase molecules altered at the carboxy-terminus are apparently translocated but not released into the periplasm (Koshland and Botstein, 1980, 1982). Thus, the carboxy -end is required to finish the secretion process, that is, to become a free periplasmic protein. Koshland, Sauer and Botstein (1982) suggested that mutations at the signal sequence of ß-lactamase (produced by Salmonella typhimurium) can affect more than one step in the secretion process, and that processing of the signal peptide is not required for the protein to be translocated (at least partially) across the inner membrane.

Recently, Müller et al. (1982) reported that ß-lactamase synthesised in vitro is co-translationally translocated into dog pancreas microsomal vesicles. Their data were consistent with those obtained for eukaryotic proteins (Walter and Blobel, 1980) and suggested that co-translational translocation of both bacterial and eukaryotic secretory proteins across the endoplasmic reticulum require identical components.

B. Protein secretion in Gram-Positive bacteria

1. Penicillinase of Bacillus licheniformis

One of the major differences between the Gram-positive and the Gram-negative bacteria is the existence of the periplasmic space in the latter. This space or compartment, contains several proteins which can be released into the exterior environment upon lysis of the cell wall. In Gram-positive bacteria, there is no periplasmic space, therefore we do not expect the existence of such compartmentalisation of proteins. However, in recent years, it has been shown that Gram-positive bacteria can retain a proportion of their extracellular proteins in a membrane-bound form.

Pollock (1961) suggested that Bacillus licheniformis strain 749/C produced constitutively, two forms of penicillinase (penicillin amido — β-lactamhydrolase) being derived from a single structural gene (Duncan and Pollock, 1965; Sherratt and Collins, 1973). In support of this, Lampen, (1967) showed that the plasma membrane enzyme can be converted to the exoenzyme by limited trypsin treatment, or incubation of cells at pH 9.0 (Sargent and Lampen, 1970). Of the two previously mentioned forms of penicillinase, one is a very hydrophilic protein (M_r 29,000) of extremely stable secondary and tertiary structure (Lampen, Bettinger and Sharkey, 1971), while the other is a hydrophobic membrane bound enzyme (M_r 33,000)

(Dancer and Lampen, 1975). Dancer and Lampen (1975) suggested that the membrane penicillinase (the membrane-bound enzyme) is an intermediate in the formation of exopenicillinase. The membrane penicillinase of B. licheniformis 749/C, differs from the exopenicillinase by having a phospholipopeptide chain of 25 amino acids at its NH_2-terminus, with the NH_2-terminal residue being phosphatidylserine and lysine as the COOH-terminal residue (Yamamoto and Lampen, 1975).

Yamamoto and Lampen (1976) proposed that the phosphatidyl tail was responsible for the hydrophobic properties of the membrane enzyme and could anchor the enzyme to the plasma membrane while leaving the major protein free to interact with substrate or antibody. Furthermore, acid hydrolysis of the peptide chain yielded only serine, glycine, glutamic acid, aspartic acid and lysine. The membrane bound enzyme was readily converted to the exoenzyme in vivo at neutral or alkaline pH (Aiyappa, Traficante and Lampen, 1977; Traficante and Lampen, 1977). Aiyappa, Traficante and Lampen (1977) suggested that release of the membrane penicillinase occurred via enzymatic cleavage to yield exoenzyme since the N-terminal residue of released penicillinase was always lysine or glutamic acid. This may indicate that a common proteolytic processing of the membrane penicillinase takes place under various conditions of

secretion (Aiyappa and Lampen, 1977). Indeed, Aiyappa and Lampen (1977) demonstrated a penicillinase releasing enzyme (penicillinase releasing protease) which was a serine protease with an approximate M_r of 21,000 dalton and a broad pH optimum of 7.0 ot 9.0. It required Ca^{2+} or Mg^{2+} for stability, its general properties and sensitivity to inhibitors being consistent with the characteristicsof penicillinase secretion.

Using discontinuous SDS-PAGE system, Izui and co-workers (1980) found that B. licheniformis 749 and 749/C produced two related hydrophilic extracellular penicillinases, an exo-large M_r 30,500 dalton and an exo-small M_r 29,500 dalton. They suggested that the exo-large is the main form and it is detectable in the early to mid expotential phase cultures of mutant 749/C while exo-small appears only as the cultures approach stationary phase. Smith, Tai and Davis (1981) reported that when labelled penicillinase was immunoprecipated from extracts of B. licheniformis 749/C, three electrophoretic peaks of molecular weights of 36,000, 33,000 and 29,000 daltonswere identified. The M_r 36,000 form exhibits moderate hydrophobicity, as expected of a precursor with an NH_2-terminal signal sequence for secretion. Part of the M_r 33,000 contained a lipid and was even more hydrophobic. They also found that [2-^{3}H] glycerol is incorporated into these molecules (36,000 and 33,000

daltons)but not into the other forms of the enzyme, thus, a mechanism was suggested which involves the incorporation of lipids and two cleavages during growth of the penicillinase chain.

On the basis of several observations, Nielsen, Caulfield, and Lampen (1981) suggested that the membrane penicillinase bears a striking resemblance to the major outer membrane lipoprotein of E. coli : it can be specifically labelled in vivo with [^{3}H] glycerol, [^{35}S-cysteine, or [^{3}H] palmitate but not by [^{32}P] orthophosphate, the labelled residues are located at or near NH_2-terminus of the membrane penicillinase, they can be completely removed by trypsin which cleaves a hydrophobic peptide from the NH_2-terminus, thereby rendering the enzyme active. They further found that the membrane penicillinase, produced by the 749/C gene carried in E. coli on phage λ was similar to the enzyme formed in strain 749/C itself. The peptide antibiotic globomycin prevented processing of the E. coli prolipoprotein and severely inhibited the attachment of [^{3}H] palmitate or [^{3}H] glycerol to the 749/C enzyme. It blocked the accumulation of penicillinase in the plasma membrane and enhanced the formation of the exoenzyme. Under the same conditions, globomycin did not prevent the attachment of palmitate or glycerol to the E. coli prolipoprotein but inhibited processing of the modified precursor to the mature lipoprotein. Hence, Nielsen, Caulfield and Lampen (1981) suggested that

globomycin blocked the derivatisation of prepenicillinase and rendered the unmodified precursor more available to the peptidase that normally produced exopenicillinase.

Chang et al. (1982) studied the biosynthesis of the early forms of B. licheniformis 749 penicillinase. They reported that (1) a primary translation product can be synthesised in vitro and in vivo (2) a detergent solubilised preparation of B. licheniformis membrane vesicles, presumably containing signal peptidase, is able to cleave the primary translation product, termed prepenicillinase, between glycine 26 and cysteine 27. Moreover, they suggested that penicillinase contains a signal sequence of 26 residues that is cleaved either during or shortly after chain translocation across the plasma membrane, whereby cysteine 27 of prepenicillinase becomes cysteine 1 of membrane-bound penicillinase. Anchorage to the plasma membrane occurs via modifications of cysteine 1, similar to those found in cysteine 1 of E. coli lipoprotein (Hantke and Braun, 1973)(Fig.6). The following series of events were envisaged in the biosynthesis of penicillinase (Chang et al., 1982):

(1) Signal sequence initiates translocation of prepenicillinase.

(2) The SH-group of cysteine 27 of nascent pre-penicillinase is modified via thioether linkage to diglyceride.

(3) Cleavage of signal sequence, with nascent

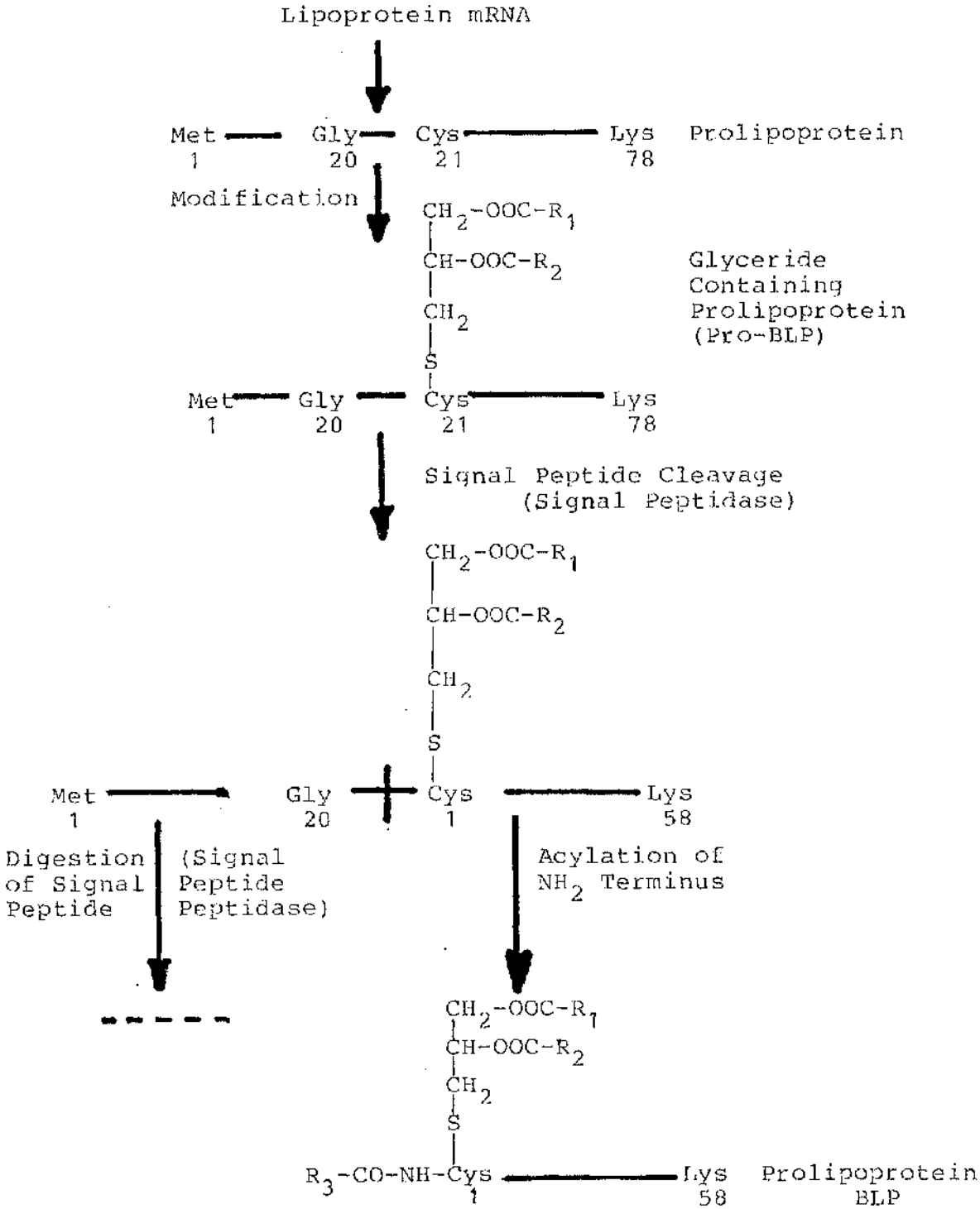


Figure 6. Biosynthesis of BLP. The S above Cys belongs to the cysteine residue. The number and length of the digested products of the signal peptide is not known.

(After Hussain, Ichihara and Mizoshima, 1982).

BLP = Braun's lipoprotein

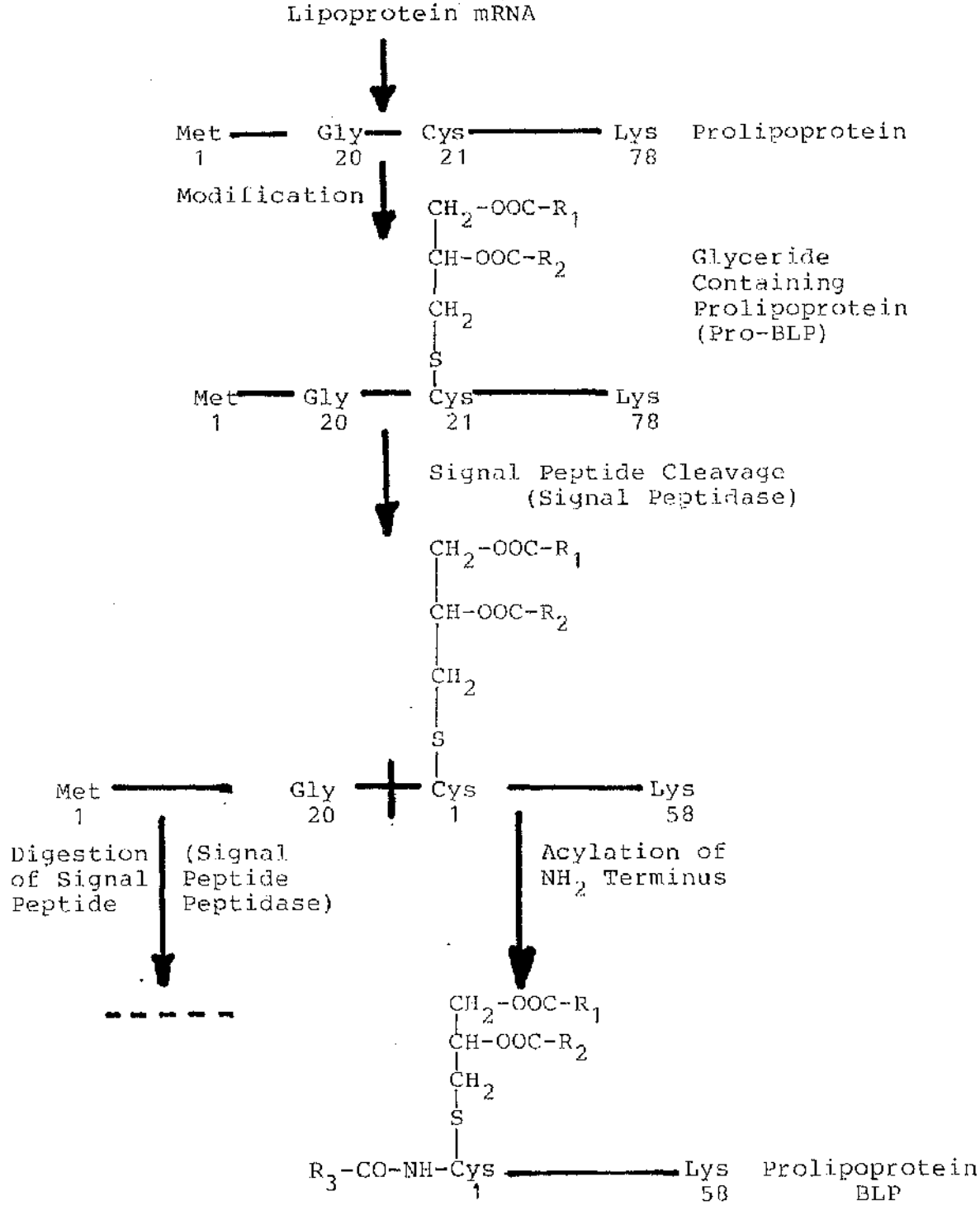


Figure 6. Biosynthesis of BLP. The S above Cys belongs to the cysteine residue. The number and length of the digested products of the signal peptide is not known.

(After Hussain, Ichihara and Mizoshima, 1982).

BLP = Braun's lipoprotein

(or completed) penicillinase to remain anchored to the outer leaflet of the plasma membrane lipid bilayer via diglyceride of cysteine 1 (formerly cysteine 27 of nascent prepenicillinase).

(4) Linkage of fatty acyl residue to the α-NH_2 group of cysteine 1, providing for additional anchorage to the membrane's outer lipid leaflet.

(5) Proteolytic processing at various sites COOH-terminal to the cysteine residue would abolish membrane anchorage and would yeild various water soluble exo-forms of penicillinase.

(6) Cleavage of prepenicillinase by signal peptidase without cysteine modification could result in secretion of the hydrophilic "processed" form produced *in vitro* and further cleavage to exo-large and then to exo-small could ensue. Finally, they concluded that membrane penicillinase may not be an obligatory intermediate for all secreted molecules and, by regulating the activities that result either in the modification of cysteine or in proteolytic cleavage, the cell may have the facility to regulate the amount of membrane-bound or secreted molecules in response to various stimuli.

Nielsen and Lampen (1982a,b) reported that the membrane penicillinases of *B. licheniformis* and *B. cereus* are lipoproteins with N-terminal glyceride thioether modification identical to that of the *E. coli* outer

membrane protein .(Fig. 6). They postulated that the lipoproteins of Gram-positive organisms are the functional equivalent of periplasmic proteins in E.coli and other Gram-negative bacteria, prevented from release by anchorage to the membrane rather than by a selectively impermeable outer membrane. Also lipoprotein modification may offer Gram-positive bacteria an efficient way of retaining a variable position of its secreted proteins in an active, releasable form.

2. The use of membrane modifying agents and protease inhibitors in the study of protein secretion

Membrane modifying agents and protease inhibitors proved to be valuable tools in the study of mechanisms of protein secretion in bacteria. Fishman, Rottem and Citri (1978) reported that the formation of penicillinase by cultures of B. licheniformis 749/C was preferentially suppressed by cerulenin [(2S) (3R) 2,3-epoxy-4-oxo-7,10-dodecadienoylamide] an antibiotic which inhibits lipid synthesis in a variety of microorganisms (Ōmura, 1976) by specifically inhibiting the β-keto-acyl carrier protein synthetase, an enzyme which is responsible for the condensation reaction between acyl and malonyl thioesters.

The effect of cerulenin was studied at concentrations which had almost no effect on growth and overall protein synthesis, but reduced the rate of [^{14}C]-acetate

incorporation by 50-70%. The levels of both the released enzyme (exopenicillinase) and its cell-bound precursor were reduced to the same extent. Enzyme formation was gradually resumed after the removal of cerulenin or the addition of a mixture of fatty acids prepared from lipid extracted from B. licheniformis. Moreover, no evidence was obtained for the accumulation of the non secreted enzyme in either cytoplasm or membrane. From these results, Fishman, Rottem and Citri (1978) suggested that de novo synthesis of fatty acids were required for the formation of both membrane bound and extracellular penicillinase and that any damage to the cytoplasmic membrane would result in an impaired synthesis of secreted enzymes. In a broader study an attempt was made to understand the role of the cytoplasmic membrane in the formation of bacterial extracellular products. Here, Fishman, Rottem and Citri (1980) used more than one membrane modifying agent. The effect of cerulenin, phenethyl alcohol, benzyl alcohol, procaine, and a series of aliphatic alcohols were tested on the production of alkaline phosphatase and penicillinase from B. licheniformis 749; haemolysin, protease and penicillinase from B.cereus 569 and alkaline phosphatase from B. subtilis. In most cases, the production of these proteins was suppressed by the membrane modifying agents. Therefore, the former group proposed that the cytoplasmic membrane had a specific role in the formation of bacterial exoproteins and that

the exoproteins differ, as a class, from cytoplasmic proteins in two ways. Firstly, the biosynthetic process of bacterial exoproteins is intimately linked with the membrane and can be disrupted by membrane modifications which have little or no effect on the synthesis of other proteins and secondly, under normal growth conditions bacterial exoproteins appear to be largely, if not entirely, dispensable.

Altenbern (1977a) found that production of staphylococcal enterotoxins B and C (SEB and SEC) was completely inhibited by concentrations of cerulenin ($4\mu gml^{-1}$ and $2\ \mu gml^{-1}$, respectively) that did not affect either growth rate or final cell density, whereas production of enterotoxin A was not similarly inhibited. In this study he used four different strains of S. aureus; ATCC 14458, S-6, 137-H2, and 2909. Both strains 14458 and S-6 were wild type strains for enterotoxin B (SEB). Strain 137-H2 was a wild type that elaborated enterotoxin C (SEC). Strain 2909 produces relatively large amounts of enterotoxin A (SEA) and was derived from strain 100 by a multistep mutagenesis procedure. A mixture of saturated fatty acids (SFA) composed of equal concentrations of lauric, myristic, palmitic, stearic, arachidic, behenic and lignoceric acids did not affect growth or toxin production in control cells, whereas they partially restored the total suppression by cerulenin of SEB formation. A mixture

of 10 unsaturated fatty acids from C16 to C24 were unable to reverse cerulenin-induced suppression of SEB production without concomitant severe inhibition of growth. Low concentrations of cerulenin, non inhibitory for growth rate or final growth density, also strongly suppressed production of α-lysin and coagulase from strain 14458. Thus, it appeared that the production of SEB, alpha-lysin and coagulase is severely inhibited by non-growth inhibitory concentrations of cerulenin.

Berkeley et al., (1978) reported that both cerulenin and quinacrine (inhibitor of the penicillinase-releasing protease) inhibited the release of enterotoxin A from S. aureus strain 772, suggesting the operation of a lipid intermediate/exoprotein-releasing protease system. In a study of the role of proteases in the secretion of exportable proteins, Altenbern (1978) examined the effect of different protease inhibitors on the production of enterotoxin B from S. aureus strain S-6. The following results were found:-

1. TPCK inhibited equally both growth and SEB formation and no differential suppression of SEB was detected.

2. In contrast PABZ (para-aminobenzamidine), over a narrow concentration range, strongly inhibited SEB yield without appreciably altering final growth density.

3. Both TLCK and antipain clearly inhibited SEB

production over a comparatively broad concentration range without suppressing growth.

4. PMSF up to saturation (10^{-3}M), had no effect on either final growth density or SEB formation. From these results Altenbern (1978) concluded that a proteolytic cleavage step may be involved in the transport of the SEB molecule to the exterior environment. Caulfield et al. (1979) reported that the secretion of levansucrase by B. subtilis strain B was inhibited by cerulenin and the protease inhibitors quinacrine and O-phenanthroline. Cerulenin (10 μgml^{-1}) totally inhibited de novo lipid synthesis, while only slightly reducing protein and RNA synthesis. Quinacrine and O-phenanthroline also reduced the release of levansucrase but they had no effect on lipid synthesis. Cerulenin inhibition suggested involvement of a lipid moiety with the enzyme when it passes through the cytoplasmic membrane, whereas protease inhibition indicated a protease cleavage mechanism in the release of the enzyme.

Tweten and Iandolo (1981) identified a putative precursor to staphylococcal enterotoxin B as a result of analysis of purified membranes of strain S-6 on agarose gel immunodiffusion. They demonstrated an immunoreactive protein that formed complete lines of identity with purified SEB. The precursor (or PSEB) was purified by immunoprecipitation and SDS-PAGE showed that it was 3,500 dalton larger than extracellular SEB. Two dimensional

peptide mapping revealed that most of the tryptic peptides were identical to that of mature SEB.

Glycerol and oleate auxotrophs of several bacterial species have proved to be useful tools in the study of the structure and function of bacterial membranes (Lillich and White, 1971; Mindich, 1970, 1971). These mutants have been particularly useful because glycerol is an essential component of both phospholipids and glycolipids and these compounds are localised in bacterial membranes (Frerman and White, 1967; White and Frerman, 1967).

V. LIPIDS OF STAPHYLOCOCCUS AUREUS

The lipid content of S. aureus strain U-71 has been characterised in detail and consisted of vitamin K_2 derivatives, glucolipids such as diglucosyldiglyceride, lysylphosphatidylglycerol (LPG), phosphatidylglycerol (PG) and cardiolipin (White and Frerman, 1967). Using gas liquid chromatography, White and Frerman (1968) separated 64 fatty acids from the complex lipids, consisting of saturated normal, iso and anteiso branched with small amounts (3 to 4%) of normal, iso and anteiso branched monoenoic acids. Further examination revealed the absence of free fatty acids, with neutral lipids accounting for 5 to 9% of the lipid fatty acids. The major portion of the fatty acids were part of the complex lipids which included mono- and diglucosyldiglyceride, phosphatidylglycerol,

lysylphosphatidylglycerol, phosphatidic acid and cardiolipin. The relative amounts of the fatty acids changed markedly between bacteria grown anaerobically and those grown aerobically (White and Frerman, 1968). Thus, the phospholipids of S. aureus (strain U-71) have been identified as phosphatidylglycerol (PG), lysylphosphatidylglycerol (LPG), phosphatidic acid (PA), cardiolipin (CL), with traces of phosphatidylethanolamine (PE) and phosphatidylglucose. The phospholipids together with carotenoids, vitamin K_2 isoprenologues and glycolipids monoglucosyldiglyceride (MG) and diglucosyldiglyceride (DG) make up the major components of the staphylococcal membrane (White and Frerman, 1967, 1968; Short and White, 1970).

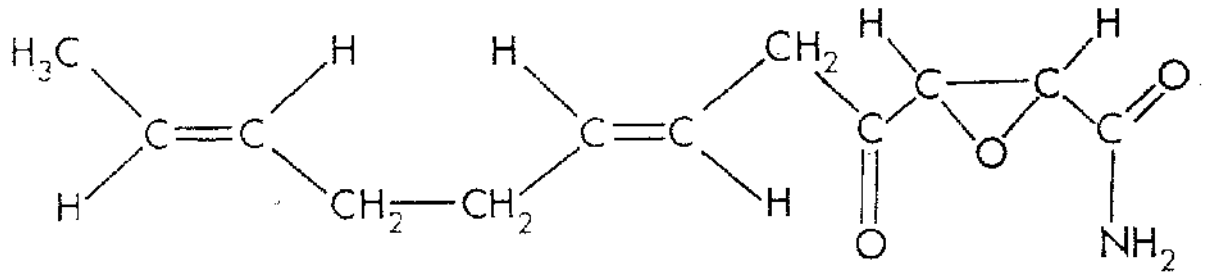
Theodore and Panos (1973) identified the fatty acid content of mesosomal vesicles and plasma membranes of S. aureus strain ATCC 6538p. They found that fatty acid content of mesosomal vesicles was 48% greater than that of plasma membrane but dominant fatty acids in both cellular components were iso and anteiso branched methyl C15, C17, and C19 and constituted at least 85% of the total fatty acids extracted. Short and White (1971) studied the metabolism of S. aureus lipids, and reported that cardiolipin (CL) accumulated while phosphatidylglycerol (PG) content decreased during stationary phase of growth. The minor lipids phosphatidylethanolamine and

phosphatidylglucose also accumulated whereas the lysylphosphatidylglycerol (LPG) content remained constant throughout stationary phase. During exponential phase the proportion and total content of phospholipids remained constant.

VI. CERULENIN

Cerulenin, an antibiotic (isolated from the culture filtrates of Cephalosporium caerulens) discovered by Hata et al. in 1960, was originally regarded as an antifungal antibiotic. It was later found that the antibiotic inhibited growth of different bacterial species and yeasts (Matsumae, Nomura and Hata, 1964). The chemical structure of the antibiotic was elucidated as (2S) (3R) 2,3-epoxy-4-oxo-6, 10-dodecadienoylamide (Ōmura et al., 1967). The structure and some properties of cerulenin are shown in Figure 7.

Cerulenin does not affect the biosynthesis of nucleic acids, proteins, cell wall or the exogenous respiration of the yeast Candida stellatoidea, whereas it significantly reduced the incorporation of [^{14}C]-acetate into fatty acids and sterols (Nomura et al., 1972). This finding provided the first indication of a natural product antibiotic that inhibited lipid synthesis. The inhibitory action of cerulenin is specifically on the condensing enzyme among the enzymes involved in the



(2S)(3R)2,3-epoxy-4-oxo-6,10-dodecadienoylamide

Molecular formula ... $C_{12}H_{17}O_3N$ (M_r 223.26)

mp ..... 93-94°C

Solubility ..... Soluble in $CHCl_3$, CCl_4, EtOAc, benzene; slightly in water

Ultraviolet ..... End absorption in H_2O

Figure 7. Structure and some properties of cerulenin.

(After Ōmura et al, 1967)

biosynthesis of fatty acids (Vance et al., 1972). D'Agnolo et al. (1973) investigated the inhibitory mechanism of cerulenin on β-ketoacyl-Acyl carrier protein synthetase isolated from E. coli. The inhibitory action of the enzyme was irreversible. One molecule of cerulenin binding to 1 molecule of the enzyme when 100% inhibitory occurs. Thus, it became clear that β-ketoacyl-ACP synthetase (ACP, acyl carrier protein), the enzyme catalysing the condensation of fatty acyl-acyl carrier protein with malonyl-acyl carrier protein was specifically inhibited by cerulenin (Fig. 8).

Both palmitate and oleate relieve the inhibition of E. coli growth caused by cerulenin (Goldberg , Walker and Bloch, 1973) also the inhibition of Saccharomyces cerevisiae sporulation, although pentadecanoate was the most effective among the saturated fatty acids (Ohno, Awaya and Ōmura, 1976). In 1976, Ōmura published a substantial review in which he described cerulenin as a novel tool for biochemistry as an inhibitor of fatty acid synthesis.

It was of interest to know whether the insertion of a newly synthesised protein into the outer membrane requires simultaneous lipid synthesis. Cerulenin was used to demonstrate that a minor protein component of the outer membrane of E. coli, which serves as a receptor for the phage lambda, can be synthesised and inserted into the

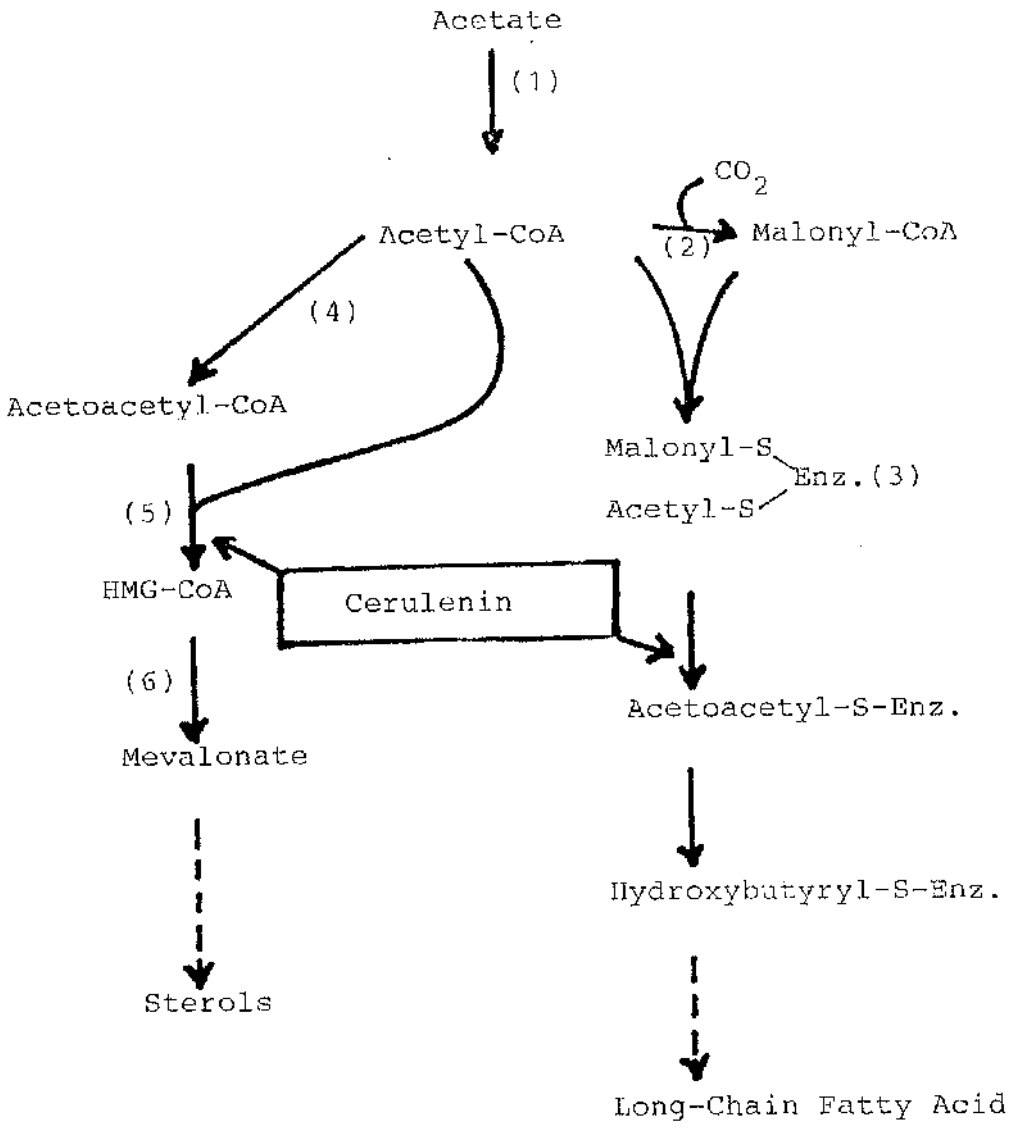


Figure 8. Targets of cerulenin in biosynthesis of sterol and fatty acid. (1) Acetyl-CoA synthase; (2) Acetyl-CoA carboxylase; (3) Fatty acid synthetase; (4) Acetoacetyl-CoA thiolase; (5) HMG-CoA synthase; (6) HMG-CoA reductase.

(After Ōmura, 1976)

outer membrane during inhibition of lipid synthesis (Randall, 1975). It was also found that cerulenin did not affect the induction of tetracycline resistance in S. aureus (Chopra, 1975). Such results indicate that at least in these cases, proteins can be incorporated into the membrane in the absence of lipids.

OBJECT OF RESEARCH

Although the diversity of the extracellular enzymes, lysins and toxins of Staphylococcus aureus is well established, the actual mechanism of secretion of extracellular products from the bacterial cell is still quite obscure. In particular, it is not clear whether the signal hypothesis of Blobel and Dobberstein (1975a,b) which was originally developed with eukaryotic cells might also apply to the staphylococcal lysins.

The study described in this thesis relates to the α-, β-, and δ-lsyins and the protease(s) of S. aureus Wood 46 with particular emphasis on α-lysin.

The specific questions to which answers were sought were:-

1. Is α-lysin secretion influenced by protease inhibitors, as might be expected if release of the lysin requires a membrane-bound protease to split off the signal sequence ?

2. Is the secretion of the lysins and protease(s) affected by the antibiotic cerulenin whose primary target is membrane lipid biosynthesis?

3. Does α-lysin exist within the staphylococcal cell as a higher molecular weight form with signal peptide still attached?

MATERIALS AND METHODS

I. ORGANISM AND MEDIA

Strain Wood 46 (NCTC 7121) of Staphylococcus aureus was used throughout for α-lysin production. The organism was maintained at 4°C (after overnight incubation at 37°C) on 10% (V/V) horse blood agar by subculture at monthly intervals (Oxoid Blood Agar Base No. 2, Oxoid Ltd., London, England).

For α-lysin production, the organism was grown in a liquid yeast extract enriched medium modified from that of Bernheimer and Schwartz (1963) and known as B S medium (see Appendix I).

II. PRODUCTION OF MEMBRANE-DAMAGING LYSINS AND PROTEASE(S) DURING GROWTH OF STAPHYLOCOCCUS AUREUS WOOD 46

Production of extracellular cytolytic toxins (lysins) and protease(s) were followed for 14h during growth of Wood 46. Sixty ml of B S medium in a 250ml Erlenmeyer flask with baffles, was inoculated with a haemolytic colony from a blood agar plate , and incubated at 37°C on an orbital shaker operating at 150 cycles per min (Gallenkamp, East Kilbride, Scotland). One ml samples were withdrawn at intervals, the cells sedimented at 10,000 'g' in a Beckman microfuge (RIIC Ltd., Glenrothes, Scotland) and the clear supernate assayed for α- β- and δ-lysins and protease(s).

III. PRODUCTION AND PURIFICATION OF ALPHA-LYSIN

1. Production of Crude Alpha-Lysin

Sixty ml of B S medium in a 250ml Erlenmeyer flask with baffles was inoculated with a haemolytic colony from a blood agar plate and incubated at 37°C for 16-18h on an orbital shaker operating at 150 cycles per min. Five ml of the overnight culture was inoculated into each of 3 x 2L Erlenmeyer flasks with baffles, containing 500ml B S medium. One ml of a 1:20 dilution of sterile Silicolapse 5000 antifoam (ICI Stevenston, Ayrshire, Scotland) was added to each flask to reduce foaming.

After 12-16h growth, the bacteria were sedimented by centrifugation at 20,000 'g' for 20 min at 4°C (Sorval RC5., Du Pont (UK) Ltd., Herts, England). Supernate was removed and protein precipitated by the addition of solid ammonium sulphate to 70% saturation, followed by stirring at 4°C overnight. A fraction of the culture supernate was removed before precipitation with ammonium sulphate and stored at -20°C for subsequent haemolytic, protein assays and peptide analysis by SDS-PAGE.

2. Preparation and Cleaning of Controlled-Pore Glass (CPG)

Cassidy and Harshman (1976a), used a combination of adsorption chromatography on controlled-pore glass and ion-exchange chromatography to yield a product estimated by electrophoresis to be 98% pure.

Sixty ml of controlled-pore glass CPG-10 (BDH Chemicals

Ltd., Poole, Dorset, England) was washed in 200ml of deaerated (by steaming) distilled water to release trapped air from the glass. After the glass had settled, the water was replaced by 0.05M potassium phosphate buffer pH 6.8 (see Appendix II). After gentle mixing the glass was allowed to settle and this washing process repeated five times. The washed glass slurry was poured into a GA 16mm x 600mm column (Wright Scientific, Kenley, England), the column being agitated during pouring as advised by the manufacturer (CPG controlled pore glass operating instructions. Electronucleonics Inc., Fairfield, New Jersey, USA). The column contents were allowed to settle overnight before application of sample. After use, the glass was removed and cleaned by heating in 15N nitric acid at 100°C in a steam bath for 2h followed by extensive washing in distilled water until the washings were close to neutrality. The glass was then washed in several changes of 0.05M potassium phosphate,pH 6.8 and stored in this buffer.

3. Chromatography of Crude Staphylococcal Alpha-Lysin on Controlled-Pore Glass

This was based on the method of Cassidy and Harshman (1976a). The ammonium sulphate precipitated protein was collected from staphylococcal culture supernate fluid by centrifugation at 55,000 'g' for 30 min at 4°C. The supernate fluid was discarded, and the precipitate re-

dissolved in a minimum volume of 0.05M potassium phosphate, pH 6.8 followed by dialysis against 4L of 0.05M potassium phosphate, pH 6.8 for 3h at 4°C. The buffer was then changed for fresh buffer, and dialysis continued for a further 3h at 4°C. After removal of insoluble material by centrifugation (60,000 'g' 20 min, 4°C) the supernate fluid was loaded onto the CPG-10 column (bed volume approximately 100ml) using a Bromma peristaltic pump (LKB 2120, LKB Insruments, Sweden) at a flow rate of 120ml h^{-1}.

After loading the sample at a rate of 120ml h^{-1}, the column was eluted by upward flow, firstly with 0.05M potassium phosphate buffer, pH 6.8 at a rate of 55ml h^{-1}. When 8 bed volumes has been pumped through the column, the buffer was changed to 1.0M potassium phosphate buffer, pH 7.5 and 5 bed volumes of this buffer pumped through the column. Fractions of 10ml and 5ml respectively were collected from the two elution buffers. The eluant fractions monitored by an LKB Uvicord II (LKB 8303) were also measured for $E_{280\ nm}$. A single peak (peak 1) was eluted from the column by 0.05M potassium phosphate pH 6.8, and fractions in this peak with an E_{280nm} of > 1.0 were pooled, and stored at -20°C. A single peak (peak 2) was eluted by 1.0M potassium phosphate, pH7.5 and fractions from this peak with E_{280nm} > 0.5 were pooled and stored at -20°C. The protein from peak II was precipitated by the addition of solid ammonium sulphate to 70% saturation during overnight stirring.

4. Preparative Electrofocusing

The technique of protein separation by their iso-electric points (pI) in a pH gradient is termed isoelectric focusing or electrofocusing. Both the theory and technique of electrofocusing have been extensively reviewed (Haglund, 1970; Peeters, 1970; Vesterberg, 1971; Arbuthnott and Beeley, 1975; Righetti, 1975; Catsimpoolas, 1976; Righetti and Drysdale, 1976; Catsimpoolas and Drysdale, 1977; Radola and Graesslin, 1977). The principle, reagents and method are outlined in Appendix III.

a. Procedure

The peak 2 material from CPG, containing α-lysin was precipitated by ammonium sulphate, and the precipitated protein collected by centrifugation (55,000 'g', 30 min, 4°C). The precipitate was redissolved in a minimum volume of 1% (W/V) glycine and dialysed against 4L of 1% (W/V) glycine for 20h at 4°C to remove inorganic salts. Even at low concentrations, salts can disturb the pH gradient in electrofocusing. After dialysis against glycine a small amount of insoluble material was removed by centrifugation (55,000 'g', 30 min, 4°C) and the supernate fluid electrofocused by the method of Winter and Karlsson (1976).

After electrofocusing, the column was emptied by gravity and fractions of approximately 3ml were collected at 4°C and the containers sealed to avoid pH changes due to CO_2 absorption.

b. Analysis of fractions from electrofocusing

The pH of each of the fractions was measured at 4°C against a standard buffer of pH 7.0 using an Intek GC51902 electrode (Graphic Controls, London, England) and pH meter (Kent, Eil 7035, Electronics Instruments Ltd., Chertsey, Surrey, England) with expanded scale and a temperature compensator immersed in a water bath at 4°C. The E_{280}^{nm} of each fraction was measured against distilled water. Each fraction was assayed for haemolytic activity against rabbit, sheep and cod erythrocytes using the rapid haemolysis test.

Fractions which constituted the protein peak and showed α-lysin activity (haemolytic for rabbit erythrocytes) were pooled and dialysed against 4L of 70% saturated ammonium sulphate at 4°C for 96h. This served the dual function of concentrating the protein and removing the ampholines. The precipitated lysin was collected by centrifugation (55,000 'g', 30 min, 4°C), resuspended in a minimum volume of 70% saturated ammonium sulphate and dialysed against 4L of 0.15M NaCl, 0.05M potassium phosphate, pH 7.4 (saline-phosphate buffer SPB. see Appendix II) at 4°C for 18h. The resulting lysin was divided into 1-2ml aliquots in dialysis tubes and stored in 70% saturated ammonium sulphate at 4°C. The lysin was stable under these conditions for more than a year. Aliquots were dialysed against SPB buffer prior to use for at least 24h.

An outline of the purification procedure is given in Fig. 9.

Figure 9. Flow diagram of the purification procedure for α-lysin

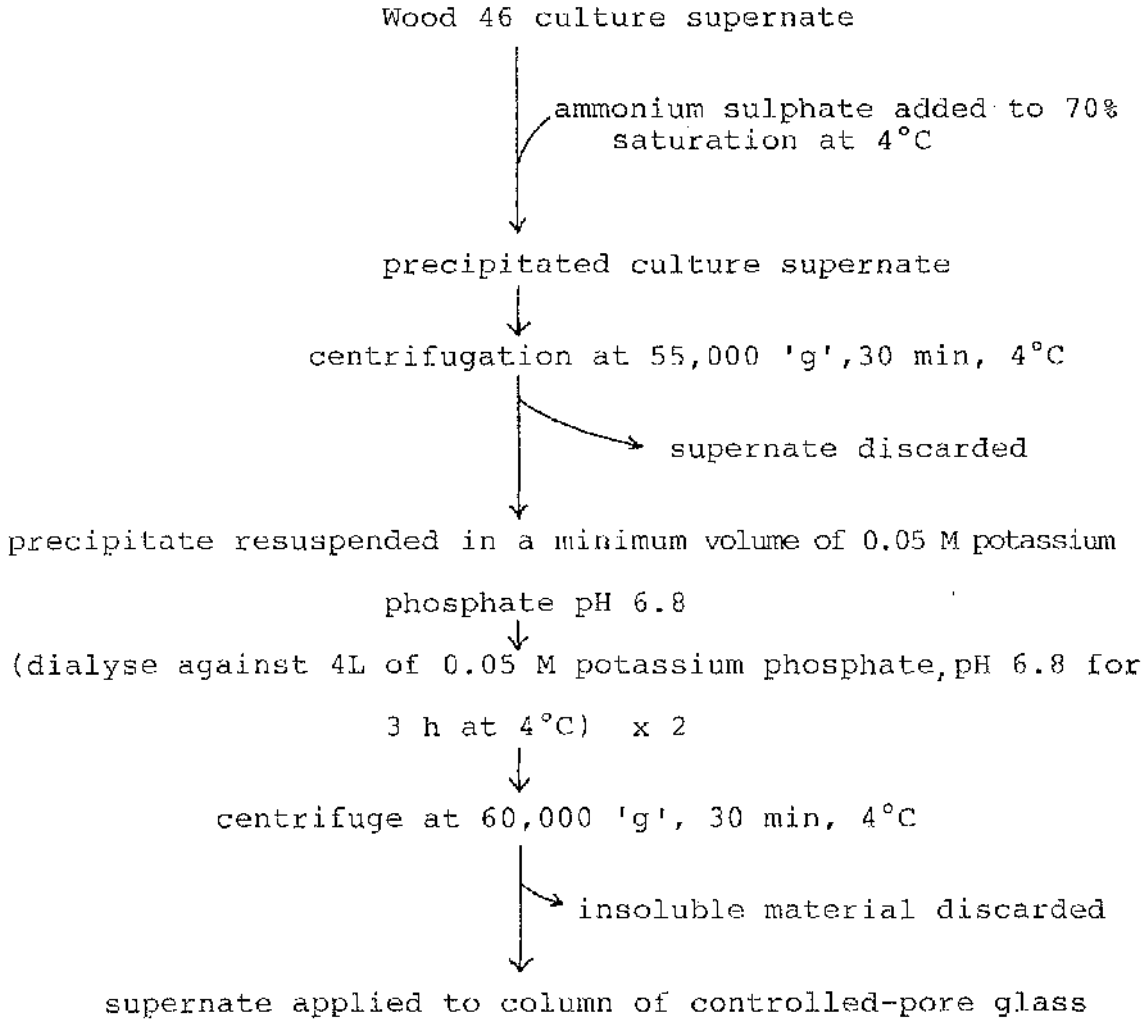


Figure 9. Flow diagram of the purification procedure for α-lysin

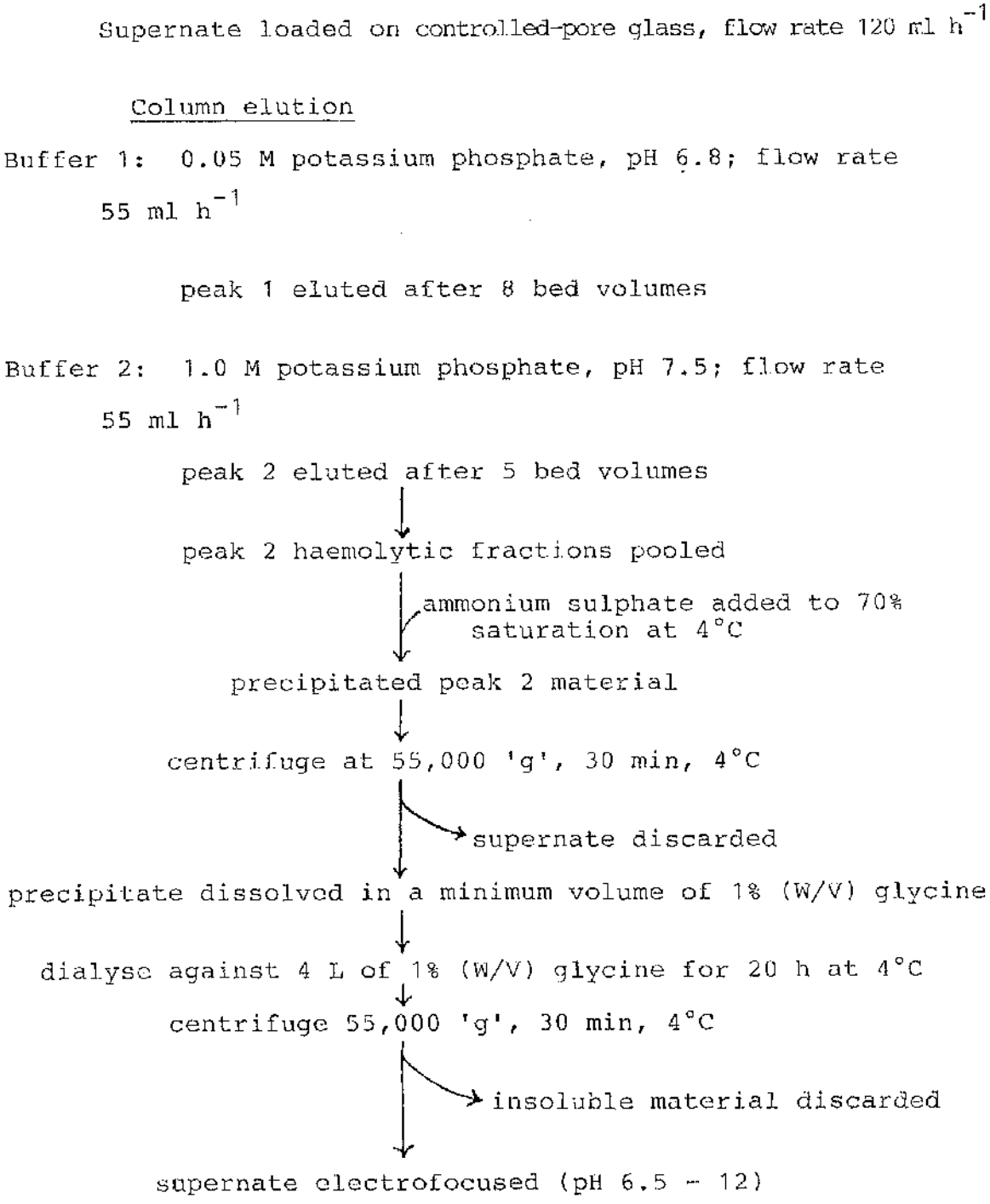
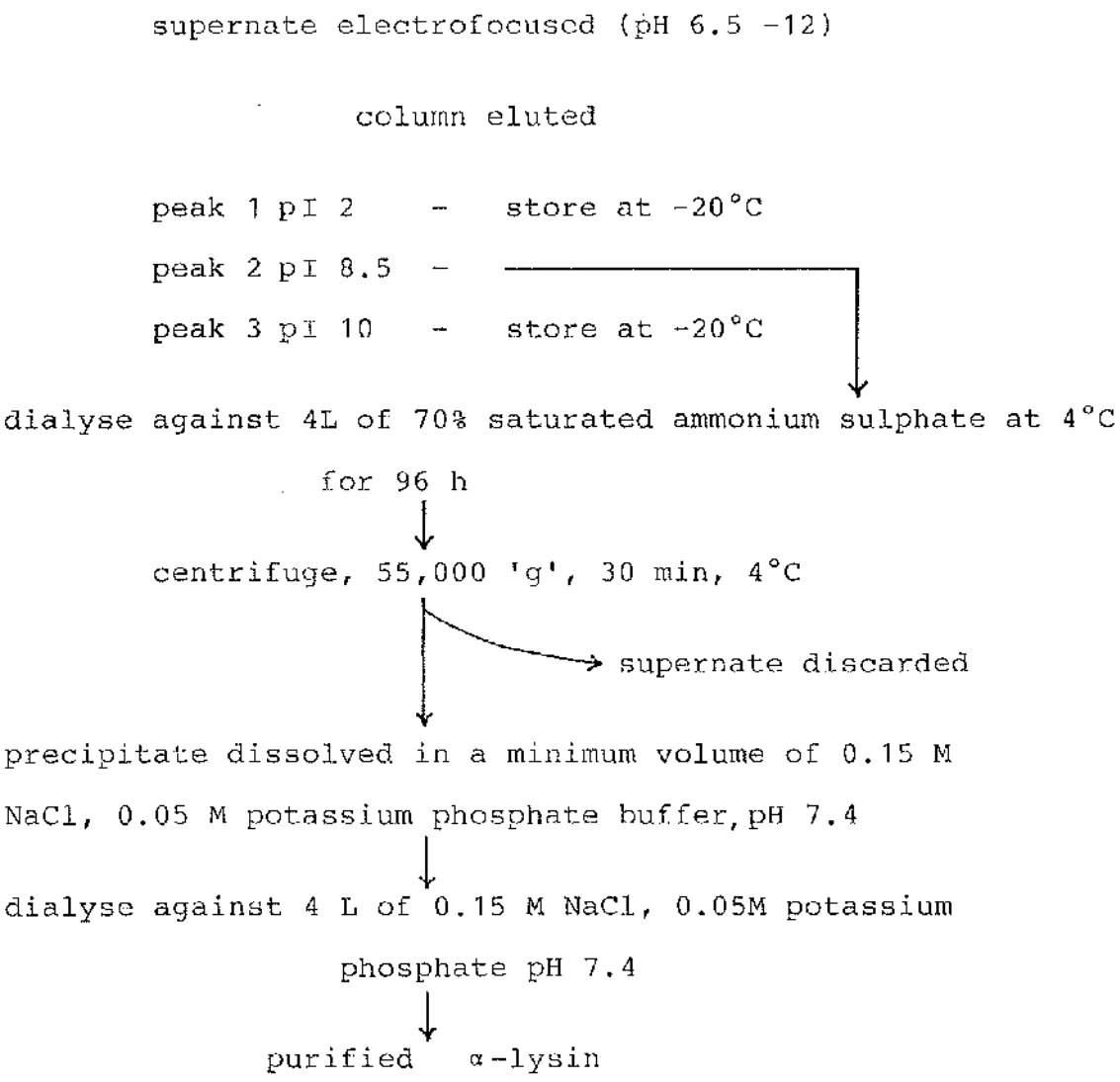


Figure 9. Flow diagram of the purification procedure for α-lysin



IV. ASSAY FOR HAEMOLYTIC ACTIVITY

1. Assay Buffers

Alpha-lysin: Phosphate Buffer Saline (Dulbecco A) pH 7.3 (PBS) see Appendix II.

Beta-lysin: Tris-Buffered Saline made 1mM with respect to Mg^{2+} (TBS + Mg^{2+}) see Appendix II

Delta-lysin: Citrate Dextrose Saline (CDS) containing 12.5% (V/V) dimethyl sulphoxide (DMSO) see Appendix II

2. Standard Erythrocyte Suspensions

Rabbit, sheep or cod blood was washed in the appropriate buffer 4 times (550 'g' for 10 min at room temp. MSE Super Minor) and the packed cell volume determined by centrifugation in graduated conical centrifuge tubes. Erythrocytes were resuspended to a final concentration of 1% (V/V) in the appropriate buffer.

3. Rapid Haemolysis Test

Fractions eluted from the electrofocusing column were assayed for haemolytic activity by adding 25 µl of each fraction to 0.5 ml of rabbit, sheep or cod standard erythrocyte haemolysis suspensions in WHO haemagglutination trays. The haemolytic assays against rabbit and sheep erythrocytes were incubated at 37°C and that against cod

at room temperature for 30 min. Haemolysis was noted as +,+/- (slight) or -. The trays were then incubated at 4°C for 15 min and read again. The assays were done in duplicate.

4. Double Dilution Titration

The haemolytic titre of lysin samples was determined by making serial doubling dilutions of lysin in 0.5ml amounts of buffer in wells in WHO agglutination trays, and adding 0.5ml standard erythrocyte suspensions to each well. Wells with buffer alone served as controls. Titrations were incubated at 37°C for rabbit and sheep, and room temperature for cod, and the highest dilution showing 50% haemolysis determined visually. The titrations were then incubated at 4°C for a further 30 min and the last well showing 50% haemolysis again noted. The dilution which released 50% haemoglobin was accepted as 1 haemolytic unit (HU_{50}) ml^{-1}. With sheep erythrocytes the difference between the titre at 37°C and that at 4°C was taken as being the titre for β-lysin.

V. PROTEIN ESTIMATION

Protein was estimated by either the method of Lowry et al. (1951) or Bradford(1976) with bovine serum albumin (Sigma London Chemical Company Ltd., Poole, Dorset, England) as standard.

VI. ASSAY FOR EXTRACELLULAR PROTEASE(S)

The method was modified from that of Charney and Tomarelli (1947) and Tomarelli, Charney and Harding (1949). Azocasein (Sigma) was dissolved in PBS at 25 mg ml^{-1}. The standard protease used for reference was subtilisin (E.C.3.2.21.14. Type VII from Bacillus amyloliquifaciens, Sigma) which was diluted in PBS over the range 2 - 25 $\mu g\ ml^{-1}$. The assay was carried out as follows:

To 0.3 ml of substrate (Azocasein) was added 0.3 ml standard protease, α-lysin preparations, or culture supernate. The assay tubes were incubated at 37°C for 30 min and the reaction was then stopped by the addition of 2.4 ml of chilled 5% (W/V) trichloroacetic acid. The precipitated unhydrolysed substrate was removed by centrifugation (850 'g', 10 min at room temperature, MSE Super Minor) and 2 ml of the clear supernate was added to 2 ml of 0.5 N sodium hydroxide (to intensify colour). The E_{440}nm was read against an appropriate blank and standard curve of E_{440}nm against protease concentration was plotted. This was used to estimate the protease in the α-lysin preparation or culture supernate as equivalent of subtilisin.

VII. IMMUNOLOGICAL ANALYSIS

Lysin preparation, supernates and membrane fractions were analysed by the double diffusion in gel method, first described by Ouchterlony (1948). Wells 3 mm in diameter

were cut out at 3 mm distances in a 5 mm thick layer of 1% (W/V) agarose (Miles Laboratories Ltd., Stoke Poges, England) in PBS, pH 7.3. See Appendix IV.

Purified α-lysin, culture supernate and purified δ-lysin (a gift from Dr. T.H. Birkbeck, Glasgow University) were tested against commercial antiserum prepared against crude α-lysin (from Wellcome Laboratories, Beckenham, England) and against antiserum prepared against purified δ-lysin (a gift from Dr. T.H. Birkbeck; Birkbeck and Whitelaw, 1980), while control and cerulenin-inhibited cell membranes and supernates were tested against antiserum prepared against purified α-lysin (Batch No. 567A, a gift from Dr. C. Adlam, Wellcome Labs., Beckenham, Kent, England) and against antiserum prepared against purified δ-lysin.

For membrane analyses, 0.01% Triton X100 was included in the gel buffers and membrane fractions. Before use, membranes were sonicated in a Soniprep 100 (MSE) for 5 min at 21°C,centrifuged in a Beckman microfuge and the supernates analysed by Ouchterlony double diffusion analyses.

VIII. POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS IN THE PRESENCE OF SODIUM DODECYL SULPHATE (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used throughout this project to determine molecular weight and the presence of other contaminating proteins in the course of α-lysin purification and also to

determine the molecular weight of the lysin and other proteins in culture supernate, cytoplasmic and membrane fractions of control and cerulenin-inhibited cells. The method used was modified from that of Laemmli (1970) and Ames (1974). The details of reagents used and the method of gel preparation are given in Appendix V. Protein samples were adjusted to 1-2 mg ml^{-1} before loading onto the gel. A volume of sample was added to an equal volume of solubilising buffer (Appendix V). Mixtures of sample and solubilising buffer (1:1) were heated at 100°C for 5 min and cooled prior to application of 20-30 µl to each track in the gel.

Proteins bind SDS in proportion to their molecular weight, this binding resulting in the loss of charge specificity of the complex, and thus migration in the electric field is based only on molecular size (Reynolds and Tanford, 1970). The ratio of the distance moved by the marker proteins to that moved by the tracking dye, when plotted against the logarithm of molecular weight of the proteins, gives a standard curve, (see Results, Fig.13) which can be used to estimate the molecular weight of α-lysin or any protein in the gel from its relative migration.

The apparatus used was based on the design of Studier (1973). The dimension of slabs were 4 x 13.8 x 0.18 cm for the stacking gel and 13 x 13.8 x 0.18 cm for the separating gel. Samples were electrophoresed at 4°C

at a constant current of 15 mA into the stacking gel, and 35 mA in the separating gel. Each run took approximately 3-4 h. Staining and destaining of the gel was done by a method based on that of Weber and Osborn (1969). Destaining involved several changes of the destaining solution (Appendix V).

For molecular weight estimations, a mixture of standard proteins was used (Sigma SDS - 6, Dalton Mark VI). This contained a mixture of Albumin, (M_r = 66,000); Ovalbumin (M_r = 45,000); Pepsin (M_r = 34,700); Trypsinogen (M_r = 24,000); β-Lactoglobulin (M_r = 18,400); Lysozyme (M_r = 14,300) and Bromophenol blue.

IX. EFFECT OF PHENYLMETHYLSULPHONYL FLUORIDE (PMSF), O-PHENANTHROLINE, QUINACRINE, PROCAINE, AND CERULENIN ON GROWTH AND HAEMOLYSIN PRODUCTION BY S.AUREUS WOOD 46

Sixty ml of prewarmed BS media in 250 ml Erlenmeyer flasks were inoculated with 0.1 ml of an overnight broth culture and incubated for 10 min at 37°C on an orbital shaker operating at 150 cycles per min prior to addition of reagents.

Phenylmethylsulphonyl fluoride (PMSF) in absolute ethanol was added to final concentrations of 50, 100, 200, 300 and 500 μg ml^{-1}. O-Phenanthroline in absolute ethanol was added to give final concentrations of 0.1, 0.2, 0.5, 0.8, 1 or 3 mM. Quinacrine (in distilled water) was added to give final concentrations of 0.1, 0.2, 0.5, 0.8 and 1 mM.

These reagents are all inhibitors of proteolytic enzymes.

Procaine (a local anaesthetic) interferes with cellular membrane fluidity. Procaine (in distilled water) was used at a final concentrations of 0.5, 2, 4, 8, 12, 16, 20 and 25 mg ml^{-1}. Cerulenin an inhibitor of lipid synthesis was dissolved in either absolute ethanol or distilled water and added to the cultures to give a final concentration of 0.5, 1.0, 1.5, 2.0 and 2.5 µg per ml in preliminary experiments.

All the above mentioned inhibitors were obtained from Sigma and were filter sterilised before injection into the cultures. For each inhibitor, cultures were grown for 15 h and cell density was followed at 600 nm. Supernates from 5 ml samples of culture were assayed for α, β- and δ-lysins by titration against 1% rabbit, cod and sheep red blood cell suspensions respectively. Proteolytic activity was determined as described previously (Section VI).

X. RELATIONSHIP BETWEEN OPTICAL DENSITY AND CELL DRY WEIGHT DURING INHIBITION OF ALPHA-LYSIN PRODUCTION BY CERULENIN

Three 250 ml Erlenmeyer flasks containing 60 ml BS medium were inoculated (0.1 ml) from an overnight broth culture and incubated at 37°C with shaking (150 cycles per min). Immediately after inoculation, cerulenin was added to two of the three flasks to give a final concentration of 8 and 20 µg ml^{-1}, the third flask was left as control.

Samples (3ml) were withdrawn at 3h intervals and optical density measured at 600 nm. At each sampling time 2 x 1 ml aliquots were spun in a Beckman microfuge for three min at 10,000 'g' and the supernates assayed for α-lysin. The sedimented cells were washed twice with distilled water and then transferred to preweighed plastic dishes before drying to constant weight at 37°C. Optical density, cell dry weight and α-lysin titres were plotted against time. The relationship between cell density and cell dry weight was found by plotting one against the other.

XI. INCORPORATION OF [^{3}H]-PHENYLALANINE DURING GROWTH OF S. AUREUS (WOOD 46) IN THE PRESENCE AND ABSENCE OF CERULENIN

L - phenyl [2,3-^{3}H] alanine (444 KBq, specific activity 1.05 TBq mmol^{-1}; Radiochemical Centre Ltd., Amersham, Bucks, England) and 0.1 ml of an overnight broth culture were inoculated into each of two 250 ml Erlenmeyer flasks, containing 60 ml BS media. Flasks were shaken at 37°C (150 cycles per min), and cerulenin (10 μg ml^{-1}, final concentration) was added at 10 min post-incubation. Samples (3 ml) were withdrawn every hour, optical density (E_{600}) recorded and 0.8 ml centrifuged at 10,000 'g' for 3 min in a Beckman microfuge. Supernates were titrated against 1% rabbit red blood cell suspension for α-lysin.

Total protein was precipitated with 10% (W/V) ice cold trichloroacetic acid, (TCA 5 ml) from duplicate 1 ml samples of cell suspensions. The precipitated protein from each sample was collected on millipore filters (0.45μm pore size, Oxoid) and each filter washed twice with 5 ml of ice cold 10% TCA, before transfer to a scintillation vial and drying under infrared light. Five ml of scintillant was added to each vial. The scintillation fluid contained: PPO 4g, POPOP 0.1g, Triton-X/100, 500 ml and sulphur free toluene, 1000 ml. Vials were counted on a Packard Tri-carb, 3255, liquid scintillation counter (Packard Instruments Ltd., Berkshire, England) over a period of 10 min. The counts per min (cpm) were corrected to disintegrations per min (dpm) by the channels ratio method, using a quench correction curve. Incorporation of [^{3}H]-phenylalanine, growth and haemolytic activities of α-lysin were plotted against incubation time for control and cerulenin-inhibited cells.

XII. INCORPORATION OF [^{14}C]-SODIUM ACETATE DURING GROWTH OF S. AUREUS (WOOD 46) IN THE PRESENCE AND ABSENCE OF CERULENIN

Experimental procedure followed was similar to that for [^{3}H]-phenylalanine incorporation except that 4440 KBq of [U - ^{14}C] sodium acetate (specific activity 2.19 GBq mmol^{-1}) was used instead of the amino acid.

Lipids were extracted from duplicate 1 ml samples

of culture by a modification of the technique described by Bligh and Dyer (1959). Samples (2 x 1 ml) of cell suspension were each added to 3.75 ml of chloroform: methanol (2:1 v/v), shaken and left for 1 h at room temperature. The lower chloroform phase (0.8 ml) was removed from each sample, transferred to a scintillation vial and dried under a stream of nitrogen. Five ml of scintillation fluid was added to each vial and the radioactivity counted on a Packard Tri-carb 3255, liquid scintillation counter over a period of 10 min. Counts per min (cpm) were corrected to dis-integration per min (dpm) as mentioned in the preceding section. Incorporation of [^{14}C] acetate, α-lysin production and growth as optical density were plotted against time of incubation for control and cerulenin-inhibited cells.

XIII. GRAVIMETRICAL ESTIMATION OF TOTAL EXTRACTABLE CELL LIPIDS

Sixty ml cultures of control and cerulenin-inhibited cells were grown for 15 h at 37°C, then optical density measured at 600 nm. Aliquots of cell suspensions (2 x 1 ml) were taken, washed twice with distilled water in a micro-fuge, and dried to constant weight. Cells were harvested from the cultures, washed 3 times with 0.05 M Tris/HCl buffer, pH 7.5, in a Sorval RC5 Centrifuge at 20,000 'g' for 15 min and freeze-dried. Supernates were assayed for α-lysin activity. Total cellular lipids were extracted from the freeze-dried cells by modification of the Bligh and Dyer method (1959) as stated by Kates (1972). To

approximately 20 mg of freeze-dried cells and 10 g of glass beads (BDH Chemical Company Ltd., Poole, England) in a 50 ml pear-shaped flask (Quickfit), 3.75 ml of Chloroform: methanol (1:2 v/v) was added and then flushed with N_2, stoppered and sealed with parafilm. After shaking for 1 h at 4°C, the flasks were allowed to stand overnight before the extracts were decanted into universal bottles. The glass beads were rinsed with 1 ml of chloroform: methanol (1:2 v/v), and the washings combined with lipid extract in the universal bottles. Pooled extracts and washings were then centrifuged (1000 'g', 5 min) and the clear supernates decanted into clean bottles. The residues were resuspended in 4.75 ml Chloroform: methanol: water (1:2:0.8 v/v) and mixtures shaken and then centrifuged. The supernates were combined with the lipid extracts and 2.5 ml of Chloroform and 2.5 ml of water were added before the mixtures were centrifuged. The lower chloroform layer was withdrawn, transferred to a quickfit flask , then brought to dryness in a rotary evaporator (Buchi Rotovapor - R, Flavi, Switzerland) at 35°C. The lipid residues were immediately redissolved in 4 ml Chloroform: methanol (1:1), transferred to preweighed test tubes and brought to dryness under a stream of oxygen-free nitrogen (BOC Limited). The tubes were dried overnight in a desiccator at 30°C and reweighed to give the weight of lipid extracted which was expressed as % lipid per mg dry weight cells.

The extracted lipids were also used for gas liquid chromatographic (GLC) and thin layer chromatographic (TLC) analyses. After the lipid residues were brought to dryness under a stream of N_2, they were stored under N_2 at -20°C until required (for GLC see following Section). For TLC, total extracted lipids from control and cerulenin-inhibited cells were dissolved in a minimum volume of chloroform: methanol (1:1 v/v) to a concentration of 1 mg ml^{-1}, and equal volumes of lipid samples (10 µl) or standard phospholipids (1 mg ml^{-1}) were applied to commercially prepared TLC plates (20 x 20 cm polygram SILG, Macherey-Nagel and Co. W. Germany), using micro-capillary tubes (Drummond Scientific Company, USA). Plates were run in an ascending solvent system consisting of chloroform: methanol: acetic acid: water (80:18:12:5 v/v/v/v) in glass tanks equilibrated before use. The plates were run for 1.5h, and individual phospholipids visualised by reaction with specific sprays (see Appendix VI), and idnetified by comparison with the mobility of standard phospholipids run on the same plate.

XIV. IDENTIFICATION OF FATTY ACID COMPONENTS OF LIPIDS BY GAS LIQUID CHROMATOGRAPHY (GLC)

Lipids were extracted from 20 mg freeze-dried cells from control and cerulenin-inhibited cultures (see Section XIII). The dried lipid residues were extracted in 5 ml petroleum ether (BP 40° - 60°C) 3 times and fatty acids

extracted from the isolated lipids were converted to methyl esters by direct methanolysis using the method of Stoffel, Chu and Ahrenus (1939) as modified by Kates and Hagen (1964). Four ml of a mixture of methanol/concentrated HCl, (14:1 v/v), were added to the flasks followed by 0.5 ml benzene. The contents of the flasks were then refluxed for 2h at 90°C,cooled and 0.5 ml water added followed by 10 ml of petroleum ether. After vigorous shaking the 2 phases were allowed to separate and the ether phase (containing methyl esters of fatty acids) separated from the lower water layer. Ether was removed and the procedure repeated with a further 5 ml of petroleum ether, which were added to the 10 ml previously separated. The solvent was then removed from the combined extract in a stream of nitrogen at 40°C and esters were stored in stoppered tubes under nitrogen at -20°C until required.

The fatty acids were separated by gas-liquid chromatography using a Packard model 428 gas liquid chromatograph (Packard Instruments Ltd., Berkshire, England) equipped with dual flame ionisation detectors and stainless steel columns (182 x 0.3 cm) of diethyl glycol succinate (DEGS 10% w/w) on chromosorb W-AW (Alltech, Carnforth, England) using nitrogen as carrier gas and a column temperature of 185°C. The methyl esters emerging from the column were detected using a flame ionisation detector. The signals from the detector were amplified and recorded by a chromatograph integrator model 308/9

(Laboratory Data Control, Shannon, Ireland) which recorded relative retention time and the peak area for each fatty acid methyl ester. The fatty acid peaks were also recorded on a Linseis type SI4 flat bed potentiometric recorder (Belmont Instruments, Glasgow, Scotland).

Fatty acid methyl esters were identified by comparing their relative retention time (R_t) with those obtained for standards and published values (Burchfield and Storrs, 1962). By plotting Log_{10} retention time against carbon numbers, separate straight lines were obtained for saturated acids and mono-enoic acids (James and Martin, 1956; Ackman, Burgher and Jangaard, 1963; Ackman, 1963). Hence, from these plots, any component separated on the column under identical operating conditions may be identified from the curve on which its Log_{10} R_t and whole carbon number intersect. Possible identities of branched chain fatty acids were derived from data reported by White and Frerman (1968).

Percentage of individual fatty acids in the samples were obtained by applying the following formula:

$$\% \text{ of fatty acid} = \frac{\text{area of peak}}{\text{effective area}} \times 100$$

$$= \frac{\text{area of peak}}{\text{total area - solvent peak area}} \times 100$$

Three sets of readings were taken and an average obtained for each sample. For every run on GLC, a separate series

of standard of unsaturated and saturated fatty acids were used.

XV. EFFECT OF EXOGENOUS FATTY ACIDS ON THE PRODUCTION OF ALPHA AND DELTA LYSINS DURING GROWTH OF S.AUREUS (WOOD 46) IN THE PRESENCE AND ABSENCE OF CERULENIN

An overnight culture (0.1 ml) was inoculated into 6 x 250 ml Erlenmeyer flasks, each containing 60 ml BS medium. Cultures were incubated at 37°C on an orbital shaker operating at 150 cycles per min for 10 min, then cerulenin was added to 3 flasks at a concentration of 8 $\mu g ml^{-1}$, and incubation continued. Samples (3 ml) were withdrawn from each culture at 3h intervals, and absorbance at 600 nm determined. After removal of the bacteria by centrifugation, the supernates were assayed for α- and δ-lysins.

After 6 h of growth, sodium oleate or sodium stearate, dissolved in distilled water and sterilised by filtration, were added at a concentration of 8.3 $\mu g ml^{-1}$ to four of the cultures, two of which had already been dosed with cerulenin. Incubation was continued with sampling and assays as described previously. Growth and α- and δ-lysin titres were plotted against time of incubation for each experiment.

XVI. ISOLATION OF TOTAL MEMBRANE FRACTIONS FROM CONTROL AND CERULENIN-INHIBITED CELLS

Membranes were isolated from control and cerulenin-inhibited cells and analysed for peptides by SDS-PAGE, dry weight and total protein determined. The method of membrane isolation was based on that of Salton and Freer (1965), but lysostaphin used instead of lysozyme.

An overnight culture of Wood 46 was inoculated (0.1 ml) into each of 3 x 250 ml Erlenmeyer flasks, containing 60 ml BS medium. Cerulenin was added to two flasks at concentrations of 8 and 15 $\mu g\ ml^{-1}$, the third flask was left as control. Cultures were shaken (150 cycles per min) at 37°C for 12 - 15 h, the optical density at 600 nm recorded. Total cell dry weight for each culture was calculated from the weights of cells (3 x washed) from two 1 ml aliquots of culture.

Cells were harvested from cultures by centrifugation at 20,000 'g' for 20 min at 4°C, washed three times with 0.05 M Tris/HCl buffer, pH 7.5, and supernates were assyed for α-lysin. After resuspension of the cells in distilled water, the optical density of each suspension was adjusted to approximately 8. Fifty ml of each cell suspension from control and cerulenin-inhibited cultures were treated with 100 $\mu g\ ml^{-1}$ lysostaphin (from Staphylococcus staphylolyticus, Sigma) and 8 $\mu g\ ml^{-1}$ DNAase (Miles Labs. Ltd., Slough, UK). During incubation of the suspensions at 37°C in a shaking water bath (Grant

Instruments Ltd., Cambridge, England) for 3-4h, samples were examined for lysis at 15 min, intervals, using phase contrast microscopy. Once the protoplasts formed quantitatively, the suspensions were centrifuged at 35,000 'g' for 45 min, the supernates removed and kept as cytoplasmic fractions.

Sedimented membranes were washed six times with distilled water at 35,000 'g' for 45 min, to remove cytoplasmic contamination. The washed membranes were resuspended in a minimum volume of distilled water and stored at -20°C. For dry weight measurement, triplicate samples of washed membranes (3 x 5ml) were transferred to preweighted plastic dishes and dried to constant weight at 45°C. The percentage of membrane in control and cerulenin-inhibited cells was determined.

XVII. PEPTIDE MAPPING BY LIMITED PROTEOLYSIS IN SODIUM DODECYL SULPHATE AND ANALYSIS BY GEL ELECTROPHORESIS

Peptide mapping was done by a modification of the method of Cleveland et al. (1977). Gels prepared as in Section VIII were first fixed, stained and destained briefly (see Appendix V), five min staining with concentrated (stock) coomassie blue R250 (Sigma) and destained by agitation for 30-40 min at 45°C. Gels were rinsed with cold water, placed on a Mylar sheet over a light box and individual bands of interest were excised with a razor blade, trimmed to a 5mm wide and then soaked for 30 min with occasional swirling in 10ml of 0.125M Tris/HCl pH 6.8, containing 0.1% SDS

and 1 mM EDTA (at this point slices were, when necessary stored at -20). Each(gel)slice was pushed(until it laid) on top of the stacking gel of the second gel.Spaces,around the slices were filled by overlaying each slice with 10-20 µl of the above buffer containing 20% glycerol. Finally, 10 µl of the same buffer containing 10% glycerol and 0.1 $mgml^{-1}$ of either trypsin, α-chymotrypsin (Sigma) or V8 protease (Miles Labs.) was overlayed on the slices.

Electrophoresis was performed as described for the first gel except the current was turned off for 30 min when the tracking dye neared the bottom of the stacking gel. The details of reagents used and the method of gel preparation are given in the Appendix VII. After protease digestion, generated peptides were stained by the ultra-sensitive silver staining method (Oakley, Kirsch and Morris, 1980).

XVIII. THE ULTRA SENSITIVE SILVER STAINING METHOD

This was modified from the method of Oakley, Kirsch and Morris (1980). The gel was first fixed in 50% methanol, 10% acetic acid for 30 min, then washed in 5% methanol, 7% acetic acid overnight. After a further period (30 min) in 10% unbuffered glutaraldehyde, the gel was given 3 rinses in 1L of distilled water (10 min each) before soaking in distilled water for more than 2h. After removing the water, fresh ammoniacal silver solution was

added and the gel stained for 15 min at 25°C with constant agitation (to prepare 500 ml of the solution, 7 ml of fresh NH_4OH was added to 105 ml of 0.36% NaOH with vigorous agitation then 20 ml of 19.4% $AgNO_3$ was carefully added. A transient brown precipitate formed, which disappeared upon addition of distilled water to the final volume). Since ammoniacal silver solution may become explosive upon drying it should be disposed of immediately after use. The gel was removed from the ammoniacal silver solution and rinsed in distilled water for 2-5 min , removed, and placed in freshly prepared 0.005% citric acid: 0.019% formaldehyde. The stained proteins became visible at this stage. The gel was removed from the solution when background began to develop, and washed in several changes of distilled water with agitation.

The gel was destained using Kodak rapid fixer (Kodak Ltd., London) 1:3 dilution in distilled water. The silver deposition on the gel surface was removed during destaining process by using a soft brush. Destaining was stopped by placing the gel in Kodak Hypo Clearing Agent ($26.6\ gL^{-1}$). Five hundred ml solution was used in each step and most of the steps were carried out with mild agitation. All the chemicals used were obtained from BDH Chemical Company.

XIX. TIME COURSE OF LYSIS OF CONTROL AND CERULENIN-INHIBITED CELLS OF S. AUREUS (WOOD 46) IN THE PRESENCE AND ABSENCE OF LYSOSTAPHIN

Cells from 60 ml of control and cerulenin-inhibited cultures, grown for 15 h, were washed twice in 0.05 M Tris/HCl buffer, pH 7.5 at 20,000 'g' for 15 min, then resuspended in 40 ml of 0.01 M Tris/HCl buffer pH 7.5. The optical density of the suspensions were adjusted to the same value (approximately 1.5) and the cell suspensions kept at 4°C until used. Autolysis was assayed at 37°C by following change in OD_{600} with time. Also the sensitivity of these cell suspensions to lysostaphin was assayed by adding different concentrations of the enzyme to the cuvette, and again following cell lysis at 37°C as a function of time at 600 nm. Cell lysis in the presence of different concentrations of lysostaphin was plotted against incubation time at 37°C and compared with a no enzyme control.

RESULTS

I. PRODUCTION OF MEMBRANE-DAMAGING LYSINS AND PROTEASE(S) DURING GROWTH OF STAPHYLOCOCCUS AUREUS WOOD 46

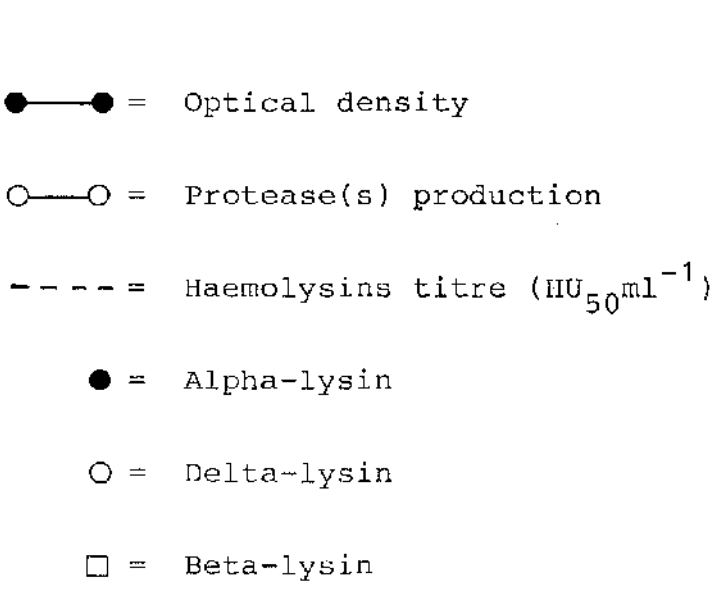
Production of α- β- and δ-lysins and protease(s) were followed for 14h during growth of S.aureus Wood 46 in shake-flask cultures. Fig. 10 shows that production of α- and δ-lysins started at the beginning of the exponential phase of growth after a lag of 3h. Beta-lysin and protease(s) production started about the 6th hour in mid exponential phase, all these products were at their maximum at the mid to late exponential phase of growth.

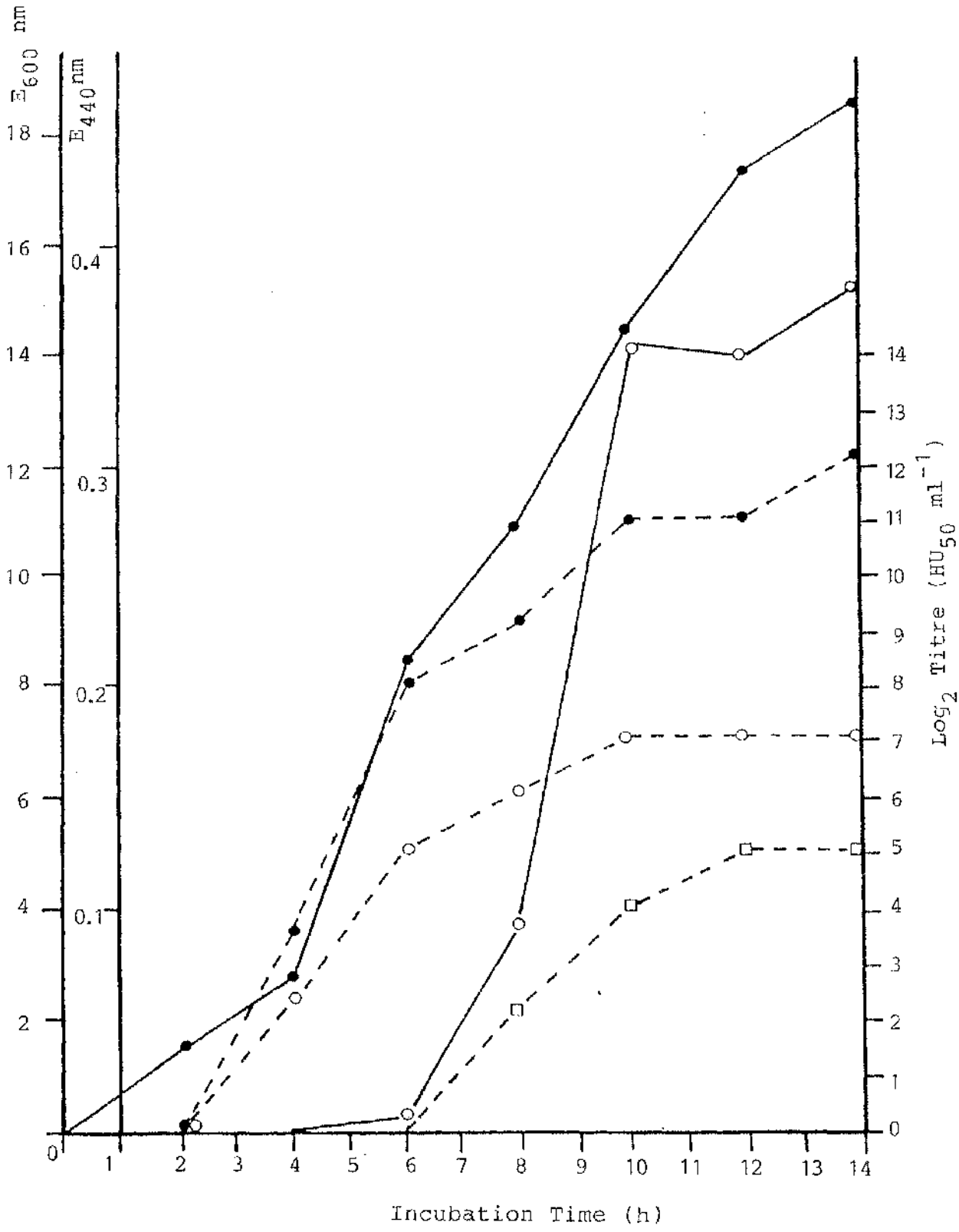
Alpha-lysin was the major extracellular product of this strain with titres of 4000 $HU_{50}ml^{-1}$ after 14h growth, δ-lysin had a titre of 128 $HU_{50}ml^{-1}$, and β-lysin a titre of 32 $HU_{50}ml^{-1}$ at the same time. As shown in Fig. 10, the curves for growth and lysin production exhibited an apparent biphasic pattern.

II. PURIFICATION OF ALPHA-LYSIN

After application of crude α-lysin to the CPG column, washing with 0.05M potassium phosphate buffer, pH 6.8 eluted a single large peak of protein with negligible haemolytic activity. A second peak of material, yellow-brown in colour and containing α-lysin activity was eluted when the buffer was changed to 1.0M potassium phosphate, pH 7.5. The colour was probably due to contamination by pigments present in the medium. An elution profile from

Figure 10. Production of haemolysins (α - β - and δ -) and protease(s) during growth of S.Aureus Wood 46.




E_{600} nm
E_{440} nm
18
16
14
12
10
8
6
4
2
0
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time (h)

CPG is shown in Fig. 11. Alpha-lysin fractions were pooled and precipitated by the addition of solid ammonium sulphate to 70% saturation and the precipitate was collected by centrifugation.

The elution profile after isoelectric focusing is shown in Fig. 12. Brown acidic material was focused at pH (2-4). The major peak corresponding to the α-lysin activity (ie. haemolytic for rabbit erythrocytes) focused at pH 8.5. The results of a typical purification are shown in Table 6.

III. ASSAY FOR STAPHYLOCOCCAL EXTRACELLULAR PRODUCTS

1. Other Staphylococcal Haemolysins

An indication of the presence of β- and δ-lysins in α-lysin preparations can be obtained by comparing the specific activity of the α-lysin against sheep or cod erythrocytes with that against rabbit. Table 7 shows the results of such titrations of the lysin from various stages in the purification procedure (N.B. no 'hot-cold' haemolysis was observed against sheep erythrocytes). The ratio of the titre against rabbit to that against sheep was increased 2.4 fold during the purification, while the ratio of the titre against rabbit cells to that against cod cells was increased 800-fold. The observed haemolytic activity against sheep erythrocytes is likely due entirely to α-lysin itself.

Figure 11. Elution profile of ammonium sulphate precipitated Wood 46 culture supernate on controlled pore glass.

Key:

Buffer 1. 0.05 M potassium phosphate pH 6.8
Flow rate 55 ml h^{-1}

Buffer 2. 1.0 M potassium phosphate pH 7.5
Flow rate 55 ml h^{-1}

●——● = Protein profile (E_{280} nm)

●- - -● = Haemolytic titre (HU_{50} ml^{-1}) against rabbit RBC

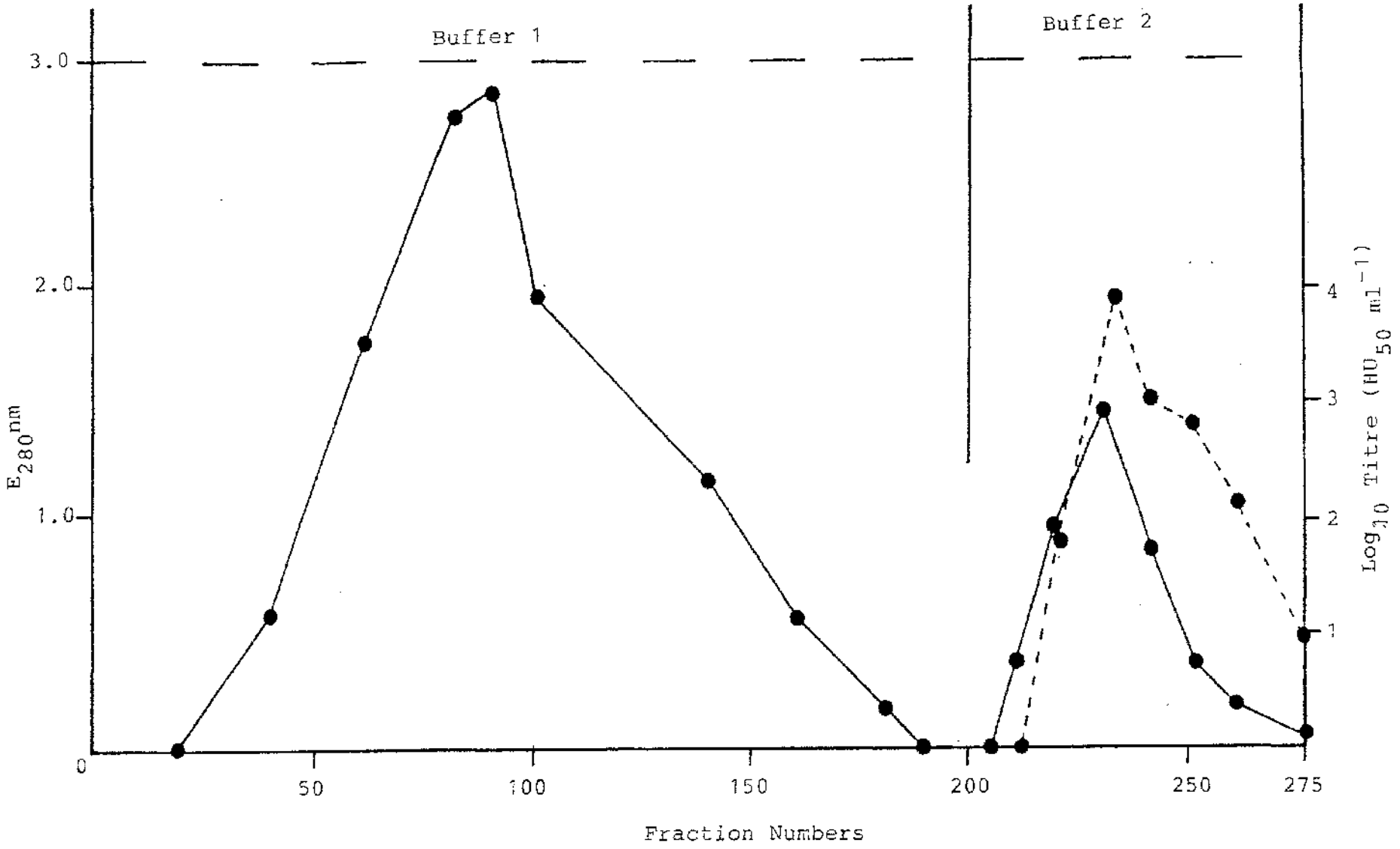

Buffer 1
Buffer 2
3.0
2.0
1.0
0
E_{280}^{nm}
50
100
150
200
250
275
Fraction Numbers
4
3
2
1
Log_{10} Titre (HU_{50} ml^{-1})

Figure 12. Electrofocusing of partially purified α-lysin.

Key:

○——○= E_{280}^{nm}

●——●= pH

●---●= Haemolytic titre ($HU_{50}ml^{-1}$) against rabbit RBC.

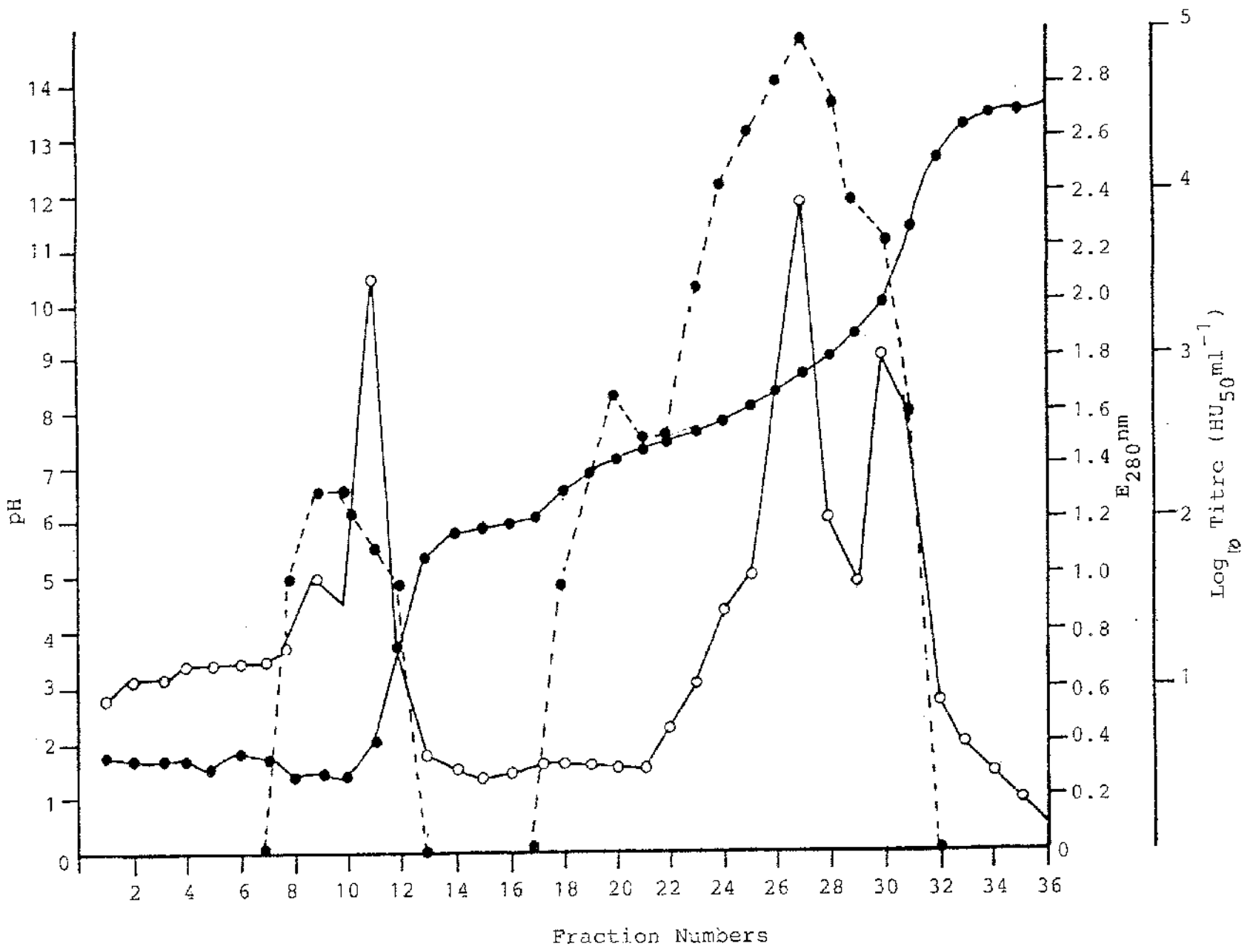

pH
E_{280}^{nm}
Log_{10} Titre ($HU_{50}ml^{-1}$)
Fraction Numbers
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Table 6. Alpha-lysin purification

Purification Step	Volume (ml)	Haemolytic Activity ($HU_{50}ml^{-1}$)	Protein ($mg\ ml^{-1}$)	Specific Haemolytic Activity ($HU_{50}mg^{-1}$)*	Total Haemolytic Activity (HU_{50})	Recovery of Activity (%)	Purification Factor
Culture supernate	1,500	8,192	8	1,024	$12.3x10^6$	100	1
Ammonium sulphate PPt before CPG Chromatography	70	32,768	15	2,184	$2.3x10^6$	19	2
Eluant from CPG	140	8,192	2	4,096	$1.1x10^6$	9	4
Pre IEF alpha-lysin	80	8,192	1	8,192	$6.5x10^5$	53	8
Electrofocused alpha-lysin after removal of ampholines and ammonium sulphate	50	81,920	0.5	163,840	$4\ x10^6$	32	160

* Against rabbit erythrocytes.

Table 7. Assay of contaminating haemolysins in the stages of purification of α-lysin

Purification Step	Specific Haemolytic Activity			Haemolytic Activity Ratio	
	Rabbit	Sheep	Cod	Rabbit:Sheep	Rabbit:Cod
Culture supernate	1024	15	40	68:1	26:1
Eluate from CGP	4096	40	12	102:1	341:1
Electrofocused α-lysin	163840	1000	8.0	164:1	20,480:1

2. Staphylococcal Extracellular Protease

The levels of protease were expressed as μg of protease per mg of protein (see Table 8). The levels of protease were decreased throughout the purification procedure until it reached .2μg protease per mg of α-lysin in the last stage of purification.

3. SDS-Polyacrylamide Gel Electrophoresis for Molecular Weight Estimation

Purified α-lysin was present as a species of molecular weight of 34K, this band also being present in the partially purified lysin from CPG, and in the culture supernate (Plate 1). There were traces of other bands of molecular weights of 27, 18 and 15K. These bands were detected in α-lysin preparations after storage for several months at 4°C in saturated ammonium sulphate (Plate 2).

4. Immunological Analysis

The results of Ouchterlony double diffusion immunological analysis are shown in Plate 3 and Plate 4. A single line of identity was evident between the purified α-lysin and Wood 46 culture supernate when run against commercial anti α-lysin (Plate 3). Purified δ-lysin gave α reaction with this antiserum. Anti δ-lsyin serum gave a single line of identity between purified δ-lysin and Wood 46 culture supernate (Plate 4).

Table 8. Levels of protease in the various stages of purification of α-lysin from supernates of S.aureus Wood 46.

Purification Step	Protein mg ml^{-1}	Protease Activity μg ml^{-1}	Level of Protease μg mg $protein^{-1}$
Culture supernate	8	12	1.5
Eluate from CPG	2	8	4
Electrofocused α-lysin	0.5	1*	2*

* figures derived by extrapolation

Plate 1. SDS-polyacrylamide gel electrophoresis of α-lysin at various steps in purification procedure

Key:

1 = Wood 46 culture supernate from overnight starter culture

2 = Wood 46 culture supernate from 1500 ml batch culture prior to the addition of ammonium sulphate

3 = Redissolved ammonium sulphate precipitate from culture supernate

4 = Peak 2 eluted from CPG

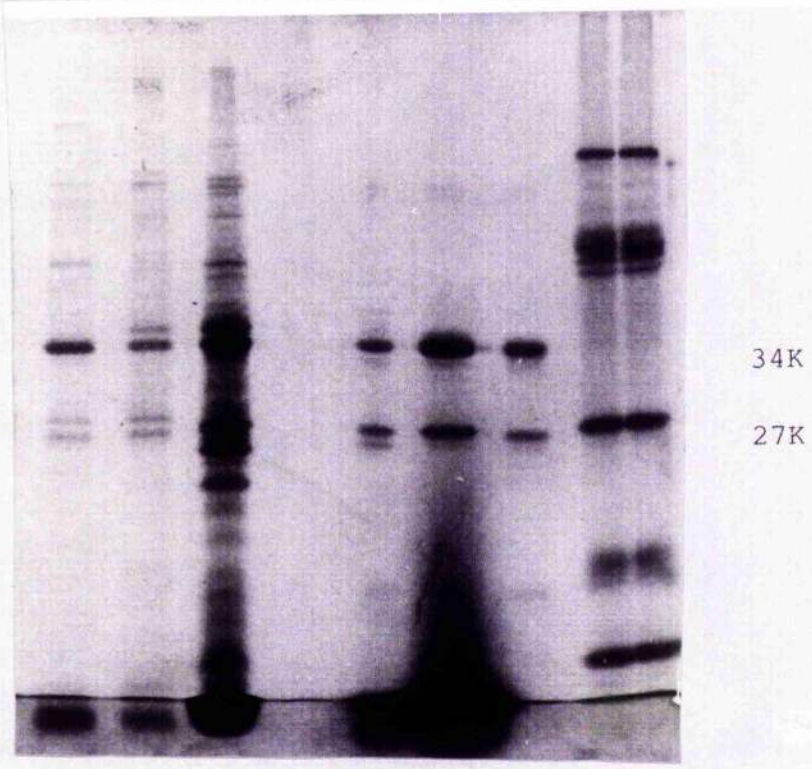
5 = Peak 2 after ammonium sulphate precipitation

6 = Alpha-lysin peak after IEF

7 = Purified α-lysin

8,9 = Sigma SDS VI standard protein mixture

10 = A fresh preparation of purified α-lysin



1 2 3 4 5 6 7 8 9

10

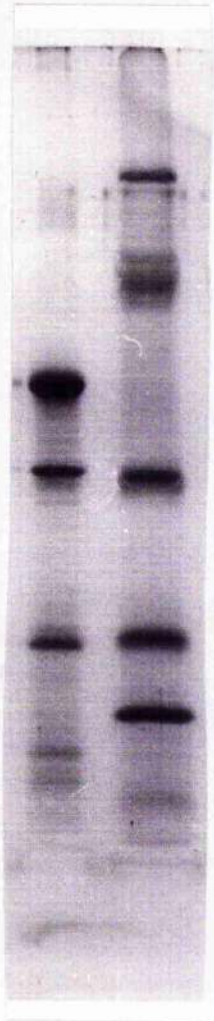


Plate 2. SDS-PAGE shows the appearance of lower molecular weight bands of 27, 18 and 15K upon storage of purified α-lysin.

Key:

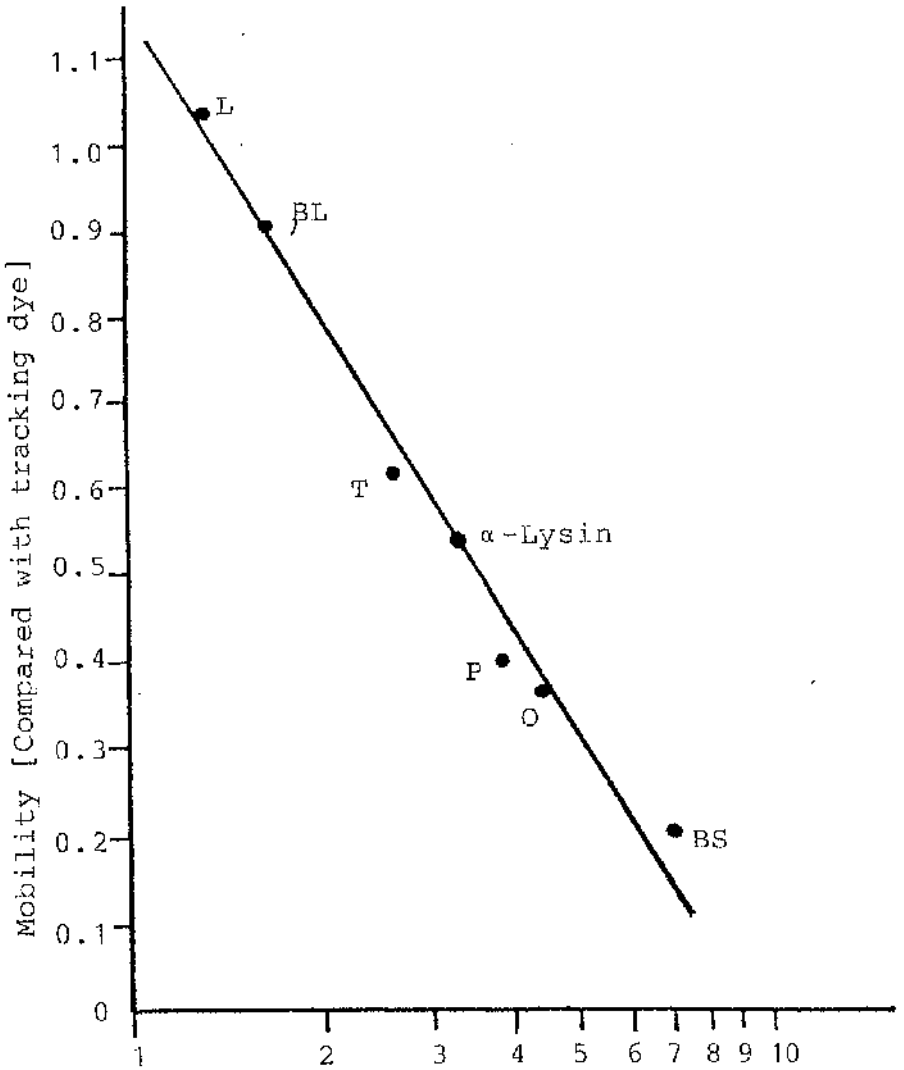
1 = Purified α-lysin preparation after storage

2 = Sigma SDS VI standard proteins

Figure 13. Plot of relative electrophoretic mobility of standard proteins in SDS-PAGE gels against logarithm of their molecular weight.

Key:

L	=	lysozyme (Molecular weight		14,300	dalton)
βL	=	β-lactoglobulin	" "	18,400	")
T	=	trypsinogen (	" "	24,000	")
P	=	pepsin (	" "	34,700	")
O	=	ovalbumin (	" "	45,000	")
BS	=	bovine serum albumin (	" "	66,000	")


Mobility [Compared with tracking dye]
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
L
βL
T
α-Lysin
P
O
BS
1
2
3
4
5
6
7
8
9
10
Molecular Weight (Dalton X 10^3)

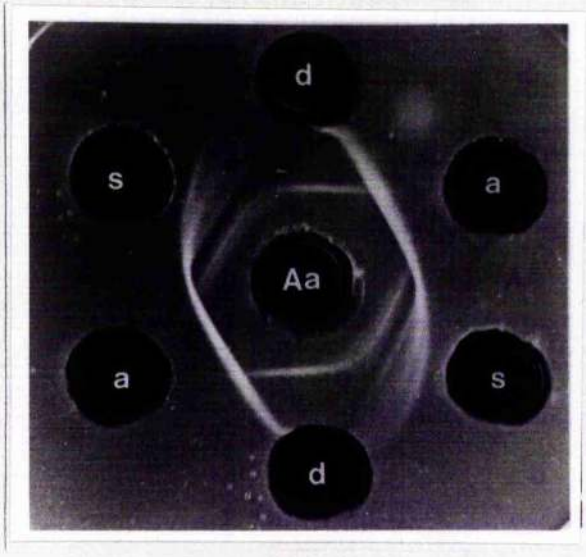


Plate 3. Double-diffusion immunological analysis of purified α-lysin and δ-lysin against commercial anti- α-lysin serum.

Key:

Aa = Anti- α-lysin

S = Wood 46 culture supernatant fluid

a = Purified α-lysin

d = Purified δ-lysin

Plate 4. Double diffusion immunological analysis of Purified α-lysin and δ-lysin against anti-δ-lysin serum.

Key:

Ad = Anti- δ-lysin

s = Wood 46 culture supernate fluid

a = Purified α-lysin

d = Purified δ-lysin

IV. EFFECT OF PHENYLMETHYLSULFONYL FLUORIDE (PMSF), O-PHENANTHROLINE, QUINACRINE, PROCAINE AND CERULENIN ON GROWTH AND HAEMOLYSIN PRODUCTION BY S.AUREUS WOOD 46

Involvement of protease(s) in the processing of precursors of extracellular proteins and enzymes from different bacteria has recently become an area of great interest. In order to investigate the significance of this phenomenon in the production of S. aureus extracellular products, the effects of different protease inhibitors on α-lysin production were studied.

PMSF was used at concentrations of 50, 100, 200, 300, 400 and 500 μg ml^{-1}; concentrations from 50 to 300 μg ml^{-1} did not affect either growth or production of α-, β- and δ-lysins. Above 300 μg ml^{-1}, growth was significantly reduced (Fig. 14). Quinacrine had no effect on either growth or the production of α- and δ-lysins within the concentration range 0.1, 0.2, 0.5, 0.8 and 1mM. A slight inhibition of β-lysin production was observed (Fig. 15). O-Phenanthroline had no effect on production of α-, β- or δ-lysins at 0.1 and 0.2 mM. However, growth inhibition occurred above 0.2 mM (Fig.16). Procaine within the concentration range of 2,4, 8, 12, 20 and 25 mg ml^{-1}, slightly inhibited growth at 12, 16, 20 and 25 mg ml^{-1} without effect on α-lysin production (Fig. 17).

Interesting results were obtained with cerulenin, an antibiotic which inhibits fatty acid synthesis. In

Figure 14. Effect of phenylmethylsulfonyl fluoride on growth and α-, β- and δ-lysin production by S. aureus Wood 46.

Key:

●——● = Growth (E_{600} nm)

●- -● = Alpha-lysin (HU_{50} ml^{-1})

○- -○ = Delta-lysin (" ")

□- -□ = Beta-lysin (" ")

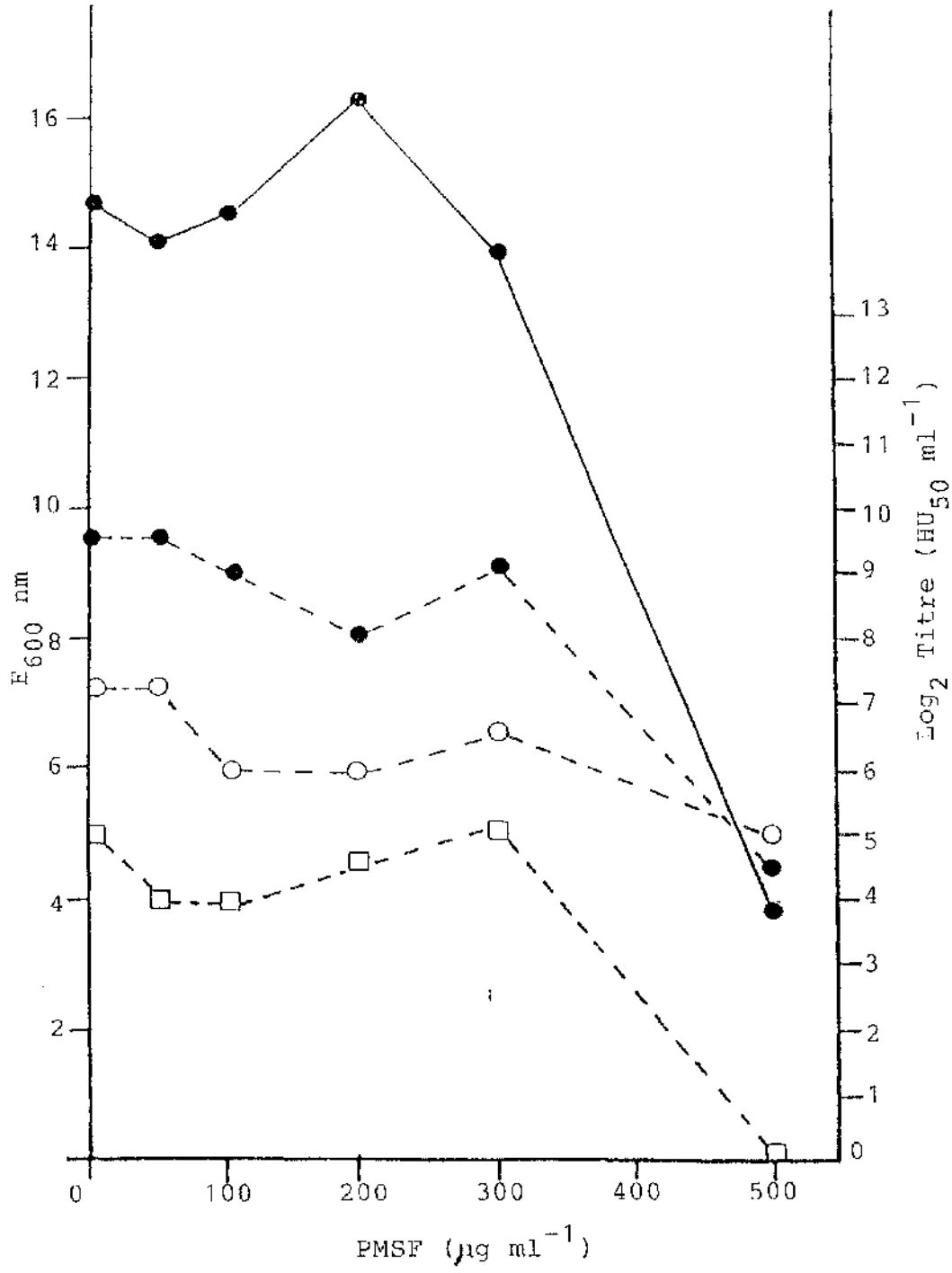

16
14
12
10
8
6
4
2
$E_{600\ nm}$
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Log_2 Titre (HU_{50} ml^{-1})
0
100
200
300
400
500
PMSF (μg ml^{-1})

Figure 15. Effect of quinacrine on growth and α-, β- and δ-lysin production by S. aureus Wood 46.

Key:

●——● = Growth (E_{600} nm)

●--● = Alpha-lysin (HU_{50} ml^{-1})

○--○ = Delta-lysin (" ")

□--□ = Beta-lysin (" ")

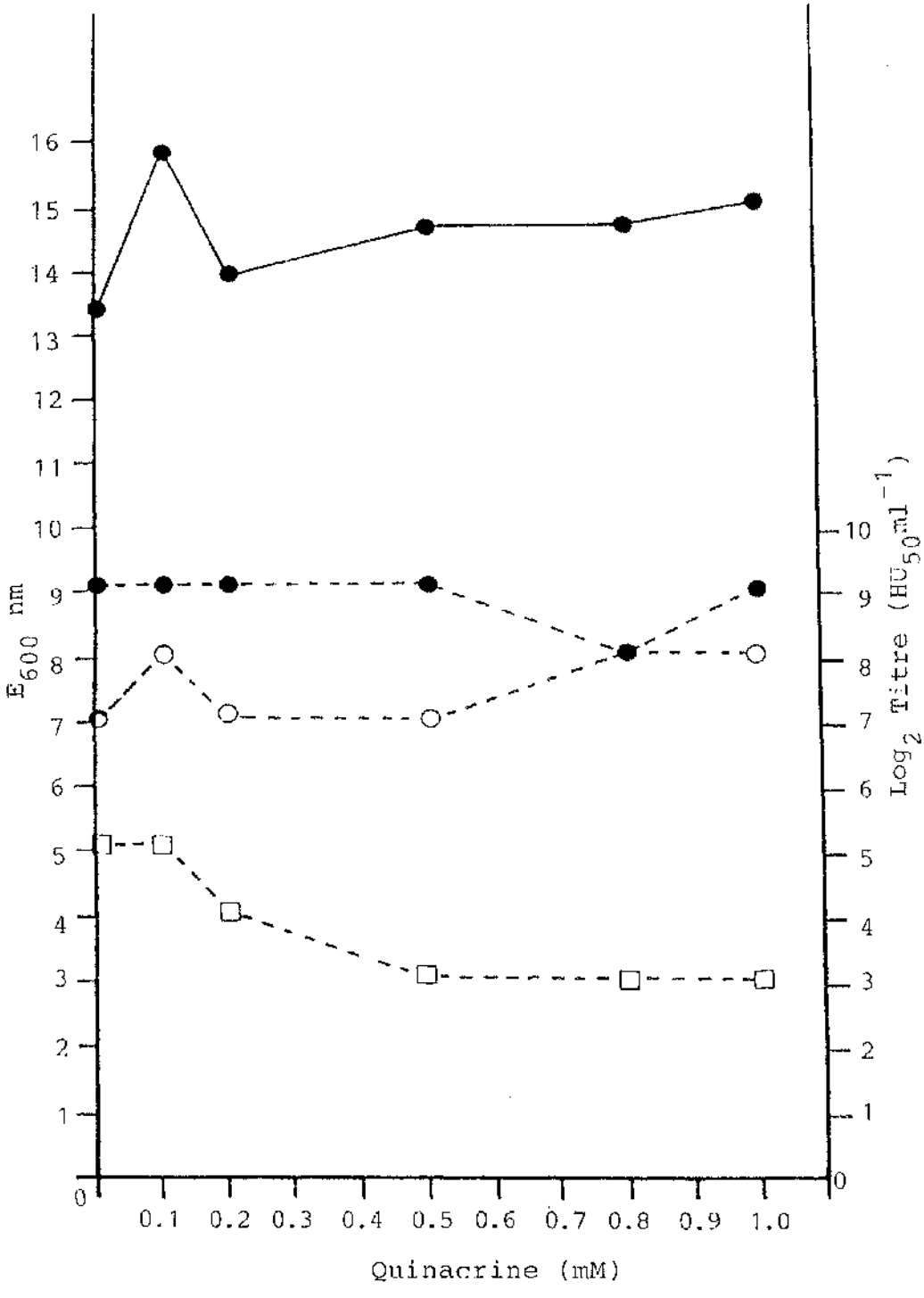

16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
E_{600} nm
10
9
8
7
6
5
4
3
2
1
0
Log_2 Titre ($HU_{50}ml^{-1}$)
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Quinacrine (mM)

Figure 16. Effect of O-phenanthroline on growth and α-, β- and δ-lysin production by S.aureus Wood 46.

Key:

●——● = Growth (E_{600} nm)

●- - -● = Alpha-lysin (HU_{50} ml^{-1})

○- - -○ = Delta-lysin (" ")

□- - -□ = Beta-lysin (" ")

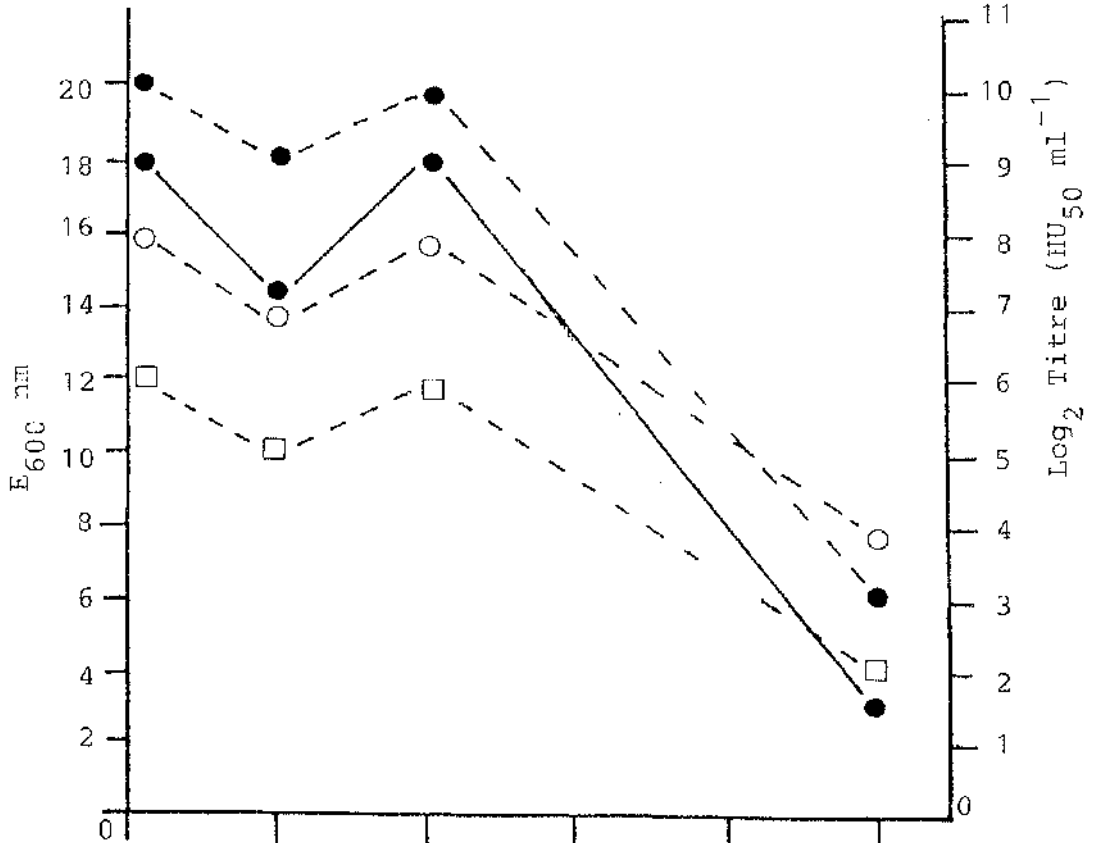
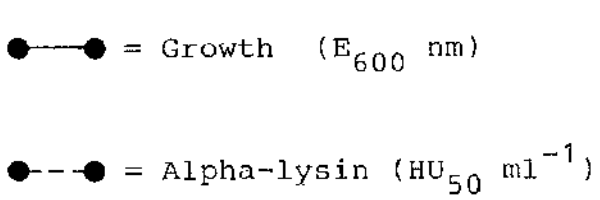
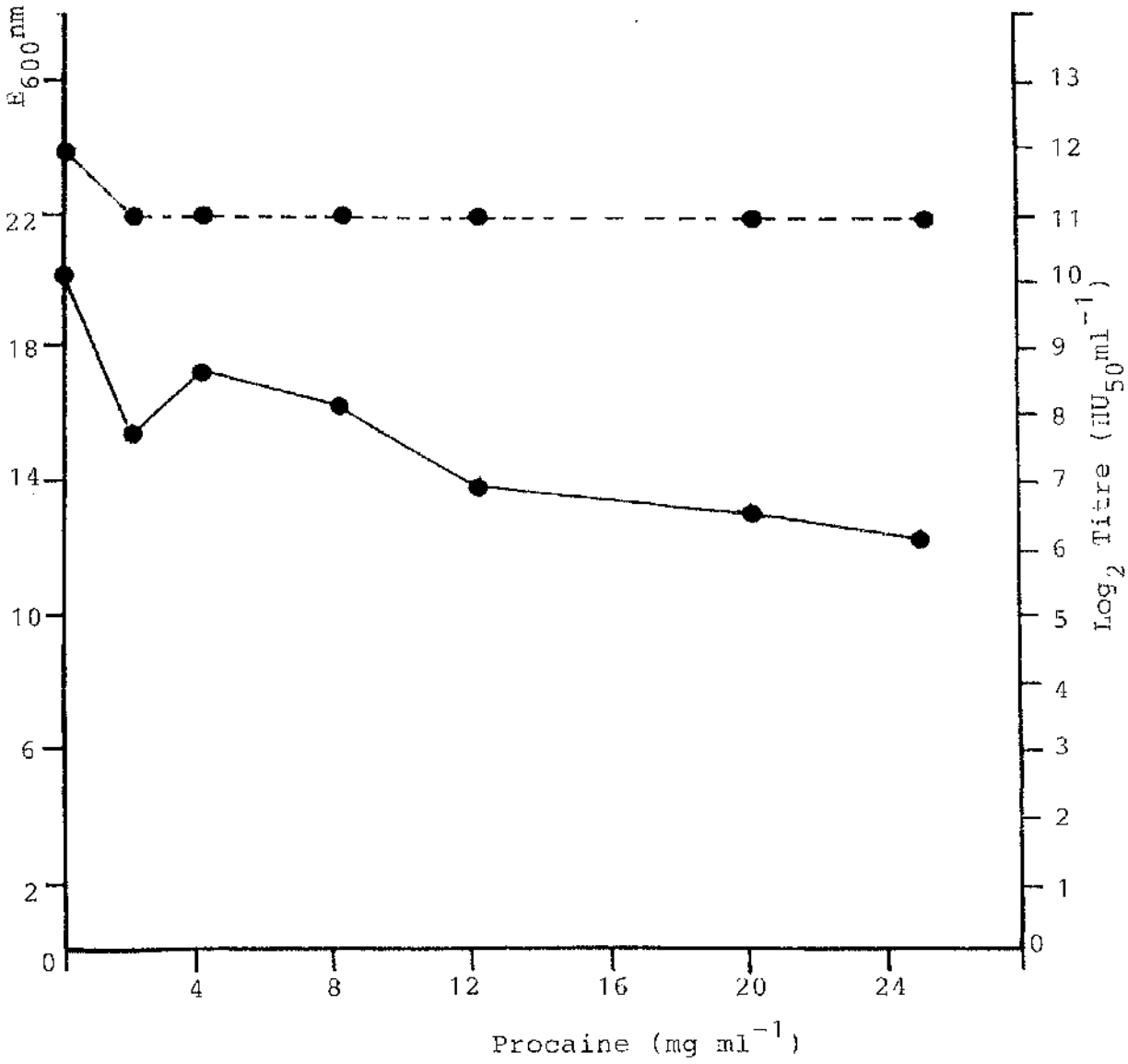

$E_{600\ nm}$
20
18
16
14
12
10
8
6
4
2
0
0.1
0.2
0.3
0.4
0.5
O-Phenanthroline (mM)
11
10
9
8
7
6
5
4
3
2
1
0
Log_2 Titre (HU_{50} ml^{-1})

Figure 17. Effect of procaine on growth and α-lysin production by S. aureus Wood 46.




E_{600}^{nm}
22
18
14
10
6
2
0
4
8
12
16
20
24
Procaine (mg ml^{-1})
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Log_2 Titre ($HU_{50}ml^{-1}$)

preliminary experiments, using 0.5, 1.0, 1.5, 2.0 and 2.5 μg ml^{-1} cerulenin, production of α-, β- and δ-lysins and protease(s) were inhibited significantly without significant effect on growth (Fig. 18). The protein most sensitive to this inhibition was α-lysin. Examination of protein profiles in culture supernates of control and cerulenin-inhibited cells showed the gradual disappearance of the 34K band (α-lsyin) with increasing cerulenin concentrations (Plate 5).

A variation in the effect of cerulenin on the production of α-lysin was observed when using different batches of yeast extract in the growth medium. Therefore, the concentrations of cerulenin required for inhibition of α-lysin secretion were determined for each batch of medium. Prior extraction of yeast extract with petroleum ether did not remove the batch to batch variation reported above.

In preliminary experiments, Difco yeast extract was used in the medium for production of α-lysin. However, in subsequent experiments, medium made with different batches of Difco yeast extract did not give a preferential inhibition of α-lysin production at low cerulenin concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 μg ml^{-1}). Alpha-lysin production in cultures grown in medium containing Oxoid yeast extract was consistently inhibited at concentrations of 8 to 10 μg ml^{-1} cerulenin.

Figure 18. Effect of cerulenin on growth, α-, β- and δ-lysin and protease(s) production by S.aureus Wood 46.

Key:

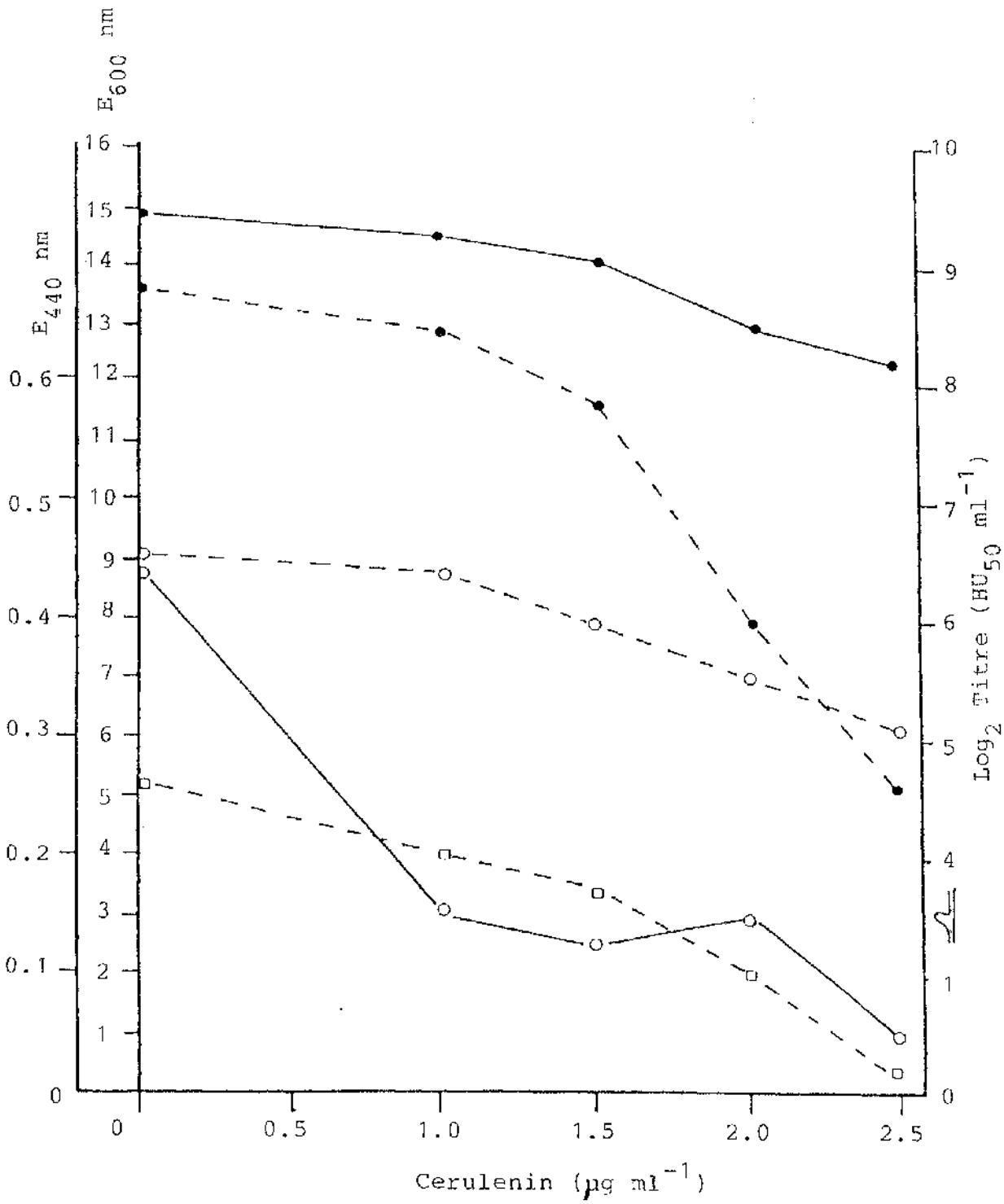
●——● = Growth (E_{600} nm)

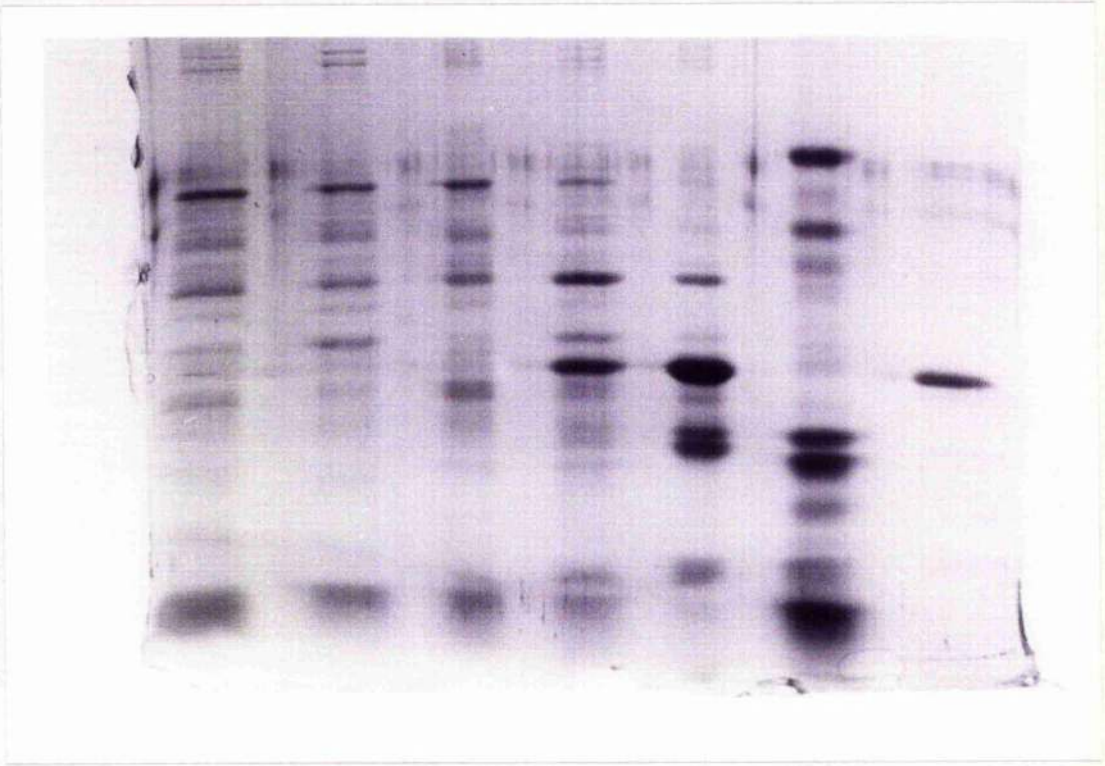
○——○ = Protease(s) production (E_{440}nm)

●- -● = Alpha-lysin (HU_{50} ml^{-1})

○- -○ = Delta-lysin (" ")

□- -□ = Beta-lysin (" ")


E_{600} nm
E_{440} nm
Log_2 Titre (HU_{50} ml^{-1})
Cerulenin (μg ml^{-1})
0
0.5
1.0
1.5
2.0
2.5
0.1
0.2
0.3
0.4
0.5
0.6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16



7 6 5 4 3 2 1

Plate 5. SDS-PAGE profile of the culture supernates of control and cerulenin-inhibited cultures.

Key:

1 = Purified α-lysin

2 = Standard protein mixture (Bovin serum albumin, Ovalbumin, Trypsinogen, Lactoglobulin, Lysozyme)

3 = Culture supernate from control cells

4 = Culture supernate from cerulenin-inhibited cells (1.5 $\mu g\ ml^{-1}$)

5 = " " " " " " (2.5 " ")

6 = " " " " " " (3.5 " ")

7 = " " " " " " (5.0 " ")

V. RELATIONSHIP BETWEEN OPTICAL DENSITY AND CELL DRY WEIGHT OF S. AUREUS (WOOD 46) DURING INHIBITION OF ALPAH-LYSIN PRODUCTION BY CERULENIN

The effects of cerulenin at 8 and 20 μg ml^{-1} on cell density, cell dry weight and α-lysin production in S.aureus (Wood 46) was studied. Cerulenin at 8 μg ml^{-1} did not affect growth whereas it significantly reduced α-lysin production. However, both growth and lysin production were inhibited significantly at 20 μg ml^{-1} cerulenin.

Alpha-lysin production began in early exponential phase (4-5th hour) with a titre of 256 HU_{50} ml^{-1} for control cells and 64 HU_{50} ml^{-1} for cells inhibited with 8 μg ml^{-1} cerulenin (Fig. 19). Meanwhile, complete inhibition of α-lysin production was achieved through growth using 20 μg ml^{-1} cerulenin, but optical density also fell by about 50%. Alpha-lysin production from control cells increased logarithmically during the exponential growth, and reached a titre of 4096 HU_{50} ml^{-1} after 12 h of incubation (Fig. 19). Alpha-lysin production continued during late exponential phase, a titre of 8196 HU_{50} ml^{-1} being evident at 18 h.Meanwhile, in the presence of 8 μg ml^{-1} cerulenin, α-lysin production did not increase during the exponential growth phase, the titre being constant (64 HU_{50} ml^{-1}) until 12 h, thereafter increasing slightly to 512 HU_{50} ml^{-1} at 18 h. (Fig. 19). No growth inhibition was observed in the exponential phase in the presence of 8 μg ml^{-1} cerulenin,

Figure 19. Effects of cerulenin at 8 and 20 μg ml^{-1} on optical density and α-lysin production during growth of S. aureus Wood 46.

Key:

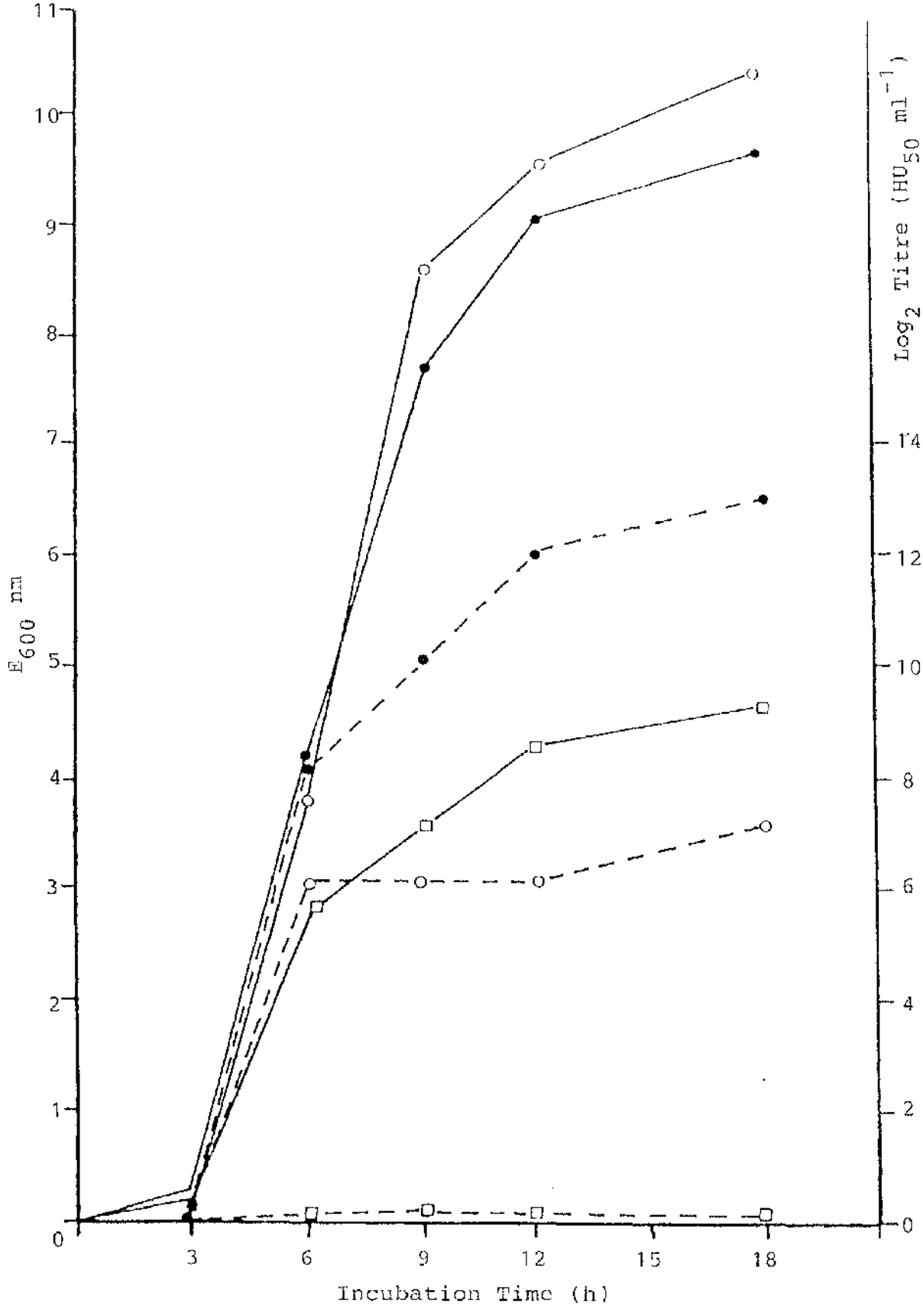
——— = Growth (E_{600} nm)

— — — = Alpha-lysin (HU_{50} ml^{-1})

● = Control

○ = 8 μg ml^{-1} cerulenin

□ = 20 μg ml^{-1} cerulenin


E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time (h)
0
1
2
3
4
5
6
7
8
9
10
11
0
2
4
6
8
10
12
14
3
6
9
12
15
18

although the optical density in the early stationary phase was slightly enhanced in the presence of the antibiotic (8 μg ml^{-1}).

In order to determine the relationships between optical density and cell dry weight in control and cerulenin-inhibited cultures, cell dry weight per ml culture and optical density were determined during the growth cycle (Fig. 19 and Fig. 20). The relationship between optical density and cell dry weight for control cells differed from that found in cerulenin-inhibited cells (Fig. 21). SDS-PAGE analysis of culture supernates of cerulenin-inhibited cells showed the disappearance of the biologically active α-lysin band from the sixth hour of growth (Plate 6).

VI. PROTEIN AND LIPID SYNTHESIS, DURING GROWTH OF S. AUREUS (Wood 46) IN THE PRESENCE AND ABSENCE OF CERULENIN

Lipid Synthesis

The incorporation of radioactivity from sodium [^{14}C]-acetate into chloroform/methanol-extractable materials was used as a measure of lipid synthesis, and followed for 10h during growth. Figure 22 shows that cerulenin at 10 μg ml^{-1} significantly reduced both α-lysin production and lipid synthesis (reduced by 50%) without significant effect on growth.

Figure 20. Effects of cerulenin at 8 and 20 μg ml^{-1} on cell dry weight and α-lysin production during growth of S. aureus Wood 46.

Key:

——— = Cell dry weight (mg ml^{-1})

– – – = Alpha-lysin (HU_{50} ml^{-1})

● = Control

○ = 8 μg ml^{-1} cerulenin

□ = 20 μg ml^{-1} cerulenin

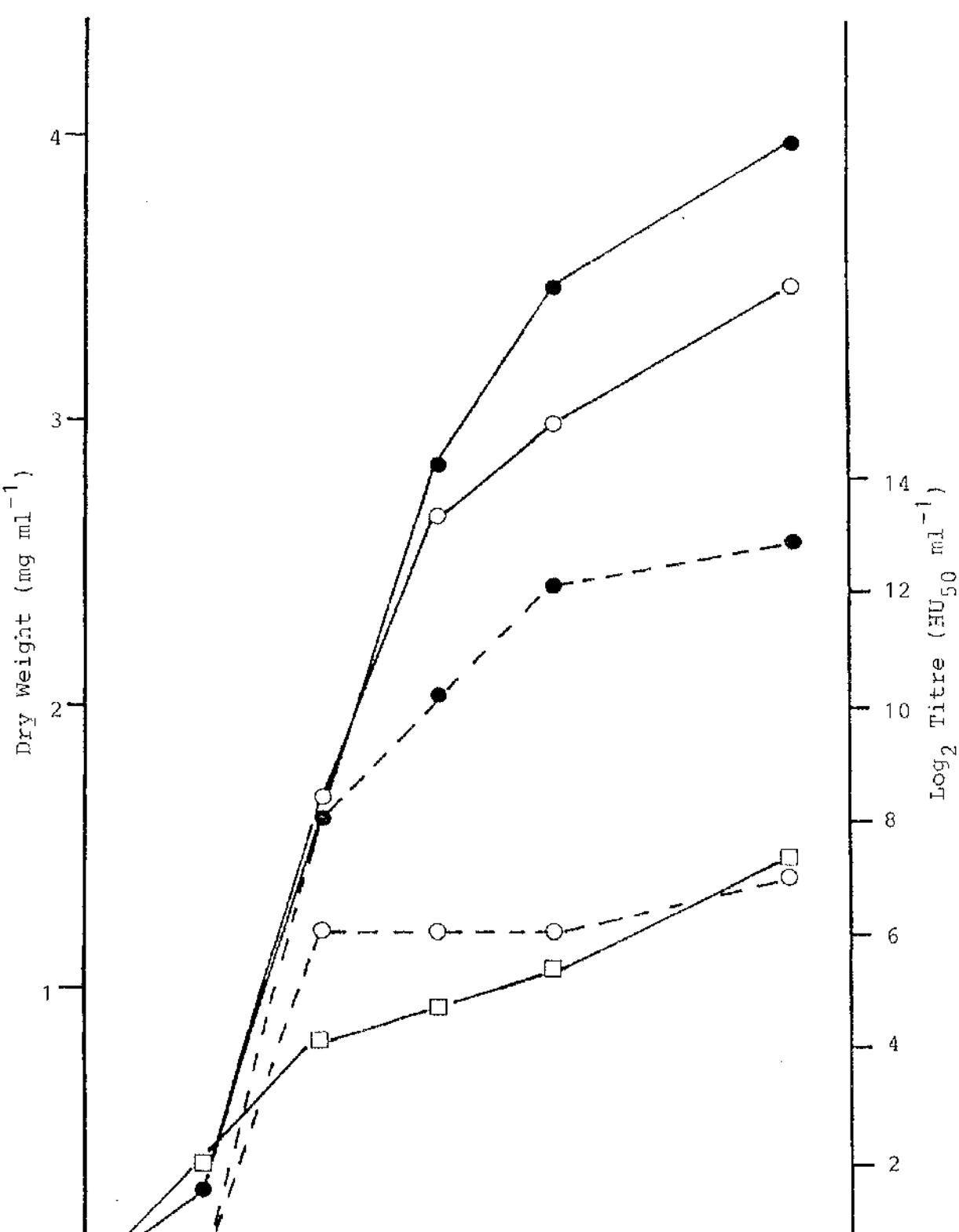

Dry Weight (mg ml^{-1})
$\log_2$ Titre (HU$_{50}$ ml^{-1})
Incubation Time (h)
0
1
2
3
4
0
2
4
6
8
10
12
14
3
6
9
12
15
18

Figure 21. Comparison of the difference between cell dry weight and optical density of cerulenin-inhibited cells with that of control cells during growth of S. aureus Wood 46.

Key:

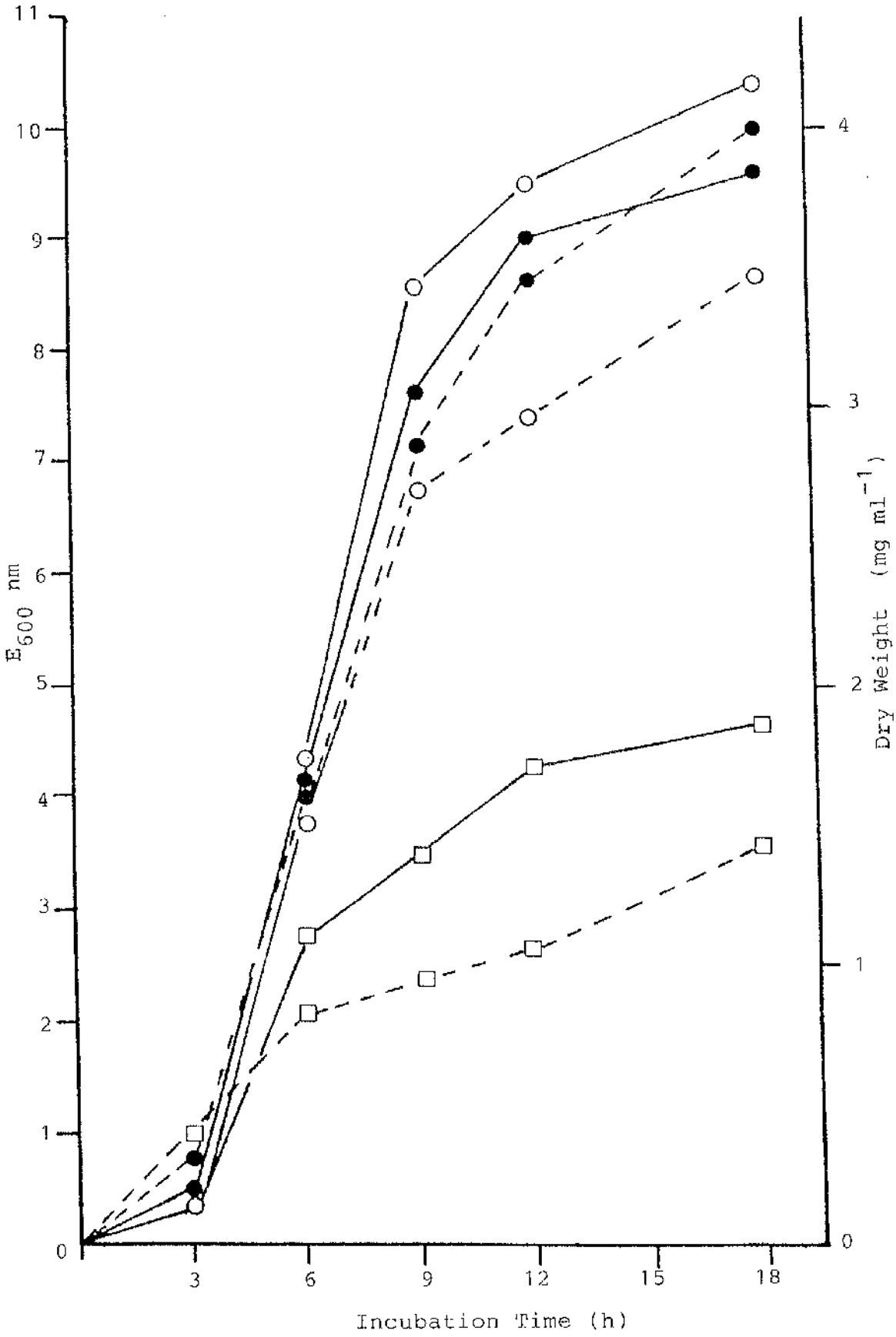
——— = Optical density at E_{600} nm

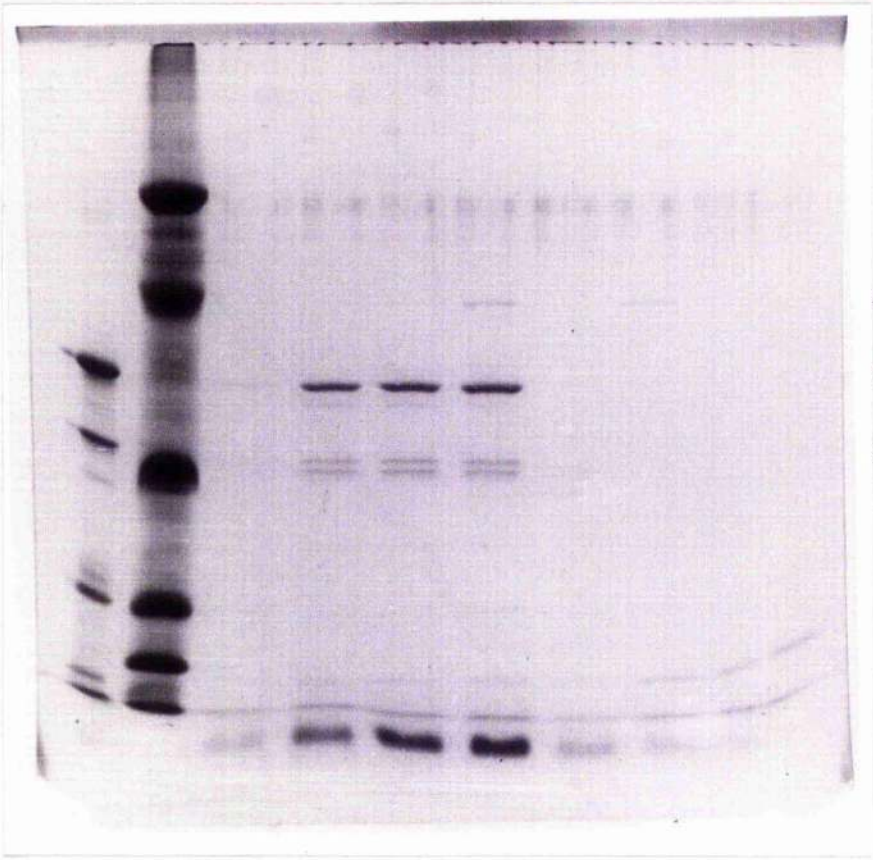
– – – – = Cell dry weight (mg ml^{-1})

● = Control

○ = 8µg ml^{-1} cerulenin

□ = 20µg ml^{-1} cerulenin


11
10
9
8
7
6
5
4
3
2
1
0
E_{600} nm
4
3
2
1
0
Dry Weight (mg ml^{-1})
3
6
9
12
15
18
Incubation Time (h)



1 2 3 4 5 6 7 8 9 10

Plate 6. SDS-PAGE of the culture supernates of control and cerulenin-inhibited cells (8µg ml^{-1}) during growth of S. aureus Wood 46.

Key:

1 = An aged purified α-lysin preparation

2 = Sigma SDS VI standard protein mixture

3-6 = Culture supernates of control cells

7-10 = Culture supernates of cerulenin-inhibited cells

3,7 = After 6 hours

4,8 = " 9 "

5,9 = " 12 "

6,10 = " 18 "

Figure 22. Incorporation of [^{14}C]-sodium acetate into control and cerulenin-inhibited cultures of S. aureus Wood 46.

Key:

▲——▲ = incorporation of [^{14}C]-acetate into control cells.

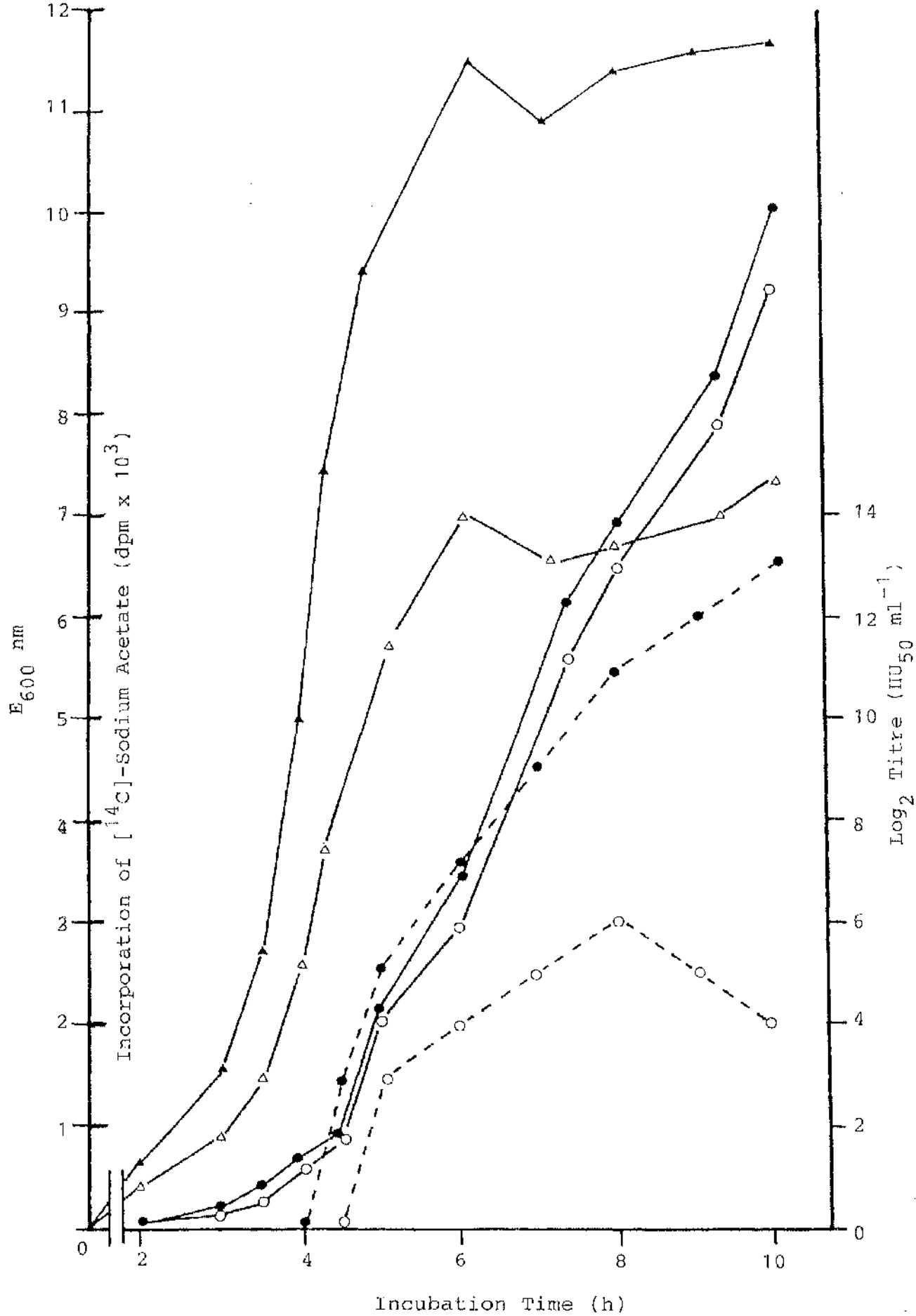
△——△ = Incorporation of [^{14}C]-acetate into cerulenin-inhibited cells

●——● = Control cell growth

○——○ = Cerulenin-inhibited cell growth (10 $\mu g\ ml^{-1}$)

●- - -● = Alpha-lysin production from control cells

○- - -○ = Alpha-lysin production from cerulenin-inhibited cells.


E_{600} nm
Incorporation of [^{14}C]-Sodium Acetate (dpm x 10^3)
Log_2 Titre (IU_{50} ml^{-1})
Incubation Time (h)
0
1
2
3
4
5
6
7
8
9
10
11
12
14
2
4
6
8
10

Protein Synthesis

The incorporation of radioactivity from [^{3}H]-phenylalanine into trichloroacetic acid (TCA) precipitable material, a measure of protein synthesis, was followed for 10 h during growth of control and cerulenin-inhibited (10μg ml^{-1}) cells of S. aureus Wood 46. Figure 23 shows that 10 μg ml^{-1} cerulenin significantly inhibited α-lysin production without any significant effect on either growth or protein synthesis.

VII. TOTAL LIPID CONTENT OF CONTROL AND CERULENIN-INHIBITED CELLS OF S. AUREUS WOOD 46

Lipids were extracted from 20 mg freeze-dried control and cerulenin-inhibited cells of S. aureus (Wood 46). Table 9 shows that total lipids extracted from control cells comprised 10% of cell dry weight whereas total lipids extracted from cerulenin-inhibited cells accounted for 5% of cell dry weight.

VIII. PHOSPHOLIPID PROFILES OF CONTROL AND CERULENIN-INHIBITED CELLS OF S. AUREUS WOOD 46

Phospholipids were separated by one dimensional thin layer chromatography (TLC). The major constituents of S. aureus strain Wood 46 phospholipids are diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG), as is shown (Plate 7). No significant qualitative changes were observed when the phospholipid profile of cerulenin-inhibited cells were compared with that of control cells.

Figure 23. Incorporation of [^{3}H]-phenylalanine into control and cerulenin-inhibited cultures of *S. aureus* Wood 46.

Key:

▲——▲ = incorporation of [^{3}H]-phenylalanine into control cells

△——△ = Incorporation of [^{3}H]-phenylalanine into cerulenin-inhibited cells

●——● = Control cell growth

○——○ = Cerulenin-inhibited cell growth (10 μg ml^{-1})

●--● = Alpha-lysin production from control cells

○--○ = Alpha-lysin production from cerulenin-inhibited cells.

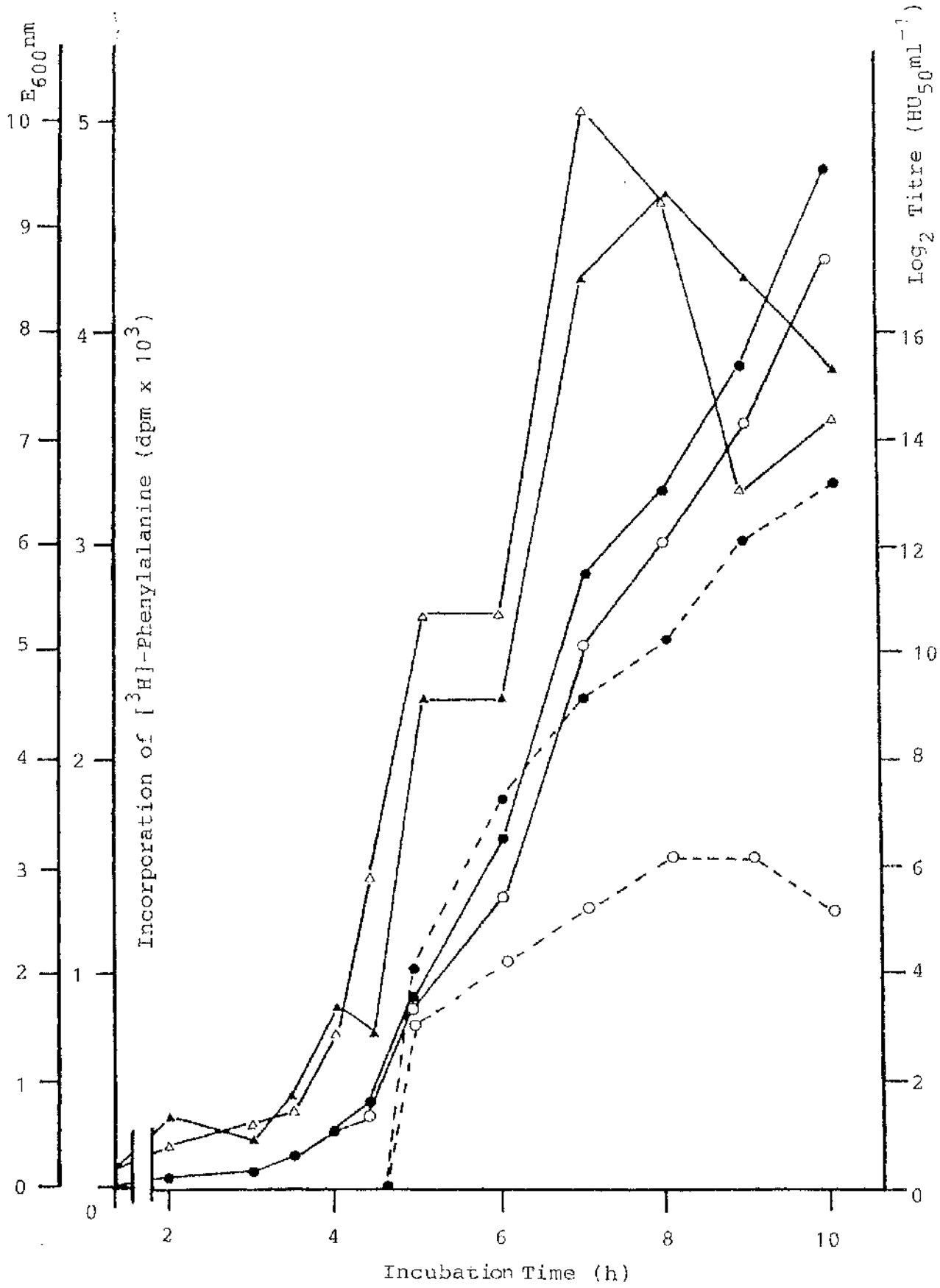
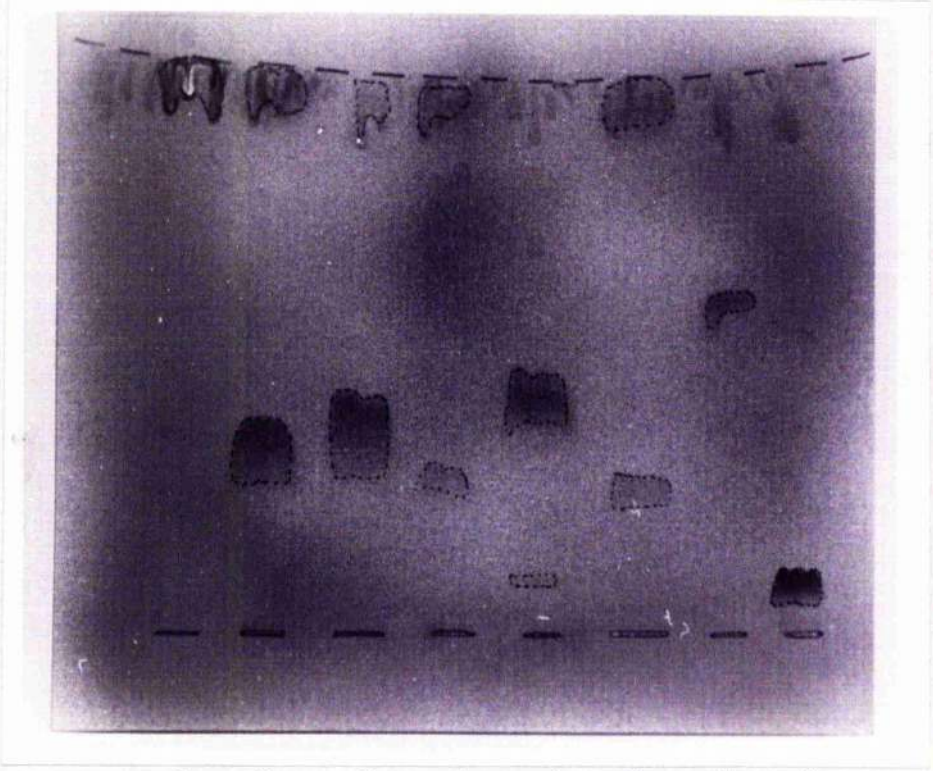

E_{600}^{nm}
Incorporation of [^{3}H]-Phenylalanine (dpm x 10^3)
Log_2 Titre ($HU_{50}ml^{-1}$)
Incubation Time (h)
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
0
2
4
6
8
10
12
14
16

Table 9. Relationship between total lipid content, alpha-lysin titre and optical density of control and cerulenin-inhibited cells of S.aureus Wood 46.

Cultural Conditions	*OD_{600}	Alpha-Lysin Titre$^{-1}_{50}$ (HU ml^{-1})	* lipid content (%)
Control	16.3 ± 0.28 (3)	8192	10.1 ± 0.9 (4)
Cerulenin (8ug ml^{-1} culture)	15.6 ± 1.95 (3)	8 to 512	4.9 ± 1.1 (5)
Cerulenin (15 ug ml^{-1} culture)	11.9 ± 1.6 (3)	4 to 32	4.2 ± 1.0 (5)

* Figures for OD_{600} and lipid content are the mean values ± standard deviation with the number of replicates in brackets.



1 2 3 4 5 6 7 8

Plate 7. Thin layer chromatography (TLC) of lipids extracted from control and cerulenin-inhibited cells of S. aureus Wood 46.

Key:

1 = Diphosphatidylglycerol (Cardiolipin)

2 = Lipids extracted from control cells

3 = Phosphatidylglycerol

4 = Lipids extracted from cerulenin-inhibited cells ($8\ \mu g\ ml^{-1}$)

5 = Phosphatidylserine

6 = Lipids extracted from cerulenin-inhibited cells ($15\ \mu g\ ml^{-1}$)

7 = Phosphatidylethanolamine

8 = Phosphatidylinositol

IX. FATTY ACID PROFILES OF CONTROL AND CERULENIN-INHIBITED CELLS OF S.AUREUS (WOOD 46) DETERMINED BY GAS LIQUID CHROMATOGRAPHY

Identification of the fatty acid components of lipid extracts of control and cerulenin-inhibited S. aureus cells, was based on retention times relative to standard fatty acids (R_t values). Figure 24 shows separate straight lines obtained by plotting Log_{10} retention time (Log_{10} R_t) against carbon numbers for saturated and unsaturated fatty acid standards. Thus, the fatty acid components in the lipids of control and cerulenin-inhibited cells were identified from the intersect of their relative retention time with the standard plots (Fig. 24). Branched chain fatty acids were tentatively identified from data given by White and Frerman (1968). Fatty acid profiles of control and cerulenin-inhibited S. aureus (Wood 46) cells are shown in Table 10.

Each fatty acid is expressed as a percentage of total fatty acids in each case. The fatty acid components of control cells are, in the order of abundance:-

C15, i > C16:0 > C17:1 > C16, i > C19, i > C13:0

Changes in fatty acid profiles of cerulenin-inhibited cells were as follows (Table 10):

(1) The appearance of fatty acids such as C20, i; C18:0 and C17:0.

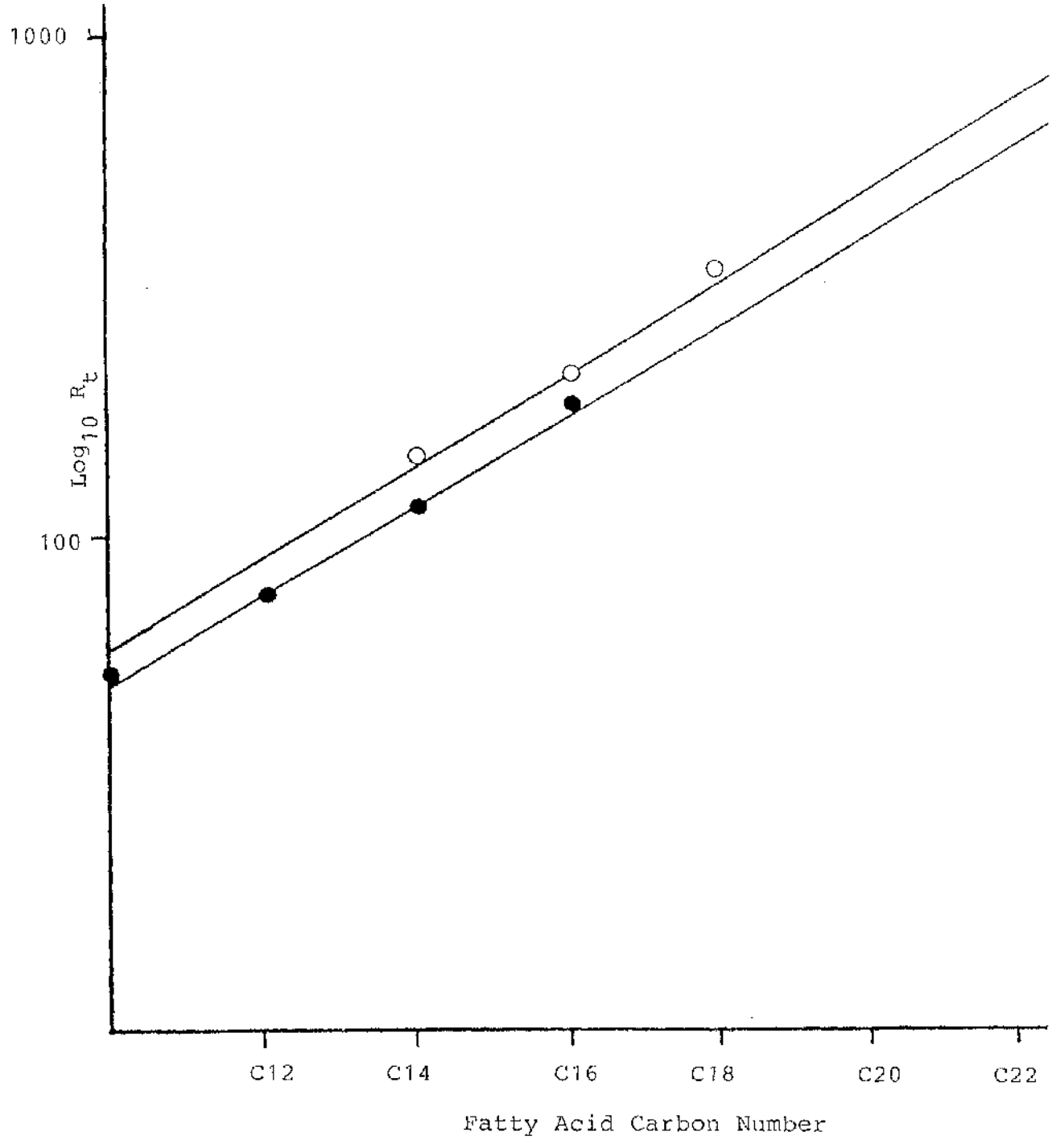
(2) A significant increase in C12:0.

Figure 24. Relationship between logarithm of relative retention times for saturated fatty acids (●) and unsaturated fatty acids (○) and carbon chain length.

Key:

●= Saturated fatty acids

○= Unsaturated fatty acids


1000
100
Log_{10} R_t
C12
C14
C16
C18
C20
C22
Fatty Acid Carbon Number

Carbon No.	% of Total Fatty Acids	
	Control	Cerulenin(2.5 μg ml^{-1})
[a]C12:O	1.23	1.9
C13:O	1.3	0.94
[b]C13:1	2.36	1.5
[c]C14,i	1.7	1.4
C15:O	3.85	2.5
C15,i	43.55	35.99
C16:O	18.06	17.76
C16,i	7.2	4.9
C17:O	0	2.11
C17:1	14.21	10.56
C18:O	0	7.48
C19,i	6.40	5.59
C20,i	0	7.66

Table 10. Fatty acid components of control and cerulenin-inhibited cells of S.aureus (Wood 46) grown in BS medium with Difco yeast extract

a : Saturated fatty acid

b : Fatty acid with one unsaturated bond

c : Saturated iso branched fatty acid.

Other fatty acids were reduced in amount compared to control cells reflecting less lipid per mg dry weight of cells. Optical density and α-lysin titres measured after 15 h incubation at 37°C were as follows:

Samples	E_{600}^{nm}	α-Lysin Titre ($HU_{50}ml^{-1}$)
Control	14.21	16382
Cerulenin (2.5 μg ml^{-1})	14.80	8

X. EFFECT OF EXOGENOUS FATTY ACIDS ON THE PRODUCTION OF ALPHA AND DELTA-LYSINS DURING GROWTH OF S.AUREUS (WOOD 46) IN THE PRESENCE AND ABSENCE OF CERULENIN

To determine the effect of exogenous fatty acid sources on the recovery of α-lysin production from cerulenin inhibition, both oleate (unsaturated monoenoic fatty acid, C18:1) and stearate (saturated fatty acid, C18:0) were used at 8.3 μg ml^{-1}. Oleate did not affect growth or the production of α-lysin from control cells (Fig. 25A), but it allowed a partial recovery of α-lysin production from cerulenin inhibition (Fig. 26A). Neither production of α-lysin from control cells (Fig. 27A) nor its inhibition by cerulenin (Fig. 28A) was affected by stearate.

Regarding δ-lysin production, oleate had no effect on the production of the lysin from either control (Fig. 25B), or cerulenin-inhibited cells (Fig. 26B). Stearate slightly enhanced δ-lysin production from control cells (Fig. 27B) however, it had no effect on lysin

Figure 25 (A,B). Effect of exogenous oleate on growth and the production of α- and δ-lysins.

Key:

——— = E_{600} nm

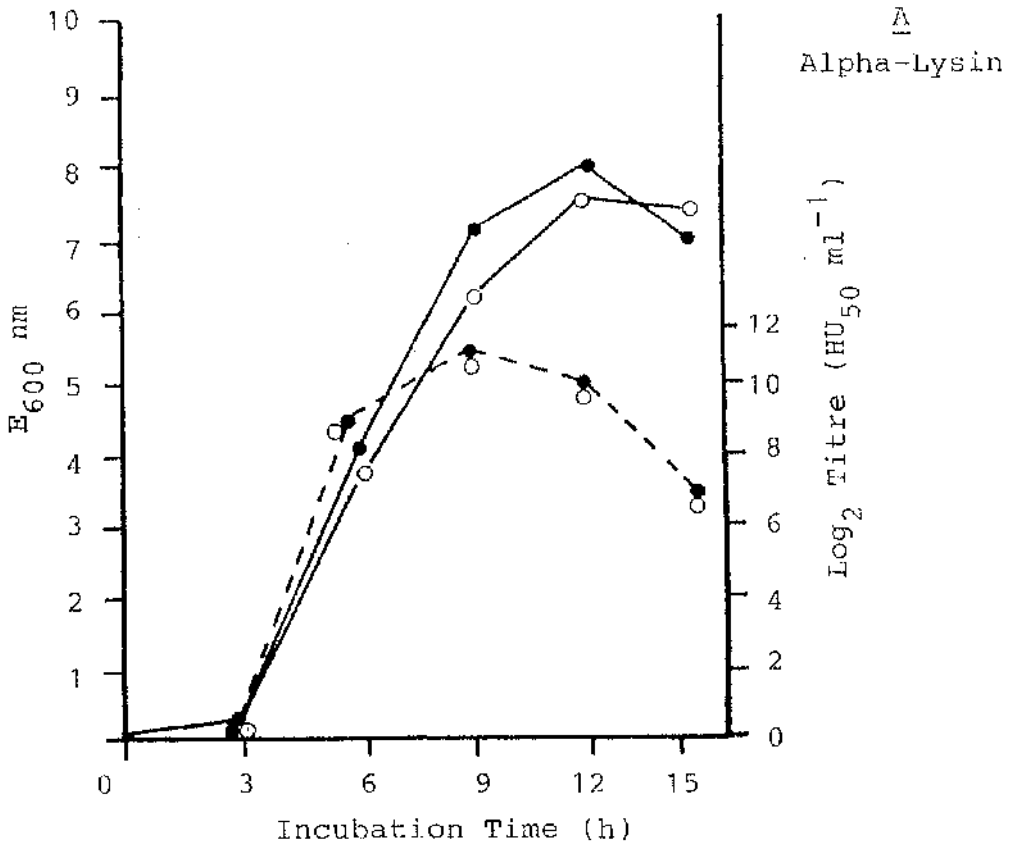
- - - - = Lysin production

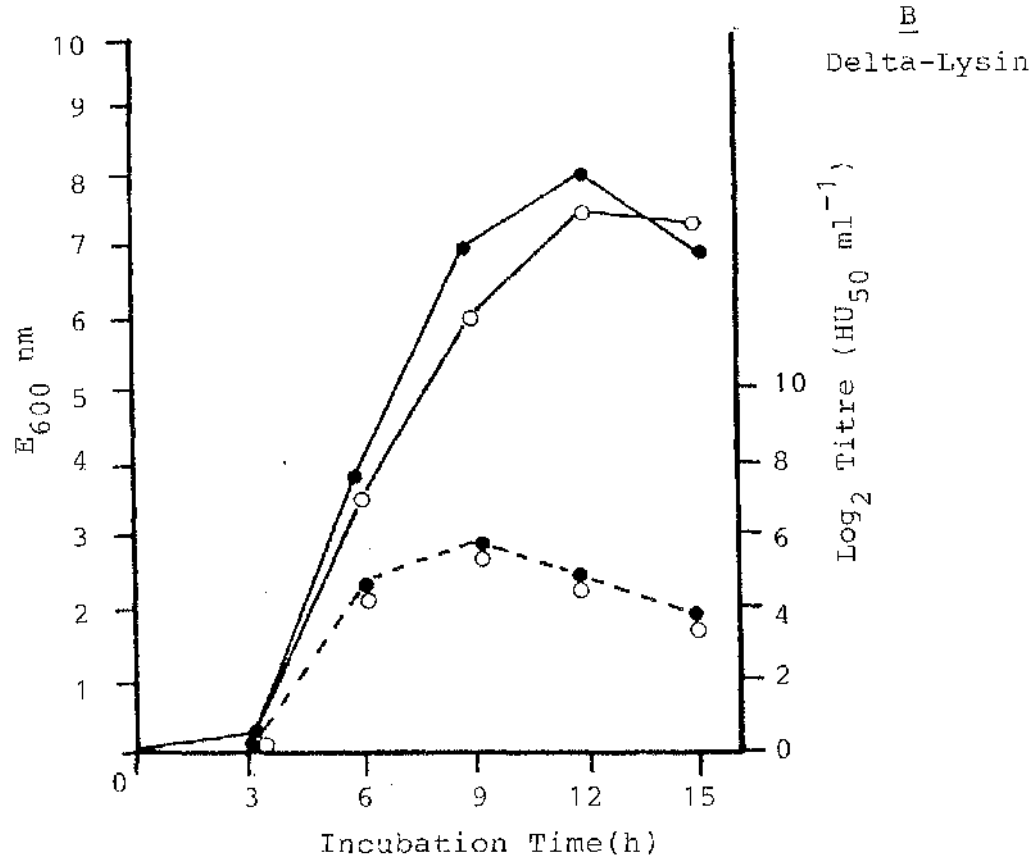
● = Control cells

○ = Oleate-supplemented cells (8.3 μg ml^{-1})

A = Alpha-lysin production

B = Delta-lysin production


A
Alpha-Lysin
E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time (h)


B
Delta-Lysin
E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time(h)

Figures 26 (A,B). Effect of exogenous oleate on growth and α-lysin production in control and cerulenin-inhibited cells.

Key:

——— = $E_{600\ nm}$

– – – – = Lysin production

● = Control cells

○ = Cerulenin-inhibited cells (8μg ml^{-1})

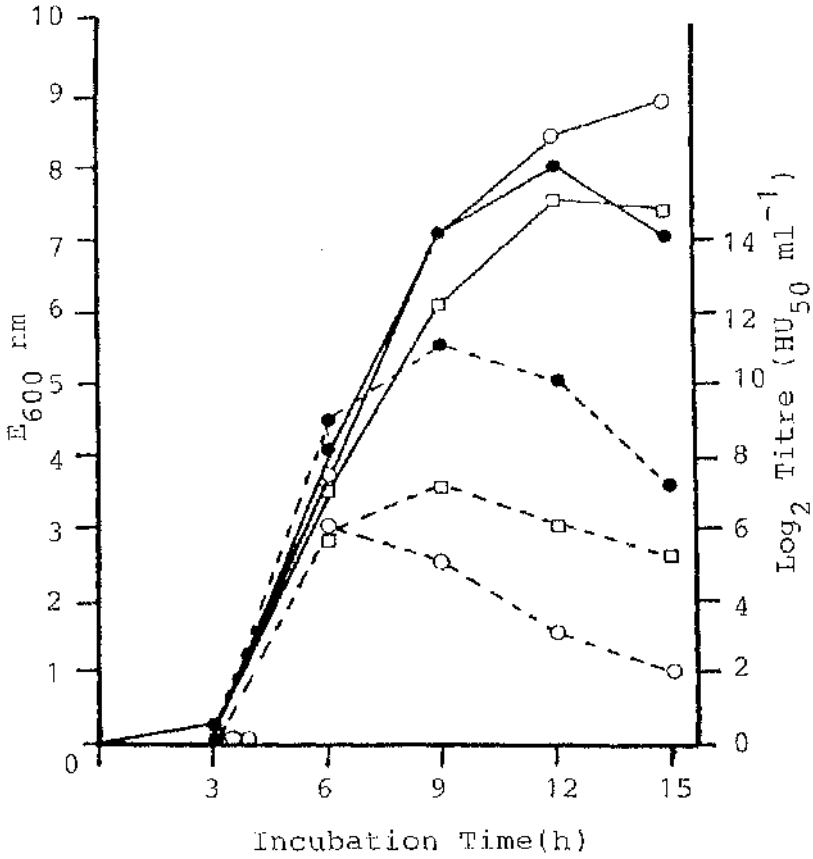
□ = Cerulenin + oleate supplemented-cells (8.3μg ml^{-1})

Λ = Alpha-lysin production

B = Delta-lysin production

A

Alpha-Lysin



B

Delta-Lysin

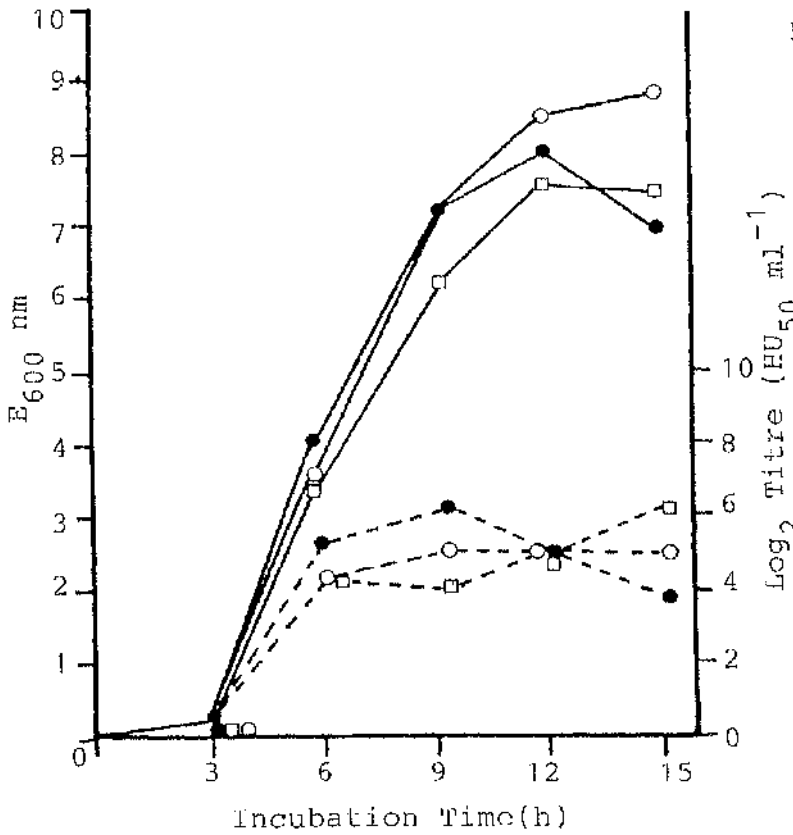


Figure 27 (A,B). Effect of exogenous stearate on growth and the production of α- and δ-lysins.

Key:

——— = E_{600}^{nm}

– – – – = Lysin production

● = Control cells

○ = Stearate-supplemented cells (8.3 μg ml^{-1})

A = Alpha-lysin production

B = Delta-lysin production

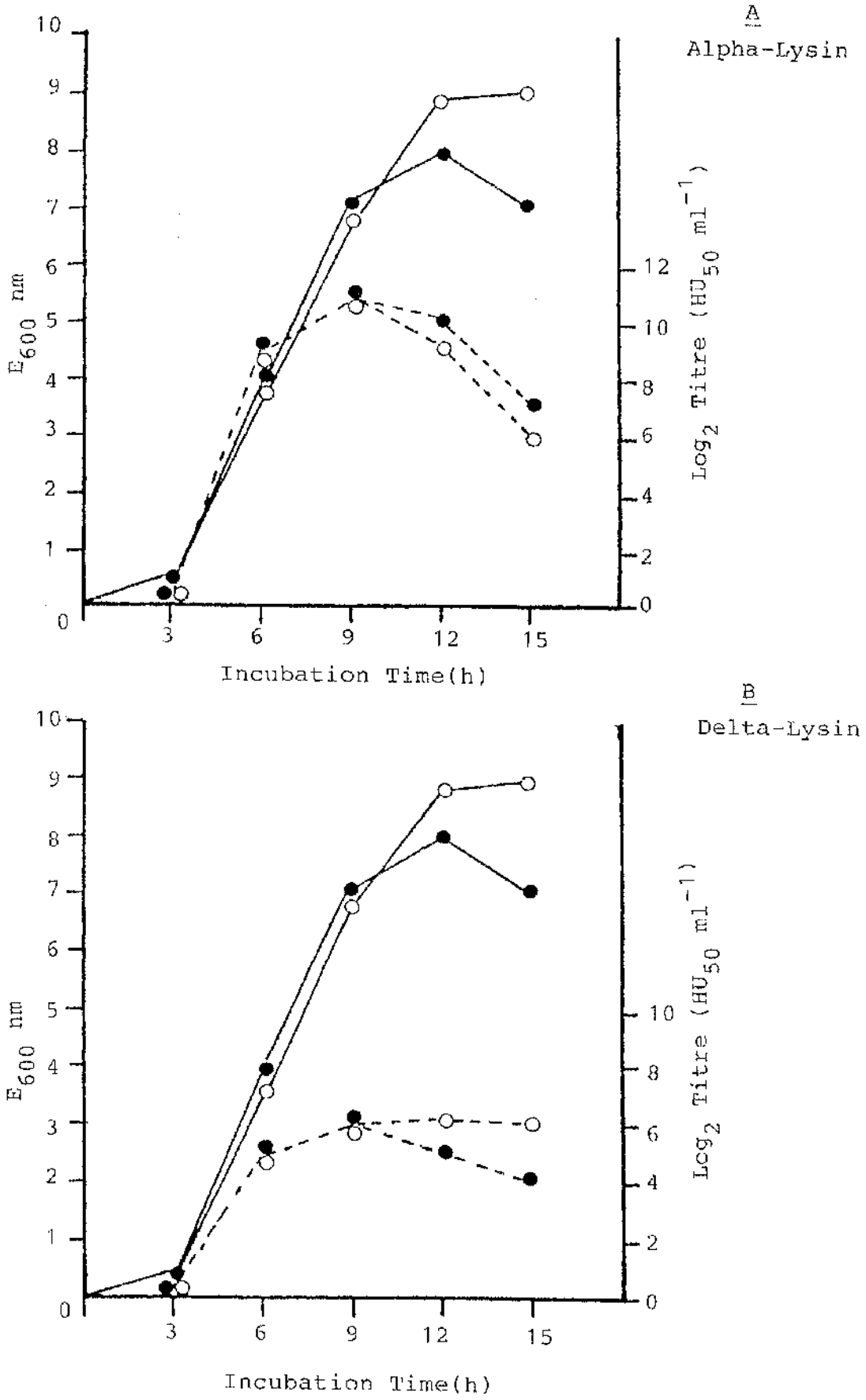

A
Alpha-Lysin
E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time(h)
B
Delta-Lysin
E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time(h)

Figure 28 (A,B). Effect of exogenous stearate on growth and production of α-lysin in cerulenin-inhibited cells.

Key:

——— = E_{600} nm

- - - - = Lysin production

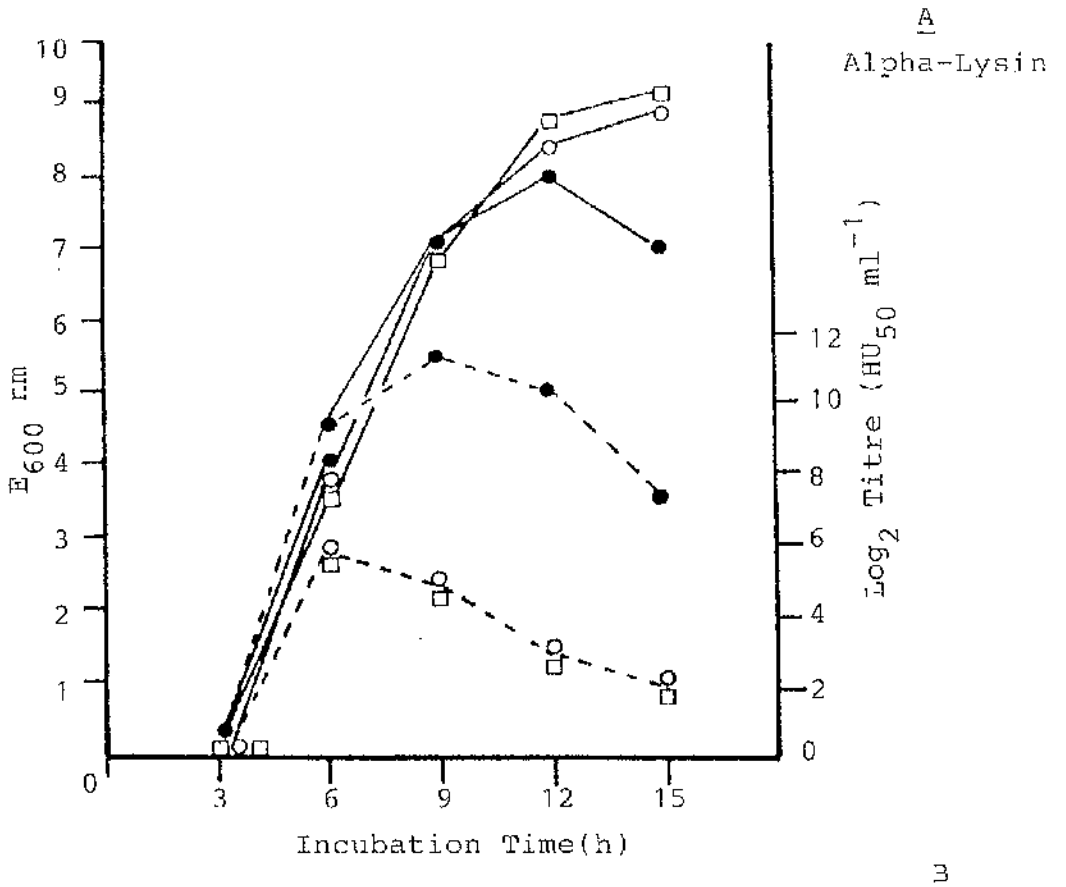
● = Control cells

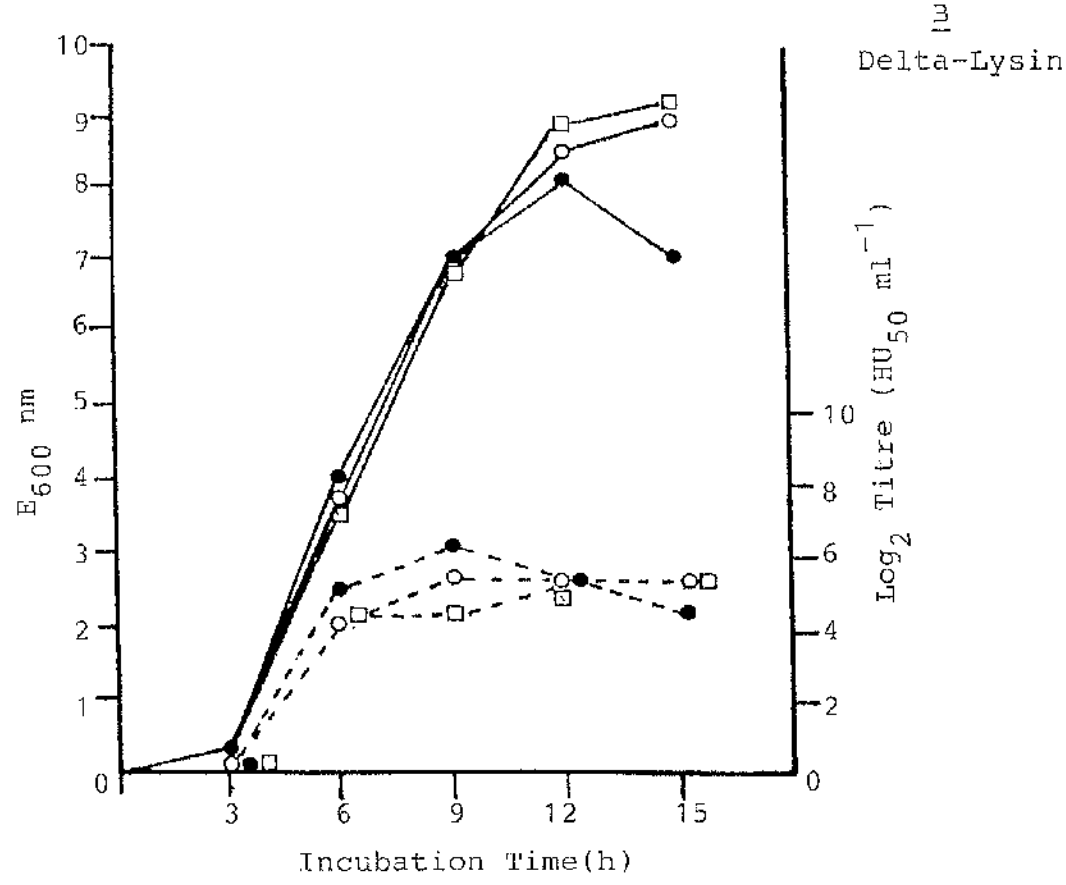
○ = Cerulenin-inhibited cells (8μg ml^{-1})

□ = Cerulenin + stearate supplemented cells(8.3μg ml^{-1})

A = Alpha-lysin production

B = Delta-lysin production


A
Alpha-Lysin
E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time(h)


B
Delta-Lysin
E_{600} nm
Log_2 Titre (HU_{50} ml^{-1})
Incubation Time(h)

production from cerulenin-inhibited cells (Fig. 28B).

In these experiments, production of both α- and δ-lysins was maximal at 9h growth, after which the titre declined, even in the course of α-lysin inhibition by cerulenin and the partial recovery of the lysin by oleate, titres declined after they had reached their maximum.

In experiments on addition of exogenous fatty acids, these were added at 6h growth. No significant effects on growth were observed in the presence of exogenous fatty acids, nor was there any lytic activity when either oleate and stearate at concentrations above 8 μg ml^{-1} were separately titrated against 1% rabbit and cod red blood cell suspensions.

XI. ISOLATION OF TOTAL MEMBRANE FRACTION FROM CONTROL AND CERULENIN-INHIBITED CELLS OF S. AUREUS WOOD 46

Membranes were isolated from control and cerulenin-inhibited cells and protein content was estimated using the method of Bradford (1976). The percentage of membrane in control and cerulenin-inhibited cells was obtained by gravimetric estimation. Table 11 shows that membranes comprised 20% of the total cell dry weight in control cells, whereas this value declined by half in the presence of 8 μg ml^{-1} cerulenin, which was without effect on growth as measured by optical density. The ratio of protein to membrane dry weight increased in the presence of cerulenin. A slight increase in protein occurred in cytoplasm in the

Table 11. Membrane protein content of control and cerulenin-inhibited cells.

Culture	E_{600}^{nm}	Titre Against 1% Rabbit Red Blood Cell Suspension (HU_{50} ml^{-1})	Total Cell Dry Weight (mg)	Membrane Content (%)	Protein: Membrane Dry Weight *	Protein in Cytoplasm *	Protein in Culture Supernate *
Control	10.3	2048	126	19.84	1	1	1
Cerulenin-inhibited (8µg ml^{-1})	10.0	512	126	9.32	1.2	1.2	0.8
Cerulenin-inhibited (20 µg ml^{-1})	7.4	8	82	6.58	1.35	0.96	0.58

* Ratios calculated assuming control value as 1.00

presence of cerulenin and was accompanied by a significant decrease in extracellular protein.

It is noteworthy that after extensive sonication of membrane fractions of both control and cerulenin-inhibited cells, and titration against 1% rabbit red blood cell suspension, titres of 40 and 10 HU_{50} ml^{-1} respectively were obtained for α-lysin.

XII. PROTEIN PROFILES OF CONTROL AND CERULENIN-INHIBITED CELLS OF S. AUREUS (WOOD 46) USING SDS-PAGE

Cerulenin at subminimal growth inhibitory concentrations reduced total extracellular protein production and the protein most sensitive to this inhibition was α-lysin.

Membranes were isolated from control and cerulenin-inhibited cells. Alpha-lysin was present as a species of molecular weight of 34K in control cell membranes (Plate 8, Track 3). However, this band disappeared from cerulenin-inhibited cell membranes (Tracks 4,5,6). The following changes were observed in cerulenin-inhibited cell membranes:

(1) The disappearance of the 34K α-lysin band.

(2) The appearance of two bands of molecular weights approximately 36 and 32K.

(3) The accumulation of a band of molecular weight of approximately 44K.

(4) The disappearance of some of the low molecular weight bands below 27K, the rest of the protein profile was similar to the profile in control membranes. The

Plate 8. SDS-PAGE analysis of membranes, supernates and cytoplasmic fractions of control and cerulenin-inhibited cells.

Key:

1 = An aged purified α-lysin preparation

2 = Sigma SDS VI standard protein

3 = Membranes isolated from control cells

4 = Membranes isolated from cerulenin-inhibited cells ($5\mu g\ ml^{-1}$)

5 = " " " " " " ($8\mu g\ ml^{-1}$)

6 = " " " " " " ($15\mu g\ ml^{-1}$)

7 = Sigma SDS VI standard proteins

8 = Supernate of control cells

9 = Supernate of cerulenin-inhibited cells ($5\mu g\ ml^{-1}$)

10 = " " " " " ($8\mu g\ ml^{-1}$)

11 = " " " " " ($15\mu g\ ml^{-1}$)

12 = Partially purified α-lysin

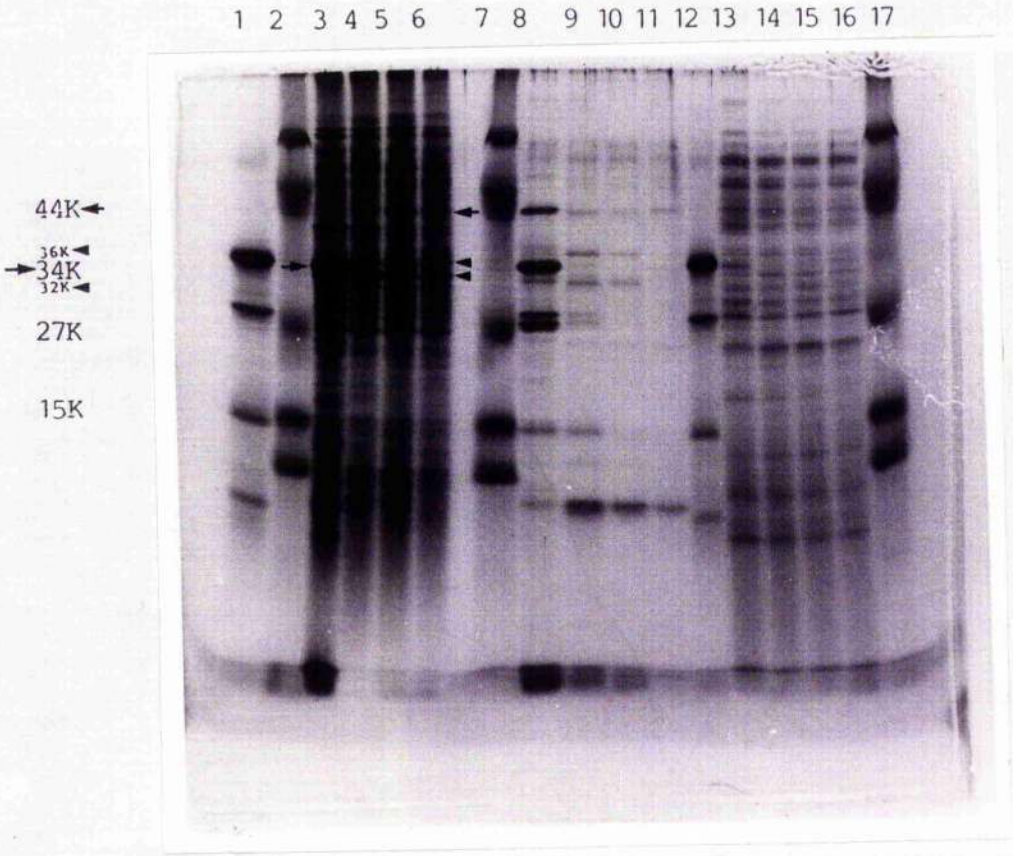
13 = Cytoplasm of control cells

14 = Cytoplasm of cerulenin-inhibited cells ($5\mu g\ ml^{-1}$)

15 = " " " " " ($8\mu g\ ml^{-1}$)

16 = " " " " " ($15\mu g\ ml^{-1}$)

17 = Sigm SDS VI standard proteins


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
44K
36K
34K
32K
27K
15K

34K α-lysin band (Track 8) also disappeared from culture supernates of cerulenin-inhibited cultures (Tracks 9,10, 11).

The cytoplasmic protein profiles of both control and cerulenin-inhibited cells were similar (Tracks, 13, 14,15,16). No protein which corresponded to the 34K α-lysin band was detected in the cytoplasm of cerulenin-inhibited cells, but there was a trace of this component in the cytoplasm of control cells. After extensive washing of control cell membranes with 1M NaCl, the α-lysin band (34K) was still present on SDS-gels.

XIII. PEPTIDE MAPPING BY LIMITED PROTEOLYSIS IN SODIUM DODECYL SULPHATE AND ANALYSIS BY GEL ELECTROPHORESIS

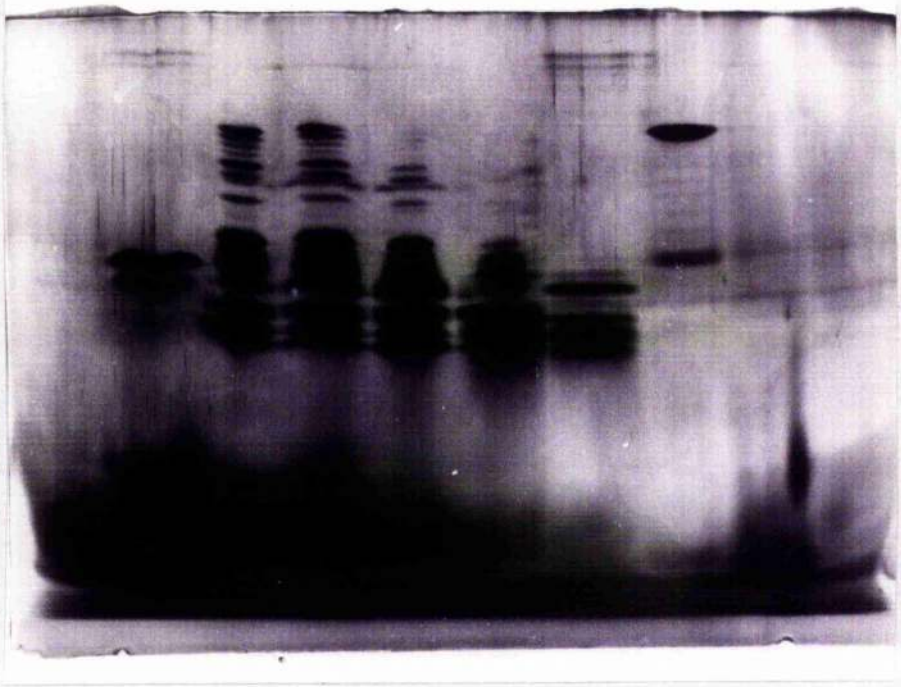
In attempts to identify possible precursor molecules of α-lysin which may have accumulated in the membranes of cerulenin-inhibited cells, bands which may have been putative precursors were excised from SDS-slab gels and subjected to comparison with authentic α-lysin by one dimensional peptide mapping after controlled proteolysis. The method, suitable for analysis of proteins isolated from SDS-gels, involved the partial digestion of proteins by any of several proteases in buffer containing SDS. The partial digests were composed of many peptides whose molecular weight was sufficiently large that their separation on 15% acrylamide-SDS gels was possible. The pattern of peptides so generated was characteristic of the

protein substrate and the proteolytic enzyme.

Peptide mapping was performed for bands in cerulenin-inhibited cell membranes including 36, 32 and 44K bands. The peptides generated from these bands by the action of proteases (α-chymotrypsin, V8 protease and trypsin) were compared to those generated from authentic α-lysin (34K) band. No overall similarity existed between the peptide maps generated from the 36, 32 and 44K bands and that of authentic α-lysin (34K). However, the 34K band in the membrane of control cells generated a similar map to that of authentic α-lysin (34K) (Plates, 9, 10, 11, 12, 13, 14, 15, 16). The degree of similarity between peptides generated from the authentic 34K α-lysin with the 27, 18 and 15K bands which appeared upon storage of purified α-lysin (34K) was investigated. Similarities were evident between the 12 peptides generated from the 34K lysin band and those generated from the 27K and 18K bands. The 27 and 18K components shared 7 and 5 of the 12 peptides generated from the authentic lysin band (34K). However, the 15K band shared only one peptide with those of the authentic α-lysin (34K), 27K and 18K bands (Plate 16).

XIV IMMUNOLOGICAL ANALYSIS FOR THE PRESENCE OF ALPHA-LYSIN PRECURSOR IN SUPERNATES OR MEMBRANES OF CERULENIN-INHIBITED CELLS OF *S. AUREUS* WOOD 46

A single precipitin line of identity was formed in double diffusion tests in agar between purified α-lysin, culture supernate and sonicated membrane of control cells



1 2 3 4 5 6 7

Plate 9. Peptide mapping of the authentic α-lysin (34K) band using α-chymotrypsin at different concentrations.

Key:

1 = Undigested standard proteins (Lysozyme 14.3K; Myoglobin 17K)

2 = Authentic α-lysin (34K) band + 0.2 µg α-chymotrypsin

3 = " " " " + 0.4 µg "

4 = " " " " + 0.8 µg "

5 = " " " " + 1.0 µg "

6 = 1 µg of α-chymotrypsin

7 = Undigested (34K) α-lysin

Plate 10. Comparison of proteolytic digests of control and cerulenin-inhibited cell membranes with authentic α-lysin (34K) band by peptide mapping using α-chymotrypsin.

Key:

1 = Standard proteins (Lysozyme, Myoglobin)

2 = 0.2 µg of α-chymotrypsin

3 = Undigested α-lysin (34K) band

4 = Authentic α-lysin (34K) band + 0.2 µg α-chymotrypsin

5 = 34K band from control cell membranes + 0.2 µg α-chymotrypsin

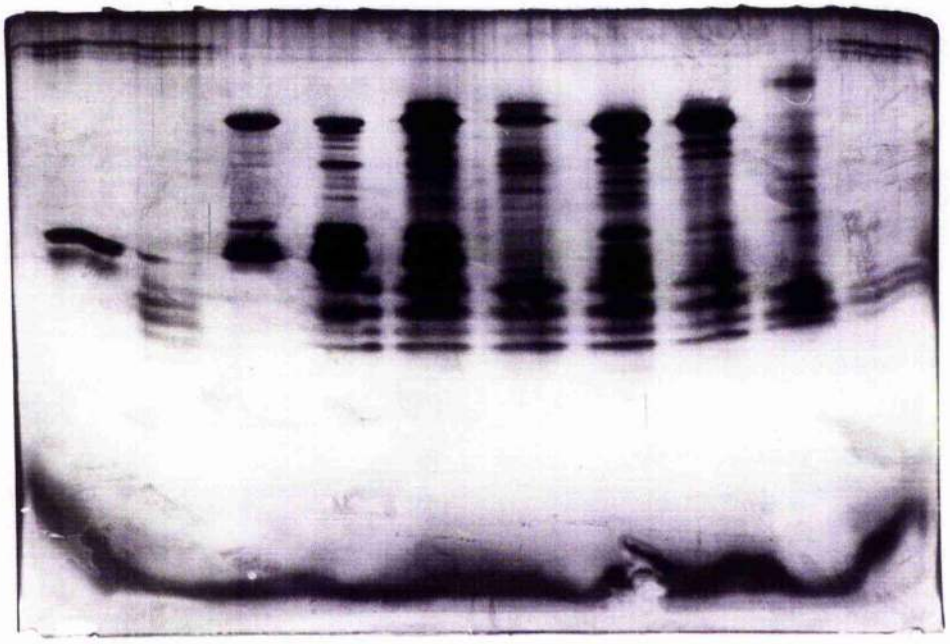
6 = 36K band in cerulenin-inhibited cell membranes (8µg ml^{-1}) + 0.2 µg α-chymotrypsin

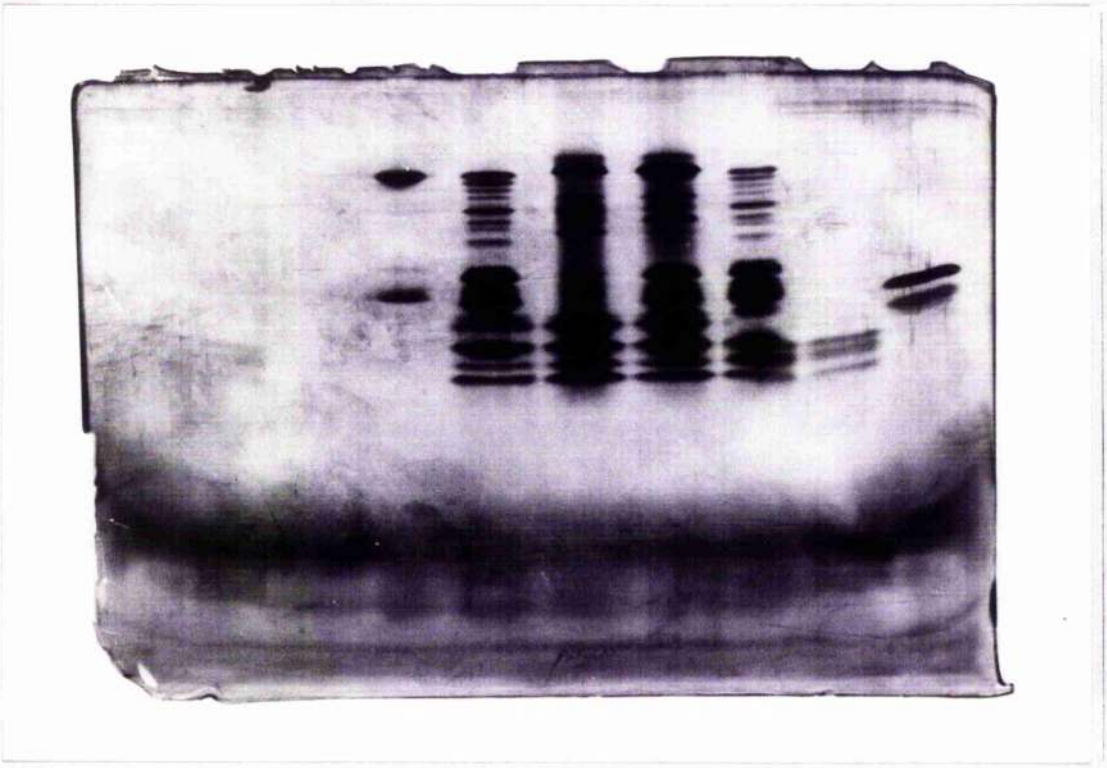
7 = 32K band in control cell membranes + 0.2 µg α-chymotrypsin

8 = 32K band in cerulenin-inhibited cell membranes + 0.2 µg α-chymotrypsin

9 = 44K band in cerulenin-inhibited cell membranes + 0.2 µg α-chymotrypsin

10 = 0.2 µg of α-chymotrypsin


34K
14K
1 2 3 4 5 6 7 8 9 10



1 2 3 4 5 6 7

Plate 11. Comparison of digests of 34K and 36K bands from control and cerulenin-inhibited cell membranes with authentic α-lysin (34K) band by peptide mapping using α-chymotrypsin.

Key:

1 = Undigested α-lysin (34K) band

2 = Authentic α-lysin (34K) band + 0.4 μg α-chymotrypsin

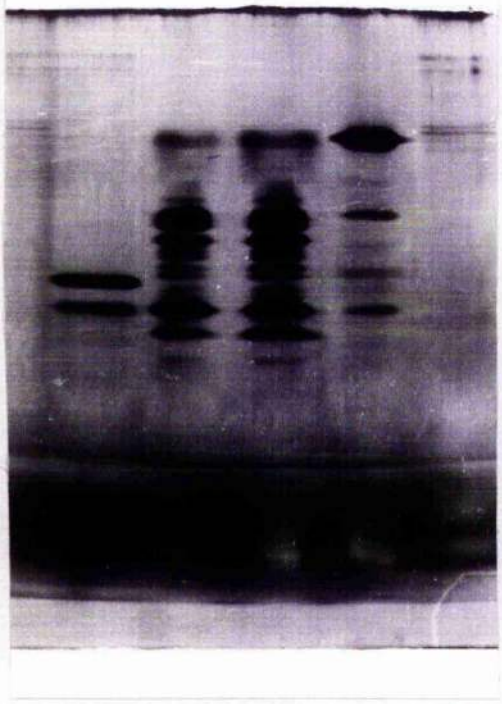
3 = 36K band in cerulenin-inhibited cell membranes ($8 \mu g\ ml^{-1}$) + 0.4 μg α-chymotrypsin

4 = 34K band in control cell membranes + 0.4 μg α-chymotrypsin

5 = Authentic α-lysin (34K band)+ 0.4 μg α-chymotrypsin

6 = 0.4 μg of α-chymotrypsin

7 = Standard proteins (Lysozyme, Myoglobin).



1 2 3 4 5

Plate 12. Peptide mapping of the authentic α-lysin (34K) band using V8 protease at different concentrations.

Key:

1 = Standard proteins (Lysozyme, Myoglobin)

2 = Authentic α-lysin (34K) band + 0.6 µg V8 protease

3 = " " " " + 0.2 µg " "

4 = Undigested α-lysin (34K) band

5 = 0.2 µg of V8 protease

Plate 13. Comparison of different bands in control and cerulenin-inhibited cell membranes with authentic α-lysin (34K) band by peptide mapping using V8 protease.

Key:

1 = Standard proteins (Lysozyme, Myoglobin)

2 = 0.6 μg of V8 protease

3,4 = Alpha-lysin (34K) band + 0.5 μg V8 protease

5 = 34K band in control cell membranes + 0.6 μg V8 protease

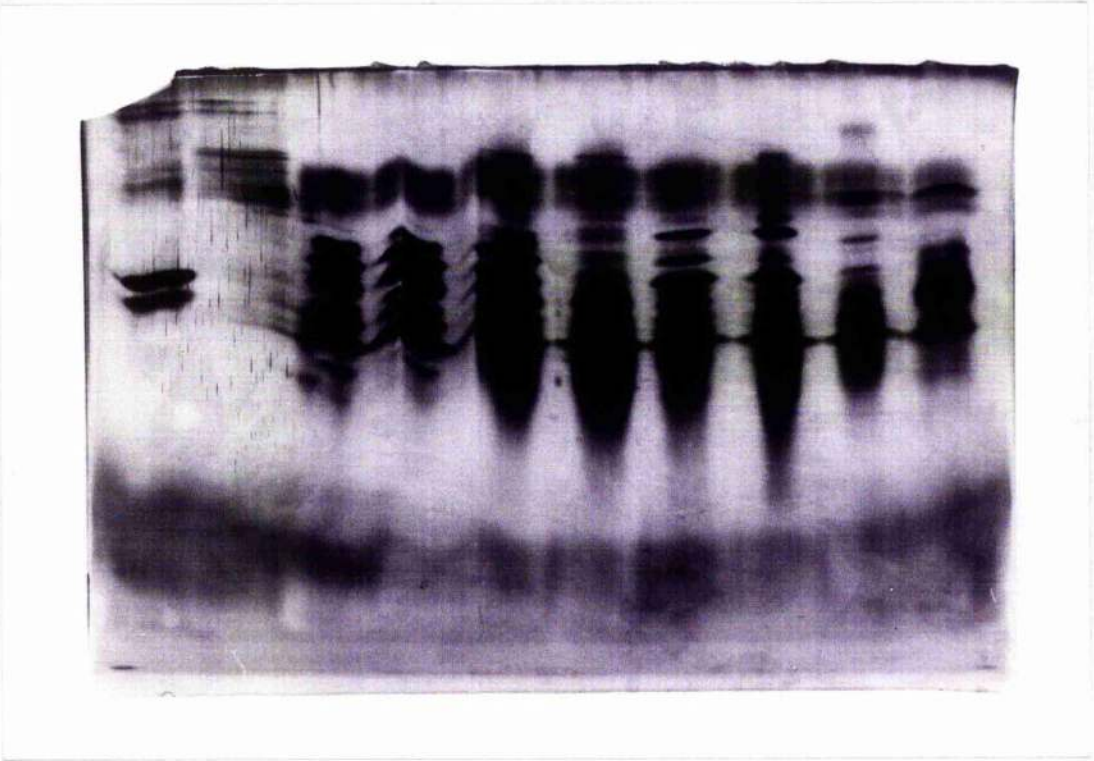
6 = 36K band in cerulenin-inhibited cell membranes (8μg ml^{-1}) + 0.6 μg V8 protease

7 = 32K band in control cell membranes + 0.6 μg V8 protease

8 = 32K band in cerulenin-inhibited cell membranes + 0.6 μg V8 protease

9 = 44K band in cerulenin-inhibited cell membranes + 0.6 μg V8 protease

10 = 44K band in lysostaphin



1 2 3 4 5 6 7 8 9 10

Plate 14. Comparison of digests of 34K and 36K bands from control and cerulenin-inhibited cell membranes with authentic α-lysin (34K) band by peptide mapping using V8 protease.

Key:

1 = Undigested aged purified α-lysin preparation.

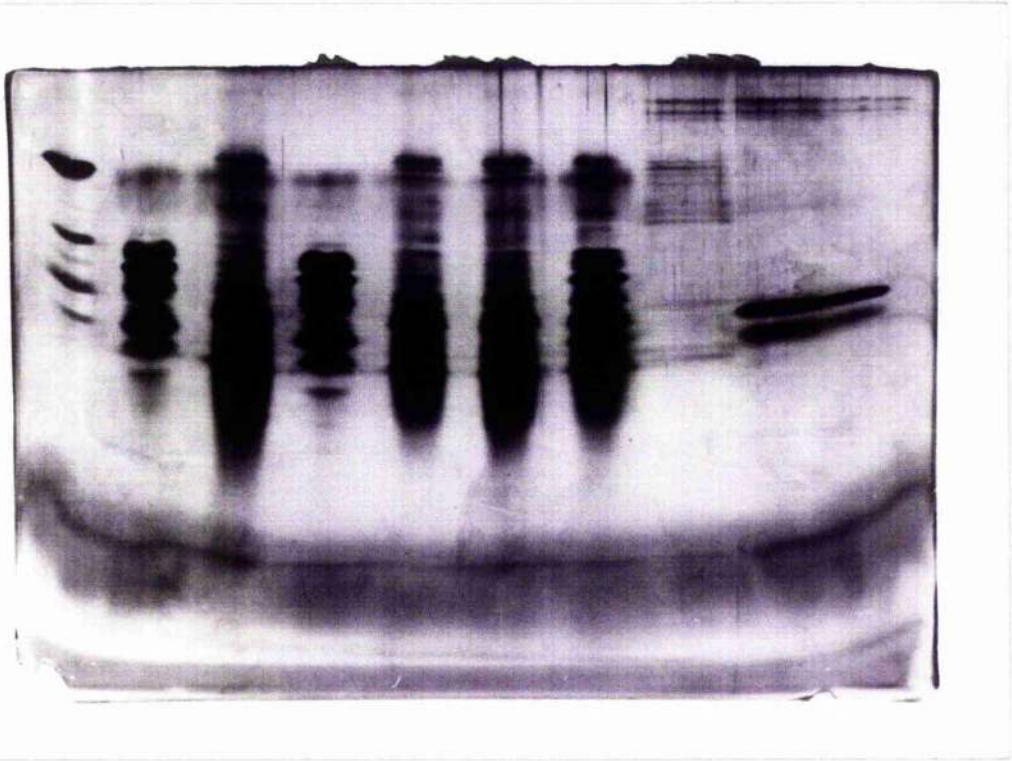
2,4 = Alpha-lysin (34K) band + 0.2 µg V8 protease

3,5,6 = 36K band in cerulenin-inhibited cell membranes (8µg ml^{-1}) + 0.2 µg V8 protease

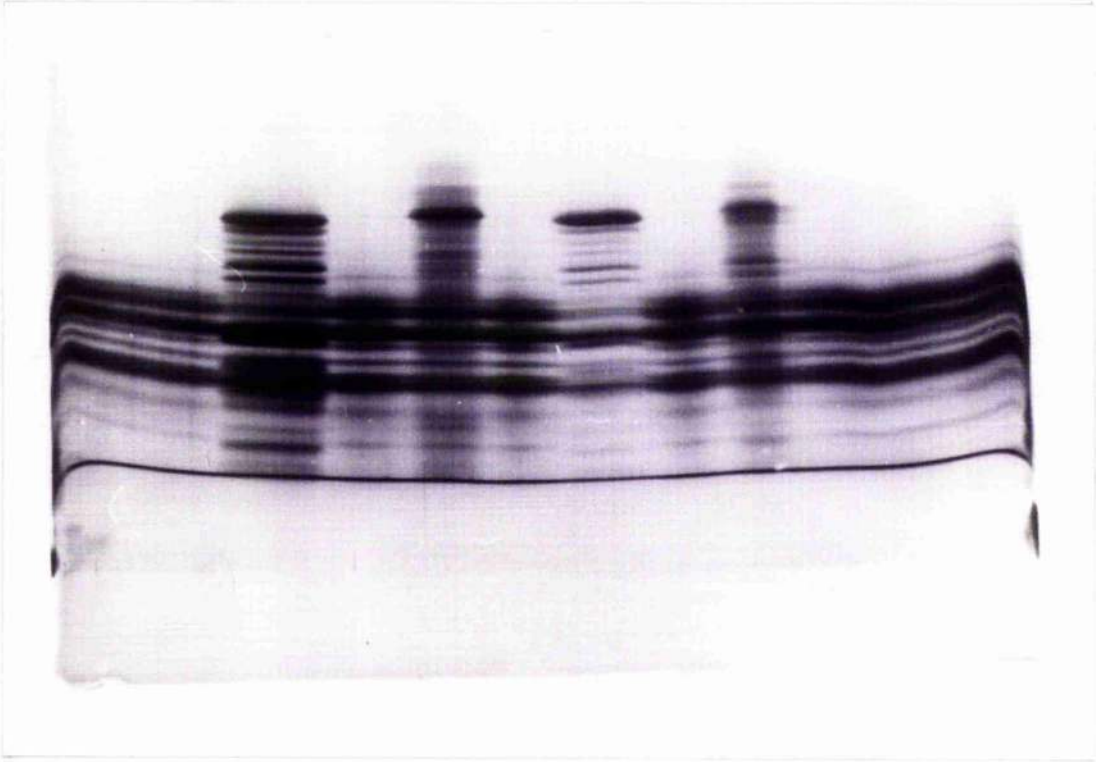
7 = 34K bands in control cell membranes + 0.2 µg V8 protease

8 = 0.2 µg V8 protease

9 = Standard proteins (Lysozyme + Myoglobin)



1 2 3 4 5 6 7 8 9



1 2 3 4

Plate 15. Comparison of digests of 34K and 36K bands from control and cerulenin-inhibited cell membranes with authentic α-lysin (34K) band by peptide mapping using trypsin.

Key:

1 = Alpha-lysin (34K) band + 0.4 µg trypsin

2 = 36K band in cerulenin-inhibited cell membranes ($8\mu g\ ml^{-1}$) + 0.4 µg trypsin

3 = Alpha-lysin (34K) band + 0.4 µg trypsin

4 = 34K band in control cell membranes + 0.4 µg trypsin

Plate 16. Comparison of different bands in cerulenin-inhibited cell membranes, control and cerulenin-inhibited cell culture supernates with authentic α-lysin (34K) band by peptide mapping using trypsin.

Key:

1 = Bovine serum albumin + 0.4 µg trypsin

2 = 44K band in cerulenin-inhibited cell membranes (8µg ml^{-1}) + 0.4 µg trypsin

3 = 36K band in cerulenin-inhibited cell membranes + 0.4 µg trypsin

4 = 32K band in cerulenin-inhibited cell membranes + 0.4 µg trypsin

5 = Authentic α-lysin (34K) band + 0.4 µg trypsin

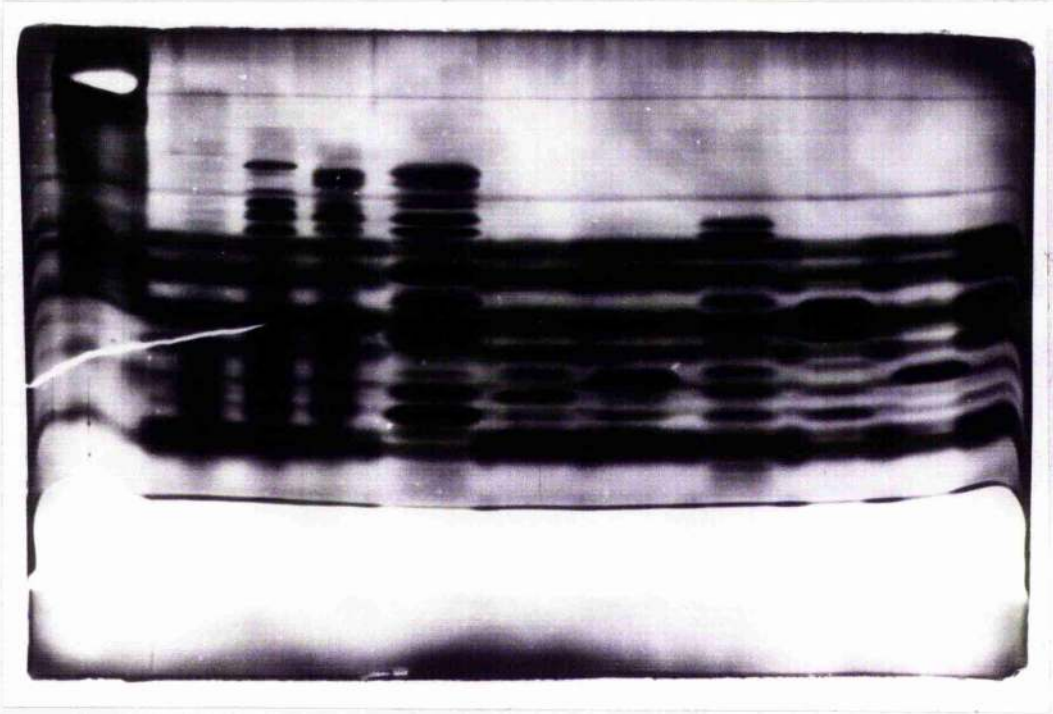
6 = 44K band in control culture supernate + 0.4 µg trypsin

7 = 15K band in purified α-lysin preparation + 0.4 µg trypsin

8 = 27K band " " " " + " " "

9 = 18K band " " " " + " " "

10 = 15K band in cerulenin-inhibited cell culture supernate + 0.4 µg trypsin.



1 2 3 4 5 6 7 8 9 10

when these were allowed to react with anti- α-lysin serum. However, neither membranes nor culture supernate of cerulenin-inhibited cells showed any precipitin reaction when allowed to diffuse against anti α-lysin serum (Plate 17). Anti δ-lysin gave a single precipitin line of identity between purified δ-lysin and culture supernate of control and cerulenin-inhibited cells, while a line of partial identity was produced between purified δ-lysin and both control and cerulenin-inhibited cell membranes (Plate 18).

XV. TIME COURSE OF LYSIS OF CONTROL AND CERULENIN-INHIBITED CELLS OF S. AUREUS (WOOD 46) IN THE PRESENCE AND ABSENCE OF LYSOSTAPHIN

Enhanced sensitivity of cerulenin-inhibited cells to lysis by lysostaphin, was an interesting feature observed in the course of membrane preparation. To investigate this phenomenon further, the time course of cell lysis was monitored at 37°C in the presence and absence of lysostaphin.

Cerulenin-inhibited cells were more resistant to autolysis than control cells (Fig. 29). This resistance was such that cells inhibited by 15 μg ml^{-1} cerulenin were more resistant to autolysis than cells inhibited with 8 μg ml^{-1} cerulenin. However, after addition of lysostaphin to these cell types, cells inhibited with 15 μg ml^{-1} cerulenin were highly sensitive to lysostaphin and reached 50% lysis within 15 to 25 min whereas control

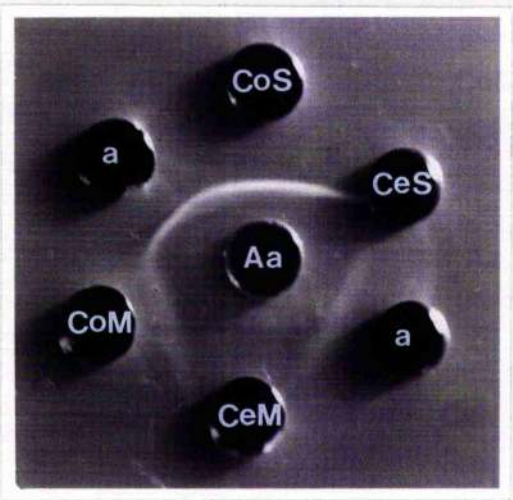


Plate 17. Double immunodiffusion in agar of α-lysin, control and cerulenin-inhibited culture supernates and membranes run against anti-α-lysin serum.

Key:

Aa = Anti-α -lsyin

a = Purified α-lysin

CoS = Culture supernate of control cells

CeS = Culture supernate of cerulenin-inhibited cells ($8 \mu g\ ml^{-1}$)

CoM = Membranes of control cells

CeM = Membranes of cerulenin-inhibited cells

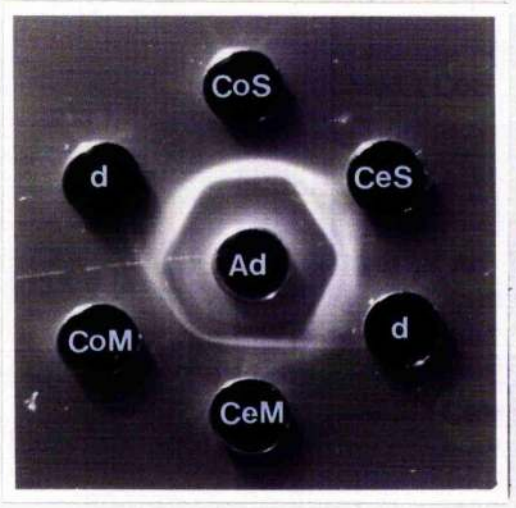


Plate 18. Double immunodiffusion in agar of δ-lysin, control and cerulenin-inhibited cell culture supernates and membranes against anti-δ-lysin serum.

Key:

Ad = Anti- δ-lysin

d = Purified δ-lysin

CoS = Culture supernate of control cells

CeS = Culture supernate of cerulenin-inhibited cells (8μg ml^{-1}

CoM = Membranes of control cells

Cem = Membranes of cerulenin-inhibited cells

Figure 29. Time course of lysis of control and cerulenin-inhibited cells in the presence (10 μg ml^{-1}) and absence of lysostaphin.

Key:

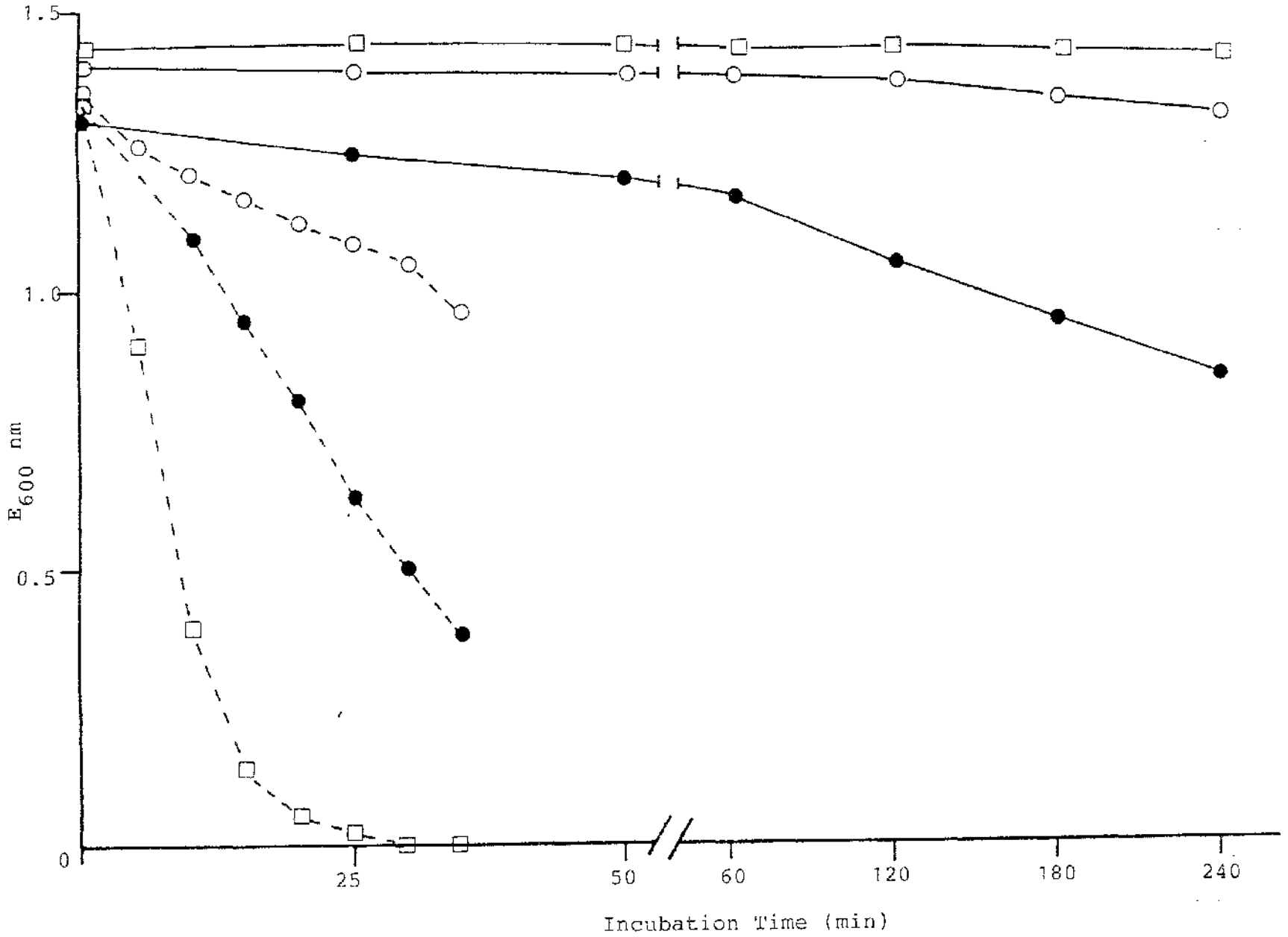
——— = No lysostaphin

– – – – = Lysostaphin (10 μg ml^{-1})

● = Control cells

○ = Cells inhibited with 8 μg ml^{-1} cerulenin

□ = Cells inhibited with 15 μg ml^{-1} cerulenin


1.5
1.0
0.5
0
E_{600} nm
25
50
60
120
180
240
Incubation Time (min)

and cells inhibited with 8 μg ml^{-1} cerulenin were relatively less sensitive to lysis by lysostaphin (Fig. 29). The sensitivity was such that control cells were relatively more sensitive to lysis by lysostaphin than cells treated with 8 μg ml^{-1} cerulenin, but this difference in sensitivity was less when the lysostaphin concentration was increased (Fig. 30).

XVI. MORPHOLOGY OF CONTROL AND CERULENIN-INHIBITED CELLS OF *S. AUREUS* (WOOD 46) UNDER PHASE CONTRAST AND ELECTRON MICROSCOPY

No significant changes in morphology were observed between control and cerulenin-inhibited cells by either phase contrast microscopy or examination of thin sections by electron microscopy (Plate 19 and 20). However, it is noteworthy that cerulenin inhibition was accompanied by loss of pigmentation and increased stickiness of cells as evidenced by their adhesion to glass.

Figure 30. Time course of lysis of control and cerulenin-inhibited cells in the presence (35 μg ml^{-1}) and absence of lysostaphin.

Key:

——— = No lysostaphin

– – – = Lysostaphin (35 μg ml^{-1})

● = Control cells

○ = Cells-inhibited with 8 μg ml^{-1} cerulenin

□ = Cells-inhibited with 15μg ml^{-1} cerulenin

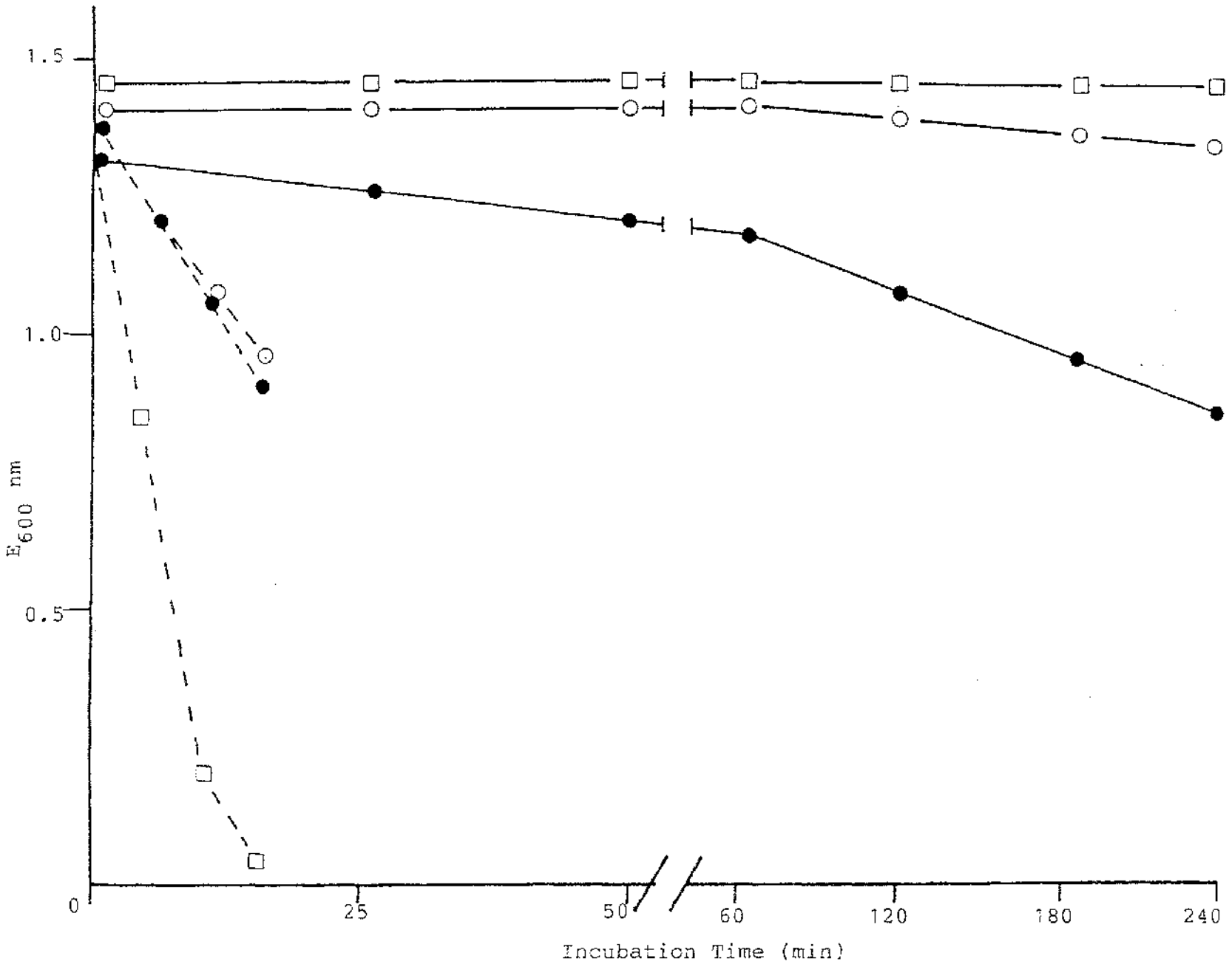

1.5
1.0
0.5
0
E_{600} nm
25
50
60
120
180
240
Incubation Time (min)

Plate 19. Electron micrographs of thin sections of control cells of *S. aureus* Wood 46.

Magnification = X 63,000

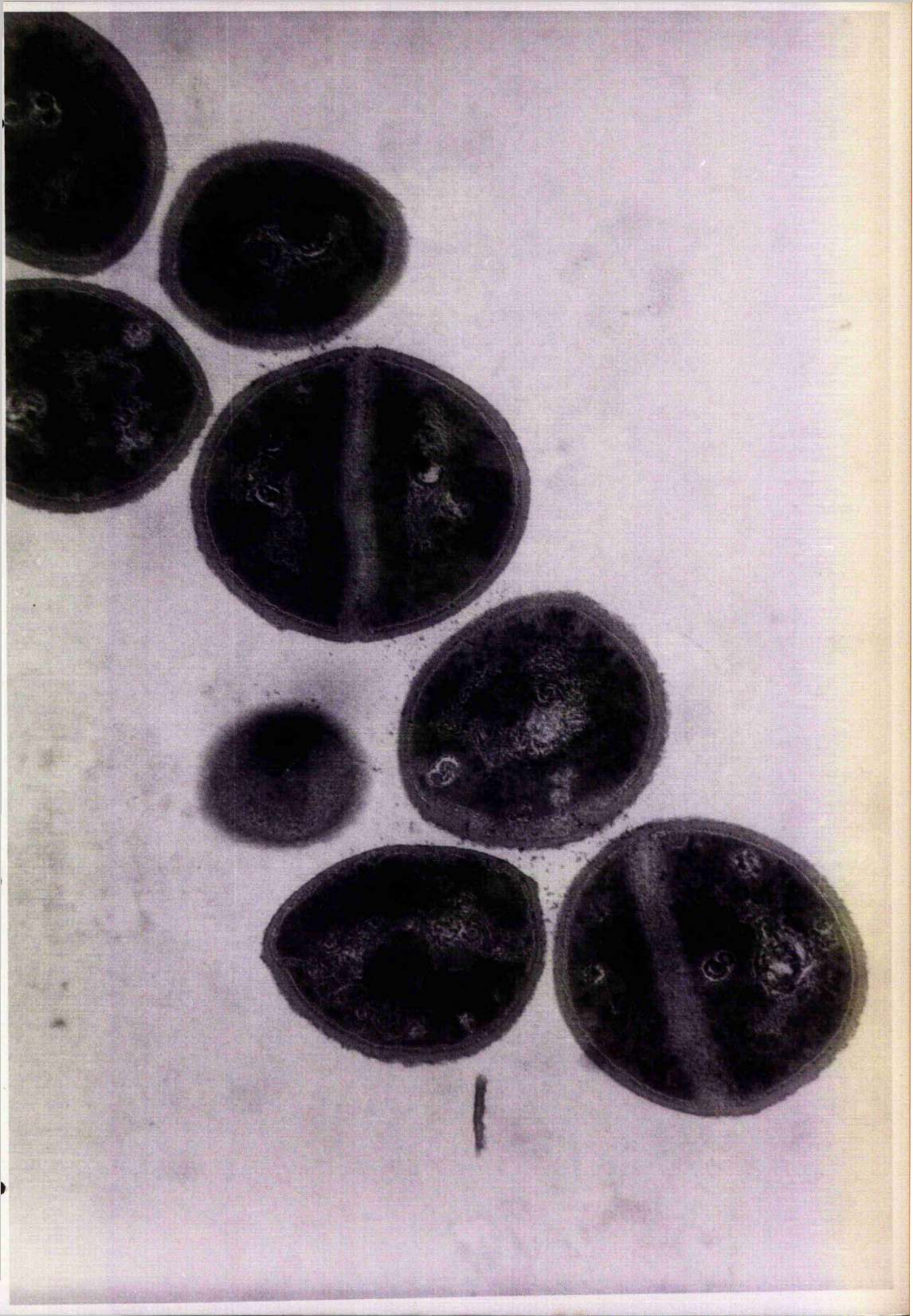
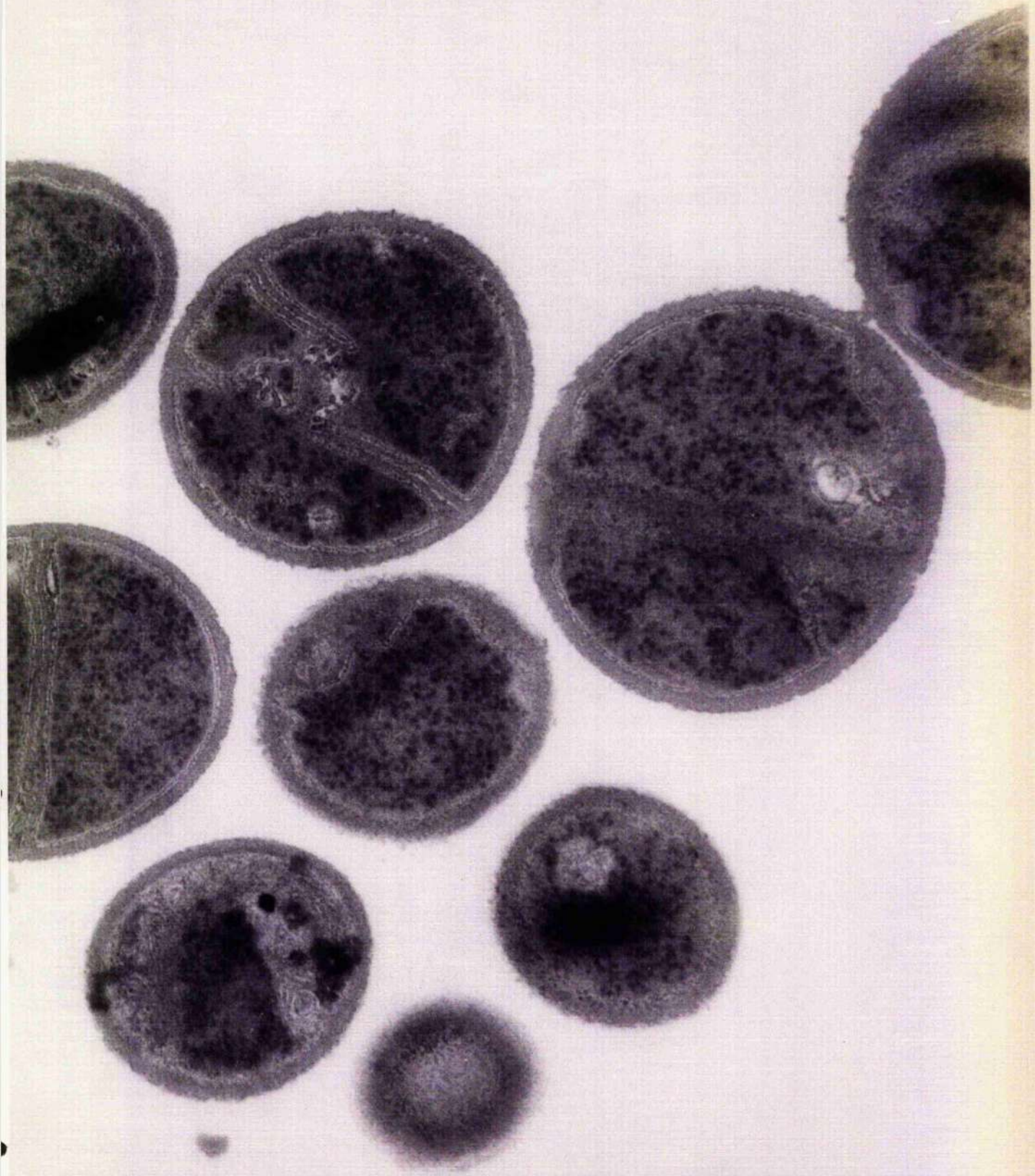


Plate 20. Electron micrographs of thin sections of cerulenin-inhibited cells (8µg ml^{-1}) of *S. aureus* Wood 46.

x 63 000



DISCUSSION

I. PRODUCTION OF MEMBRANE DAMAGING LYSINS AND PROTEASE(S) DURING GROWTH OF S. AUREUS WOOD 46

The experiments described show that the growth characteristics of S. aureus (Wood 46) in batch culture consist of an initial lag period before a relatively extended period of exponential growth, which is followed by a period of slower growth which precedes stationary phase.

Production of both α- and δ-lysins started at the beginning of exponential growth and continued to increase with maximum titres of biologically-active lysins in culture supernates during the post-exponential phase of growth, with a marked increase just prior to the onset of this phase. This pattern of lysin production was also confirmed by SDS-PAGE of culture supernates, which showed a sudden increase in the intensity of bands corresponding to both α- and δ-lysins at approximately 9h of growth. Production of both β-lysin and protease(s) started at mid-exponential growth reaching their maximum titre in early stationary phase similar to α-lysin. These results are in agreement with those of Mangalo, Pillet and Raynaud (1954) who reported that α-lysin is liberated into the culture medium during the exponential phase of growth, after a lag phase of approximately 3h. Gladstone and Glencross (1959) also noted that the course of α-lysin production lagged slightly behind growth. Production of α-lysin followed an apparent biphasic pattern.

This was also reported by Duncan and Cho (1971) and McNiven and Arbuthnott (1972).

The present results showed that α-lysin and total extracellular protein production of Wood 46 occurs during the post-exponential period of slower growth suggesting that lysin production, particularly α-lysin, does not simply parallel growth of the culture. Coleman and Abbas-Ali (1977) reported that at higher growth rates and accompanying higher rates of cellular protein synthesis, there is a corresponding reduction in the availability of biosynthetic machinery for extracellular protein synthesis.

It has been reported that α-lysin is released rapidly after synthesis without intracellular accumulation (Duncan and Cho, 1971; McNiven and Arbuthnott, 1972). SDS-PAGE analysis of the cytoplasmic proteins of Wood 46 grown for 15h showed no band of α-lysin. This is compatible with current ideas on the synthesis and release of exported proteins (Davis and Tai, 1980), which may involve concurrent synthesis on membrane bound polysomes and export through the membrane.

In some experiments, a decline in the activities of both α- and δ-lysins occurred after they had reached their maximum titre at the end of exponential growth. This was also observed by others (Gladstone and Glencross, 1959; Duncan and Cho, 1972). Duncan and Cho (1972)

suggested that the observed decline in α-lysin activity could be a result of surface denaturation. No doubt a contribution towards the decline of α-lysin activity in culture supernates comes from the activity of extracellular protease(s). Indeed the inclusion of protease inhibitors in the buffers during purification of α-lysin leads to an increase in total activity of the purified lysin.

II. PURIFICATION OF ALPHA-LYSIN

The procedure used, although based on the method of Cassidy and Harshman (1976a), differed from the original method in several respects. Firstly, proteins of the culture supernate were precipitated by ammonium sulphate prior to chromatography on controlled pore glass (CPG). This served two purposes:

a. It acted as a purification step, by separating the proteins from the lower molecular weight materials.

b. It reduced the time required to load the crude lysin on the CPG column, thus decreasing the exposure of the lysin to extracellular proteases. Secondly, chromatography on CPG yielded a four-fold increase in the specific haemolytic activity of α-lysin, and reduced levels of contaminating β-lysin, δ-lysin and protease(s). To reduce these levels further, Cassidy and Harshman (1976a) employed ion-exchange chromatography on DEAE-Sephadex.

The present procedure replaced the ion-exchange step with isoelectric focusing. This method has the advantage of being both preparative in separating α-lysin from contaminating material and analytical in determining the pI of the purified α-lysin. The electrofocusing step increased the specific haemolytic activity of the α-lysin another four-fold, and the levels of δ-lysin and protease(s) were reduced further. The "Rabbit:Cod" haemolytic ratio of 20,000, if calculated on an equal weight basis, gives the level of contamination of purified α-lysin by δ-lysin at < 0.7 %. Some protease activity could be detected in purified α-lysin at 0.5 $mgml^{-1}$, the level of detection of protease in the assay used being 2 μgml^{-1}. The acidic material of pI 4.5-5.5 which was eluted from the CPG column contained the highest proteolytic activity. This pI is close to two of the three reported staphylococcal proteases, Protease I of pI 4.0 and protease III of pI 5.0 (Arvidson, Holmes and Lindholm, 1973). Of these two, the observed proteolytic activity is more likely due to protease I, as protease III is a metalloprotease, requiring Ca^{2+} for activity (Arvidson, 1973).

No 'hot-cold' haemolysis, characteristic of β-lysin, was observed at any purification stage, suggesting that the apparent increase in β-lysin-like activity after electrofocusing was probably due to α-lysin, which also lyses sheep erythrocytes, although not as well as it does

rabbit erythrocytes.

A single precipitin line was formed when the purified α-lysin was analysed by immunodiffusion with antiserum against crude α-lysin and purified α-lysin. Purified α-lysin was present as a species of molecular weight of 34,000 dalton plus a minor band of 27,000 dalton when analysed by SDS-PAGE. Storage of purified α-lysin in saturated ammonium sulphate at 4°C over a period of 10 months caused an increase in the intensity of the component of 27,000 dalton plus the appearance of components with molecular weights of 18,000 and 15,000 daltons. These bands probably represent proteolytic degradation products of α-lysin as reported by Dalen (1975). Results of peptide mapping revealed overall similarities in the patterns of peptides generated from the 34,000, the 27,000 and 18,000 dalton components. The peptides generated from the 15,000 dalton band were less clear, but a major peptide was similar to a major peptide of both the 27,000 and 18,000 dalton components.

The estimation of the molecular weight of purified α-lysin (34,000 dalton) is in general agreement with published work (Forlani, Bernheimer and Chiancone, 1971; McNiven, Owen and Arbuthnott, 1972; Six and Harshman, 1973b; Watanabe and Kato, 1974; Dalen, 1975), as is the pI (8.5) estimation (Wadström, 1968; McNiven, Owen and Arbuthnott, 1972; Six and Harshman, 1973b; Good and Baldwin, 1973; Dalen, 1975).

III. EFFECT OF PROTEASE INHIBITORS (PHENYLMETHYLSULFONYL FLUORIDE, O-PHENANTHROLINE AND QUINACRINE), PROCAINE AND CERULENIN ON GROWTH AND HAEMOLYSIN PRODUCTION BY S.AUREUS WOOD 46

The mechanism of export of most extracellular bacterial proteins is, in general, obscure. However, our overall understanding of the details of synthesis and secretion of exoproteins has been significantly deepened since the formulation of the signal hypothesis of Blobel and Dobberstein (1975 a,b). As described in detail in the introduction, the essence of this theory is that translation of messenger RNA yields a polypeptide chain longer than that of the mature, extacellular form of the protein. During translation a specific protease, bound to the membrane, cleaves the nascent polypeptide chain, removing a 23-25-amino acid hydrophobic peptide anchor, thus permitting discharge of the final mature exoprotein to the exterior.

This general mechanism seems to apply to the formation of several proteins in both eukaryotes and prokaryotes. To test this theory, the effect of several protease inhibitors on the production of bacterial extracellular proteins was explored. Phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) and other protease inhibitors stop secretion of enterotoxin B in S. aureus (Altenbern, 1978). Quinacrine interferes with the action of penicillinase-releasing activity in Bacillus lichenformis 749/C (Traficante and Lampen, 1977). Production of

levansucrase by Bacillus subtilis is inhibited by both quinacrine and O-phenanthroline (Caulfield et al., 1979). Quinacrine also inhibits the production of enterotoxin A and coagulase by S.aureus (Berkeley et al., 1978; Engels and Kamps, 1981). Production of cytolytic toxins (haemolysins) by S.aureus also involves the synthesis and export to the cell exterior of sizable polypeptides.

Following the investigations quoted above, the effect of certain protease inhibitors on produciton of haemolysins by S.aureus (Wood 46) was studied. Phenylmethylsulfonyl fluoride (PMSF), quinacrine and O-phenanthroline (a metalloprotease inhibitor), were used in this study. The production of α-, β- and δ-lysins were not affected by any of these three protease inhibitors at concentrations below those which inhibited growth.

The lack of any effect of PMSF, quinacrine or O-phenanthroline on production of haemolysins by S.aureus may indicate the absence of a proteolytic cleavage mechanism in the processing of precursor haemolysins of S.aureus. Alternatively, a proteolytic mechanism may be required but the proteases involved may have unusually narrow substrate specificity and therefore the inhibitors used here may not have been active in the system.

Lazdunski, Baty and Pages (1979) reported that treatment of E.coli strain K12 with 0.5% procaine (a local anaesthetic) resulted in the accumulation of precursors

and mature forms of the two periplasmic proteins alkaline phosphatase and glutamine-binding protein. They suggest that an increase in molecular packing of the lipid bilayer in the membrane induced by procaine may have caused a change in the physical state of the membrane sufficient to impair the transport of these proteins. Procaine was also reported to selectively suppress the production of alkaline phosphatase and penicillinase by Bacillus lichenformis 749 at 3 $mgml^{-1}$ and also penicillinase by Bacillus cereus 569 at 6 $mgml^{-1}$ without further effects on growth (Fishman, Rottem and Citri, 1980). Concentrations of procaine from 2 $mgml^{-1}$ to 25 $mgml^{-1}$ had no effect on α-lysin production, but concentrations above 5 $mgml^{-1}$ significantly reduced growth. Again these results are in keeping with an export mechanism which differs from that for alkaline phosphatase and penicillinase in other Gram-positive bacteria.

Some recent observations suggest a possible link between lipid synthesis and secretion of proteins in bacteria. Cerulenin, a specific inhibitor of "fatty acid synthetase" (Ōmura, 1976) inhibits the secretion of enterotoxins A, B and C, and Staphylocoagulase by S.aureus (Altenbern, 1977a; Berkeley et al., 1978; Engels and Kamps, 1981), penicillinase by B.lichenformis (Fishman, Rottem and Citri, 1978) and levansucrase, α-amylase and protease by B.subtilis (Caulfield et al., 1979; Paton, May and Elliott, 1980; Mäntsälä, 1982). In these studies cerulenin was

used at sub growth inhibitory concentrations. Based on the above reports, the effect of cerulenin on the production of α-, β- and δ-lysins and protease(s) by S.aureus (Wood 46) was tested. Cerulenin, at low concentrations (0.5-2.5 $\mu g ml^{-1}$), significantly reduced the production of α- and β-lysins and protease(s) with a relatively lower effect on δ-lysin production. At these concentrations, the effect on growth was insignificant.

Variation in the degree of inhibition of α-lysin production by similar concentrations of cerulenin was observed when different batches of growth medium were prepared using yeast extract from the same manufacturer. However, greater variation in the degree of inhibition of α-lysin production by similar concentrations of cerulenin was found between cultures in media prepared from yeast extract obtained from different suppliers such as Oxoid and Difco. This variation could be due to the presence of different levels of exogenous fatty acids in the yeast extract diffusate which partially relieved the inhibition of α-lysin secretion by cerulenin. Gas liquid chromatography (GLC) showed certain short chain saturated and unsaturated fatty acids in yeast extracts obtained from both Oxoid and Difco. During a study of the effect of moderate concentrations of saturated and unsaturated fatty acids on growth and enterotoxin B production by S.aureus 14458, variability in SEB/OD ratios

and in the inhibitory activity of various fatty acid mixtures, were attributed to variable amounts of phospholipids in yeast extract (Altenbern, 1977b).

Prolonged solvent extraction of yeast extract with petroleum ether prior to use in BS medium did not totally eliminate the variable degree of inhibition of α-lysin production by cerulenin. Cultures growing in medium containing Oxoid yeast extract showed reproducible inhibition of α-lysin production between 8 and 10 $\mu g\ ml^{-1}$ cerulenin without a significant effect on growth whereas, those grown in medium containing Difco yeast extract showed a more variable response to the same levels of cerulenin as observed by Altenbern (1977a) for several other strains of S.aureus.

At sub-growth-inhibitory concentrations, cerulenin occasionally caused a small increase in OD_{600} relative to that of the control culture and also promoted attachment of bacteria to the wall of the growth vessel. However, the increase in OD_{600} in the presence of cerulenin did not correspond to any increase of cell dry weight compared to that of control cultures. The increase in OD_{600} values in the presence of cerulenin may result from an increase in cell size or cell aggregation. It has been shown that cerulenin is a potent inhibitor of lipoteichoic acid synthesis in Streptococcus faecalis ATCC 9790 and blocks a critical step in cell division that takes place about 10 minutes before the

termination of a round of DNA synthesis (Carson and Daneo-Moore, 1978). Higgins, Carson and Daneo-Moore(1980) studied the morphological effect of cerulenin on S.faecalis ATCC 9790 by three-dimensional reconstructions of cells based on measurements from axial ultra-thin sections. They concluded that cerulenin interfered with cell division by inhibiting normal construction of the division furrow and centripetal growth of the cross wall in envelope growth sites. Rather than form a division septum, many of the septum initiation sites in peripheral wall continued to elongate and produce abnormally large amounts of peripheral wall surface instead of cross wall. These data were interpreted as indicating the participation of a lipid-containing inhibitor of autolytic capacity, which was necessary for normal septation. Insufficient amount of this inhibitor in cerulenin-inhibited cells would result in their cross wall continuing to separate into new peripheral wall for a much longer period of time than that seen in uninhibited cells. Furthermore, since new sites are initiated throughout the period of inhibition, it would seem that the effect of the drug is on the completion, rather than the initiation of new growth sites (Higgins, Carson and Daneo-Moore, 1980).

Bearing these conclusions in mind, it is reasonable to suppose that an increase in cell size may explain the increase in OD_{600} values noted in cerulenin inhibited cells.

However, thin sectioning electron microscopy (EM) did not show any significant change in the morphology of cerulenin-inhibited cells compared to those of control cells. It is difficult to detect changes in cell size or cell volume from the thin-sectioning EM results, since S.aureus divides in 3 alternating planes, making it more difficult to study volume changes in this organism as a consequence of cerulenin. Cerulenin inhibits the synthesis of lipoteichoic acids (Carson and Daneo-Moore, 1978) which maintain a polyanionic surface on the cell, providing an integrated cation-exchange system between the exterior of the cell, through the walls to the membrane. This retains a high concentration of Mg^{2+}, required for membrane stability and for activity of a variety of enzymic reactions (Baddiley, 1972). Such imbalanced physical properties may have resulted in a change in cell size and loss of polyanionic property which, in turn, may have led to increased stickiness of cells with consequent increased aggregation and adhesion to glass.

It was curious that cerulenin inhibited cells were more resistant to autolysis than control cells, yet more sensitive than control cells to lysostaphin. This altered response, upon cerulenin-inhibition may reflect the relative absence of the proposed "lipid-associated inhibitor" of autolytic activity, lipoteichoic acid (LTA).

In turn, such cells may be depleted in autolytic enzyme complement, which is reflected in their high stability. A tight association between LTA and autolysins would be expected to result in loss or depletion of both components if the synthesis of one of them is inhibited. The increased sensitivity of cerulenin-inhibited cells to lysostaphin may also reflect an absence of LTA, since this is one of the primary charged components on the cell surface. Altered surface charge may explain not only increased accessibility of substrate (peptidoglycan) to the lysostaphin enzyme, but also the altered adhesion characteristics noted in these cells. This aspect warrants further investigation.

Clearly, the cell surface of S.aureus (Wood 46) is a very complex mosaic of components, including lipoteichoic acid (LTA), wall teichoic acid (TA), peptidoglycan and proteins. Alterations in the levels of any one of these components would affect others. To further investigate the effect of cerulenin on cell lysis by endogenous muralytic enzymes, its effect on LTA synthesis must be determined.

Interestingly, besides affecting fatty acid synthesis, cerulenin also causes a major reduction in the carotenoid content of S.aureus Wood 46. The exact relationship between this phenomenon and lipid synthesis remains obscure.

IV EFFECT OF CERULENIN ON CELLULAR PROTEIN AND LIPID SYNTHESIS AND ON TOTAL LIPID CONTENT, PHOSPHOLIPID AND FATTY ACID PROFILES OF S.AUREUS WOOD 46

Cerulenin at 10μgml^{-1} had no significant effect on growth rate or protein synthesis as measured by the rate of incorporation of [^{3}H]-phenylalanine. However, it markedly reduced α-lysin production (98% inhibition) and lipid synthesis (40-50% inhibition). At this concentration, cerulenin caused a 50% reduction in total cell lipid as determined by dry weight of chloroform/methanol extracts. These results may indicate a close association between α-lysin secretion and lipid synthesis. However, the degree of inhibition of α-lysin secretion by cerulenin was not directly proportional to the degree of inhibition of lipid synthesis. A 40-50% reduction in lipid synthesis was sufficient to almost stop secretion of α-lysin. Proportional correlations between the degree of lipid synthesis and secretion of penicillinase (B.lichenformis), levansucrase (B.subtilis), and α-amylase and protease (Bacillus amyloliquefaciens) have been reported (Fishman, Rottem and Citri, 1978; Caulfield et al., 1979; Paton, May and Elliott,1980).

Qualitative analysis of phospholipids by 1 dimensional thin layer chromatography (TLC) showed that the major phospholipid constituents of Wood 46 were phosphatidyl-glycerol and cardiolipin. No qualitative changes were detected under cerulenin inhibition. Qualitative analysis

of fatty acid components of cell lipids revealed saturated, monoenoic, and iso-branched chain saturated fatty acids of 12 to 20 carbon atoms. The most abundant were iso-branched saturated C15 (43%). Next most abundant were saturated C16 (18%) followed by monoenoic C17 (14%). Previously published analyses of fatty acids of S.aureus showed normal, iso, and anteiso saturated fatty acids of carbon numbers from C10-C21, the most abundant being C15 anteiso branched (White and Frerman, 1968; Theodore and Panos, 1973). Minor differences between results published by different groups for the same strain probably reflect differences in growth conditions.

Cerulenin at sub-growth inhibitory concentrations reduced α-lysin secretion and caused significant changes in the fatty acids of the cells. The major significant changes in fatty acid profiles of inhibited cells were: monoenoic C13 reduced by 36%, saturated C15 and C12 reduced by 35% and saturated isobranched C16 reduced by 32%. These results suggest that these fatty acids play a part in the process of α-lysin secretion. Meanwhile, certain fatty acids which were not present in control cells appeared in cerulenin-inhibited cells. These were saturated C17, saturated C18 and saturated isobranched C20. These results confirmed that, at the concentrations used here, cerulenin inhibited fatty acid synthesis as reported by Ōmura (1976).

Exogenous stearic acid did not affect either growth or the production of α-lysin from control cells nor did it relieve inhibition of α-lysin secretion by cerulenin, whereas oleic acid partially restored α-lysin secretion from cerulenin-inhibited cells while not affecting growth or α-lysin secretion in control cells. Both palmitate and oleate relieved growth inhibition of E.coli caused by cerulenin (Goldberg, Walker and Bloch, 1973). Also the inhibitory effect of cerulenin on secretion of B.lichenformis penicillinase and B.amyloliquefaciens α-amylase and protease could be reversed by the addition of a mixture of fatty acids obtained from these bacteria respectively (Fishman, Rottem and Citri, 1978; Paton, May and Elliott 1980). The lack of effect by stearate and its absence in control cells indicates that this fatty acid is not required in the process of α-lysin secretion. The incomplete recovery of α-lysin secretion after addition of oleate during cerulenin inhibition may be due to poor uptake of this fatty acid.

Previous results strongly suggested that de novo lipid synthesis is obligatory in the process of α-lysin secretion. Lipid synthesis may be involved in the process of protein translocation through the membrane in two possible ways: either lipids are directly involved in intermediates in the process of lysin translocation through the membrane, or since bacterial extracellular products

have to traverse the lipid bilayer, any biophysical or biochemical changes which alter the physical state of this structure would have a subsequent effect on the transport of the extracellular product. In S.aureus, cerulenin inhibits the secretion of enterotoxin B (Altenbern, 1977a) and both cerulenin and quinacrine inhibit the secretion of enterotoxin A as well as levansucrase in B. subtilis (Berkeley et al., 1978; Caulfield et al., 1979). Based on these findings Caulfield et al. (1979) suggested that the cerulenin effect may provide preliminary evidence for the involvement of a lipid intermediate in the release of these exoproteins, while the quinacrine effect may indicate a protease cleavage mechanism in the release of exoproteins. Thus, a lipid intermediate/proteolytic-releasing mechanism similar to that of B.lichenformis penicillinase (Traficante and Lampen, 1977) is possibly applicable to protein secretion in S.aureus. However, recent investigations of the structure of cell-bound penicillinase of B.lichenformis (Simons et al.,1978), revealed that covalently bound phospholipopeptide is not present but the membrane penicillinase contains an amino-terminal extension which is hydrophobic in character.

More recently, Nielsen and Lampen (1982a,b) reported that the membrane penicillinases of B.lichenformis and B.cereus are lipoproteins with N-terminal glyceride thioether modifications identical to that of E.coli outer

membrane protein (murien lipoprotein). The absence of half-cystine in amino acid analyses of α-lysin does not eliminate the possible presence of a thioether linked lipid intermediate being involved in secretion, since these analyses are all done on cell-free lysin. The presence of half-cystine in membrane bound lysin would be strong circumstantial evidence favouring a lipid-linked intermediate of the type found in membarne bound penicillinase. Available evidence suggest that α-lysin secretion is of the second type mentioned above, i.e. a membrane property effect rather than an effect on a covalent lipid-linked intermediate of α-lysin. This type of effect on the physical properties of the membrane may also account for the observation that the production of staphylococcal enterotoxin B (SEB) by non-growing cells (Altenbern, 1978) and hyaluronidase in growing cells (Berkeley and Pepper, 1979) cannot be inhibited by cerulenin. Other examples of an effect on membrane properties come from the work of Bocquet-Pages, Lazdunski and Lazdunski (1981) who reported that cerulenin inhibited the synthesis or assembly of the E.coli outer membrane proteins OmpA, OmpC, and OmpF, none of which is a lipoprotein. It appears that the above examples represent exoproteins where a lipid intermediate in transport is not involved. The lack of the effect of protease inhibitors on production of haemolysins by S.aureus (Wood 46) is in keeping with the

absence of a mechanism of secretion involving lipid intermediate/proteolytic-release in the secretion of α-lysin. However, the possibility that releasing proteases may have very high substrate specificity must be recognised.

The profound effect of cerulenin on the production of α-lysin illustrates the importance of lipid synthesis in the process of lysin secretion, since significant reduction in total lipid synthesis and changes in fatty acid profiles were evident in cerulenin - inhibited cells. Moreover, the connection between lipid synthesis and lysin production was further strengthened by the fact that inhibition of lysin secretion was overcome by addition of exogenous oleate. However, the difference in the extent of inhibition of α-lysin secretion (95%) and total lipid synthesis (40-50%) suggests a relatively specific lipid requirement for the process of secretion.

Petit-Glatron and Chambert (1981) reported that synthesis of fatty acids and production of levansucrase and α-amylase in B.subtilis were not inhibited to equal extents by cerulenin and concluded that the inhibition of fatty acid synthesis by cerulenin is unrelated to the inhibition of protein secretion. Moreover, they suggested that cerulenin inhibition of exoenzyme secretion in B.subtilis is a result of physicochemical interaction with the membrane rather than the result of interference with intracellular lipid synthesis. However, at the concentrations used in the present work, there was no

doubt that cerulenin strongly inhibited α-lysin secretion and at the same time reduced lipid synthesis by ~ 50%.

Recovery of protein secretion by addition of exogenous fatty acids in Bacillus amyloliquefaciens was not accompanied by a change in the lipid to protein ratio in the membrane suggesting alterations in the lipid content of the membrane were not responsible for inhibition (Paton, May and Elliott,1980). Although there are a few contradictions regarding the action of cerulenin, the present work shows without doubt that cerulenin inhibits lipid synthesis and lysin secretion.

V. ISOLATION OF TOTAL MEMBRANE FRACTION FROM CONTROL AND CERULENIN-INHIBITED CELLS OF S.AUREUS WOOD 46

In an attempt to investigate changes in the membrane induced by cerulenin, membranes were isolated from both control and cerulenin-inhibited cells. Cerulenin, at concentrations without effect on growth, caused a 50% reduction in total membranes isolated, and the ratio of protein to membrane dry weight in cerulenin-inhibited cells was greater than that for control cells.

There are two possible explanations for the observed decrease in the membrane dry weight in the presence of cerulenin. There may be a fall in surface to volume ratio as a result of an increase in cell size due to inhibition of septation in cell division, as was found for

Streptococcus faecalis (Higgins, Carson and Daneo-Moore, 1980). Alternatively, and more likely, there may be loss of membrane components during their washing and purification due to reduced membrane stability. Micrococcus luteus (lysodeikticus) grown in the presence of diphenylamine, which inhibits carotenoid synthesis, had a tendency to autolyse on harvesting and the membranes from these organisms were unstable on further washing in Tris buffer. The instability of several membrane preparations from the diphenylamine-grown cells was manifested by the greatly diminished yield of the final membrane fraction and the detection of non-sedimented cytochromes during the washing (Salton and Freer, 1965). Thus, similar loss of membrane components may have occurred as a consequence of cerulenin effect on lipid synthesis.

The ratio of membrane protein to total membrane dry weight in cerulenin-inhibited cells was greater than that for control cells. Also under inhibition of lipid synthesis, protein synthesis proceeded normally. This suggested that cerulenin did not affect translocation or synthesis of integral membrane proteins. Certainly, cerulenin did not affect the synthesis of integral membrane proteins in Bacillus amyloliquefaciens at concentrations which significantly reduced the production of protease and α-amylase (Paton, May and Elliott,1980). This result is surprising since current theories of protein secretion

(Blobel and Dobberstein, 1975 a,b) and assembly of membrane proteins (Rothman and Lenard, 1977) envisage that both classes of proteins are synthesised on membrane bound ribosomes, the only difference being that membrane proteins do not completely transverse the bilayer but become fixed within it. The initial steps of entry into the membrane for the two classes of proteins are envisaged as identical. Therefore, either the complete passage of secreted proteins requires an additional step, inhibited in the presence of cerulenin, or else the two processes are different in some other way not envisaged in current theories.

VI. PROTEIN PROFILES OF CONTROL AND CERULENIN-INHIBITED CELLS OF S.AUREUS WOOD 46

Analysis of protein profiles of control and cerulenin-inhibited cell membranes using SDS-PAGE revealed that α-lysin was present as a species of molecular weight 34K in control cell membranes, but absent from cerulenin-inhibited cell membranes. The rest of the protein profile in both control and cerulenin-inhibited cell membranes were rather similar. Moreover, no α-lysin band was detected in cytoplasm of either control or cerulenin-inhibited cells.

The finding that α-lysin was tightly associated with control cell membranes and not removed by washing with 1M NaCl led to the assumption that this membrane-

associated lysin was a possible intermediate in the secretion of the lysin. Examples of membrane bound forms of extracellular proteins are: the penicillinase of B.licheniformis (Sawai and Lampen, 1974), α-amylase and protease of B.subtilis (Mäntsälä and Zalkin, 1979, 1980), and enterotoxin B (Tweten and Iandolo, 1981) and coagulase (Engels and Kamps, 1981) of S.aureus. These membrane-bound forms are thought to be intermediates in the secretion of extracellular proteins. However, most of the membrane-bound forms have a molecular weight 3000-5000 daltons greater than the active extracellular proteins. This additional sequence of amino acids corresponds to a stretch of 15-30 residues in the signal sequence which functions to initiate protein secretion through membrane (Blobel and Dobberstein, 1975 a, b). Since the membrane-associated α-lysin has the same molecular weight as the extracellular protein and produced similar peptide maps to those of authentic 34K lysin, the possibility that the membrane-associated lysin represents a precursor form is unlikely. However, it more likely represents the ultimate form of the lysin while in the process of translocation through the membrane.

Assuming that α-lysin has a precursor with an N-terminal signal sequence,a possible reason why a heavy form of the lysin was not detected in the membrane may be due to the action of endogenous protease converting

the precursor into the ultimate form during the prolonged incubation of the cells at 37°C in the course of membrane isolation. In accordance with this possibility, it has been reported that protein translocation through membranes is highly susceptible to protease or high salt washes (Warren and Dobberstein, 1978; Walter et al., 1979). A second possibility is that the precursor form may be converted to the ultimate form very rapidly, and hence not in sufficient amount to be detected in the membrane. Tweten and Iandolo (1983) in a study of the transport and processing of staphylococcal enterotoxin B, reported that subcellular fractionation of pulse-labelled S.aureus S6 cells revealed that all of the pro SEB (PSEB) and some SEB were associated with the membrane fraction. They consistently observed mature SEB on the membrane in amounts approximately equivalent to PSEB. Therefore, they suggested that factors other than a hydrophobic signal sequence can stabilise the association of PSEB and SEB with the membrane. They further showed that both proteins could disappear from the membrane at almost identical rates during a pulse-chase experiment and concluded that possibly the membrane-associated mature SEB represents co-translationally processed PSEB which is in transit across the membrane and in a stable association with it. Therefore, such factors must be taken into account in the failure in detecting a higher

molecular weight α-lysin precursor in control cell membranes.

Another possibility is that α-lysin may not have a precursor with N-terminal signal sequence but may contain an internal sequence similar to that of Ovalbumin. Ovalbumin is not synthesised as a larger presecretory protein but contains an uncleavable internal signal (Palmiter, Gagnon and Walsh, 1978). An internal signal sequence would not be detected by the methods of analysis used in the present study. More importantly, there have been reports that some bacterial proteins are exported or incorporated in the form in which they are synthesised, that is, without a need for a precursor form. These include the colicin E1 and E3 (Jackes and Model, 1979), several products of sex factors (Achtman et al., 1979) and δ-lysin (K. Y. Lee personal communication). However, it is not yet known whether these proteins are synthesised on free or membrane bound ribosomes but it is significant that the E1 type colicins mentioned have a similar mode of membrane disruption to Staphylococcal α-lysin (Schein, Kagan and Finkelstein, 1978; Bhakdi, Füssle and Tranum-Jensen, 1981) and probably to δ-lysin (Freer, Birkbeck and Bhakoo, 1984). All three proteins have a high affinity for membranes, and available evidence suggests that they share the common property of pore-formation. This high membrane affinity appears to reflect

amphipathic properties of the proteins which are not structurally equivalent to signal sequence but may serve similar functions in terms of membrane affinity.

In the search for possible α-lysin precursors which may have accumulated in the membranes as a result of inhibition of lipid synthesis by cerulenin, peptide mapping was performed on several new bands from membranes isolated from inhibited cells. None of these bands however, produced closely similar maps to that for authentic 34K α-lysin apart from the 34K band taken from control membranes. These results suggested that cerulenin did not cause accumulation of α-lysin precursor in the membranes.

Immunological analysis of membranes and culture supernates of cerulenin-inhibited cells using agarose gel diffusion in the presence of anti-α-lysin serum did not reveal any α-lysin precursor. However, control membranes gave complete lines of identity with purified α-lysin confirming that the 34K membrane band present in control cell membranes is α-lysin. Using anti-δ-lysin serum, both cerulenin-inhibited and control membranes produced partial lines of identity with purified δ-lysin, while cerulenin-inhibited culture supernates produced complete lines of indentity with purified δ-lysin. This showed that the production of δ-lysin was not as sensitive to the effect of cerulenin as α-lysin and that the mechanism of α-lysin export may differ from that of δ-lysin.

No significant band of α-lysin was found in cytoplasm from either control or cerulenin-inhibited cells. However, the weakly staining band was present at 34K in control cytoplasmic fractions, which did not produce any haemolysis upon titrating against rabbit red blood cells. This may have arisen from traces of non-sedimentable residual membrane fragments. The virtual absence of α-lysin from control cytoplasm indicated that this protein was probably synthesised co-translationally on membrane bound ribosomes.

Thus, in conclusion there was no detectable quantities of α-lysin or its precursor in either membranes or cytoplasm of cerulenin-inhibited cells. Also it is noteworthy that cerulenin, at sub-growth inhibitory concentrations inhibited the secretion of penicillinase by B.lichenformis (Fishman, Rottem and Citri, 1978), levansucrase and α-amylase by B.subtilis (Caulfield et al., 1979; Mäntsälä, 1982) but did not cause intracellular accumulation of these products.

In the present study protein profiles of cerulenin-inhibited cells were very similar to those of control cells when analysed by SDS-PAGE, with the exception of the α-lysin band being absent in inhibited cell membranes. Cerulenin also had no effect on total protein synthesis but, through inhibition of lipid synthesis, may have induced morphological and topological changes in the

membrane structure sufficient to change the putative binding sites for the nascent ribosome-associated α-lysin peptide and perhaps those of other extracellular products. Signal recognising particles (SRP) have been isolated from microsomal membranes in eukaryotic cells. These specifically bind and arrest the translation of the signal sequence of nascent secretory proteins, but not of the cytoplasmic proteins. This arrest is released upon SRP-mediated binding of the elongation-arrested ribosomes to the microsomal membranes where a 'docking protein' releases this arrest and translation is then coupled to translocation (Meyer, Krause and Dobberstein, 1982; Meyer, Louvard and Dobberstein, 1982). In a very important study, it was recently reported that β-lactamase synthesised *in vitro* was co-translationally translocated into dog pancreas microsomal vesicles giving the first solid evidence that co-translational translocation of both bacterial and eukaryotic secretory proteins across membrane requires identical components (Müller *et al.*, 1982). Bearing this in mind, the disappearance of α-lysin band from membranes of cerulenin-inhibited cells could be interpreted as a result of blockage of translation of α-lysin mRNA.

The membrane trigger hypothesis of Wickner (1979) and the helical hairpin hypothesis of Engleman and Steitz (1981) both emphasise the role of spontaneous insertion of the proteins into the lipid bilayer without

specific binding sites. The fact that cerulenin inhibits lipid synthesis and causes changes in membrane composition may result in the arrest of protein insertion into the membrane, thereby stopping its export. However, this explanation is less likely since no precursor form of α-lysin was detected in the cytoplasmic fraction of cerulenin-inhibited cells.

The data presented in this work indicates the importance of lipid synthesis in the secretion of α-lysin. The results are assessed in relation to other theories of protein secretion in the current literature, such as the signal hypothesis of Blobel and Dobberstein (1975 a,b) and the membrane trigger hypothesis of Wickner (1979).

If the success or failure of a hypothesis can be judged by the interesting experiments it has provoked, then each school of thought has achieved a great measure of success.

CONCLUSIONS

The main conclusions from the present investigation are:

1. Alpha-lysin secretion is associated with lipid synthesis.
2. Exogenous fatty acids partially restore lysin production from cerulenin inhibition.
3. Cerulenin-inhibition does not allow lysin or precursors of lysin to accumulate in the plasma membrane or cytoplasm.
4. The lack of effect of protease inhibitors could reflect the absence of a proteolytic cleavage mechanism in the export of this lysin.
5. The increased sensitivity of cerulenin - inhibited cells to lysostaphin may reflect loss of lipoteichoic acid (LTA), whereas their lack of autolysis may reflect a depletion in autolytic enzyme complement.

Further work is required before the mechanism of export of α-lysin is elucidated. In particular the following areas warrant further attention:

1. The effect of other exogenous fatty acids (radiolabelled) on the recovery of α-lysin secretion from cerulenin-inhibition.
2. The nature of the translation products of α-lysin mRNA in cell-free systems from other bacteria.
3. The effect of a wider range of protease inhibitors

on the production of the lysin and other extracellular products of Wood 46.

4. The effect of cerulenin on LTA synthesis and the influence of such membrane associated components on export of proteins.

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APPENDICES

APPENDIX I

B S MEDIUM [Modified from Bernheimer & Schwartz (1963)]

Yeast extract dialysate	2000 ml
Casamino acids (Difco Technical)	64.0 g
Glucose (BDH)	8 g
Aneurine hydrochloride	0.4 mg
Nicotinic Acid (BDH Analar)	3.7 mg

Distilled water to 3200 ml

medium adjusted to pH 7.1

Sterilisation: 30 min at 121°C ($70 kg m^{-2}$ steam pressure)

preparation of yeast extract dialysate:

Yeast extract (Oxoid and Difco)	200 g
Distilled water	500 ml

Yeast extract was dissolved in distilled water by steaming for 20 min, allowed to cool, and poured into a 760 mm length of 66mm washed visking tubing. The dialysis sac was then immersed in 1500ml of distilled water in a 5 litre beaker and dialysis allowed to take place for 72h at 4°C with stirring.

The sac contents were discarded and the outside solutions (diffusate) of two such dialyses were combined to give a volume of approximately 2000 ml of dialysate. To minimise the risk of contamination during the 72h dialysis, the dialysis tubing and beaker were rinsed in 70% absolute alcohol prior to use.

APPENDIX II

Buffers

a. Potassium Phosphate Buffer

Stock solutions

A 1.0 M KH_2PO_4 136.1 gL^{-1}

B 1.0 M $K_2HPO_4.3H_2O$ 228.2 gL^{-1}

x	y	pH
92.0	8.0	5.8
87.7	12.3	6.0
81.5	18.5	6.2
73.5	26.5	6.4
62.5	37.5	6.6
51.0	49.0	6.8
39.0	61.0	7.0
28.0	72.0	7.2
19.0	81.0	7.4
13.0	87.0	7.6
8.5	91.5	7.8
5.3	94.7	8.0

X ml of A and y ml of B were mixed to give 1.0 M potassium phosphate buffer at the required pH. 8.7 g NaCl was added per litre to the appropriate dilution to give 0.15M NaCl, 0.05M potassium phosphate-saline buffer (SPB) at the required pH.

b. Tris-HCl Buffer

Stock solutions: A 0.2M Tris (hydroxymethyl) aminomethane ($24.2 g L^{-1}$)

B 0.2M HCl (17.15 ml concentrated HCl made up to 1L with distilled water)

x	pH
5.0	9.0
8.1	8.8
12.2	8.6
16.5	8.4
21.9	8.2
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

50ml of A + Xml of B to a total of 1000ml gives 0.01M Tris-HCl at the required pH.

8.7g NaCl was added per litre to the appropriate dilution to give 0.145M NaCl, 0.01M Tris-HCl pH7.2.

c. Haemolytic Assay Buffers

α-lysin: Phosphate Buffered Saline (PBS)

1 tablet Dulbecco A (OXoid Ltd., London, England) dissolved in 100ml.

Composition of buffer	gL^{-1}
NaCl	8.0
KCl	0.2
Disodium hydrogen phosphate	1.15
potassium dihydrogen phosphate	0.2

final pH 7.3

β-Lysin: Tris-buffered saline made 1mM with respect to Mg^{2+}

Stock solutions A 0.2M Tris ($24.2gL^{-1}$)

B 0.2M HCl (17.15 ml conc made up to 1L with distilled water) } Tris-HCl

C 1.0M NaCl ($58.5\ gL^{-1}$)

D 0.1M $MgCl_2.6H_2O$ ($20.3\ gL^{-1}$)

1 litre of Tris-buffered saline (TBS) + Mg^{2+} made up as follows:

125ml solution A + 107ml solution B + 154ml solution C + 10ml solution D made up to 1L with distilled water.

Final pH 7.4

δ-lysin: Citrate-dextrose-saline (CDS) containing 12.5% (V/V) Dimethyl Sulphoxide

Glucose	20.5g
Tri sodium citrate	8.0g
NaCl	4.0g

Distilled water to final volume of 1 litre.

12.5ml dimethyl sulphoxide (DMSO) added per 100 ml CDS to give 12.5% DMSO in CDS.

APPENDIX III

Electrofocusing: Principle,Materials and Methods

a.Principle

Proteins, polypeptides and amino acids are examples of ampholytes, i.e. they can behave as acids and bases since they contain both carboxyl and amino groups. On dissociation these groups may become negatively charged or positively charged. The net charge of an ampholyte is thus dependent on the number and dissociation constants of these groups and also on the pH of the solution. The isoelectric point (pI) of an ampholyte is the pH at which the net charge on the molecule is zero. Low molecular weight ampholytes have been produced by LKB produkter (Stockholm, Sweden), called carrier ampholytes. In an electrical field, these carrier ampholytes migrate to and focus at their pIs,and as they have a strong buffering capacity and conductance at their pIs, a buffered pH gradient is set up from anode to cathode. The smoothness of this gradient is dependent on the number, buffering capacity, relative amounts and pIs of the carrier ampholytes used to form the gradient. The gradient can be maintained after the removal of the electrical field if mixing is prevented by the use of a stabilising density gradient composed of a highly water soluble non-electrolyte such as sucrose or sorbitol or the ampholytes are present in a hydrated gel such as Sephadex. This is

a natural pH gradient, generated by the electric current itself. If proteins are present in this system they will migrate to the pH value in the system where they carry no net charge and will focus in narrow zones where pH of the gradient = pI of the protein.

b. Carrier Ampholytes

The carrier ampholytes used must fulfil a number of requirements as defined by Svensson (1961, 1962, a,b). They should have:

1) good buffering capacity at their isoelectric point so that they are capable of maintaining the pH at that point.

2) good conductivity at the isoelectric point to permit passage of current when they have reached their isoelectric point.

For practical reasons they should also have:

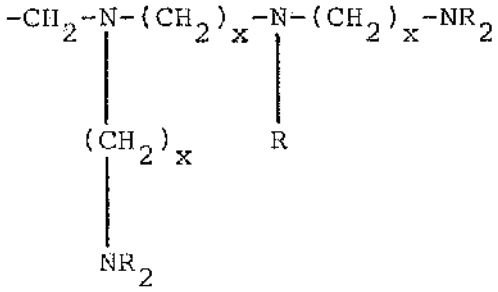
3) low molecular weight so that they may be easily separated from the proteins to be focused.

4) chemical composition that is different from substances to be separated so that they will not mask the identification of those substances, e.g. a low E_{280}

5) finally, a carrier ampholyte should not react with or denature the substances to be fractionated.

LKB manufacture carrier ampholytes under the trade name of 'Ampholines' and Pharmacia under the name 'Pharmalytes' which fulfil the above criteria. They

consist of a mixture of aliphatic polyamine polycarboxylic acids obtained by coupling acrylic acid to a mxiture of polyalkalenc-polyamines under conditions where no amide bonds form. The reaction leads to the formation of a large number of homologues and isomers with molecular weights between 300-1000 and pI values graded from 3-10. The generalised formula of carrier ampholytes is:



where x = 2 or 3

and R = H or $-CH_2-CH_2-COOH$

Further details of the physical and chemical properties of ampholytes are discussed by Davies (1970), Haglund (1970, 1975) and Vesterberg (1976)

C. Equipment and Electrofocusing Solutions

1) Column A diagram of the column given in Fig. 31 A 8100-1 column (LKB produkter AB, Stockholm, Sweden) having a capacity of 110ml was used. The electrofocusing compartment (17) is cylindrical and is cooled by water on either side in compartments (shaded areas) which are connected with tubing (33) via nipples (22) and (23). The electrodes are platinum, the upper electrode (32) consists of a loop which is connected to terminal (30), while the central electrode is wound round a teflon rod (20) in the central tube (19) and is connected to a terminal at the top of the column (29). The teflon rod as well as supporting the central electrode also acts as a lever for valve (26). This valve is open during the electrofocusing and is closed by means of a setting device (25) prior to emptying the column to prevent mixing of the electrode solution in the central tube (19) with the separated proteins in the electrofocusing compartment.

2) Electrofocusing solutions

Electrode solutions: Cathode at top of column

Anode solution

Sorbitol (Merck Lab. Chemicals, Darmstadt) 12g

or Sucrose (BDH Analar)

Distilled water 14ml

Glacial acetic acid or Sulphuric acid (BDH,Analar) 0.2ml

pH. Approx 2.6

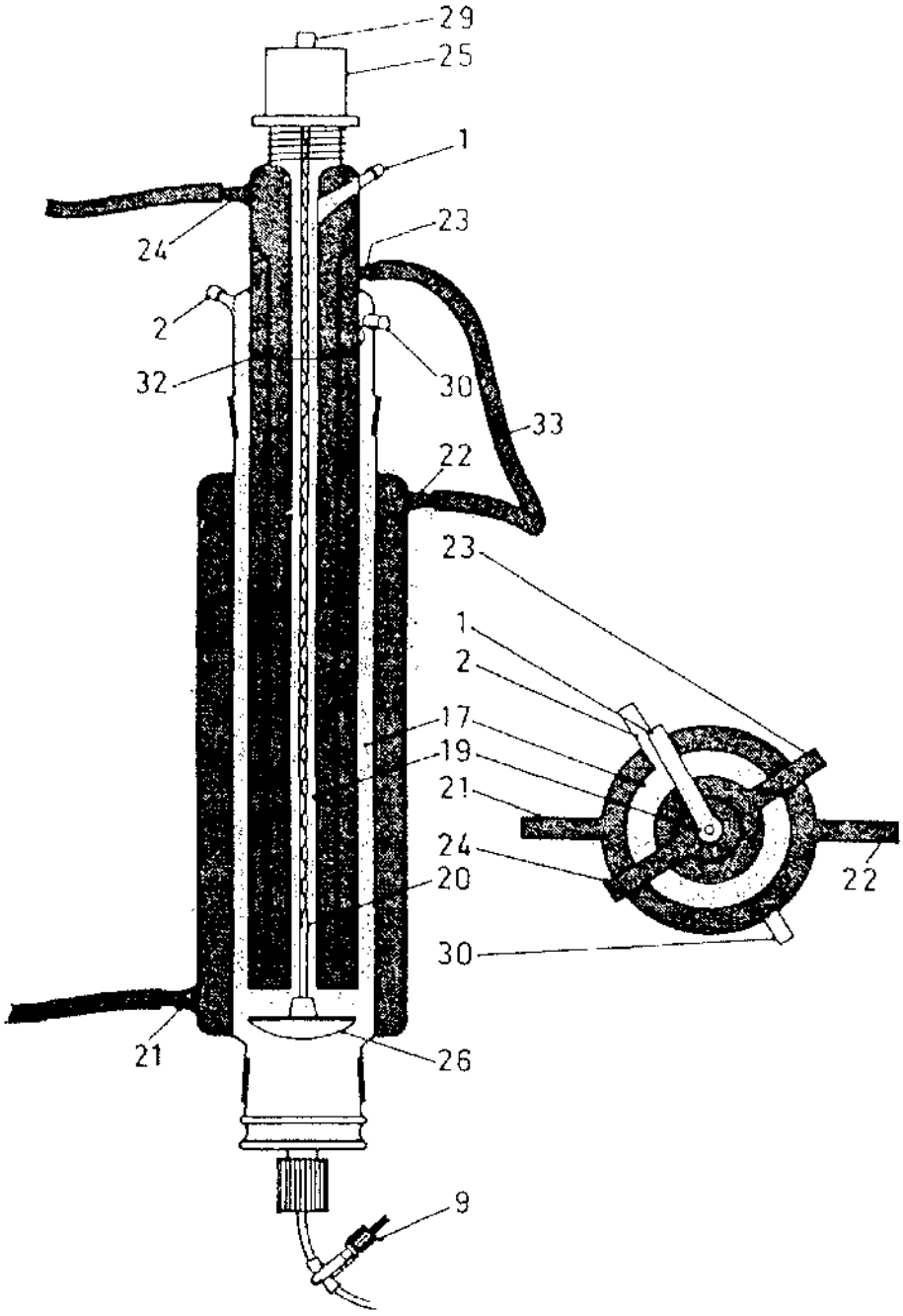


Figure 31. Diagram of an LKB electrofocusing column.

Cathode solution

Distilled water	20ml
Sodium hydroxide	0.2g

pH Approx 12.8

Gradient Solutions:

Dense solution

Sorbitol	27.0g
volume of distilled water (+sample if added)	37.0ml
Ampholines pH 8-9.5	1.5ml
Ampholines pH 9-11	0.2ml

Light solution

Sorbitol	2.7g
Volume of distilled water(+sample if added)	52.0ml
Ampholine pH 6-8	0.2ml
Ampholine pH 7-9	1.3ml

3) Accessory equipment

The power source was an LKB 3371 power supply having a voltage range of 0-1200V.

Electrofocusing was run at 4°C, which was maintained by the use of a thermostatically controlled water bath (Type S.B.2 Grant Instrument Co. Cambridge, England) and Circulating pump (Type Q2, Grant Instrument Co., Cambridge, England).

The sorbitol density gradient was prepared from the appropriate solutions with a linear gradient mixer (LKB 8121) fitted with a stirrer motor (LKB 8121).

4) Procedure

The column was mounted vertically with the aid of a plumb line in a framework of Lab-lox (Gallenkamp, London, England). The cooling water was turned on to enter the system via nipple (21) and leaves via nipple(24). With valve (26) open, the anode solution was loaded into the central compartment (19) via nipple (1). The lysin sample, ampholines and sorbitol were mixed by means of the gradient mixer and loaded onto the column under gravity, via nipple (2) to fill the electrofocusing compartment (17). This pushed the anode electrode solution up into the central compartment (19). The cathode electrode solution was then layered onto the gradient (via nipple 2).

Electrical current was applied to the column via the terminals (29) and (30). An initial potential of approximately 400V was applied giving an initial current of 28mA. The voltage was increased over 24 hour period to a maximum of 1150 V to maintian the current at 28 mA. The electrofocusing was run for a total of 72h by which time the current had decreased to 1.2 mA. In the end of the 3 days period the power was turned off, and valve (26) closed by means of the setting device (25). The electrode solution was withdrawn via nipple (1) and the column drained under gravity by opening the clamp(9).

APPENDIX IV

Double Diffusion in Agarose

1% agarose in PBS

Agarose (Miles Labs. Ltd) 1.0g

PBS (Oxoid Ltd., England)

pH 7.4 (PBS)

The agarose was dissolved by boiling in the buffer, then dispensed in 10ml amounts, sterilised at 121°C 0.07 kg cm^{-2} for 10 min and stored at 4°C.

APPENDIX V

Polyacrylamide Gel Electrophoresis

a. Stock Solutions

1) Lower buffer (4 x strength) pH 8.9	For 100 ml
Tris/HCl (1.5M)	18.1g
SDS	0.4g

2) Upper buffer (4 x strength) pH 6.8	
Tris/Hcl (0.5M)	6.06g
SDS	0.4g

3) Acrylamide/Bis	
Acrylamide	30g
Bis	0.8g

4) Temed

Undiluted stock

5) Ammonium persulphate

10% (W/V) made up freshly from crystals kept in desiccator. (Use 50mg in 0.5ml water).

6) Running buffer (100ml). pH 8.3	
Tris	3.03g
glycine	14.4g
SDS	1.0g

7) Solubilising Buffer

Glycerol	10ml
β-Mercaptoethanol	5ml
SDS	3g
Tris-buffer,pH 6.8, 0.0625 M (dilute upper buffer 1 in 8)	to 100ml
Bromophenol Blue	0.01g

Samples (1-2mg ml^{-1}) were boiled for 2 min before loading. For re-use, they were stored at -20°C for few days or -70°C if longer period.

b. Preparation of Separating Gel (Lower Gel)

The following reagents were added to a Buchner conical flask

Lower gel buffer (4x)	10ml	
Water	13.4ml	(12.5%)
Acrylamide/Bis	16.6ml	

mixed and degased for 10 min then:

Ammonium persulphate	200µl
Temed	20µl

were added, the flask contents mixed gently and pipetted into the gel plate carefully up to the appropriate level (13cm), then overlayed with butanol (saturated with water). The gel was allowed to polymerise then the butanol was drained and the interface washed with distilled water several times before the stacking gel solution was added.

c. Preparation of Stacking Gel (Upper gel)

The following reagents were added to a Buchner conical flask

Upper gel buffer	2.5ml
Water	6.0ml
AC/Bis	1.5ml

mixed and degased for 10 min then:

Ammonium persulphate	30µl
Temed	20µl

were added and the flask contents mixed gently. The sample comb was placed between the gel plates and the upper gel solution (stacking gel) was poured. The gel was allowed to polymerise then the comb was removed carefully under the running buffer in the gel tank.

d. Fixing and Staining Solution

Coomassie blue R250	1.25g
50% methanol	454ml
Glacial Acetic acid	46ml

The above stock solution was diluted 1:10 with:

50% MeOH	454ml
Glacial HAc	46ml

The gel was stained (with the dilute stain) overnight at 37°C on a shaking water bath at slow speed, then destained at 37°C with shaking. The gel was left overnight in 5% glacial acetic acid followed by 1h washing in distilled

water before drying on a slab gel dryer (Model SGD-200, Savant Instrument, Inc. USA).

e. Gel Drying

The gel was laid on 3mm Whatman chromatography paper then put onto the gel dryer. The air was excluded from between gel and teflon overlay then heat and vaccum applied for 1.5h.

f. Electrophoresis Conditions

The sealing tape was removed from the base of the gel plate then the plate was fixed on the gel tank(Fig 32). Running buffer was poured into the top tank until the surface of the top gel had submerged under the buffer, the bottom tank was already filled with buffer. The gel was run at 15mA until the tracking dye had passed into the lower gel, then the current was increased to 35mA until the tracking dye was within 1cm from the base of the gel. The power was then switched off and the gel removed from between gel plates, stained and destained as described above.

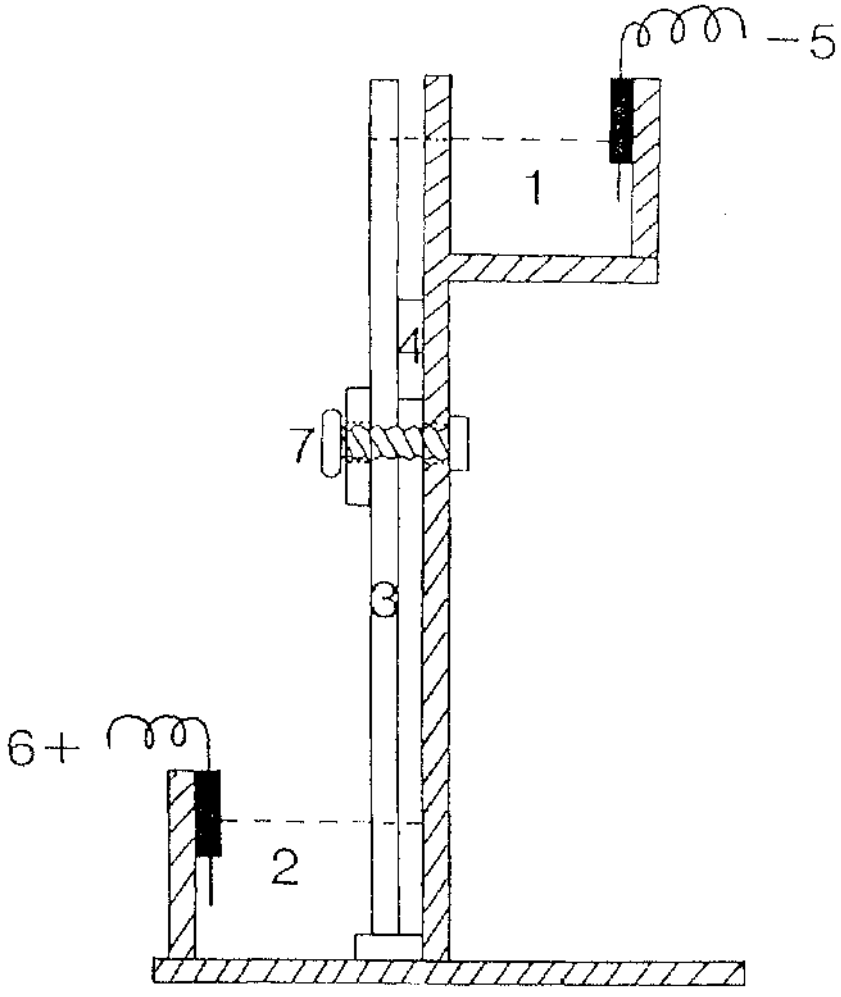


Figure 32. SDS-PAGE apparatus (side view)

1,2 = Running buffer
3 = Gel plate
4 = Gasket
5 = Cathode electrode
6 = Anode electrode
7 = Adjustable clamp

(After Studier, 1973).

APPENDIX VI

Zinzadze Reagent (Molybdenum blue)

Specific for phospholipids (Dittmer and Lester, 1964)

Reagent:

Soln.1

40.1g MoO_3 boiled in 1 litre of 25N H_2SO_4 until dissolved.

Soln.2

1.78g powdered molybdenum was added to 500ml of solution 1. The mixture was boiled gently for 15 min, cooled and the residues decanted. Equal volumes of soln. 1 and 2 were mixed and diluted with 2 volumes of water. The final solution was greenish yellow.

Plates were sprayed with solution and phosphate containing compounds appeared as blue spots.

Ninhydrin (specific for amino groups in lipids)

0.6g of ninhydrin was dissolved in 297.5ml of butanol and 12.5ml of 10% acetic acid. Plates were sprayed with solution and heated at 105°C for 10 min to detect any amino containing compounds.

APPENDIX VII

Polyacrylamide Gel Electrophoresis with Proteolysis

a. Stock solutions:

1) Lower buffer (4 x strength) pH 8.9

	For 100 ml
Tris/HCl (1.5M)	18.1g
SDS	0.4g
EDTA (1mM)	0.037g

2) Upper buffer (4 x strength) pH 6.8

Tris/HCl (0.5M)	6.06g
SDS	0.4g
EDTA (1mM)	0.037g

3) Acrylamide/Bis

Acrylamide	30g
Bis	0.8g

4) Temed

Undiluted stock

5) Ammonium persulphate

10% (W/V) made up freshly from crystals kept in desiccator.

(use 50mg in 0.5ml water).

6) Running buffer (1000ml) pH 8.3

Tris	3.03g
glycine	14.4g
SDS	1.0g

b. Preparation of Separating Gel (lower gel)

The following reagents were added to a Buchner conical flask.

Lower gel buffer (4 x)	10ml	
Water	10ml	(15%)
Acr/Bis	20ml	

mixed and degased for 10 min then:

Ammonium persulphate	200µl
Temed	20µl

were added, the flask contents mixed gently and pipetted carefully into the gel plate up to the appropriate level (8cm) i.e. less than normal separating gel by 5cm. The gel was overlayed with a small amount of butanol, allowed to polymerise then the butanol was drained and washed away with distilled water before the stacking gel solution was added.

c. Preparation of Stacking Gel (upper gel)

The following reagents were added to a Buchner conical flask

Upper gel buffer (4 x)	5ml	
Water	11.68ml	(5%)
Ac/Bis	3.32ml	

mixed and degased for 10 min then:

Ammonium sulphate	60µl
Temed	40µl

were added and the flask contents mixed gently.

The gel solution was

pipetted into the gel plate up to 1.5cm distance from the top edge of the plate, overlayed with butanol and allowed to polymerise. The butanol was drained and washed away with distilled water. Gel slices from the previous gel were placed on this gel with proteolytic enzyme and buffer overlay before electrophoresis.

d. Other Buffers Required

1) Buffer required for washing slices of the first gel (1 x strength) pH 6.8

	For 100 ml
Tris/HCl (0.125M)	1.51g
SDS	0.1g
EDTA (1mM)	0.037g

2) Glycerol buffer required for filling the spaces between the slices of the first gel which were layered on the top of the second gel pH 6.8

Tris/HCl (0.125M)	1.51g
SDS	0.1g
EDTA (1mM)	0.037g
Glycerol	20ml

3) Glycerol buffer with given amount of trypsin required to overlay on the slices pH 6.8

Tris/HCl (0.125M)	1.51g

SDS	0.1g
EDTA (1mM)	0.037g
Glycerol	10ml
+	
Trypsin (0.1mg ml^{-1})	