PREFACE

This thesis is the original work of the author. He wishes to acknowledge that electron microscopy was done in collaboration with Dr. J.H. Freer and polyacrylamide gel electrophoretic analysis of purified lysin was made in collaboration with Dr. R. Parton.

Duncan K. R. Low.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

STAPHYLOCOCCAL β-LYSIN

DUNCAN K.R. LOW M.A.

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Department of Microbiology

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SUMMARY

Three strains of S. aureus for the production of β-lysin (sphingomyelinase C), R-1, G128 and IL7s were examined. Of these, S. aureus G128 was deemed to be the most suitable for further studies. Enzyme production in YDB medium was maximal in the late logarithmic phase of growth, when cell numbers were at a maximum. The culture was freed of cells by centrifugation. The supernatant was made 85% with respect to ammonium sulphate and allowed to stand overnight. The resulting precipitate was fractionated by gel filtration on Biogel P60, ion exchange chromatography on carboxymethyl cellulose and finally electrofocusing in an LKB 110 ml electrofocusing column, pH range 7 - 11, in a sorbitol density gradient.

The main peak of β-lysin activity focussed with a pI of 9.3. After removal of ampholines, the product had a specific activity of 62.5×10^6 HU/mg against sheep cells indicating a 38,000 fold purification. The product was free from α- and δ-lysin, and protease, nuclease, hyaluronidase, phosphatase, coagulase, lipase and leukocidin activities. It gave a single symmetrical peak in the ultracentrifuge, with an S_{20w} of 3.1, and a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis. The molecular weight was estimated to be 32,500 by gel chromatography and electrophoresis.

The highly purified sphingomyelinase C released water-soluble organic phosphorus from aqueous dispersions of sphingomyelin, and bovine and human erythrocyte ghosts in the presence of Mg^{2+}, in amounts indicating extensive sphingomyelin degradation. Extensive

ultrastructural changes were evident in both human and bovine freeze-etched ghosts after sphingomyelinase C treatment. Pools of apparently solid particle-free material, possibly the ceramide product of hydrolysis, accumulated in the hydrophobic region of the bilayer. These observations could be explained in terms of a membrane in which sphingomyelin is preferentially located in the outer half of the bilayer. Sphingomyelinase C is an extremely powerful probe of membrane structure. For this purpose it is critical that the degree of freedom from contaminating proteins should be known.

The hot-cold lytic activity of sphingomyelinase C was examined using phase-contrast microscopy, and an explanation of this phenomenon in terms of lipid phase transitions was proposed.

β-lysin was found to be non-toxic for mice in doses up to 7.5 mg/kg and was not dermonecrotic. Intravenous injections of Evans blue showed that there was no local increase in vascular permeability to the dye at the site of intracutaneous injection of β-lysin. The enzyme did not act as a single virulence factor and its role in pathogenicity may well be to increase the sensitivity of normally resistant cells to attack from the other extracellular products of staphylococci.

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LIST OF ABBREVIATIONS

Greek letters:

α	alpha	η	eta
β	beta	μ	mu
γ	gamma	ν	nu
δ	delta	ρ	rho

Prefixes for multiples and submultiples of units

k	kilo	10^3	μ	micro	10^{-6}
c	centi	10^{-2}	n	nano	10^{-9}
m	milli	10^{-3}			

Abbreviations

A amps

Å ångström (1Å = 0.1 nm)

BSA bovine serum albumin

CL cardiolipin

CM carboxymethyl (cellulose)

cm centimetre

c.f.u. colony forming units

Co A coenzyme A

^{o}C degrees Celsius

D diffusion coefficient

DEAE diethylaminoethyl (cellulose)

DNA deoxyribonucleic acid

E extinction coefficient

$E^{1cm}_{650\ nm}$ extinction value of 650 nm solution in a cell of 1 cm light path at a wavelength of 650 nm

EDTA ethylene diamine tetra acetate

et al	et alios (and others)
EYF	egg yolk factor
fig (with reference numeral)	figure
g	gram
g	gravitational field, unit of centrifugation
h	hour
HU	haemolytic unit
IU	international unit
kg	kilogram
l	litre
LD_{50}	median lethal dose
lysoPC, PE, PG, PI, PSer	see below
m	metre
mA	milliamp
mg	milligram
ml	millilitre
mm	millimitre
mM	millimolar
µg	microgram
µl	microlitre
M	molar
m_{eff}	effective mass
min	minute
MW	molecular weight
N	normal
NADH	nicotinamide adenine dinucleotide, reduced
nm	nanometre

no.	number
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pH	negative logarithm of the hydrogen ion concentration
PI	phosphatidylinositol
pI	isoelectric point
PLC	phospholipase C
PSer	phosphatidylserine
R	gas constant per mole
rev	revolutions
RNA	ribonucleic acid
S	sedimentation coefficient
S_{20w}	sedimentation coefficient corrected to 20°C in distilled water
SDS	sodium dodecyl sulphate
sec	second
Sph	sphingomyelin
T	temperature
TBS	tris buffered saline
TEMED	N,N,N',N' tetramethylethylenediamine
V	volts
vol	volume
v/v	volume for volume
Vo	void volume
Ve	elution volume
W	watt
wt	weight
w/v	weight for volume

YDB	yeast diffusate broth
$\bar{\nu}$	partial specific volume
ρ	density
$\propto$	proportional to
η	viscosity
%	per cent
+	plus
-	minus
=	equals
&	and
"	inches
<	less than

INTRODUCTION

I GENERAL

Staphylococcus aureus is a pathogenic bacterium which produces a variety of toxic and potentially toxic factors. The purification and characterisation of the individual toxins is a basic requirement for determining the contribution of these products to the mechanisms of pathogenicity.

In spite of much information on the biological properties of individual toxins, their mode of action at the molecular level often remains obscure and, in most cases, their function in bacterial metabolism is unknown.

Staphylococci produce at least four haemolytic toxins, α, β, γ and δ. The toxicity of heated broth cultures of staphylococci recovered from lesions in man was first demonstrated by de Christmas (1888) and the haemolytic activity of such cultures against rabbit erythrocytes was first observed by Van de Velde (1894) and confirmed by Kraus & Clairmont (1900) and Neisser & Wechsberg (1901).

The discovery by Bordet (1897) that the heart blood of rabbits which had died of a staphylococcal infection was frequently lysed, suggested that this haemolytic activity was of importance in the pathogenicity of staphylococci.

There have been many reviews on the staphylococci and their toxins and other extracellular products in the last 25 years including those of van Heyningen (1950), Elek (1959), Gladstone (1966),

Bernheimer (1968, 1970, 1974), Arbuthnott (1970), Bergdoll (1970, 1972), Wiseman (1970, 1975), Woodin (1970, 1972), Abramson (1973), Jeljaszewicz (1972), Wadström & Möllby (1972) and Wadström, Thelestam & Möllby (1974). The points relevant to this thesis are reviewed below.

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1. Discovery

Staphylococcal β-lysin was first described as serologically distinct from α-toxin by Glenny & Stevens (1935). They examined culture filtrates from two different strains of staphylococci and found that with the first, dermonecrotising activity and haemolytic activities against sheep and rabbit erythrocytes were neutralized by roughly equal amounts of standard serum as was expected. However, with the filtrate from the second strain it was found that from twice to ten times as much standard serum was required to prevent haemolysis of sheep cells as of rabbit cells. The neutralization point determined by intracutaneous injection into guinea pigs or by intravenous or intraperitoneal injection into mice agreed closely with that determined by haemolysis of rabbit cells. They suggested that there was a second lysin haemolytic to sheep cells, but not to rabbit cells, and which was not necrotic to guinea pigs nor lethal to mice in the amounts tested. They further noted that the haemolytic effect of this second toxin was greatly intensified by standing at $4^{\circ}C$ after incubation at $37^{\circ}C$, suggesting that its identity with the hot-cold lysin of Walbum (1921) and Bigger, Boland & O'Meara (1927). These antigenically distinct lysins were referred to by Glenny & Stevens as α-toxin and β-toxin.

Bryce & Rountree (1936), examining strains of both human and animal origin, found that β-lysin was produced mainly by the bovine strains. They also found that the erythrocytes of sheep and ox were particularly susceptible to its action whereas the erythrocytes of rabbit, rat, guinea pig, ferret and koala were very resistant. Human cells were, however, slightly susceptible. Elek & Levy (1950) confirmed that β-toxin was produced primarily by coagulase-positive strains from animals, particularly from bovine mastitis (Slanetz & Bartley, 1953).

β-lysin has been shown to require Mg^{2+} or Co^{2+} ions for optimal activity, Co^{2+} being more effective than Mg^{2+}. Ca^{2+} inhibits the toxin (Jackson & Mayman, 1958; Robinson, Thatcher & Gagnon, 1958; Haque & Baldwin, 1964; Chesbro et al., 1965; Doery et al., 1965; Wiseman, 1965; Maheswaran & Lindorfer, 1967; Gow & Robinson, 1969).

2. Production

Many workers have reported enhancement of β-lysin production by growth in an atmosphere of 10 - 25% CO_2 (for review, see Wiseman, 1975), seemingly most effective as a mixture with oxygen rather than air. As with α-toxin production, however, it seems that the incorporation of yeast diffusate in the medium obviates the requirement for carbon dioxide, as two separate laboratories (Wadström & Möllby, 1971a; Bernheimer, Avigad & Kim, 1974) have reported good yields in liquid media without extra CO_2 in the gas phase. The role of carbon dioxide in the enhancement of β-lysin production is unknown, but effects upon growth do not appear to be responsible (Wiseman, 1970).

There is little information about the influence of nutritional factors on β-lysin production. However, the amino acid requirements for growth and β-lysin production by the R-1 strain have been investigated by Wiseman (1970) who showed an absolute requirement for proline, arginine and glycine. Cystine and methionine were required in the presence of each other, but not separately. The growth factors thiamine and nicotinamide were indispensible. Unfortunately there was no distinction between amino acids required for growth and those required for lysin production; however, Chesbro et al. (1965) achieved higher yields on supplementing their medium with 0.5% arginine. Using chemically defined media, Sharma & Haque (1973a) showed that the growth requirements of the 681C strain for lysin formation in air differed from those in carbon dioxide. Glutamine, proline and cystine were required both under air and carbon dioxide incubation and glycine, leucine, histidine, isoleucine, methionine, phenyl-alanine and tyrosine were required in air only. They confirmed the requirement for thiamine and nicotinamide. In a subsequent paper (Sharma & Haque, 1973b) tryptophan analogues were shown to be inhibitory to growth by interfering with tryptophan metabolism, despite the fact that strain 681C of S. aureus does not require this amino acid. Free fatty acids increase the rate of growth of S. aureus without increasing β-lysin production (Fritsche & Zitz, 1973).

β-lysin is produced at a maximal rate at a neutral pH during the early logarithmic growth phase (Wiseman, 1970).

3. Purification

The first attempts to purify β-lysin were in 1936 when Bryce

& Rountree selectively inactivated α-toxin by heating their preparations at 60°C for 15 min. Kodama & Kojima (1939) demonstrated that β-lysin could be precipitated with ethanol, methanol or acetone. This was partially confirmed by Fulton (1943), who obtained maximal recovery by precipitation in acetone at pH 9.0. Since then several methods of purification have been reported (Table 1) but since it is only recently that rigorous criteria of homogeneity have been applied, it is not surprising that there are some discrepancies in the reported activities of β-lysin. Table 1 shows that in purifying β-lysin, Gow & Robinson (1969) observed an increase in total haemolytic activity. This has also been reported by Haque & Baldwin (1969) and Wadström & Möllby (1971a). However, Bernheimer, Avigad & Kim (1974) were unable to duplicate this phenomenon, which was suggested to be due, at least in part, to the removal of inhibitors (Gow & Robinson, 1969). This activation always occurred during ion exchange chromatography, which was not included in Bernheimer's procedure. The most recently reported preparation procedure for β-lysin, that of Zwaal et al. (1975), gave a very high specific activity of sphingomyelinase C activity, but no data on β-lysin activity was reported. They suggested that this high specific activity, compared to those of Wadström & Möllby (1971a) and Bernheimer, Avigad & Kim (1974) was due to their procedures being carried out in 50% (v/v) glycerol, which stabilises some enzymes. Their product did not haemolyse human erythrocytes after 1 h at 37°C. They point out that the earlier purification procedure published by their group (Colley et al., 1973) was not reliable.

Purified β-lysin has been reported as stable in 50% glycerol at -20°C (Zwaal et al., 1975) or in the lyophilized state at -20°C

Table 1 Purification of β-lysin

Reference	Strain	Procedures[1]	Specific activity Haemolytic units/mg protein	Recovery	Purification (HU/mg protein)
Robinson, Thatcher & Gagnon (1958)	L16	A,C,A,D			670
Jackson (1963)	J32A	A,A,C			255
Chesbro et al. (1965)	UNH-Donita	C	6×10^4		
Doery et al. (1965)	1061-17	A,A,C	3.7×10^4		4
Wiseman (1965)	R1, 252F	C,A	1.2×10^6	17	30
Maheswaran et al. (1967)	J19	A,B,C,C	6.8×10^4		245
Wiseman & Caird (1967)	R-1, 252F	C,A,B			70
Gow & Robinson (1969)	MB534	A,B,C,D[2]	5.2×10^8	5200	180,800
Haque & Baldwin (1969)	681	A,C	6.6×10^6	2900	4,500
Wadström & Möllby (1971a)	R-1	C,E,B	$10^7 - 10^8$ (2 IU/mg)	15,000	40,000
Colley et al. (1973)		A,B	(1 IU/20 µl)		
Bernheimer, Avigad & Kim (1974)	G-128,R-1,234	A,B,E	4.7×10^5 (140 IU/mg)	27	81
Zwaal et al. (1975)	269HH	A,B,C,C	(1900 IU/mg)		

[1] A, precipitation; B, gel filtration (molecular sieve); C, ion exchange chromatography; D, electrophoresis (starch block); E, isoelectric focusing.

[2] D, Electrophoresis (sucrose gradient)

[3] Figures in brackets indicate specific activity of sphingomyelinase in IU/mg.
IU = International Unit

(Jackson, 1963; Maheswaran, Smith & Lindorfer, 1967; Wiseman & Caird, 1967; Wadström & Möllby, 1971a) although it has been reported elsewhere that lyophilisation leads to a substantial loss of activity (Bernheimer, Avigad & Kim, 1974).

4. Physicochemical characteristics

Despite the variety of purification procedures reported, relatively few contain any information on the physical properties of the molecule. However, it is known to be a protein (Haque & Baldwin, 1969; Jackson, 1963; Maheswaran, Smith & Lindorfer, 1967; Wiseman, 1965). The reported molecular weights are not consistent, nor do they correlate well with the reported sedimentation coefficients of 1.7 and 1.8 (Table 2). There are two reports of amino acid composition (Bernheimer, Avigad & Kim, 1974; Fackrell, 1973, Ph.D. Thesis, University of Manitoba); these are given in Table 3 along with the amino acid composition of α-toxin (Six & Harshman, 1973b) for comparison. There are a few discrepancies, notably the absence of proline and methionine and the presence of cystine in Fackrell's data as opposed to those of Bernheimer, Avigad & Kim (1973). The significance of this is not clear, especially since β-lysin prepared from the two strains in question (R-1 and G128) behave identically when analysed by polyacrylamide gel electrophoresis (Bernheimer, Avigad & Kim, 1974). The molecular weight by summation as reported by Fackrell (1973) agrees with the value of 15,500 obtained by Chesbro & Kucic (1971), but this is a minimal molecular weight and on summation of the composition as adjusted in Table 3, one obtains a molecular weight of 42,595.

Table 2 Some characteristics of β-lysin

Reference	Strain	Molecular Wt.	pI	S_{20w}
Chesbro et al. (1965)	UNHO Donita	59,000[a]	8.6 - 8.9	
Gow & Robinson (1969)	MB 534			1.7
Chesbro & Kucic (1971)	UNH-15	15,500[ab]		
	243-B1	15,500[a]		
		13,800[b]		
	243-B2	13,600[a]		
		11,000[b]		
Wadström & Möllby (1971a)	R-1	38,000[b]	9.4	
		33,000[b]		
Maheswaran & Lindorfer (1971)			9.5	
Bernheimer, Avigad & Kim (1974)	G128, R-1, 234	30,000[b]		
		29,000[c]	9.0	
		29,000[d]		
Fackrell & Wiseman (1976b)		26,000[b]	9.5	1.8
		16,100[d]		

(a) Ultracentrifugation; (b) Gel filtration; (c) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; (d) Amino acid analysis.

Table 3 Comparison of amino acid composition of α-toxin and β-lysin

Amino acid	Residues		
	α-toxin B (Six & Harshman 1973b)[a]	β-lysin (Bernheimer, Avigad & Kim 1974)[b]	β-lysin (Fackrell 1973)[c]
Aspartic acid	43	44	44
Threonine	23	14	17
Serine	19	23	33
Glutamic acid	20	25	38
Proline	9	10	0
Glycine	24	21	39
Alanine	11	12	25
Valine	14	12	18
Cystine/Cysteine	0	0	4
Methionine	6	4	0
Isoleucine	14	9	17
Leucine	14	12	20
Tyrosine	10	14	1
Phenylalanine	8	8	13
Lysine	23	28	33
Histidine	4	8	8
Tryptophan	4	6	
Arginine	8	6	13
Ammonia			143

a. Histidine set at 4 in original data.

b. Methionine set at 4.

c. Histidine set at 8.

Staphylococcal β-lysin is markedly unstable; its activity can be lost with standing at room temperature, when subjected to protracted agitation or when filtered through sintered glass or membrane filters (Chesbro et al., 1965). Crude β-lysin is relatively heat stable, being unaffected by heating at 60°C for 30 min but highly purified β-lysin loses 50% of its activity if treated in this fashion, and is inactivated by subsequent heating to 100°C for 10 min (Gow & Robinson, 1969).

Wadström & Möllby (1971a) have suggested that β-lysin starts to inactivate rapidly after it reaches a certain stage of purity. Crude β-lysin has a half life of 120 days at -20°C, but purified β-lysin has a half life of 1-2 days at the same temperature. Zwaal et al. (1975) purified and stored their sphingomyelinase preparation in 50% (v/v) glycerol and claimed that this was responsible for the high specific activity of their enzyme.

Chesbro et al. (1965) reported that β-lysin is resistant to digestion by trypsin (1 mg/ml, 24 h exposure) although α-toxin and nuclease were destroyed in 120 min. The trypsinized lysin had changed properties, however, in no longer being strongly retarded on cellulose phosphate columns. Chymotrypsin, pepsin and papain rapidly destroyed the activity of β-lysin. Wiseman (1965), however, found β-lysin to be sensitive to trypsin. β-lysin was also sensitive to the thiol-inactivating agents p-chloromercuribenzoate and iodoacetate (Chesbro et al., 1965).

5. Evidence for multiple forms

There is evidence that β-lysin can exist in more than one form.

While studying a strain from canine furunculosis by serological methods, Thaysen (1948) described a toxin which he called β_2 which differed from β-lysin in its antigenic properties and kinetics of haemolysis. Both toxins were present in culture filtrates and normal serum possessed significant levels of anti-β_2.

Further support for two forms of the toxin came from Haque & Baldwin (1964) and Maheswaran, Smith & Lindorfer (1967), both groups reporting the separation of a cationic (major) form and an anionic (minor) form on both DEAE- and CM-cellulose chromatography. Chesbro et al. (1965) have demonstrated two components by both immunodiffusion and electrophoresis in β-lysin purified by gradient elution chromatography on cellulose phosphate. In some of the strains they used they also noted a haemolysin which haemolysed rabbit erythrocytes at 37°C and sheep erythrocytes after a 'hot-cold' sequence, and was similar in activity to the anionic β-lysin reported by Haque & Baldwin (1964). They suggested that these lesser haemolytic activities were the basis of reports of multiple β-lysins.

Two antigenic components have also been detected in the β-lysin preparations of Doery et al. (1965) and Wiseman & Caird (1967). Haque & Baldwin (1969) showed that S. aureus 681 also produced an anionic and a cationic form.

Wiseman (1965) noted that there were slight differences in the heat sensitivity of β-lysins prepared from two different strains of staphylococcus (R-1 and 252F) although antiserum to β-lysin from 252F could neutralize the R-1 lysin. He proposed that this reflected the evidence of more than one kind of β-lysin. Similar suggestions were put

forward by Haque (1967) and Ali & Haque (1974), from studies of the electrophoretic behaviour of β-lysin from different strains of staphylococci.

Isoelectric focusing studies have also shown the existence of more than one form. Wadström & Möllby (1971a) found two peaks of activity on electrofocusing of crude β-lysin, a major cationic peak with a pI of 9.4 and a minor anionic peak with a pI of 3, the latter containing less than 5% of the total activity. In addition they showed microheterogeneity of the cationic peak, which on refocusing in narrow pH gradients (pH 8.0 - 10.0) showed peaks at pH 8.8, 9.2, 9.4 and 9.8.

Bernheimer, Avigad & Kim (1974) did not report the anionic form after the isoelectric focusing of a partially purified β-lysin preparation. However, they described a second protein peak at pI 9.5 (compared with 9.0 for β-lysin) which had a low degree of activity (2-3% of the specific activity of the main β-lysin. They suggested that this might be an inactive form of β-lysin capable of conversion to fully active material.

6. Mode of action

Bernheimer (1974) has described β-lysin as "the cytolytic toxin par excellence. It is both an enzyme and a toxin at the same time."

The discovery by Jackson & Mayman (1958) that the haemolytic activity of β-lysin was activated by divalent ions was the first evidence that suggested that it could be an enzyme. Then Doery et al. (1963, 1965) noted that β-lysin was capable of releasing water soluble phosphorus from rabbit and sheep red cell stroma. They found that this was due to the action of a phospholipase C acting only on sphingomyelin and lyso-

phosphatidylcholine. This activity was inhibited by commercial antitoxin which contained anti-β-lysin.

The degradation of sphingomyelin as suggested by Doery is thought to proceed as follows:

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

This observation was based on the loss of sphingomyelin from phospholipid extracts examined by paper chromatography and confirmed by the appearance of phosphorylcholine in the supernatant. These findings have since been confirmed (Wiseman & Caird, 1966, 1967; Maheswaran & Lindorfer, 1966, 1967; Fritsche, 1970; Wadström & Möllby, 1971a), and the enzyme described as a sphingomyelinase C or sphingomyelin choline-phospho-hydrolase E.C.3.1.4.12.

Wiseman & Caird (1967) examined the activity of the toxin on several phospholipids and reported that it was quite specific since only sphingomyelin and lysophosphatidyl choline were attacked. β-lysin did not hydrolyse phosphatidylethanolamine, phosphatidylcholine or the phosphate bonds of RNA, β-glycerophosphate and phenyl phosphate. In the same study, these workers explained the differences in susceptibility of various erythrocytes from different species (Smith & Price, 1938; Marks & Vaughn, 1950; Jackson & Mayman, 1958) by showing correlation between sensitivity to β-lysin and the sphingomyelin content of the membrane. This is supported by the work of Wadström & Möllby (1971b) and Bernheimer, Avigad & Kim (1974) who showed that sheep, ox and goat erythrocytes, where more than 40% of the phospholipid is sphingomyelin

(van Deenen & de Gier, 1964; Rouser et al., 1968) were more sensitive than human erythrocytes by several orders of magnitude (Table 4). It has been suggested that rabbit erythrocytes could be totally resistant to β-lysin and that their lysis is caused by contamination with small amounts of α-toxin, since as little as 1% contamination of β-lysin by α-toxin could account for the lysis (Bernheimer, Avigad & Kim, 1974). This may also apply to the erythrocytes of some other species.

According to White (1973) no mammalian tissues contain nearly such a high percentage of sphingomyelin as do ruminant erythrocyte membranes. It is worth noting that the sphingomyelin of the plasma membrane fraction of bovine mammary gland is 22% (Keenan et al., 1970). The significance of these observations will be discussed later.

Maheswaran & Lindorfer (1967) have investigated some aspects of the kinetics of hydrolysis of sphingomyelin by purified β-lysin. They found that the degradation of sphingomyelin from erythrocyte ghosts by β-lysin was directly proportional to temperature between 37°C and 45°C, although degradation of purified sphingomyelin was maximal at 41°C. Degradation of 'native' sphingomyelin in sheep erythrocyte membranes was much more rapid than that of purified ox-brain and sheep erythrocyte sphingomyelin. When lysin concentration was plotted against liberation of phosphorus, a straight line was obtained indicating a first order reaction.

7. The hot-cold haemolysis reaction

The most striking feature of staphylococcal β-lysin is its ability to produce "hot-cold" haemolysis in susceptible cells (Table 4). Incubation

Table 4 Sensitivity of erythrocytes from different animals to lysis by purified β-lysin

	"Hot-cold" Haemolytic titre (HU/ml)[a]	% Phospholipid as sphingomyelin[b]
Sheep	10^9	51.0
Ox	10^8	46.2
Goat	10^5	45.9
Rabbit	10^2	19.0
Pig	10	26.5
Cat	10	26.1
Chicken	10	22.7*
Fowl	10	21.4**
Human	10	20.1
Horse	10	13.5
Guinea pig	10	11.1

a. From Wadström & Möllby (1971b)

b. From Rouser et al. (1968) except *Kleinig et al. (1971)

**Kates & James (1961)

at 37°C of sheep or bovine erythrocytes with small amounts of lysin in the presence of Mg^{2+} leads to no haemolysis despite phospholipid hydrolysis, but when the erythrocytes are chilled after treatment with lysin at 37°C, rapid lysis ensues. This effect was early recognised as a characteristic of β-lysin (Bigger, Boland & O'Meara, 1927; Glenny & Stevens, 1935). This property is also exhibited by the α-toxin from Clostridium perfringens, which is a phospholipase C capable of hydrolysing phosphatidylcholine in addition to sphingomyelin (McFarlane & Knight, 1941; Smyth, 1972). Erythrocytes are lysed by Cl. perfringens enzyme and the haemolytic titre is increased approximately 4-8-fold in erythrocytes with a high sphingomyelin content if incubation at 37°C is followed by chilling.

The nature of the hot-cold phenomenon has been the subject of some speculation. Pulsford (1954) found that red cells incubated with β-lysin could be lysed at 37°C by rapid alteration of the pH or sodium chloride concentration above or below 0.85%. Wiseman (1965) also found that concentrations of NaCl below 0.80% were lytic, but concentrations above this level were not. He also found that rapid lowering of the pH from 6.9 gave rapid lysis of the treated cells while the change from 6.9 to 9.8 did not. Higher concentrations of sodium chloride (2.5%) and glucose (0.5 - 3.0%) inhibited lysis of treated sheep cells. Wiseman (1970) explained this increase in sensitivity to changes in pH and osmotic pressure as due to β-lysin causing small perforations in the cell membrane which were enlarged by a decrease in sodium chloride concentration or by rapid changes in pH. The hot-cold phenomenon could be affected by a decrease in temperature causing contraction of the cell membrane which would have the same result, causing an increase in the size of the perforations.

A variety of agents are now known to attack this weakened structure, including the phospholipase C from Bacillus cereus (Colley et al., 1973), staphylococci α- and δ-haemolysins (Möllby et al., 1974), the haemolytic toxin of Prymnesium parvum (Bergmann & Kidron, 1966) and ethylene diamine tetracetate (EDTA) (Smyth, Möllby & Wadström, 1975).

Bernheimer (1974) has suggested that sphingomyelin located on or near the membrane surface is extensively hydrolysed by β-lysin, giving "essentially lipid monolayers" which, although sufficiently stable to persist at 37°C, collapse through thermodynamic instability on cooling. This explains both "hot-cold" haemolysis and the internal vesiculation of treated ghosts and intact erythrocytes.

Meduski & Hochstein (1972) showed that treatment of sheep, human and rat erythrocytes with the triiodide (I_3^-) ion also gave a hot-cold effect.

The lytic effects of I_3^- depend on its concentration and lysis is complete at 37°C within 60 min. The extent of haemolysis is reduced if suboptimal concentrations of I_3^- are used, or if the length of incubation is decreased. However, additional leakage of haemoglobin is observed on cooling to 0°C, in proportion to the dose of I_3^- used. This cold lysis was inhibited by dipalmitoyl lecithin which was shown by thin-layer chromatography to bind the I_3^-. The authors suggested that a similar interaction of I_3^- with exposed choline groups in membrane phospholipids leads to hot-cold lysis.

The erythrocytes of sheep, man and rat showed both a decreasing order of sensitivity to the lytic effects of I_3^- and decreasing proportions

of sphingomyelin as part of their cell membrane phospholipids (Table 4) and Meduski & Hochstein (loc cit) proposed that sphingomyelin may well be the most active substrate for interaction with I_3^-. They argued that the fixed positive charge of phospholipids, particularly sphingomyelin, permits leakage of haemoglobin through the membrane which once initiated, proceeds to equilibrium during the 'hot' phase. When cells are transferred into the ice bath before the equilibrium is attained the passive movement of haemoglobin proceeds until equilibrium is reached. Their overall view is that "hot-cold haemolysis is a common feature of the response of erythrocytes to any agent which alters or removes $-\overset{+}{N}(CH_3)_3$ groups of membrane phospholipids."

Smyth, Möllby & Wadström (1975) have suggested that divalent cations, particularly magnesium, are important for the stability of sphingomyelin-depleted membranes since addition of ethylene diamine tetraacetate (EDTA) to sheep erythrocytes incubated with β-lysin induced rapid haemolysis at 37°C. Chelators related to EDTA were the most effective, whereas chelators more specific for Ca^{2+}, Fe^{2+} and Mg^{2+} were without effect. It has been reported (Romero, 1974) that the K^+ permeability of human resealed ghost membranes is mainly determined by the amount of Mg^{2+} associated with the membrane, so it is not surprising that removal of magnesium from the membrane structure can result in an increase in membrane permeability and lysis. It may be, however, that intra-membrane cations are more important to the impermeability of the membrane than those at the surface. β-lysin perturbation may make the former cations more accessible to the chelator and/or the erythrocyte membrane more sensitive to changes in divalent cation content. The authors propose that hot-cold haemolysis may be a consequence of the

temperature dependence of divalent cation stabilization of the ruminant erythrocyte membrane at 37°C.

8. Toxic properties

This is certainly the most controversial issue concerning β-lysin, the majority of the controversy arising from much of the early work having been done with impure preparations and so dosage levels are not really relevant. The presence of δ-lysin in staphylococcal culture filtrates was not demonstrated until the work of Williams & Harper (1947). Glenny & Stevens (1935) stated that β-lysin was lethal for rabbits but not for mice. In addition their preparation was non-dermonecrotic for rabbits although giving mild erythrema when injected subcutaneously. This response of rabbits was confirmed by Bryce & Rountree (1936). Thatcher & Matheson (1955) claimed that β-lysin caused emesis in cats, but it could be inactivated by boiling and subsequent incubation with ascorbic acid. However, Robinson, Thatcher & Gagnon (1958) later separated a fraction emetic for cats from a hot-cold haemolytic fraction in staphylococcal supernatants. Heydrick & Chesbro (1962) claimed that intraperitoneal injection of β-lysin is only lethal for guinea-pigs in the presence of Mg^{2+} ions. Wiseman (1965) found that partially purified preparations were non-lethal for rabbits, guinea pigs and mice in the presence or absence of Mg^{2+} and that subcutaneous injections in rabbits caused only mild erythrema. Maheswaran, Smith & Lindorfer (1967) found β-lysin to be non-dermonecrotic in both the presence and absence of Mg^{2+}. Toxicity studies with the more recent purifications of β-lysin of Gow & Robinson (1969) and Wadström & Möllby (1971a,b) are in agreement so far as both groups found

β-lysin to be lethal for rabbits. Wadström & Möllby (1971b) found β-lysin to be lethal in the same range (10 - 100 μg) for mice and guinea-pigs and also for chicken embryos (0.25 - 10 μg). The LD_{50} of α-toxin for mice is 1 μg (Bernheimer & Schwartz, 1963; Lominski, Arbuthnott & Spence, 1963; Fackrell & Wiseman, 1976b) so it would appear that β-lysin is 10 - 100 times less toxic than α-toxin. It could also be argued that this toxicity represents a level of contamination of 1 - 10% α-toxin, or some other similarly toxic component, assuming no synergistic interaction between toxins.

Much of the more recent work on toxicity has been done using cells in tissue culture. Bernheimer & Schwartz (1964) were unable to demonstrate β-lysin disruption of rabbit leukocytes or liver lysosomes, but in a later publication (1965) found that β-lysin attacked rabbit blood platelets. Chesbro et al. (1965) showed that their preparation was leukocidal for guinea-pig macrophages. Their preparation also showed carbohydrase activity. Korbecki & Jeljaszewicz (1965) showed that β-lysin exerted a toxic effect on KB and monkey kidney cells, causing detachment of the former from glass and vacuolation and disintegration. Monkey kidney cells became granular. They further showed by histochemical methods (Jeljaszewicz et al., 1965) that KB cells were altered in their ability to hydrolyse β-naphthyl acetate and 5-bromoindoxyl acetate.

Gladstone & Yoshida (1967) found that crude β-lysin had no effect on HeLa, L, HL, FL, HeP, chick fibroblasts and rat heart connective tissue. Hallander & Bengtsson (1967) found no effect on uptake of neutral red by human, bovine and monkey kidney cells after incubation with β-lysin for 6 h.

Wiseman (1968) reported that β-lysin was toxic for human amnion, KB and monkey kidney cells as measured by dye uptake, absence of acid production in the medium and ability to attach to glass surfaces. Wadström & Möllby (1971b) found that β-lysin lysed human thrombocytes and their α-granules, and was also toxic for HeLa cells, monkey kidney cells and diploid human fibroblasts.

However, more recent studies (Wadström, Thelestam & Möllby, 1974) revealed that fibroblasts and HeLa cells were highly resistant. It is, however, not possible to compare the effects of toxins in laboratory animals with the effects on cells in tissue culture since the dose per cell is far greater in the second case than the first.

9. In vivo mode of action

There is no evidence at present that β-lysin hydrolyses sphingomyelin in vivo. This is possibly the case with the phospholipase C (α-toxin) from Cl. perfringens as well, since it was noted that in a patient suffering from a clostridium septicaemia the osmotically fragile cells had a grossly altered protein composition but little or no change in phospholipid (Simpkins et al., 1971).

Corkill (1955) showed that injection of β-lysin into rabbits increased the blood sugar levels. Smith (1965) found that the blood glucose level in human patients dying of staphylococcal infections was significantly elevated the day before death. However, carcass analysis of mice killed by a staphylococcal infection showed decreases in total glucose. This is not contradictory to earlier reports if one assumes that the elevated blood sugar levels indicate glucose loss from tissues

passing into the urine via the blood stream. Bergman, Gutman & Chaimovitz (1965) reported biphasic changes in blood pressure in cats and mice injected with β-lysin. However, both Bergman, Gutman & Chaimovitz and Corkill were using preparations which were toxic and of unspecified purity. β-lysin can pass the blood brain barrier, but does not cause specific EEG changes before the death of the animal and it has been proposed that the animals die from a cardiovascular shock (Kwarecki et al., 1973), possibly mediated by the release of vasoactive substances.

III OTHER STAPHYLOCOCCAL TOXINS AND ENZYMES

Strains of S. aureus are known to produce a large number of extracellular toxins and enzymes both in vivo and in vitro. Bernheimer & Schwartz (1961) found that potentially pathogenic strains could produce as many as 12 - 14 proteins detectable by starch gel electrophoresis, whilst non-pathogenic strains produced about half as many. Wadström, Thelestam & Möllby (1974) reported with some strains, as many as 30 protein bands in isoelectric focusing in polyacrylamide gel; in addition, different strains revealed different patterns of bands.

Most staphylococcal extracellular proteins are basic in charge and have similar molecular weights (Tables 5 & 6) so it is not surprising that many of the earlier preparations of β-lysin used for biological studies had differing properties, caused by contamination. For example, the purified β-lysin of Chesbro et al. (1965) was shown to contain glycosidase activity and was probably contaminated with endo-β-N-acetylglucosaminidase (Wadström & Hisatsune, 1970).

Table 5 Extracellular toxins from S. aureus

Toxin	pI	MW	Reference
α-toxin	8.55	26-36,000	Wadström (1968); McNiven, Owen & Arbuthnott (1972; Six & Harshman (1973a,b).
β-lysin	9.4 9.5 9.0	30-38,000 30,000	Wadström & Möllby (1971a); Maheswaran & Lindorfer (1971; Bernheimer, Avigad & Kim (1974).
δ-lysin	4.65 6.7 9.0	103,000 195,000	Kantor, Temples & Shaw (1972)
γ-lysin	9.8	26,000	Taylor & Bernheimer (1974)
lymphocyte mitogen	5.6, 8.8		Kreger, Cuppari & Taranta (1972)
epidermolytic toxin	7.0	33,000	Kondo, Sakurai & Sarai (1973); Arbuthnott, Billcliffe & Thompson (1974)
enterotoxins A-E	6.8 - 8.6	28,000 - 35,000	Bergdoll (1972)

Table 6 Extracellular enzymes from S. aureus

Enzyme	pI	Molecular Wt.	Reference
Protease	9.4	12,500	Arvidson, Holme & Lindholm (1973)
Hyaluronidase	7.9	82,000	Wadström & Möllby (1972); Abramson & Rautela (1971)
Lipase	9.5	100,000	Vesterberg (1972)
Endo-β-N-acetyl glucosaminidase	9.6	70,000	Wadström & Hisatsune (1970)
Coagulase	5.9-6.1	44,000	Tirunarayanan (1969) Duthie & Haughton (1958)
Nuclease	10.1		Wadström (1967)
Phosphatase	3.8-4.2	58,000	Tirunarayanan (1969) Malveaux & San Clemente (1969)
Amidase	9.8		Wadström & Vesterberg (1971)

1. The staphylococcal haemolysins

S. aureus produces at least three other haemolysins in addition to β-lysin. These have been designated α, δ, and γ (Glenny & Stevens, 1935; Smith & Price, 1938; Williams & Harper, 1947). The discovery of α-toxin was made in studies of the lethal and haemolytic effects of cultures on rabbits (Van de Velde, 1894). δ-lysin was detected by Williams & Harper (1947) in strains of S. aureus grown on sheep blood agar to which α and β antihaemolysins had been added. γ-lysin was demonstrated by Smith & Price (1938) following the observations of Morgan & Graydon (1936) on two antigenically distinct rabbit red cell lysins. As pointed out earlier, it is impossible to consider the properties of any single component of staphylococcal filtrates without considering the properties of the other components which may be contaminating the "purified" protein, and so some aspects of the haemolysins will be briefly reviewed.

i. Staphylococcal α-toxin

α-toxin is certainly the staphylococcal haemolysin which has been characterised most fully. It would appear that the view expressed by Burnet (1929), that a single substance is responsible for the dermonecrotic, haemolytic and lethal activities is substantially correct. These effects, combined with the particular sensitivity of rabbit erythrocytes for the toxin, are the characteristics which differentiate it from the other staphylococcal products.

a. Physicochemical characteristics: The purified toxin is a protein (Arbuthnott, 1970). Several amino acid analyses have been

reported (Bernheimer & Schwartz, 1963; Coulter, 1966; Fackrell & Wiseman, 1967b; Six & Harshman, 1973b) and the composition is markedly similar to that of β-lysin (Table 3). The reported molecular weights vary between 10,000 - 45,000 depending on the method used (Wiseman, 1975). There are two sedimentation coefficients quoted for alpha-toxin, one at approximately 3.0S and the second at 12.0S or 16.0S (Bernheimer & Schwartz, 1963; Lominski, Arbuthnott & Spence, 1963). The 12S peak is apparently composed of inactive polymerized toxin and can be disaggregated by 8 M urea to yield active 3S toxin (Arbuthnott, Freer & Bernheimer, 1967). The 12S toxin appears as rings 90 - 100 Å in diameter when examined by negative staining in the electron microscope. These rings are composed of six subunits, 20 - 25 Å in diameter. McNiven, Owen & Arbuthnott (1972) estimated the molecular weight of 12S toxin to be 170,000, suggesting that each of the six subunits is a 3S monomer with a molecular weight of 28,000.

b. Evidence for multiple forms: In addition to the 3S and 12S forms described above, the 3S form of the toxin reveals further micro-heterogeneity in electrophoretic mobility, pI and molecular weight. The variations in molecular weight may be due to two forms of 3S toxin, one of molecular weight 22,000 and the second a dimer of molecular weight 44,000 and that estimates in the 30,000 region represent mixtures of the two (McNiven, Ph.D. Thesis, 1972). Purified α-toxin has been found repeatedly to be electrophoretically heterogeneous and up to four charge isomers have been described (Bernheimer & Schwartz, 1963; Wadström, 1968; McNiven, Owen & Arbuthnott, 1972; Dalen, 1975). The main component has a pI of around 8.5 and accounts

for 80 - 90% of the haemolytic activity of the strains which produced it (Wadström, 1968; McNiven, Owen & Arbuthnott, 1972).

c. Mode of action: The mode of action of α-toxin has been discussed in reviews by Arbuthnott (1970), Bernheimer (1974) and Wiseman (1975) but its mechanism of action is still controversial. From its interactions with intact erythrocytes, isolated membranes and artificial membranes, it has been suggested that α-toxin interacts with phospholipids in sensitive membranes via hydrophobic moieties located within the molecule (Weissmann, Sessa & Bernheimer, 1966; Arbuthnott, 1970). In this context it has been noted that α-toxin is sufficiently surface active to be capable of forming monolayers at the air-water interface (Buckelew & Colacicco, 1971). This does not, however, explain the differences in the sensitivity of erythrocytes from different mammalian species to α-toxin (Bernheimer & Schwartz, 1963; Cooper, Madoff & Weinstein, 1966), since Cassidy, Six & Harshman (1974) have shown that spherules composed of human erythrocyte phospholipids and rabbit erythrocyte phospholipids were equally sensitive to lysis by α-toxin.

Another view of the mode of action is that the membrane activates the toxin which then acts as a protease hydrolysing membrane proteins (Wiseman & Caird, 1970, 1972). The sensitivity of erythrocytes to α-toxin correlates with the level of proteolytic activity in the membranes. However, Freer, Arbuthnott & Billcliffe (1973) were unable to find evidence for a proteolytic mechanism and concluded that membrane proteins were not involved as a substrate or receptor for α-toxin. Kato et al. (1975) have shown that flavin

mononucleotide is capable of inhibiting haemolysis of rabbit erythrocytes by α-toxin, and suggest that this is due to interaction with specific glycoprotein or glycolipid binding sites for α-toxin. Kato & Naiki (1976) have postulated that an N-acetylglucosamine containing ganglioside is the membrane receptor for α-toxin, which is similar to the sialidase sensitive and sialidase resistant gangliosides which are suspected of being the receptor sites for tetanus and cholera toxins respectively (van Heyningen, 1963; van Heyningen et al., 1971; Holmgren, Lonnroth & Svennerholm, 1973).

What is clear, however, is that the addition of 3S α-toxin to erythrocytes from a variety of mammalian sources results in the formation of ring-shaped structures as seen by electron microscopy. These ring polymers (12S) also result from interaction of α-toxin with liposomes, but not with bacterial membranes (Bernheimer, 1974; Wiseman, 1975). These rings often appear in an ordered fashion. Remsen, Watson & Bernheimer (1970) suggested this may be due to a pre-existing regular arrangement of phospholipid but it now appears that this may be due to contamination with δ-toxin (Bernheimer et al., 1972). Freer, Arbuthnott & Billcliffe (1973) have shown by the freeze-etching technique that α-toxin is capable of disrupting the hydrophobic inner core of the membrane.

d. Toxic properties: In addition to the haemolytic effect, α-toxin has been found to be lethal to all species of experimental animals tested so far. The LD_{50} is between 25 - 50 μg/kg for mice and approximately 2 μg/kg for rabbits (Arbuthnott, 1970; Lominski, Arbuthnott & Spence, 1963; Watanabe & Kato, 1974). Injection of

small amounts of α-toxin into the skin of experimental animals results in extensive necrosis, sloughing of tissue and scab formation (Wiseman, 1975). α-toxin also shows a variety of toxic effects against cells in tissue culture (Jeljaszewicz, 1972).

ii. Staphylococcal δ-lysin

δ-lysin is the most frequently produced haemolysin by both coagulase positive and coagulase negative staphylococci and is recognisable by its broad haemolytic spectrum (Elek, 1959; Kreger et al., 1971). It is commonly assayed against human or horse erythrocytes, although it has been found that fish erythrocytes are particularly sensitive (Birkbeck, Chao & Arbuthnott, 1974).

a. Physicochemical characteristics: δ-lysin is a protein, but the wide variety of physicochemical and biological properties reported for the lysin suggests that different studies have not always involved the same substance. The molecular weight has been reported as 12,000 by gel filtration by Kayser (1966) and 200,000 by Hallander (1963). The sedimentation coefficient has been quoted as low as 1.45 (Kayser, 1966) and as high as 11.9 (Kreger et al., 1971). Most workers state that the main form is basic with a pI of around 9.5 (Kreger et al., 1971; Möllby & Wadström, 1970; Fackrell & Wiseman, 1976). However, Maheswaran & Lindorfer (1970) found three components with a main form isoelectric at pH = 3.75. Whitelaw, Chao & Birkbeck (unpublished observations) report a main form with pI between 4.5 and 5.

b. Evidence for multiple forms: Jackson & Little (1958) separated the activities of δ-lysin and α-toxin by heating at 60°C for 10 - 15 min.

They observed a sharp drop in δ-lysin activity in addition to the inactivation of α-toxin. They suggested the existence of two δ-lysin compounds, one heat labile and the other heat stable, which also differed in ethanol solubility. However, Guyonnet & Plommet (1970) thought that only the heat labile component was δ-lysin. Hoffman & Streitfeld (1965) separated two forms of δ-lysin by paper chromatography. Caird & Wiseman (1970) reported two forms in the ultracentrifuge and Kreger et al., (1971) found both soluble and insoluble types. Isoelectric focusing studies have also shown that δ-lysin is molecularly heterogeneous (Maheswaran & Lindorfer, 1970; Kreger et al., 1971; Kantor, Temples & Shaw, 1972).

c. Mode of action: The mode of action is unclear. Wiseman & Caird (1968) have proposed that δ-lysin possesses a phospholipase C activity, probably specific for phosphatidylinositol. This would confirm the results of Doery et al. (1965) who found similar activities in their toxin preparations. Kreger et al. (1971), however, failed to detect phospholipase C activity in their preparations and phospholipase C specific for phosphatidylinositol has been purified from S. aureus and has been shown to be distinct from δ-lysin activity (Low & Finean, 1976).

It has been postulated that the lysin acts as a surfactant (Marks, 1951; Bernheimer, 1970; Galston, 1971) which would account for its low specificity and wide spectrum of activity. It has been reported that δ-lysin can be neutralised by phospholipids (Kapral, 1967) and it may be that δ-lysin behaves as a surface active polypeptide.

However, recent work by Kapral et al. (1976) showed that δ-lysin inhibited water absorption in the ileum of rabbits and guinea pigs. It was shown to have an action similar to cholera toxin, increasing cyclic AMP levels in isolated guinea pig ileum (O'Brien & Kapral, 1976). These workers suggested that it could have a possible role in staphylococcal enteritis.

d. Toxic properties: In addition to acting as a haemolysin, δ-lysin has been shown to have some leukocidal activity (Gladstone & van Heyningen, 1957; Jackson & Little, 1957) and is capable of damaging cells in tissue culture (Gladstone & Yoshida, 1967; Jackson & Little, 1956, 1957; Kreger et al., 1971; Thelestam & Möllby, 1975a,b). However, the lethal effects of δ-lysin are very low indeed compared to those claimed for α-toxin. It has been reported as dermonecrotic (Marks & Vaughn, 1950; Gladstone, 1966; Fackrell & Wiseman, 1976b) although there are reports to the contrary (J.P. Arbuthnott, unpublished data).

The LD_{50} for mice, rabbits and guinea pigs has been quoted as between 4 and 10 mg/kg (Fackrell & Wiseman, 1976b; Gladstone, 1966) although Wadström & Möllby (1972) have stated that it is as high as 5,000 mg/kg. Thus it would seem unlikely that it plays a major part in animal or human disease, especially since it is inhibited by normal serum (Marks & Vaughn, 1950). However, there are reports that δ-lysin can act synergistically with other lysins, potentiating their effects (Kreger & Bernheimer, 1971; Wadström, Thelestam & Möllby, 1974).

iii. Staphylococcal γ-lysin

γ-lysin was described in 1938 by Smith & Price, but for a long time it was thought to be identical with the α_2 and γ-lysins of Elek & Levey (1950) and Elek (1959). However, it is now recognised as separate and has been recently obtained in a highly purified form (Möllby & Wadström, 1971; Taylor & Bernheimer, 1974; Fackrell & Wiseman, 1976a,b).

a. Physicochemical characteristics: With the exception of Fackrell & Wiseman (1976a), who report a pI of 6.0, all other workers agree that γ-lysin is a basic protein with a pI between 9.5 and 9.9 (Guyonnet, Plommet & Bouillane, 1968; Guyonnet & Plommet, 1970; Möllby & Wadström, 1971; Taylor & Bernheimer, 1974). There may also be an anionic form since Möllby & Wadström (1971) reported that γ-lysin bound to DEAE-sephadex. The molecule has an $S_{20w} = 2.6$ and for the two forms the molecular weight has been reported as 26,000 and 29,000 by Taylor & Bernheimer (1974) and 45,000 (Fackrell & Wiseman, 1976a). γ-lysin is inhibited by agar (Jackson, 1963) and a variety of sulphonated polymers (Wadström & Möllby, 1972) and also by lipids (Taylor & Bernheimer, 1974; Fackrell & Wiseman, 1976b).

b. Evidence for multiple forms: Evidence for multiple forms of γ-lysin is scant due to the lack of data on highly purified material. Taylor & Bernheimer (1974) quoted the existence of two components of different molecular weight and pI, confirming the earlier findings of Guyonnet (1970). These two proteins are extremely close in pI (9.8 and 9.9) which may explain why other workers have not resolved

separate forms (Fackrell & Wiseman, 1976a,b; Möllby & Wadström, 1971).

c. Mode of action: The mode of action of γ-lysin is unknown. Its reaction kinetics favour an enzymic mode of action, as does its cation requirement. It is capable of releasing acid soluble nitrogen and phosphorus from erythrocyte ghosts (Fackrell & Wiseman, 1976b). However it does not degrade the extracted phospholipids of human erythrocyte membranes (Taylor & Bernheimer, 1974; Fackrell & Wiseman, 1976b). Gamma-lysin acts on human, rabbit and sheep erythrocytes but not on horse erythrocytes (Guyonnet & Plommet, 1970).

d. Toxic properties: Möllby & Wadström (1971) reported that γ-lysin was lethal for mice and rabbits in doses of less than one milligram and that it was cytotoxic for a variety of cell types in tissue culture. Guyonnet (1970) also reported that the lysin was toxic to mice. However, Fackrell & Wiseman (1976b) found γ-lysin to be non-toxic to mice in doses up to 100 µg. Guinea pigs, on the other hand, were killed instantly if they were injected with 50 µg amounts intracardially. The lysin is non-dermonecrotic.

2. The staphylococcal enterotoxins

There are five serologically distinct enterotoxins, designated A, B, C, D and E (Bergdoll, 1972). They are produced in rather small amounts under normal cultural conditions and all 5 have molecular weights between 28,000 and 35,000. Their pI's range from 6.8 for enterotoxin A to 8.6 for enterotoxin B (Bergdoll, 1970). They are not normally lethal for humans although deaths have been reported in

the very young (Kienitz, 1964). The minimal amount required to produce the emetic symptoms in man is thought to be 1 µg, but laboratory animals are generally more resistant. Large doses (1 mg/kg) of enterotoxin B in rhesus monkeys causes death (Crawley et al., 1966). The enterotoxin is thought to have a direct effect on the gastro-intestinal tract (Bergdoll, 1972).

3. Epidermolytic toxin

This toxin causes intra-epidermal splitting and extensive exfoliation of the epidermis. It is a protein with a molecular weight of between 24,000 and 33,000 (Kapral & Miller, 1971; Arbuthnott et al., 1972; Melish, Glasgow & Turner, 1972; Kondo, Sakurai & Sarai, 1973) and a pI of approximately 7.0 (Arbuthnott, Billcliffe & Thompson, 1974).

4. Leucocidin

Panton-Valentine leucocidin specifically kills polymorpho-nuclear leucocytes and macrophages of rabbit and man and no other cell type. It consists of two protein components designated f and s, which act synergistically. The molecular weights are 32,000 and 38,000 respectively (Woodin, 1970, 1972) and the complex has a pI of 9.0 (Wadström, Thelestam & Möllby, 1974). It is not a very toxic substance alone, as injected leucocidin acts by killing circulating leucocytes, which are soon replaced. It interacts with the leucocyte cell membrane, interfering with the potassium ion pump.

5. Extracellular enzymes

There are a number of exoenzymes produced by staphylococci with important biological activities which may contribute to the pathogenic process. These have been reviewed by Abramson (1972) and only a brief outline of their properties will be given here.

i. Coagulase

Free coagulase is an acidic protein with a pI of 5.9 - 6.1 (Tirunarayanan, 1969) and a molecular weight of approximately 44,000 (Duthie & Haughton, 1958) and its biological activity is the clotting of plasma.

ii. Hyaluronidase

Hyaluronidase hydrolyses the cell cementing substance hyaluronic acid, thus increasing the permeability of tissues. There are as many as four to five forms of the enzyme (Vesterberg et al., 1967) with pI's of 4, 6, 6.4, 8.2 and 9.7. The major fraction of Abramson & Rautela (1971) had a molecular weight of 82,000.

iii. Phosphatase

Phosphatase has a pI of 3.8 - 4.2 (Tirunarayanan, 1969) and a molecular weight of 58,000 (Malveaux & San Clemente, 1969). It is capable of degrading a variety of sugar phosphates important to the metabolic activity of cells.

iv. Nuclease (deoxyribonuclease)

The enzyme is a phosphodiesterase which can cleave DNA and RNA to produce nucleosides. It has a molecular weight of 17,000 (Heins et al., 1967) and a pI of 10.1 (Wadström, 1967; Tirunarayanan et al., 1969).

v. Proteases

The proteases are a heterogeneous group of enzymes with molecular weights between 45,000 and 50,000 (Tirunarayanan & Lundblad, 1966) and pI values of approximately 4, 9.5 and 5 (Arvidson, 1973; Vesterberg et al., 1967). They are recognised by their ability to hydrolyse a variety of substrates including gelatin, casein, human serum and fibrin.

vi. Lipase

There are a variety of enzymes from staphylococci with lipolytic activity, such as the egg yolk factor. Lipase activity has been found with a pI in the region of 9.5 (Vesterberg et al., 1967) and molecular weights ranging from 100,000 (Vadehra & Harmon, 1967) to 1,000,000 (Tirunarayanan & Lundbeck, 1968).

vii. Endo-β-N-acetylglucosaminidase

This enzyme has a pI of 9.5 and can contaminate preparations of β-lysin (Wadström & Hisatsune, 1970). Care must therefore be taken not to interpret the lytic activity of β-lysin preparations against intact bacteria as due to the lysin itself.

IV THE ACTION OF PHOSPHOLIPASES ON CELL MEMBRANES

1. Introduction

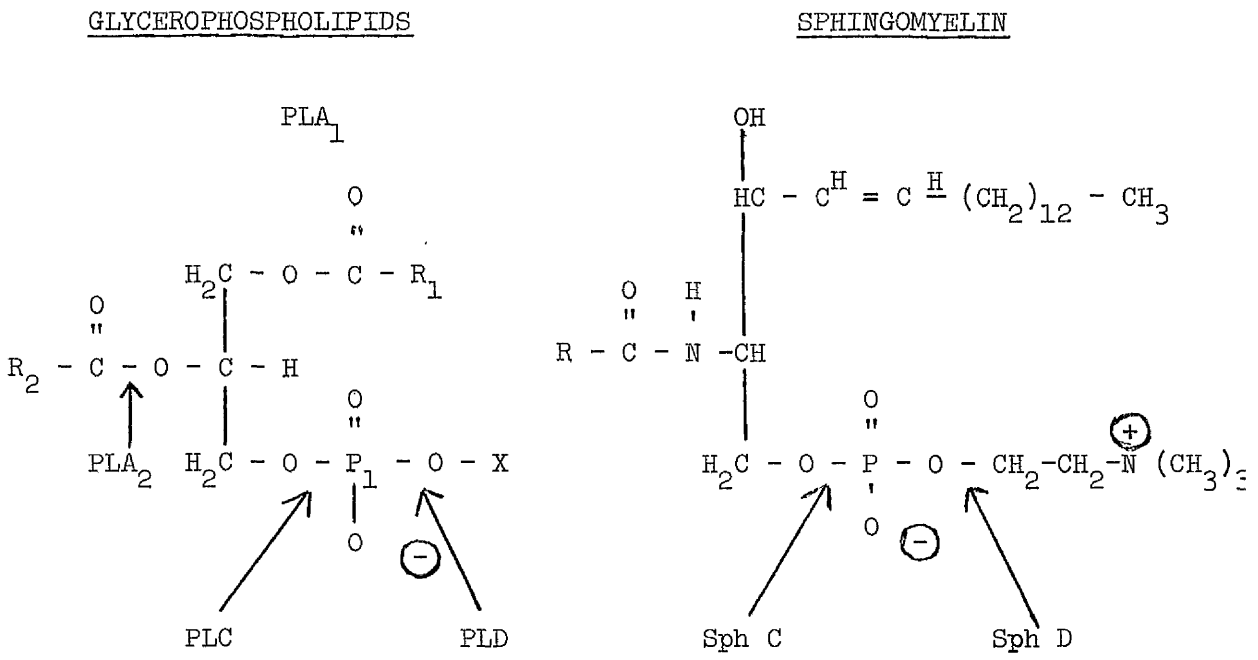
The phospholipases are classified as hydrolases and are widespread in nature. There are four established types which are classified by their site of attack on phospholipids (Figure 1).

Phospholipases A_1 and A_2 catalyse the release of a fatty acid from the 1 and 2 positions respectively. Phospholipase C catalyses the release of the phosphorylated base and phospholipase D the release of the nitrogenous base only. There is also a phospholipase B activity which acts primarily on monoacylphosphoglycerides ("lyso" compounds) which are the products of phospholipase A activity, and a phosphatidate phosphatase which splits inorganic phosphate from phosphatidic acid.

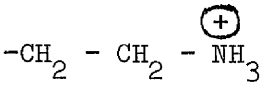
i. Phospholipase A

Phospholipase A_2 was discovered long ago as an active principle of snake venoms and has been studied extensively by Hanahan and his group (Hanahan, Brockerhoff & Barron, 1960). Enzymes of this type are widely distributed among animal tissues (Gallai-Hatchard & Thompson, 1965), and De Haas et al. (1968) have purified the pancreatic enzyme and found that it has a molecular weight of 14,000, less than half that of the Crotalus adamanteus enzyme (Wells & Hanahan, 1969). The enzyme has also been purified from bacteria (Scandella & Kornberg, 1971). The snake venom enzyme preferentially hydrolyses lecithin whilst the pancreatic enzyme rapidly attacks the acidic phospholipids, hydrolysing lecithin rather slowly (De Haas et al., 1968).

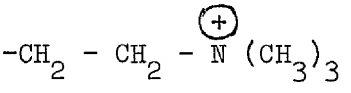
Figure 1. Site of action of various phospholipases

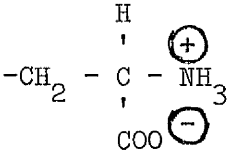


X = phosphatidylethanolamine

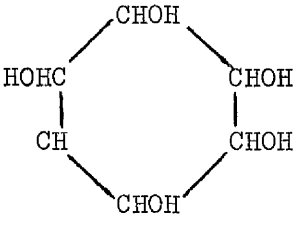


phosphatidylcholine





phosphatidylinositol



phosphatidic acid -H

R, R_1, Ra = fatty acids

PLA_1, PLA_2, PLC, PLD = phospholipases

Sph C, Sph D = sphingomyelinases

Phospholipase A_1 was characterised by Waite & van Deenen (1967) from rat liver. Phospholipase A_1, like A_2 requires Ca^{2+} for activity. Bacteria have also been used as a source of phospholipase A (Kent & Lennarz, 1971; Gaal & Ivanovics, 1972; Bernard et al., 1973; Doi and Nojima, 1973; Nishijima, Akamatsu & Nojima, 1974). There are a number of other reports on the sources and purification of phospholipases A, but it is only worth mentioning here the discovery of phospholipase A activity in staphylococcal culture filtrates by Magnusson, Doery & Gulasekharam (1962). However, Nygren, Hoborn & Wahlen (1966) showed that this was not a very common extracellular product, appearing in only 5 out of the 97 strains they tested.

The phospholipase A enzymes are often membrane-associated (Kent & Lennarz, 1972; Kramer, Jungi & Zaher, 1974; Zwaal et al., 1974) and are usually involved in phospholipid metabolism and turnover and the transfer and exchange of fatty acid residues between different phospholipids. The products of hydrolysis, namely lysophospholipids, are effective detergents capable at low concentrations of disrupting membranes. Therefore phospholipase A is of limited use as a probe in studies of phospholipid distribution and lipid-protein interactions.

ii. Phospholipase B

This enzyme also known as lysophospholipase, is also of importance in phospholipid catabolism and is widespread in its distribution. It has not been used at all in structural studies of membranes since its substrate is the product of phospholipase A digestion, and as stated before, the lysophosphatides are effective

membrane-disrupting agents. However, it is important to note that phospholipase A can show phospholipase B activity (Van Deenen & De Haas, 1963; Shiloah et al., 1973) and that the sphingomyelinase (β-lysin) from S. aureus hydrolyses lysophosphatidylethanolamine (Doery et al., 1965).

iii. Phospholipase D

Enzymes of this type have not been found in animal cells but are present in plant tissues. However, extensive purification has rarely been achieved and so reports on its substrate specificity are somewhat contradictory. Ono & White (1970) have reported the preparation of a cardiolipin-specific phospholipase D from an extract of Haemophilus parainfluenzae. A similar enzyme has also been reported in Escherichia coli homogenates (Cole, Benns & Proulx, 1974) and extracts from E. coli, Salmonella typhimurium, Proteus vulgaris and Pseudomonas aeruginosa (Cole & Proulx, 1975). An enzyme of broader specificity hydrolysing phosphatidylethanolamine, phosphatidylcholine, cardiolipin, sphingomyelin, phosphatidylserine and lysophosphatidylcholine has recently been purified from the culture medium of Streptomyces hachijoensis (Okawa & Yamaguchi, 1975a). Corynebacterium ovis, C. haemolyticum and C. ulcerans produce a phospholipase D which preferentially attacks sphingomyelin and lysophosphatidylcholine (Soucek, Michalec & Souckova, 1967, 1971). This enzyme is believed to be the most important dermonecrotic toxin and lethal factor in these species (Souckova & Soucek, 1972; Soucek & Souckova, 1974). It also has the unusual property of inhibiting the activity of the sphingomyelinase C of S. aureus and the phospholipase C

of Cl. perfringens (Soucek, Michalec & Souckova, 1971; Souckova & Soucek, 1972; Möllby et al., 1974).

iv. Phospholipase C

This enzyme is found primarily in bacteria and has been studied extensively (Ottolenghi, 1969). The most frequent sources have been Cl. perfringens and B. cereus.

Cl. perfringens α-toxin was the first bacterial toxin to have its mode of action defined as an enzyme activity (Macfarlane & Knight, 1941). A lecithinase in B. cereus was also suspected of acting as the lethal toxin (McGauchey & Chu, 1948; Chu, 1949). However, the lethal, haemolytic and phospholipase C activities of B. cereus supernatants have been separated (Ottolenghi, Gollub & Ulin, 1961; Johnson & Bonventre, 1967). There are now various methods suitable for the purification of these two enzymes. Since phosphorycholine is water soluble and the residual diglyceride does not have detergent properties, phospholipase C enzymes are of far greater use than the other types of phospholipase in membrane studies. Several species of Bacillus produce phospholipase C (van Heyningen, 1970). The best known is that from B. cereus which has been purified to apparent homogeneity using a variety of methods (Zwaal et al., 1971; Otnaess et al., 1972; Little, Aurebekk & Otnaess, 1975). The method giving the highest recovery and activity is that of Little, Aurebekk & Otnaess, based on affinity chromatography. The enzyme purified in this fashion is only slightly haemolytic to human erythrocytes and is totally inactive against sphingomyelin although it can hydrolyse phosphatidylcholine and ethanolamine (Van Deenen, 1964;

Kleiman & Lands, 1969; Zwaal et al., 1971). There is good agreement on the physical properties of this enzyme; it appears to have a molecular weight between 21,000 and 25,000 and a pI of 6.5 (Otnaess et al., 1972; Zwaal et al., 1971). There are reports that B. cereus may produce two or more distinct phospholipases with different substrate specificities (Slein & Logan, 1963, 1965) but these data have not been corroborated. Studies have been carried out on the divalent metal ion dependence of this enzyme which appears to be a zinc metalloenzyme (Ottolenghi, 1965; Little & Otnaess 1975).

There are a great variety of techniques for the preparation of partially purified phospholipase C from Cl. perfringens which are reported (Möllby, Nord & Wadström, 1973; Smyth & Arbuthnott, 1974) as failing to meet the high standards of purity required for use in membrane research. There are, however, a few methods which claim to yield a homogeneous preparation (Casu et al., 1971; Stahl, 1973a; Möllby & Wadström, 1973; Takahashi, Sugahara & Ohsaka, 1974; Bird, Low & Stephen, 1974; Smyth & Arbuthnott, 1974). Such products still show some variation in their properties, despite the fact that they appear to be highly purified preparations of a single enzyme. It would appear that there are between one (Möllby & Wadström, 1973) and four molecular forms of Cl. perfringens phospholipase C (Takahashi, Sugahara & Ohsaka, 1974; Bird, Low & Stephen, 1974). The main component focuses at around pH 5.5, but there is poor correlation between different workers for the molecular weight which has been quoted as 30,000 (Möllby & Wadström, 1973); 43,000 (Takahashi, Sugahara & Ohsaka, 1974); 48,000 (Bird, Low & Stephen, 1974) and

53,800 (Smyth & Arbuthnott, 1974). All these preparations contained the lethal, haemolytic and phospholipase C activities in a single peak and all were capable of hydrolysing both lecithin and sphingomyelin. This enzyme also requires divalent metal ions and may well be another zinc metalloenzyme (Ispolatovskaya, 1970).

Other clostridia are also known to produce phospholipase C (Ispolatovskaya, 1971). The only other clostridial phospholipase C to have been extensively purified is that from Cl. noyvi type A. It is capable of hydrolysing both sphingomyelin and lecithin and requires divalent cations for activity but is immunologically distinct from the enzyme from Cl. perfringens (Taguchi & Ikezawa, 1975).

Phospholipase C has also been purified from Aeromonas hydrophila (Bernheimer, Avigad & Avigad (1975), Ps. fluorescens (Sonoki & Ikezawa, 1975), Streptomyces hachijoensis (Okawa & Yamaguchi, 1975b), Acinetobacter calcoaceticus (Lehmann, 1972) and of course, the sphingomyelinase C and phosphatidylinositol-specific phospholipase C from S. aureus (Doery et al., 1965; Low & Finean, 1976). These enzymes vary considerably in their properties, which are summarised in Table 7. It can be seen that with the exception of the enzyme from S. hachijoensis, only the phospholipases capable of degrading sphingomyelin are haemolytic. Sheep erythrocytes have a high sphingomyelin content (Table 4). It is noteworthy that the phospholipase C from B. cereus, generally regarded as being non-haemolytic, lyses guinea pig erythrocytes, which have a high lecithin and low sphingomyelin content (Bult & Zwaal, 1973). Haemolysis is also a function of the availability of phospholipids to the

Table 7 Substrate specificities of some bacterial phospholipases C

	PC	Sph	PE	PSer	PG	PI	lyso PC	lyso pI	CL	Haemolysis (sheep) at 37°C	Reference
Clostridium perfringens	+	+	+	±	-		-		-	+	Van Deenen (1964)
Clostridium noyvi	+	+	+				+			+ (horse)	Taguchi & Ikezawa (1975)
Staphylococcus aureus Sph. C	-	+	-	-	-		+		-	-*	Doery et al. (1965)
Bacillus cereus	+	-	+	+	+		-	-	+	-	Van Deenen (1964) Zwaal et al. (1971)
Acinetobacter calcoaceticus	+	+	+	+						+	Lehmann (1972)
Pseudomonas aureofaciens	+	-	+	-	-	-	±		-		Sonoki & Ikezawa
Pseudomonas fluorescens	+		+	±	±				±		Doi & Nojima (1971)
Streptococcus hachijoensis	+	+	-	-		+	+		+	-	Okawa & Yamaguchi (1975)
Staphylococcus aureus PIC	-	-	-	-		+	-	+	-	- (human)	Low & Finean (1976)
Aeromonas hydrophila	+	-	+	+			-				Bernheimer, Avigad & Kim (1975)

* = Extensive haemolysis results if cells are incubated for 30 min at 4°C

+ = hydrolysis or haemolysis

- = no hydrolysis or haemolysis

PC = phosphatidylcholine; Sph = sphingomyelin; PE = ethanolamine; PSer = serine; PG = glycerol; PI = inositol lysoPC = lysophosphatidylcholine; lysoPI = lysophosphatidylinositol; CL = cardiolipin.

phospholipase (McFarlane, 1950). Haemolysis can be one of two types, hot haemolysis, which occurs at 37°C and 'hot-cold' haemolysis, discussed earlier. Hot-cold haemolysis appears to be a property of enzymes hydrolysing sphingomyelin since it is shown by the enzymes from Cl. perfringens (van Heyningen, 1941), some strains of A. calcoaceticus (Lehmann, 1973) and S. aureus (Glenny & Stevens, 1935). The effect of chilling was not tested for in the case of the enzymes from S. hachijoensis (Okawa & Yamaguchi, 1975b) and Cl. novyi (Taguchi & Ikesawa, 1975).

2. Phospholipases as specific membrane probes

Much experimental evidence has been gathered on the organisation of components in the bulk of the membrane by a variety of physical techniques such as optical rotatory dispersion, circular dichroism, X-ray diffraction, nuclear magnetic resonance, electron spin resonance, infra-red and laser raman spectroscopy and differential scanning calorimetry (recent Physical Studies of the Structure of Bio-Membranes, Ed. D. Chapman, Chemistry and Physics of Lipids, Vol. 8, No. 4, May 1872). However, most physical techniques are essentially averaging techniques and it is advantageous to work with specific probes and electron microscopy since this allows the study of specific sites in specific membranes.

One such approach is via the use of proteases which have shown the asymmetrical distribution of membrane bound enzymes (Wallach, 1972; Bretscher, 1973; Juliano, 1973; Steck, 1974; Singer, 1974). The enzymic hydrolysis of phospholipids has also been studied in

relation to the distribution of components within the membrane, membrane integrity, permeability and the activity of membrane bound components. A variety of membrane systems have been studied and these are discussed in turn.

i. The erythrocyte membrane

The action of phospholipases on erythrocytes can be studied in a number of systems; the intact cell, the resealed ghost, the non-sealed ghost and liposomes made from the extracted lipids. Experiments with the intact cell and resealed ghost will yield information on components accessible only from the outside of the bilayer whilst the non-sealed ghost yields information on phospholipids accessible from both sides of the bilayer. Studies with liposomes reveal parameters of enzymes such as pH optimum, substrate specificity, temperature dependence.

Although the composition of the erythrocyte membrane is approximately 50 wt% protein, 40 wt% lipid and 10 wt% carbohydrate, there are nevertheless variations in both the lipid and protein composition of membranes from different species (Van Deenen & De Gier, 1964; Rouser et al., 1968; Zwaal & Van Deenen, 1968). The lipid fraction is composed of approximately equimolar amounts of phospholipids and cholesterol. The sum of the choline containing phospholipids, phosphatidylcholine and sphingomyelin, from a variety of mammalian species is 46 - 60% of the total membrane phospholipids (Van Deenen & De Gier, 1964; Nelson, 1967). However, the ratio of phosphatidylcholine to sphingomyelin varies markedly from species to

species (Table 8) (Zwaal, Roelofsen & Colley, 1973). This partly explains why the reported effects of phospholipases on membranes vary, according to the type of membrane and substrate specificity of the phospholipase studied (McFarlane, 1950; Colley et al., 1973).

a. The erythrocyte ghost membrane: Phospholipase C from both Cl. perfringens and B. cereus degrades approximately 70% of the phospholipids of erythrocyte 'ghosts' from a variety of sources (Lenard & Singer, 1968; Casu et al., 1968; Gordon, Wallach & Straus, 1969; Glaser et al., 1970; Ottolenghi & Bowman, 1970) apparently without interfering with protein structure. The work of Simpkins, Panko & Tay (1971a,b,c) and Berengo & Simpkins (1972) confirms these observations. Thus it is clear that not only are phospholipids orientated with their head groups towards the outside (hydrophilic) part of the bilayer of ghosts but that they are also available for hydrolysis by phospholipases and not masked by protein as suggested by some of the earlier membrane models such as the Davson-Danielli model refined by Robertson (1964) (unless one assumes phospholipases to be capable of perturbing the protein monolayer sufficiently to penetrate to the level of the phospholipids). It also suggests that most of the phospholipid is independent of protein but the 30% which resists hydrolysis is in a different physical state from the rest of the lipid in ghost membranes - perhaps involved in a more tightly coupled interaction with membrane proteins.

Phospholipase A_2 from various snake venoms and pancreas is also capable of degrading the majority of phospholipid in ghosts (Ibrahim & Thomson, 1965; Condrea, Barzilay & Mager, 1970). Unlike

Table 8 Approximate ratios of phosphatidyl choline (PC) : sphingomyelin (Sph) in the erythrocytes of various species

Species	PC : Sph ratio
Rat, dog, guinea pig	4 : 1
Horse	3 : 1
Human, rabbit	3 : 2
Pig, cat	1 : 1
Cow, sheep, goat	1 : 12

phospholipase C, however, phospholipase A_2 alters the circular dichroism spectrum of 'ghost' proteins towards the ultraviolet. It would seem that the lipid and protein portions of the membrane suffer a different fate after the different phospholipase treatments (Simpkins, Panko & Tay, 1971a,b,c; Bernengo & Simpkins, 1972). Roelofsen and co-workers, using highly purified preparations of pancreatic phospholipase A_2 and B. cereus phospholipase C (Roelofsen et al., 1971; Zwaal & Roelofsen, 1973) have stated that the cessation of degradation of phospholipid beyond 70% is due to the inability of these enzymes to hydrolyse sphingomyelin. They also pointed out that the phospholipase C of Cl. perfringens similarly had little effect on phosphatidylethanolamine and phosphatidylserine.

The degradation of phospholipids in erythrocyte ghosts by phospholipase C from B. cereus and Cl. perfringens leads to the formation of dense droplets as observed by phase contrast microscopy (Coleman et al., 1970; Ottolenghi & Bowman, 1970) coupled with some 45% shrinkage of the cells. This was assumed to represent the accumulation of diglyceride (and also ceramide in the case of Cl. perfringens), the product of hydrolysis. Pancreatic lipase cleared these black dots (Colley et al., 1973) confirming that they were mainly diglyceride. Colley et al. (1973) also reported that 'black dots' were not formed in ghosts treated with sphingomyelinase C from S. aureus and concluded that the produced ceramides remained in position in the membrane.

Treatment of ghosts with phospholipase A_2 from porcine pancreas produced no marked change in appearance under phase contrast

microscopy (Colley et al., 1973) although treatment of ghosts with the enzyme from Naja naja venom altered the freeze-fracture plane (Speth et al., 1972). Treatment of ghosts with a combination of pancreatic phospholipase A_2, B. cereus phospholipase C and pancreatic lipase resulted in the total breakdown of the cell membrane lipids, with less than 1% release of membrane protein but the ghost surprisingly still maintained a spherical structure (Zwaal, Roelofsen & Colley, 1973).

b. Intact erythrocytes and resealed ghosts: Purified pancreatic phospholipase A_2 does not hydrolyse phospholipids in the intact cell, despite being able to degrade glycerophospholipids in ghosts (Roelofsen et al., 1971). It is non-haemolytic.

On the other hand, phospholipases A_2 from snake venom (Ibrahim & Thompson, 1965) and bee venom (Colley & Zwaal, 1973) catalyze lecithin breakdown without causing haemolysis. Naja naja venom phospholipase A_2 can cause the breakdown of as much as 68% of the lecithin in human erythrocytes without causing haemolysis or changing the freeze-fracture plane of the membrane (Verkleij et al., 1973). It is surprising that the production of lysolecithin in situ does not lead to haemolysis, while exogenous lysolecithin is known to be a lytic agent. However if the cells are subsequently incubated with bovine serum albumin, which removes the fatty acids from the membrane, the cells lyse (Gul & Smith, 1972).

Crude venom phospholipase A_2 preparations often show lytic properties: this is due to the synergism between the enzyme and a

direct lytic factor (Condrea, De vries & Mager, 1964; Condrea et al., 1964; Condrea, Barzilay & Mager, 1970; Condrea, Barzilay & De Vries, 1971; Mollay & Kreil, 1974) and similar effects have been observed with bee venom phospholipase A_2 and peptides such as viscotoxin and prymnesin (Lankisch & Vogt, 1971, 1972).

Purified B. cereus phospholipase C is not haemolytic (Zwaal et al., 1971) whereas crude preparations are, probably due to a heat labile haemolysin (Ottolenghi, 1969). Roelofsen et al. (1971) reported that the enzyme was incapable of hydrolysing phospholipids in intact membranes, a lack of susceptibility which was not altered by treatment with trypsin, pronase or neuraminidase.

Purified phospholipases C from Cl. perfringens, A. calcoaceticus and Cl. noyvi are haemolytic (Table 7), degrading phospholipids in intact cells. Pure sphingomyelinase from S. aureus degrades sphingomyelin in intact membranes without causing haemolysis at 37°C (Colley et al., 1973) but the treated cells subsequently lyse if chilled to 4°C (hot-cold effect). Phosphatidylinositol-specific phospholipase C from S. aureus degrades 60% of the membrane phosphatidylinositol at the outer surface of human erythrocytes without haemolysis (Low & Finean, 1976).

In the absence of haemolysis, phospholipases can only be acting at the surface of the cell. Since the exchange of phospholipid molecules from one side of vesicle membranes to the other is extremely slow (Kornberg & McConnell, 1971) then one must assume that the phospholipids degraded are localized in the outer half of the bilayer of treated (non-haemolysed) erythrocytes. Thus at

least two-thirds of the lecithin in human erythrocytes is located on the outside of the bilayer according to the results of Verkleij et al. (1973). Sphingomyelinase can degrade 80 - 85% of the sphingomyelin in human and pig erythrocytes and 50 - 60% of the sphingomyelin in cow and sheep erythrocytes, without causing lysis. Subsequent treatment of these cells with B. cereus phospholipase C leads to lysis. Cow and sheep erythrocytes are unaffected (Colley et al., 1973). Kahlenberg, Walker & Rohrlick (1974) have shown that when inside-out vesicles derived from human erythrocytes are treated with snake venom phospholipase A_2 and Cl. perfringens phospholipase C, phosphatidylethanolamine and phosphatidylserine were preferentially degraded. The lysis of human and pig erythrocytes is presumably due to the hydrolysis of lecithin by phospholipase C, which was previously unable to interact with its substrate. There is no breakdown of glycerophospholipid in ox and sheep red cells, which explains the lack of haemolysis. Presumably there is insufficient lecithin relative to the large excess of sphingomyelin. It is interesting to note that guinea-pig erythrocytes which have a high lecithin:sphingomyelin ratio, are lysed by pure phospholipase C from B. cereus (Bult & Zwaal, 1973).

Casu et al. (1969) and Bretscher (1972) have suggested that the outer part of the bilayer of erythrocytes consists of predominantly choline-containing phospholipids. The results of Colley et al. (1973), Verkleij et al. (1973) and Kahlenberg, Walker & Rohrlick (1974) support this suggestion and the results of Low & Finean (1976) also indicate that the greater portion of phosphatidylinositol is located in the

outer portion of the membrane. Pancreatic phospholipase A_2 hydrolyses glycerophospholipids in sphingomyelinase-treated human erythrocytes (Colley et al., 1973). However, it does not cause extensive lysis unless the fatty acids produced are removed by bovine serum albumin. Bee venom and pancreatic phospholipases A_2 and B. cereus phospholipase C can also act on glycerophospholipids in cells sensitised by osmotic swelling (Lankisch & Vogt, 1972; Woodward & Zwaal, 1972; Colley & Zwaal, 1973) and the bee venom enzyme and phospholipase C cause haemolysis. Pancreatic phospholipase A_2 can degrade only 14% of the phosphatidylethanolamine of sphingomyelinase treated cells indicating that phosphatidylethanolamine is largely absent from the exterior of the bilayer.

Resealed ghosts can be lysed by pure pancreatic phospholipase A_2 and B. cereus phospholipase C, due to lipid degradation by these enzymes (Woodward & Zwaal, 1972). It has been suggested that the B. cereus enzyme only acts if it is introduced when the ghost is still sufficiently permeable to allow its penetration into or through the membrane so that it may attack the inner surface (Low, Limbrick & Finean, 1973). Surface active agents at sub-lytic concentrations potentiate phospholipase A_2-induced haemolysis (Roelofsen et al., 1971; Lankisch, Jacobi & Schoner, 1972) and B. cereus phospholipase C haemolysis of human and pig, but not sheep erythrocytes (Zwaal et al., 1973).

ii. Other membrane sources

Phospholipases have been used on a variety of other membranes.

Op den Kamp, Kauerz & Van Deenen (1972) showed that both pancreatic phospholipase A_2 and B. cereus phospholipase C hydrolysed phospholipids in protoplasts prepared from B. subtilis. Phospholipase A_2 lysed the protoplasts, but phospholipase C did not. Nanninga, Tijssen & Op den Kamp (1973) showed that the phospholipase-treated protoplasts differed little in electron microscopic appearance from controls, there being no appearance of diglyceride droplets. It is possible that the far higher protein:lipid ratio in bacterial membranes results in protein contributing far more to the stability of the bilayer. Pure phospholipases A_2 and C also hydrolysed phospholipids of E. coli, provided they had first been treated with tris and EDTA, to make the phospholipids accessible (Duckworth et al., 1974). The cells were killed but not lysed by this treatment. The EDTA treatment disrupts the barrier function of the outer membrane (Butler & Kornberg, 1966).

Cardiolipin is only slightly degraded by phospholipase treatment and may be located in such a way that it is inaccessible to enzymes (Mavis & Vagelos, 1972). Phospholipases do not hydrolyse phospholipids in intact bacteria because of the cell wall. The report of Chesbro et al. (1965) that β-lysin acts as a carbohydrase can be discredited on the grounds of contamination by staphylococcal lysozyme. Prasad, Kalra & Brodie (1975a) have shown that phospholipase A from snake venom produces gross structural changes in the lipid region of vesicles derived from Mycobacterium phlei, without significantly altering protein structure.

Cl. perfringens phospholipase C, with its broader substrate

specificity and haemolytic properties may be expected to have a greater effect against protoplasts. However, Bernheimer, Avigad & Grushoff (1968) found protoplasts from both Strep. faecalis and Sarcinia lutea as well as spheroplasts from E. coli to be completely resistant to lysis.

Many other cell membrane types have been studied, both in tissue culture and in whole organs. However, work with phospholipases of unspecified purity must be interpreted with caution, since crude commercial sources often contain contaminating biological activities which may interfere with the breakdown of membranes (Möllby, Nord & Wadström, 1973; Smyth, Freer & Arbuthnott, 1975). Highly purified phospholipase C causes membrane damage to human diploid embryonic lung fibroblasts, hydrolysing some 46% of the choline containing phospholipids and causing release of 40% of the ^{3}H-uridine labelled nucleotides which were used as a marker (Möllby, Thelestam & Wadström, 1974). These cells are resistant to staphylococcal sphingomyelinase (Thelestam, Möllby & Wadström, 1973). Phospholipases also interfere with cell adhesion between chick embryo retinal cells (Curtis, Campbell & Shaw, 1975). Cl. perfringens phospholipase C hydrolyses phospholipid and appears to interfere with the metabolic activities of intact tissues such as thyroid (Macchia & Pastan, 1967) and muscle fibres (de Mello, 1973; Boethius et al., 1973). B. cereus phospholipase C and phospholipase D hydrolyse phospholipids in isolated myelin (Guarnieri et al., 1975) but phospholipid depletion does not appear to alter myelin structure significantly, as assessed by tetrodotoxin binding (Villegas, Barnola & Camejo, 1973). Snake venom phospholipase A_2 and Cl. perfringens phospholipase C may

penetrate through connective tissue and the Schwann cell to reach the axolemma of squid giant axons (Rosenberg, 1975).

Membranes from sub-cellular organelles are also susceptible to treatment with phospholipases, including microsomes (Jothy, Tay & Simpkins, 1973; Scherphof, Scarpa & van Toorenenberger, 1972), mitochondria (Ottolenghi & Bowman, 1970; Burnstein, Kandrach & Racker, 1971; Packer et al., 1974) and chloroplasts (Tuquet, 1972a,b).

3. The effect of phospholipases on membrane bound enzymes

Phospholipases have also been used to determine the phospholipid dependence, or constraint, of membrane bound enzymes (Hallinan, 1974). The lipid dependence of several membrane bound enzymes in E. coli was investigated by Mavis, Bell & Vagelos (1972). They showed that Acyl-CoA:glycerol 3 phosphate acyl transferase and NADH oxidase were inactivated at a rate very similar to that of phospholipid degradation. However, glycerol-3-phosphate dehydrogenase and succinic dehydrogenase remained completely active after hydrolysis of 95% of phospholipid. Thus it would seem that different enzymes exhibit different sensitivities to phospholipases.

Erythrocyte acetylcholinesterase is thought to be located in the phospholipoprotein matrix, although it probably is not dependent on phospholipids for activity, since the total activity can be liberated from the matrix by phospholipases (Grafius, Bond & Millar, 1971; Whittaker & Charlier, 1972).

Phospholipases have been used to study transport systems

such as those for amino acids (Prasad, Kalra & Brodie, 1975b), glucose (Kahlenberg & Banjo, 1972; Kahlenberg, Dolansky & Rohrick, 1972; Banjo et al., 1974), palmitic acid (Mahadevan & Sauer, 1974) and the electron transport system (Vidal et al., 1966; De Pierre & Ernster, 1975; Prasad, Kalra & Brodie, 1975a) and there is much evidence for the lipid-dependence of certain components of these systems. Most work has, however, centred on the Mg^{2+} dependent and $(Na^+ + K^+)$-stimulated ATPases of the human erythrocyte membrane (Zwaal, Roelofsen & Colley, 1973). Treatment of human erythrocyte ghosts with purified pancreatic phospholipase A_2 or B. cereus phospholipase C results in a large drop in the activity of both these ATPases. S. aureus sphingomyelinase has no effect on the Mg^{2+} - ATPase, but activates the $(Na^+ + K^+)$ - ATPase. The loss in activity of $(Na^+ + K^+)$ - ATPase following phospholipase C treatment could be recovered by the addition of phosphatidylserine, but phosphatidylethanolamine and phosphatidylcholine had no effect. Roelofsen & van Deenen (1973) showed that phosphatidylserine decarboxylase, which converts phosphatidylserine to phosphatidylethanolamine (Kanfer & Kennedy, 1964), decreases the activity of ATPase. However, 85% of phosphatidylserine had to be degraded before any effect was detectable. On the other hand, Stahl (1973b) using the enzyme from Cl. perfringens found no specificity in the type of phospholipids capable of reactivating the partially inactivated enzyme. This was also observed by Goldman & Albers (1973) who pointed out that their phospholipase C treatment with the Cl. perfringens enzyme did not reduce the phosphatidylserine content. De Pont et al. (1973) showed that $(Na^+ - K^+)$ - ATPase from cattle brain microsomes was unaffected by phosphatidylserine decarboxylase and

pointed out that the pretreatment of (Na^+ - K^+) ATPase with anhydrous ether may have caused the subsequent loss of activity observed by Roelofsen & van Deenen (1973) after phosphatidylserine decarboxylase treatment. It is probable, however, that both phosphatidylserine and (Na^+ + K^+) ATPase are associated on the inside of the erythrocyte membrane (Zwaal, Roelofsen & Colley, 1973).

Certain lipid-associated enzymes are specifically constrained by phospholipids e.g. the liver microsomal enzymes glucose 6-phosphatase and UDP glucuronyl transferase (Zakim, 1970; Vessey & Zakim, 1971; Zakim, Goldenberg & Vessey, 1973). Enzyme activity could easily be masked by separation of enzyme and substrate in the bilayer (Berry, Stellon & Hallinan, 1974). Phospholipase treatment might release the enzyme, thus increasing activity by increasing the number of active sites available for catalytic interaction with the substrate. Cater, Trivedi & Hallinan (1975) have shown that if latency of glucose-6-phosphatase is destroyed, then inhibition of the enzyme by phospholipid degradation rapidly follows.

OBJECT OF THE RESEARCH

Staphylococcal β-lysin (β-toxin, sphingomyelinase C) is one of the few cytolytic toxins to have had its mode of action defined. Doery *et al.* (1963, 1965) showed that β-lysin was a phospholipase C with a very narrow substrate specificity, hydrolysing only sphingomyelin and lysophosphatidylcholine. It has the noteworthy property of causing the "hot-cold" haemolysis of ruminant erythrocytes. However there is still much uncertainty about some of the other properties of β-lysin, for example its toxicity and physicochemical characteristics.

The objects of this research were:

a. To obtain β-lysin in a highly purified form, essentially free from the other biologically active products of staphylococci.

b. To characterise the protein physicochemically.

c. To examine the toxic properties of β-lysin.

d. To investigate the biochemical and ultrastructural effects of β-lysin on intact erythrocytes and erythrocyte ghost membranes.

MATERIALS AND METHODS

I β-LYSIN PRODUCTION

1. Strains

Three strains of S. aureus were examined for lysin production. Strains G128 and R1 were obtained from Dr. G.M. Wiseman and were originally isolated from fomites and a case of bovine mastitis respectively (Wiseman, 1965). Strain IL7S was an old laboratory strain obtained from Dr. J.P. Arbuthnott and was originally isolated from a human source by Professor I. Lominski. These strains were selected because they produce predominantly β-lysin with only minor amounts of other toxins or enzymes. Cultures were maintained both in the freeze dried state in ampoules and as frozen (-20°C) glycerinated (10%) log-phase broth cultures.

2. Media

The culture medium (YDB) used for toxin production was that of Bernheimer, Avigad & Kim (1974). Details of the preparation are as follows. The diffusate of yeast extract was prepared by the method of Bernheimer & Schwartz (1963). One litre of a 40% (w/v) solution of yeast extract (Difco Laboratories, Detroit, Michigan, U.S.A.) was dialysed against 4000 ml of distilled water at 4°C with mixing. After 6 days the contents of the dialysis sac was discarded and the outside solution (diffusate, final volume approximately 2.6 litres) was used as indicated below.

The medium contained 220 ml of yeast extract diffusate, 20 g Casamino acids (Difco Laboratories, Detroit, Michigan, U.S.A.), 33 µg thiamine, 1.2 mg nicotinic acid (both Analar grade, BDH Chemicals, Poole,

Dorset, England) and distilled water up to 1000 ml. Four ml of a 1/20 dilution of "Antifoam" (Silcolapse 5000, I.C.I., Stevenston, Ayrshire) were added per litre and the pH was adjusted to 7.2. Sterilization was carried out for 20 min at 121°C.

3. Cultural conditions

It was essential to subculture the strain on to sheep blood agar plates (Oxoid Blood Agar Base No. 2, Oxoid Ltd., London, England) before cultivation in broth. Plates were inoculated from a loopful of thawed glycerinated broth culture and incubated overnight at 37°C. After incubation, 50 ml of YDB medium in a 250 ml conical flask were inoculated as a starter culture from an isolated colony which showed good haemolysis at 4°C. After incubation overnight at 37°C on an orbital shaker operating at 200 cycles per minute, the starter culture was added as a 5% (v/v) inoculum to 500 ml aliquots of YDB medium in 2-litre conical flasks and incubation was continued at 37°C for 5-6 h, when the bacterial population reached an $E^{1cm}_{650\ nm}$ of 7.5 to 10.5. The cultures were then centrifuged at 16,000 g for 30 min at 4°C in an MSE 18 or MSE 25 centrifuge (supernatant fluids were pooled at 4°C).

4. Measurement of bacterial growth

Growth was estimated by measuring the $E^{1\ cm}_{650}$ of samples of culture withdrawn at ½-hour or 1 h intervals. Where the E_{650} exceeded 0.40 the culture was diluted with YDB medium to bring the extinction value to within the range 0.00 - 0.40. Extinction values were measured on an SP 600 spectrophotometer (Pye Unicam, Cambridge, England). Uninoculated YDB medium served as a blank.

II ASSAYS

1. Haemolytic assay for β-lysin

Haemolytic activity was determined using serial two-fold or ten-fold dilutions of β-lysin in Tris-buffered saline (0.85 per cent (w/v) NaCl in 0.05 M Tris-HCl buffer, pH 7.4) made 1 mM with respect to $MgCl_2$ (see Appendix I). Pipettes were changed every 4 transfers, or where greater accuracy was required, after every transfer. To each dilution was added an equal volume (0.5 ml) of sheep red blood cells in Tris-buffered saline (TBS), standardised to give an $E_{545\ nm}^{0.5\ cm} = 0.7$ after lysis with saponin. After incubation at 37°C for 30 min followed by 4°C for 2 h, the dilution of enzyme giving 50 per cent haemolysis was estimated visually. This amount of enzyme was defined as constituting one haemolytic unit.

2. Other haemolytic assays

Haemolytic assays for α-toxin and δ-lysin were performed in a similar manner to assays for β-lysin with the following exceptions.

Diluents	α-toxin :	0.05 M phosphate buffered saline pH 7.0 containing 1 mg/ml bovine serum albumin (BSA).
	δ-lysin :	citrate-dextrose saline (see Appendix I).
Erythrocytes	α-toxin :	0.7% (v/v) rabbit erythrocytes.
	δ-lysin :	0.7% (v/v) cod erythrocytes.

Incubation was at 37° for 30 min with α-toxin and 22°C for 30 min with δ-lysin.

3. Sphingomyelinase C activity

Sphingomyelinase C activity was assayed by measuring phosphorus release from 4 ml of an aqueous dispersion of sphingomyelin (1 mg/ml) in 0.05 M TBS pH 7.4 made 1 mM with respect to $MgCl_2$ (see Appendix I). After incubation at 37°C for 30 min the reaction was stopped by adding 1.6 ml of 5% (w/v) BSA and 8 ml ice cold 10% (v/v) trichloracetic acid (TCA). Reaction mixtures were centrifuged at 1000 g for 10 min, the pellet washed twice with 1.0 ml volumes of 10% (v/v) TCA and the pooled supernatant assayed for phosphorus by a modification of the method of Allen (1940) (see Appendix V). Sphingomyelinase C was also assayed by the substrate decrease method of Zwaal & Roelofsen (1974), substituting chloroform for diethyl ether in the reaction mixture (see Appendix V) and assaying for phosphorus by a modification of the method of Allen (1940).

4. Assays for staphylococcal extracellular products

i. Lipase

Saline agar (1.5% (w/v) agar (Oxoid), 0.85% (w/v) NaCl) containing 0.01% (v/v) thiomersal was made 1% (v/v) with tributyrin and dispensed in 0.5 ml amounts in 5.0 cm x 0.5 cm tubes. 0.1 ml volumes of serial doubling dilutions of lysin sample in saline were layered onto the surface of the agar and the tubes incubated at 37°C for 18 h. Lipase activity was indicated by clearing due to the hydrolysis of tributyrin. Alternatively the same reaction could be performed in wells in agar plates made up as above. Activity was again indicated by a zone of clearing.

ii. Phosphatase

0.5 ml amounts of a 0.1% (v/v) solution of phenolphthalein phosphate were dispensed in 4" x ½" test tubes. 0.5 ml volumes of dilutions of lysin sample in 0.05 M TBS + Mg^{2+}, pH 7.4 were added and the tubes incubated at 37°C for 30 min. Solutions were made alkaline by the addition of 0.1 ml of 0.1 M sodium hydroxide to each tube. Phosphatase activity was indicated by a pink colouration due to free phenolphthalein. This was measured at 550 nm in an SP 600 spectrophotometer in cells of 5 mm light path. Alkaline phosphatase from E. coli (Sigma Chemical Co., London) was used as a standard.

iii. Coagulase

Citrated rabbit plasma (0.4 ml) was dispensed in 4" x ½" test tubes. Palitsch buffer (0.2 ml) (see Appendix I) was added to each tube. Serial doubling dilutions of the lysin sample in saline were added (0.2 ml) and the tubes incubated at 37°C for 6 h. Coagulase activity was indicated by the formation of a clot.

iv. Fibrinolysin

Human plasma was diluted 1:10 in saline and made 0.01% (v/v) with thiomersal and was dispensed in 0.8 ml volumes in 4" x ½" test tubes. The plasma was clotted by addition of 0.1 ml (2 units) of thrombin (Sigma, London) solution to each tube. 0.2 ml volumes of serial doubling dilutions of the lysin sample in 0.05 M TBS, pH 7.4 were added and the tubes incubated at 37°C overnight. Fibrinolysin activity was indicated by lysis of the clot.

v. Egg yolk factor

An egg yolk extract was made from 20 ml of fresh hen egg yolk

by mixing with 10 volumes of 0.05 M phosphate buffer pH 7.3 containing 5% w/v NaCl and allowing the mixture to stand for 30 min at room temperature, followed by centrifugation at 1000 g for 20 min. This led to the formation of a pellet, a middle clear layer and a lipid scum on the surface. The middle layer was made 0.01% (v/v) with thiomersal and 2 ml amounts were dispensed in 6" x $\frac{5}{8}$" test tubes. Serial doubling dilutions of the lysin sample in saline were added (0.2 ml) and the tubes incubated at 37°C for 18 h. The degree of turbidity in the egg yolk extract was measured at 540 nm in an SP 600 spectrophotometer in 0.5 mm cells. Phospholipase C prepared by the method of Smyth & Arbuthnott (1974), was used as a standard (to test for sensitivity).

vi. Hyaluronidase

Hyaluronidase was assayed by the method of Dorfmann (1955). Bacterial samples were compared with an International Standard preparation of ovine testicular hyaluronidase (Miles Seravac Ltd., Slough, England), standardised by the method of Humphrey (1957).

Suitable dilutions of test solution were made in hyaluronidase diluent (see Appendix I). To 0.5 ml volumes of standardised hyaluronic acid solution (see Appendix I), 0.5 ml of test solution was added. Tubes were then incubated at 37°C for 45 min, after which 5 ml of acid albumin was added to each tube. The $E^{\frac{1cm}{600}}$ of each solution was read in an SP 600 spectrophotometer against an appropriate diluent/acid albumin blank.

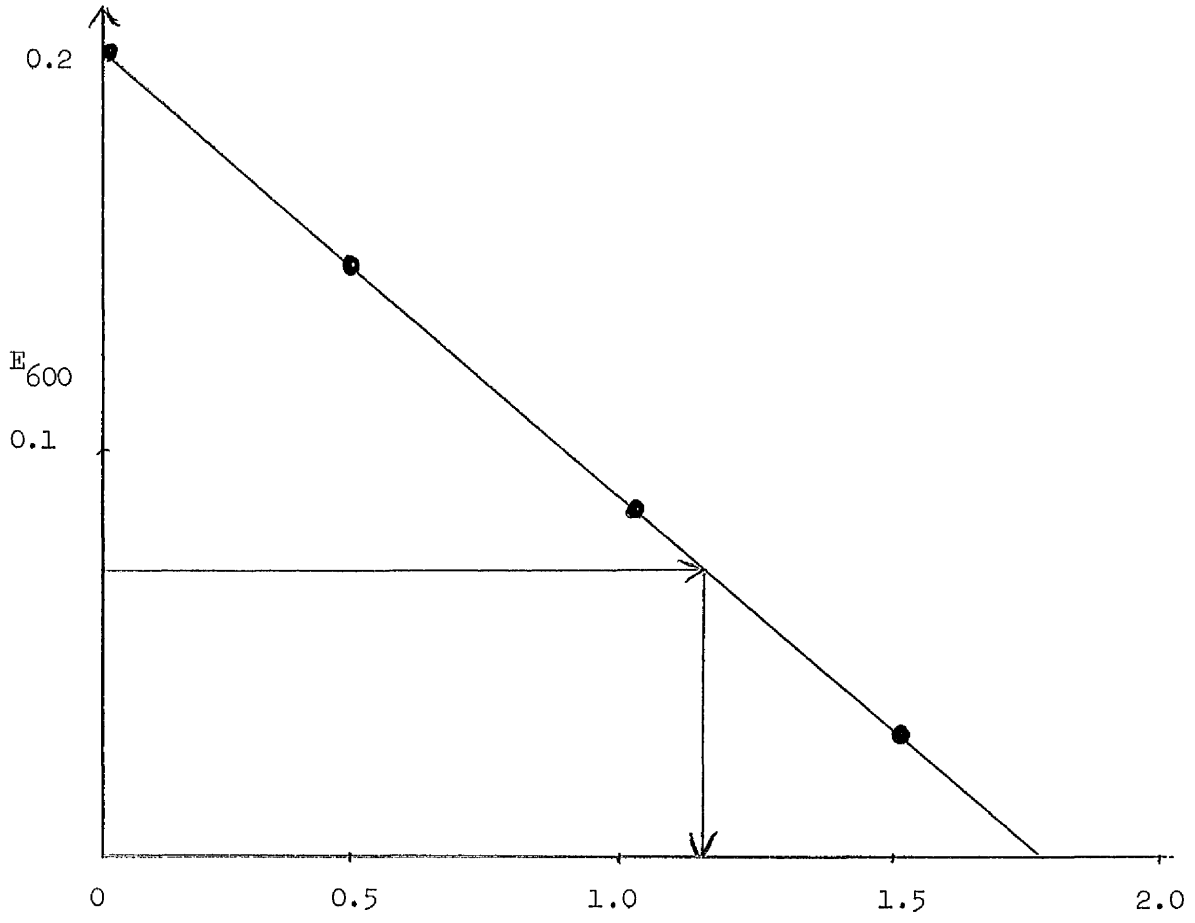
A standard curve was obtained by plotting optical density against enzyme concentration using an International Standard preparation of ovine hyaluronidase and unknown samples were read against this curve (figure 2).

Figure 2 : Assay for hyaluronidase activity

Determination of hyaluronidase activity with reference to standard hyaluronidase preparation

The resultant E_{600} of samples of a standardised hyaluronic acid solution after digestion with increasing amounts of ovine testicular hyaluronidase are shown in the figure opposite.

A 1:10 dilution of crude β-lysin preparation of unknown hyaluronidase activity, assayed as described in the text, reduced the turbidity of the standard solution to an $E_{600} = 0.075$. As shown in the figure, this corresponds to the activity of 1.08 IU of hyaluronidase. The preparation thus contained 1.08 x 10 x 2 IU/ml = 21.6 IU/ml.


0.2
E_{600}
0.1
0
0.5
1.0
1.5
2.0
International Units of Hyaluronidase

vii. Protease

Phosphate agar (1.5% (w/v) agar (Oxoid), 0.05 M phosphate pH 7.3) containing 1% (w/v) casein (BDH) was made 2 mM with KH_2PO_4 and 1 mM with $CaCl_2$. The solution was made 0.01% (v/v) with thiomersal and dispensed in 5 cm diameter Petri dishes. Wells of 5 mm diameter were cut in the agar and were filled with test volumes of lysin sample in buffer. The plates were incubated at 37°C overnight. Protease activity was detected as a precipitated paracasein band. Subtilisin (Protease, type VIII, Sigma Chemical Co., London) was used as a standard (to test for sensitivity).

viii. Leukocidin

Fresh human blood was allowed to form a clot on a chemically cleaned coverslip. The blood clot was washed off with phosphate buffered saline (0.05 M phosphate, pH 7.5 with 0.85% NaCl) and was placed on top of a drop of the lysin sample in saline. The specimen was examined for dead leukocytes, as described by Woodin (1972).

ix. Nuclease

Tris-HCl agar (1.5% w/v agar (Oxoid) 0.05 M Tris/HCl) pH 7.4, 12.5 mM with respect to $CaCl_2$ and $MgCl_2$ was made 1% (w/v) with salmon testes DNA (Type III, Sigma Chemical Co., St. Louis). The agar solution was made 0.01% (v/v) with thiomersal and dispensed in 5 cm Petri dishes. Wells of 5 mm diameter were cut and filled with test volumes of lysin sample; the plates were incubated at 37°C overnight and then flooded with 1.0 M HCl. Nuclease activity was detected as a zone of clearing in the agar surrounding the wells. Micrococcal nuclease (Sigma Chemical Co., London) was used as a standard (to test for sensitivity).

5. Protein estimations

Protein estimations were carried out by the method of Lowry et al., (1951) using bovine serum albumin (Fract. V, Sigma Chemical Co., St. Louis) as a standard. The protein content of fractions from chromatographic columns was monitored by measuring E_{280}^{1cm} using a Pye Unicam SP 500 spectrophotometer with a quartz 1 cm flow-through cell linked to an SP 40P Automatic Sample Changer and an SP 22 Chart Recorder. The E_{280}^{1cm} of fractions from isoelectric focusing columns was measured manually.

III PURIFICATION OF β-LYSIN

1. Preparation of crude lysin

To each 100 ml of supernatant fluid 56 g of ammonium sulphate were added to give about 80% saturation, and after the salt had dissolved the mixture was allowed to precipitate overnight at 4°C. The precipitate was harvested by centrifugation at 16,000 g for 20 - 40 min in an MSE18 or MSE 25 centrifuge at 4°C. The pellet was resuspended in approximately 100 ml of 80% saturated ammonium sulphate and centrifuged at 27,000 g for 15 min in an MSE 25 at 4°C. After discarding the supernatant, the pellet was stored at -20°C. Purification of lysin was based on the method of Bernheimer, Avigad & Kim (1974), all procedures being carried out at 4°C. The material from ammonium sulphate precipitation of culture supernatants was transferred to a dialysis sac in a minimal volume of 0.05 M phosphate buffer, pH 7.2, and dialysed overnight against 50 volumes of the same buffer.

2. Gel filtration

Five to seven ml aliquots of crude lysin (approximately 375mg protein) were fractionated on a 2.5 x 90 cm column of Biogel P-60, 100-200 mesh (Bio-Rad Laboratories, Richmond, California) equilibrated at 4°C with 0.05 M phosphate buffer, pH 7.2. Fractions of about 5 ml were collected during elution. In order to reduce contamination by α-toxin, only fractions in the middle and right hand limb of the peak showing greatest activity against sheep cells were pooled and further purified. This pool was dialysed overnight against saturated ammonium sulphate and the precipitate was stored at -20°C, along with other fractions, until required.

3. Ion exchange chromatography

Ion exchange chromatography against carboxymethyl (CM) cellulose was introduced prior to electrofocusing to remove the large amount of acidic material in the pool at this stage of purification. Without ion exchange chromatography a large precipitate formed at the anode (bottom) of isoelectric focusing columns.

The ammonium sulphate precipitate was reconstituted with, and dialysed against starting buffer (0.05 M phosphate, pH 6.5) and then fractionated on a 2 x 22 cm column of Whatman Microgranular CM cellulose (CM 32, Whatman Biochemicals Ltd., Kent). After 100 ml of starting buffer had been passed through the column, 300 ml of a linear gradient of phosphate buffer (0.05 M phosphate buffer, pH 6.5; 0.2 M phosphate buffer, pH 6.5 + 0.2 M NaCl) formed in a 440 ml LKB Gradient Mixer (LKB 8122, LKB Producter AB, Stockholm, Sweden) was applied to the column.

Fractions (4 ml) active against sheep cells were pooled and dialysed against saturated ammonium sulphate and stored at -20°C.

4. Isoelectric focusing

The terms isoelectric focusing or electrofocusing are used to describe the technique of separating proteins by their isoelectric points (pI) in pH gradients. The theory and technique of isoelectric focusing have been reviewed by Peeters (1970), Haglund (1970), Vesterberg (1971), Drysdale (1974) and Arbuthnott & Beeley (1975).

i. Principle

Amino acids, polypeptides and proteins are examples of ampholytes. Ampholytes can behave as acids or bases since they contain both carboxyl and amino groups. Thus on dissociation they may become negatively charged (carboxyl groups) or positively charged (amino groups). The net charge on an ampholyte is dependent on the number of carboxyl and amino groups, the dissociation constants of these groups and the pH of the solution. The isoelectric point (pI) of an ampholyte is that pH where the net charge carried on the molecule is zero.

LKB Producter (Stockholm, Sweden) have synthesised a special class of low molecular weight ampholytes called carrier ampholytes. These carrier ampholytes have good buffering capacity and conductance at their pI's. Thus if they are placed in an electrical field they migrate to their respective pI's where they determine the pH by their buffering capacity and a pH gradient is set up from anode to cathode. This gradient can be maintained after the electrical field is removed, if mixing is prevented by the use of a stabilizing density gradient of a highly water-soluble non-electrolyte such as sucrose or sorbitol.

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.

ii. Carrier ampholytes

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

They should have:

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

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

For practical reasons they should also have:

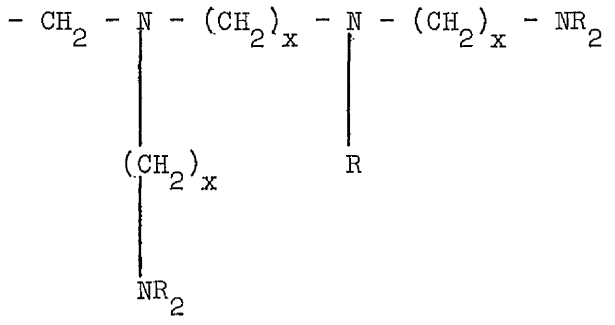
c) low molecular weight so that they may be easily separated from the proteins to be focused.

d) 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}

e) 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' which fulfil the above criteria. They consist of a mixture of aliphatic polyamino polycarboxylic acids obtained by

coupling acrylic acid to a mixture of polyalkalene-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 $- CH_2 - CH_2 - COOH$.

For further details, Davies (1970) and Haglund (1975) discuss the physical and chemical properties of Ampholines.

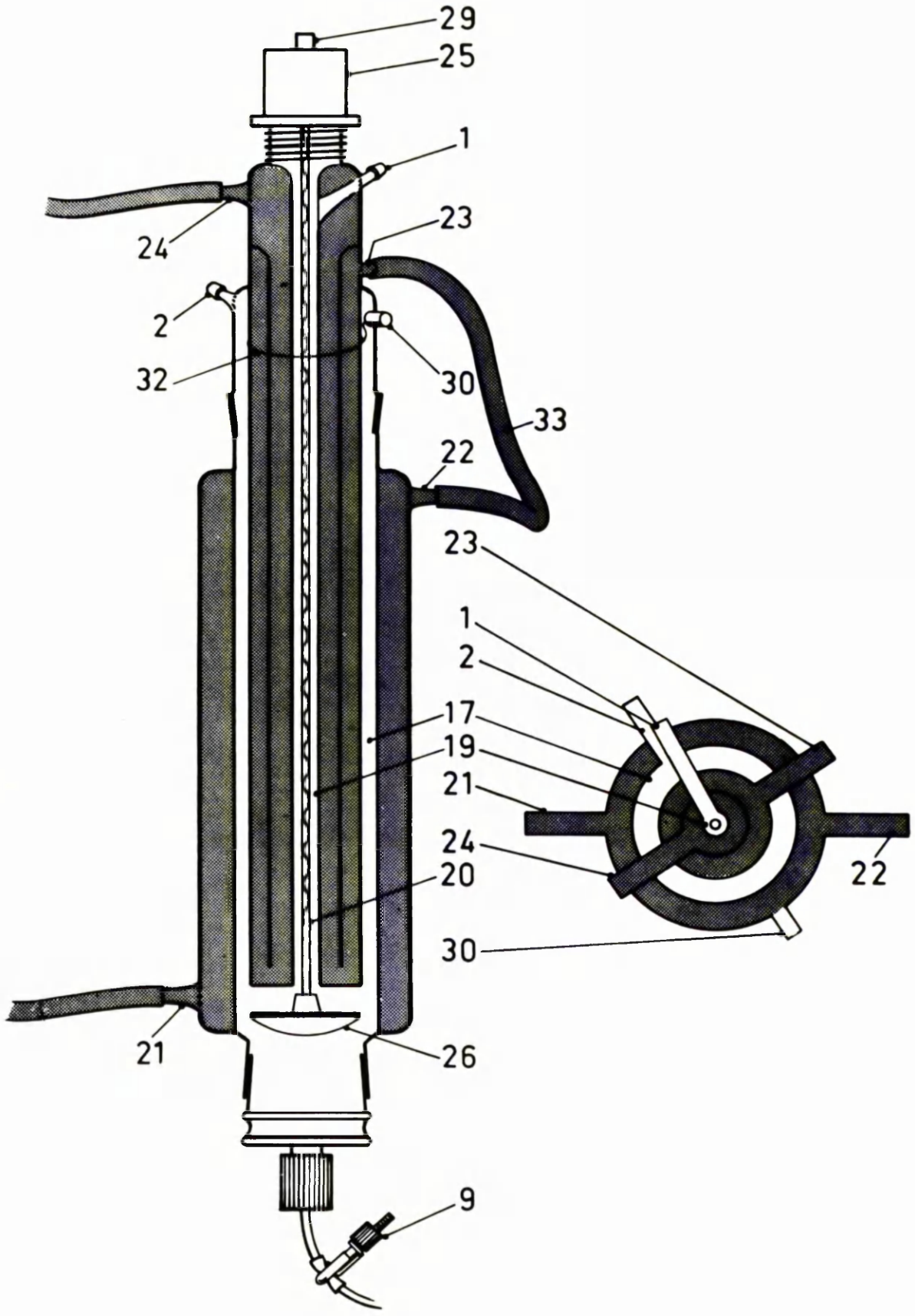
iii. Apparatus

The columns used were supplied by LKB Produkter AB. Figure 3 is a diagram of the apparatus. The electrofocusing compartment (17) is cylindrical in shape and cooled by water on either side in compartments (shaded areas) connected with tubing (33) via nipples (22) and (23).

The electrodes are platinum. The upper electrode consists of a loop (32) located at the top of the electrofocusing compartment and is connected to terminal (30). The central electrode is wound round

Figure 3. A diagram of the LKB electrofocusing column

The electrofocusing compartment (17) has a cylindrical cross section and is thermostatically controlled all around by cooling water compartments (shaded areas).


29
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a Teflon rod ~~and~~ which also acts as a lever for a valve (26) which closes the central compartment (19) before commencing the emptying procedure.

iv. Accessory equipment

The LKB 3371 Power Supply having a voltage range of 0 - 1200V was used as a power source for both column electrofocusing and the Multiphor Apparatus (below).

Electrofocusing was done at 4°C, which was maintained by a thermostatically controlled water bath (Type S.B.2, Grant Instrument Co., Cambridge) fitted with a cooling coil (Grant Instrument Co.) and pump (Type Q2, Grant Instrument Co.) for circulating cooling water.

v. Column electrofocusing

The sucrose or sorbitol density gradients were prepared, using the appropriate solutions (Appendix II) by a linear gradient mixer (LKB 8121) fitted with a stirrer motor (LKB 8121).

Peristaltic pumps were employed for filling and emptying columns to ensure a constant flow rate. The Perpex peristaltic pump (LKB 10200) fitted with a 9:100 SG ratio gear box (LKB 10233) giving a flow rate of 80 ml/hour was used. All fractions were collected on an LKB 7000 UltroRac fraction collector. Fractions were monitored for pH at 4°C with a micro-electrode (Activion model M27DP, Kinglassie, Fife) coupled to a Vibret model 46A pH meter (EIL, Richmond, Surrey) fitted with a temperature compensator in the water bath at 4°C (Activion, Kinglassie, Fife).

a. Procedure: Since salts may disturb the pH gradient, all lysin samples were dialysed against an excess volume of 1% glycine (BDH,

Analar) at 4°C. The maximum permissible salt concentration in the sample loaded is 0.5 mM for the 110 ml column. The level of protein (Lowry et al., 1951) and haemolytic activity against sheep erythrocytes was determined for each sample prior to electrofocusing.

The column was mounted vertically with the aid of a plumb line on a Lab-lox (Gallenkamp, London) scaffolding and assembled according to the instructions in the LKB electrofocusing manual, with valve 26 in the open position, as seen in Figure 3. Anode solution was loaded through nipple (1), the gradient and cathode solution through nipple (2). For the composition of these solutions see Appendix II. The dense and light solutions used to make up the density gradient were originally prepared by the method of Bernheimer, Avigad & Kim (1974) but were later prepared with sorbitol instead of sucrose, which tends to become hydrolysed at high pH values (Haglund, 1975). Dialysed lysin and 0.5 - 1.0% w/v ampholine was used as the light solution; the dense solution consisted of 45% w/v sucrose or sorbitol and 1.5 - 3.0% w/v ampholines. Details of the composition of these solutions is given in Appendix II. In all experiments the electrode polarity was such that the anode filled the central compartment (19) and the cathode was layered on top of the gradient in compartment (17). This allowed the precipitation of acidic material to occur in the lower part of the column, thus avoiding possible sedimentation and disturbance of other protein zones.

Electrofocusing was performed using a final potential of 800V. Initial potentials in the order of 300-400V were applied. The maximum power at the start was 0.2 - 0.6W and the period of focusing was from 42 - 96 hours at 4°C.

At the end of the run, when the current had fallen to a steady value, the voltage was switched off, valve (26) closed and the electrode solution removed from compartment (19) to prevent possible leakage interfering with the effluent. Valve (9) was opened and the column drained with a peristaltic pump. Fractions of 2 ml were collected in 4" x ½" glass test tubes, which allowed an adequate amount for subsequent analysis whilst maintaining resolution.

All glassware was cleaned prior to use by soaking in a bath of Divolab (Diversey Ltd., Barnet, Herts) and dried at 37°C after thorough rinsing in distilled water.

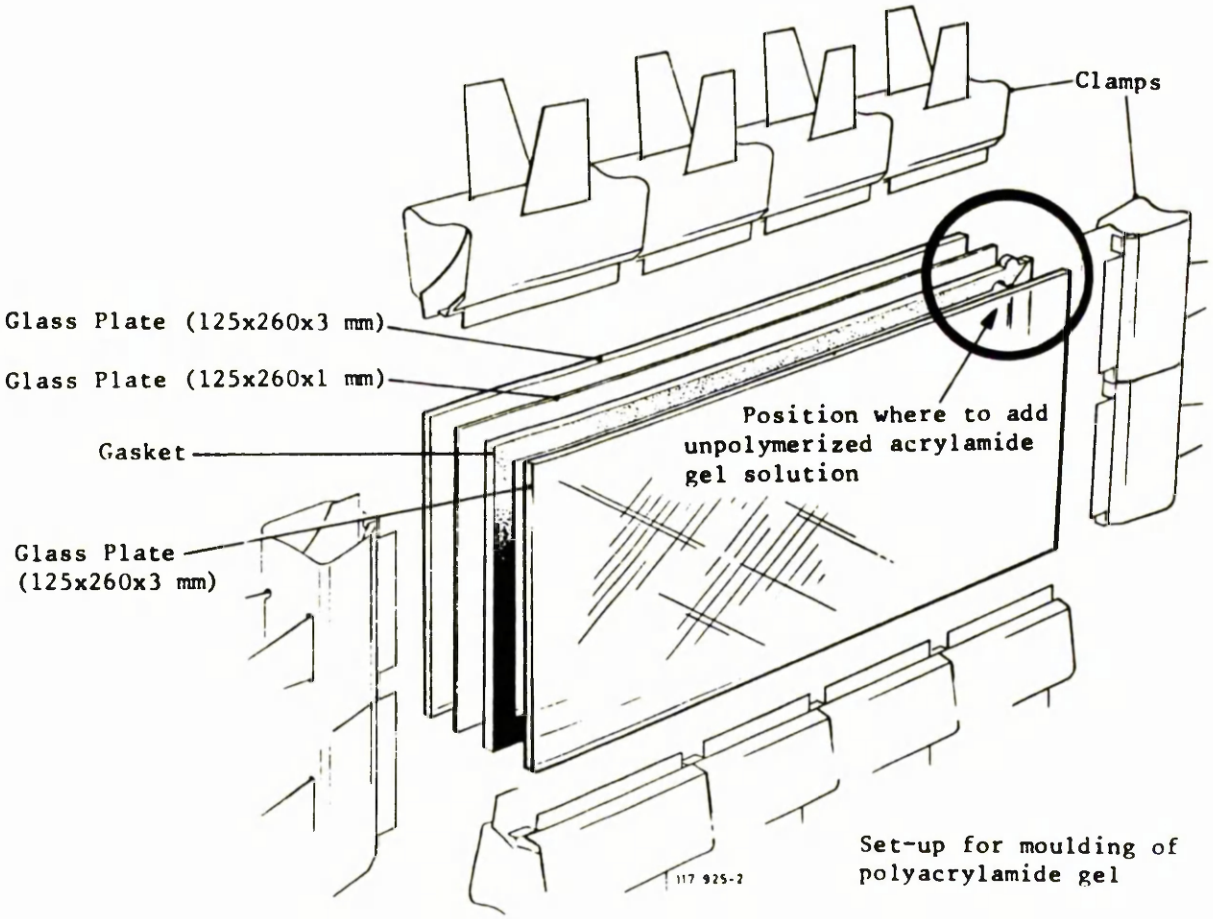
b) Analyses of column fractions: Fractions were placed in the cooling water bath as soon as they were collected and allowed to equilibrate for 10 min prior to pH measurement. The pH meter was calibrated against standard buffers of pH 7.0 and 9.2 (E.I.L., Richmond, Surrey) cooled to 4°C with the fractions under test. The calibration of the meter was checked after every fourth fraction. Approximately ½ min was allowed for equilibration after immersion of the electrode before the pH value was read off the appropriate expanded scale.

The protein content of column fractions was estimated from E_{280} values in an SP 500 UV spectrophotometer using quartz semi-micro cells of 1.0 cm light path. Distilled water was used as a blank.

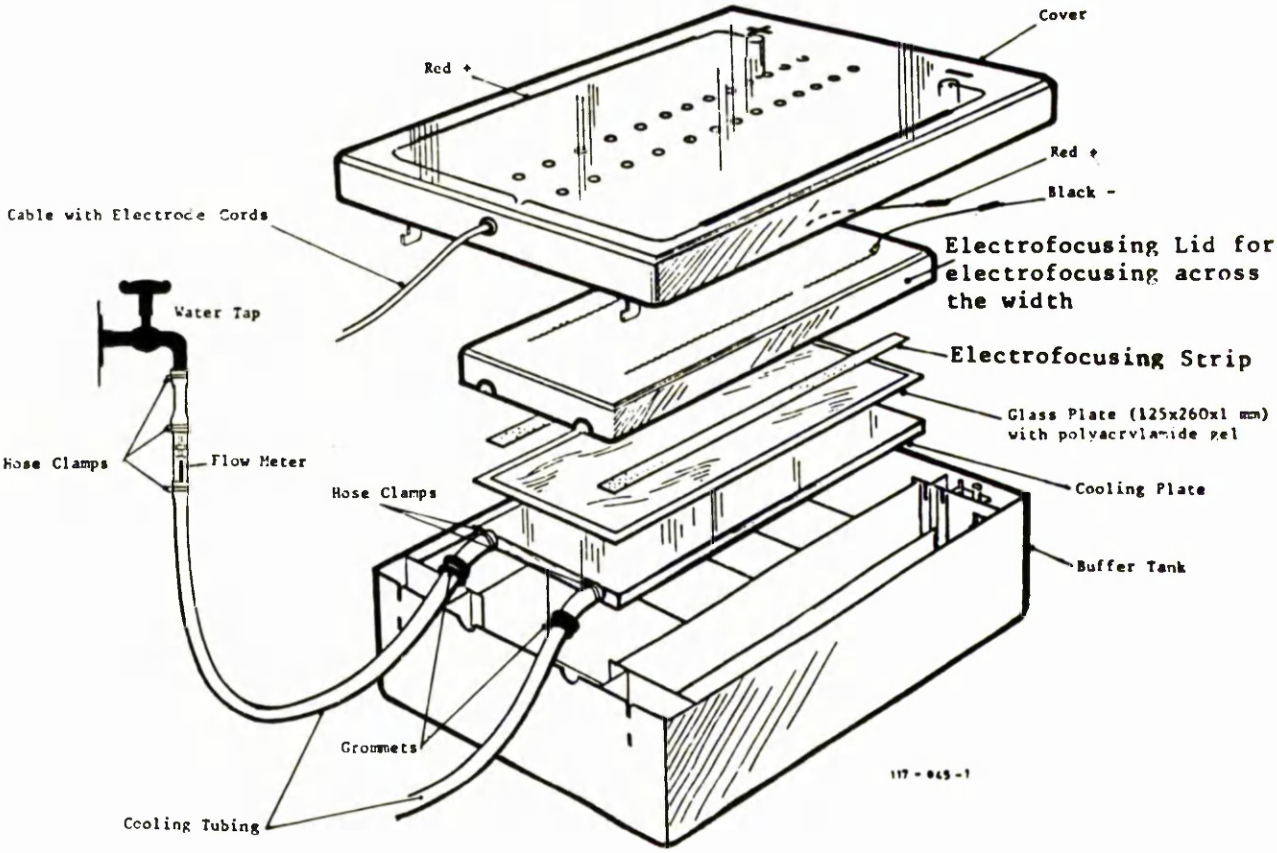
vi. Electrofocusing in polyacrylamide slabs

Electrofocusing experiments in polyacrylamide gel slabs were performed using the LKB 2117 Multiphor apparatus (see Figure 4). Here the electrofocusing run was performed in a thin layer of polyacrylamide

Figure 4. A diagram of the LKB 'Multiphor' apparatus for electrofocusing in polyacrylamide gel slabs.



Set-up for moulding of polyacrylamide gel



Multiphor mounted for thin layer celelectrofocusing in polyacrylamide gel. Electrofocusing across the width.

gel on a glass plate mounted on a cooling plate maintained at 4°C by circulating water. The electrical field was applied via two strips of paper soaked in the appropriate anode or cathode solutions and connected to the electrical supply by the specially designed safety cover and electrofocusing lid. The whole apparatus was mounted level on the buffer tank supplied for electrophoresis experiments.

Gel slabs were prepared with the solutions of Karlsson et al. (1973) (see Appendix II) and, after thorough degassing under reduced pressure, were polymerized between glass plates separated by a gasket (see Figure 4) using a fluorescent light source.

a) Procedure: Samples were applied to the gel surface in 5 x 10 mm strips of filter paper (Whatman 3MM, Whatman Ltd., Kent) soaked in a solution (approximately 1 mg/ml) of sample and applied to the surface of the gel slab with the aid of a template, spacing between adjacent samples being at least 5 mm. The electrofocusing strips were soaked with the appropriate electrode solutions (see Appendix II) and were also applied to the surface of the gel and the protruding end was cut off. The gel plate was then placed on the cooling plate using approximately 5 ml of a 1% w/v detergent solution to improve thermal contact, and the electrofocusing lid was laid carefully on top, ensuring good electrical contact. The safety cover was lowered to start the experiment. The current dropped rapidly, which allowed an increase in voltage every 10 min until maximum power had been reached. The method of cooling determined the maximum power, and using a cold water bath at 4°C, this was 40W. For a pH range in the gel of 3.5 to 9.5, an initial voltage of 210V was applied, with a final voltage of 1080V. The period of focusing was from $1\frac{1}{2}$ - $2\frac{1}{2}$ h.

The run was terminated 40 min after bands of haemoglobin from freshly lysed sheep erythrocytes had migrated together and fused from opposite sides of the gel. The pH gradient was determined with an antimony surface electrode (Beeley, Stevenson & Beeley, 1972) (Activion Ltd., Kinglassie, Fife). Measurements were made while the gel plate was still on the cooling plate at the end of the experiment. The electrofocusing strips were then removed before the gel slab was fixed and stained.

Gels were stained using the method of Billcliffe & Arbuthnott (1974) (see Appendix II). Gels were placed in stain while still on the glass support slab for 15 min at 60°C, or 1 h at room temperature. The gel was then removed and transferred to tap water. Frequent changing of the tap water led to a rapid increase in the intensity of bands which began to appear after 4 - 6 h. Bands were fully visible after soaking overnight in tap water.

IV CHARACTERIZATION OF β-LYSIN

1. Molecular weight determination

i. Slab gel electrophoresis

The method used was that of Parton (1975) and was based on that of Laemmli (1970) as modified by Ames (1974). Separating and stacking gels contained 10% (w/v) and 5% (w/v) acrylamide (BDH., Poole), respectively. Both the gels and the buffer were made 0.1% (w/v) with sodium dodecyl sulphate (SDS). The total dimensions of the slabs were approximately 8.0 x 7.0 x 0.3 cm, the separating gels being

6.0 x 7.0 x 0.3 cm. Details of the gel buffers and catalysts are in Appendix III.

Samples were adjusted to approximately 1 mg protein/ml as determined by the method of Lowry et al. (1951) and a 0.5 ml sample was added to 0.5 ml of 0.125 M tris-HCl buffer pH 6.8 containing 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mixture was heated at 100°C for 5 min prior to application of between 25 and 100 µl to the gel.

Electrophoresis was carried out in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd., London) at a constant current of 15 mA/gel for approximately 2 h. Staining and destaining was by the method of Weber & Osborn (1969). Destaining was accomplished with several changes of destaining solution (see Appendix III). For molecular weight estimations, the following mixture of protein standards was made up and run under identical conditions: bovine serum albumin (mol. wt. 67,000, Sigma Chemical Co., London), ovalbumin (43,000, Sigma Chemical Co., London), bovine pancreatic chymotrypsinogen (25,700, Miles Seravac, Maidenhead, Berkshire), trypsin (23,000, Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and horse-heart cytochrome C (11,700, Koch-Light, Colnbrook, Buckinghamshire).

ii. Gel filtration on Biogel P-60

The molecular weight of the partially purified material from gel filtration was estimated from its movement on a column of Biogel P-60.

The column (2.5 x 90 cm) was equilibrated with 0.05 M phosphate buffer pH 7.2 at 4°C. The void volume (V_o) was determined by application of 2 ml buffer containing 10 mg dextran blue 2000 (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden). The standard proteins, ovalbumin (MW = 43,000), myoglobin (MW = 17,000) and cytochrome C (MW = 11,700) were applied individually as 10 mg/ml solutions in 2 ml of phosphate buffer. Myoglobin was obtained from Sigma Chemical Co. Ltd., London; the other proteins were obtained from the sources described in the previous section.

A 2 ml sample containing approximately 5 mg of partially purified β-lysin was applied to the column. The protein content of the fractions was monitored as described earlier with the wavelength set at 280 nm for protein samples, 625 nm for dextran blue and 412 nm for cytochrome C. The ratio of elution volume (V_e) to void volume (V_o) was plotted against the logarithm of the molecular weights of the standard proteins. The molecular weight of β-lysin was estimated from its elution behaviour.

2. Estimation of S_{20w}

Sedimentation studies were performed at 60,000 rev/min on an MSE analytical ultracentrifuge with a 20 mm double sector overfilling cell. Samples of purified β-lysin (2 ml, concentration 1 - 2 mg/ml) were dialysed overnight against 500 ml of 0.1 M KCl, buffered with 0.01 M Tris-HCl, pH 7.4 prior to centrifugation. During the run the dialysate was used as a blank.

Photographs were taken at a Schlieren angle of 40° at 4 min

intervals on Kodak Tri-X film. After development in Kodak Microdol X developer the film was fixed and washed prior to measurement being made from projected enlargements.

A value of 0.714 was calculated for the partial specific volume by the method of Cohn & Edsall (1943) using the amino acid composition of Bernheimer, Avigad & Kim (1974) and the sedimentation coefficients were corrected to 20°C in distilled water (see Appendix VI).

3. Immunological analysis

i. Antiserum

Antiserum to crude β-lysin was prepared as follows. The ammonium sulphate precipitate from a culture of S. aureus G128 was dialysed against 0.05 M TBS + Mg^{2+}, pH 7.4, for 12 h and was toxoided by adding formaldehyde to a final concentration of 0.2% (w/v) and leaving the mixture to stand for 96 h at 4°C (Bernheimer et al., 1968).

The mixture was spun on a bench centrifuge to remove the slight precipitate which formed and 2 ml of the toxoid (protein content 4.7 mg/ml) was injected intramuscularly into the hind quarters of adult New Zealand White rabbits. A booster injection of the same mixture was given 4 weeks later. A further 10 days later the rabbits were anaesthetized with 0.4 ml/kg (body weight) of intravenously injected Nembutal (Abbott Laboratories Ltd., England) and bled out by cardiac puncture. The blood was allowed to clot at 37°C for 30 min, and was then placed at 4°C for 18 h and the serum removed and stored at -20°C.

ii. Neutralization of β-lysin

The antiserum, diluted 1 in 100 using 0.05 M TBS + Mg^{2+}, pH 7.4, was titrated using doubling dilutions in 0.5 ml volumes of 0.05 M TBS + Mg^{2+}; 0.2 ml volumes of 0.05 M TBS + Mg^{2+} containing 8 haemolytic units of β-lysin were added to each tube in the series of dilutions. After incubating the toxin/antitoxin mixture for 45 min in a water bath at 37°C, 0.3 ml of 0.7% (v/v) sheep erythrocyte suspension was added to each tube and the titrations were incubated for a further 30 min at 37°C followed by 2 h at 4°C. The end point was taken as the last tube in the series showing no haemolysis.

iii. Double diffusion tests

Double diffusion tests were carried out by a modification of the technique of Elek (1948) and Ouchterlony (1948, 1949). Wells (5 mm diameter) were cut at 5 mm distance in a 3 mm thick layer of 1% Ionagar (Difco) in borate/sodium chloride buffer at pH 8.3 (see Appendix IV). After addition of appropriate reagents to the wells, the plates were incubated at 37°C for 24 h and the precipitin lines which developed were clearly visible.

V BIOLOGICAL EFFECTS OF β-LYSIN

1. Effects on erythrocyte membranes

i. Preparation of ghost membranes

Erythrocyte ghosts were prepared from fresh human and bovine blood samples by a modification (Freer, Arbuthnott & Billcliffe, 1973),

of the method of Hoogeveen et al. (1970). Fresh citrated blood was centrifuged at 1500 g for 10 min at room temperature and the plasma and buffy coat layer were carefully removed by aspiration. The pellet was resuspended in 20 vol of 0.85% (w/v) NaCl and the centrifugation and aspiration process was repeated until the erythrocytes had been washed three times.

Erythrocytes were lysed by slow dropwise addition to 200 volumes of ice-cold 0.01 M phosphate buffer, pH 7.4, which was stirred throughout this step. Centrifugation of the lysate at 35,000 g for 30 min at 4°C gave a light buff coloured membrane pellet which was resuspended and washed twice in 200 vol of 0.01 M Tris HCl buffer, pH 7.4, and finally in 200 vol of 0.01 M Tris HCl buffer, pH 7.4 made 0.001 M in $MgCl_2$ and sedimented as before.

Erythrocyte ghosts made in this fashion were haemoglobin free as assessed by polyacrylamide gel electrophoresis (Freer, Arbuthnott & Billcliffe, 1973) and were free of inorganic phosphate as determined by the methods of Chen, Toribara & Warner (1956) and Allen (1940).

For analysis, ghost membranes were prepared as described above, except that the final wash was carried out in 0.01 M Tris-HCl buffer, pH 7.4, without $MgCl_2$. After removal of an aliquot for phosphorus and protein determinations, the remaining ghosts were dialysed for 12 h against distilled water (three changes of 5 litres) at 4°C after which the preparation was freeze dried and weighed.

ii. β-lysin treatment of erythrocyte ghost membranes

Freshly prepared erythrocyte ghosts (40 mg dry wt) were

suspended in a volume of 5 - 10 ml 0.01 M Tris-HCl buffer, pH 7.4, made 0.001 M in $MgCl_2$. β-lysin (100 µg) was added and the sample incubated at 37°C in a water bath for 1 h. Samples were removed at 5 and 60 min for biochemical analysis, examination by phase contrast microscopy and by freeze-etching and the remaining membranes were centrifuged at 100,000 g for 1 h at 4°C. Aliquots of the pellet and supernatant were assayed for protein and phosphorus.

iii. Freeze-etching

Drops of fresh sample (approximately 10 µl) without added cryoprotective agent were placed on paper discs (extracted three times with Arklone P (ICI Chemicals Ltd., Glasgow), and were frozen rapidly by immersion in Freon 22 at liquid nitrogen temperature for 2 - 3 sec. Specimens were then rapidly transferred into liquid N_2 and stored until used (always less than 24 h). Freeze-fracturing was performed in a Balzers 360 M Freeze-Etching Unit with a specimen temperature of -100°C; specimens were etched for 30 sec. Platinum-carbon replicas were floated on water and cleaned by transfer into 5% (w/v) sodium dodecyl sulphate for approximately 18 h, followed by transfer into 70% (v/v) H_2SO_4 for approximately 5 h. After washing in water, replicas were transferred to grids covered with carbon coated formvar and examined in a Philips EM 300 electron microscope operated at 60 kV. Electron micrographs of freeze-etched replicas in this thesis are labelled according to the conventions suggested by Branton et al. (1975), where the half-membrane is labelled P or E according to whether it is the half which is closest to the protoplasmic (P) or the extracellular space (E). The true surface of the membrane, labelled PS or ES, is

the hydrophilic portion of the membrane usually exposed by the etching process; the fracture face, labelled PF or EF, is the hydrophobic portion of the membrane revealed by the fracture process.

iv. Extraction of lipids

Freeze-dried ghost preparations (40 mg) were hydrated and extracted three times with 10 ml of a chloroform:methanol (2:1) mixture at 37°C for 1 h. The pooled extracts were washed according to the method of Folch, Lees & Sloane-Stanley (1957) with 0.73% (w/v) NaCl and the solvent was removed by evaporation under vacuum. The lipid residues were extracted with chloroform and after removal of chloroform under a stream of nitrogen, the dry weights were determined. For estimation of lipid phosphorus, the dried lipids were dissolved in a known volume of chloroform. The pellet from β-lysin treated erythrocyte ghost membranes was extracted as above, using three changes of 2:1 chloroform:methanol.

v. Thin-layer chromatography

Thin-layer chromatography was carried out on Silica gel G (Nach Stahl, Merck, Darnstadt, Germany). A slurry was made by mixing 36 g Silica Gel G, 48 ml distilled water and 24 ml 'Analar' methanol in a stoppered jar for 1 min. The slurry was spread using a Shandon semi-automatic Unoplan Leveller on glass plates 20 cm x 20 cm. After air-drying, the plates were stored in a desiccator cabinet and were activated prior to use by heating in a drying oven at 110°C for 20 - 30 min. Plates were developed with chloroform:methanol:water (65:35:4 by vol) and the spots of the separated lipids were visualized by charring with 50% (v/v) sulphuric acid for 15 min at 105°C, or by spraying with the acid molybdate reagent of Dittmer & Lester (1964).

2. Effects in mice

i. Intravenous injection

Dilutions of β-lysin were made using 0.05 M TBS + Mg^{2+} (see Appendix I). Each test was performed with twenty male mice. The mice were of a closed colony bred from the Charles River HAM 1 CR strain and were 3 - 4 weeks old at the time of the injections. Four groups of five mice, each weighing 20 - 22 g, were injected intravenously via the caudal veins at the base of the tail as follows: group A - 0.1 ml of diluent (control); group B - 0.1 ml containing 0.1 mg/ml β-lysin; group C - 0.1 ml containing 1 mg/ml β-lysin; group D - 0.15 ml containing 1 mg/ml β-lysin. Injections were made using a 23 gauge 1.1/4" needle and a 1 ml sterile polypropylene syringe (Becton & Dickinson, Drogheda, Ireland). Diluted β-lysin was kept in an ice bath throughout the experiment. The maximum dosage corresponded to the injection of 400,000 HU of β-lysin.

The weight of each mouse was taken every 24 h for a period of 7 days, when the experiment ended.

ii. Intradermal injection

Dilutions of β-lysin were made in 0.05 M TBS + Mg^{2+}. Each test was performed with three male hairless mice from a closed colony of hr/hr strain which were 3 - 4 weeks old at the time of the injection. Each mouse was injected intradermally in four sites on the back with 5, 10, 25 and 50 μg of purified β-lysin in 0.05 ml of TBS + Mg^{2+}. The mice were examined after 24 h for signs of dermonecrosis, and were then

injected with 0.1 ml of 0.5% (w/v) Evans blue in phosphate buffered saline. After a further four hours, the animals were killed and skinned and the injection sites were examined for evidence of any increase in local vascular permeability.

3. Pathogenicity of S. aureus G128 for sheep

Cells were harvested in the late log phase of growth from YDB medium and washed three times in 0.05 M phosphate buffered saline (PBS), pH 7.5 (see Appendix I). The cells were prepared for challenge by suspension in PBS. The viable count was estimated by the method of Miles, Misra & Irwin (1938) by making serial ten-fold dilutions in PBS with vigorous mixing between steps. Each dilution (0.2 ml) was dropped from a sterile graduated pipette onto mannitol salt agar (Oxoid Ltd.) in Petri dishes. The plates were incubated at 37°C for 24 - 48 h and the viable count was estimated from the first countable dilution.

Five ml of suspension (21.5×10^{10} c.f.u./ml) was injected intravenously into a Scottish Blackface ewe. A sample of blood was taken immediately before injection, and a second sample was taken 6 h later (approximately 1 h after death). The blood samples were examined by phase contrast microscopy, and the viable count was estimated as above. The lipids were extracted by the method of Reed et al. (1960) and examined by thin layer chromatography on Silica gel G.

RESULTS

I LYSIN PRODUCTION

1. Lysin production during the growth cycle

The relationship between lysin production and growth phase in the 3 strains of S. aureus was studied in shake flask cultures of 500 ml of YDB medium at 37°C. The inoculum was 1 ml of frozen glycerinated cells. Aliquots (10 ml) were withdrawn at intervals and the supernatant was assayed for β-lysin after centrifugation at 16,000 g for 30 min at 4°C in an MSE 18 centrifuge. The results, which are summarised in Figure 5, show that:

i. Lysin production was at a maximum at the end of the logarithmic growth phase.

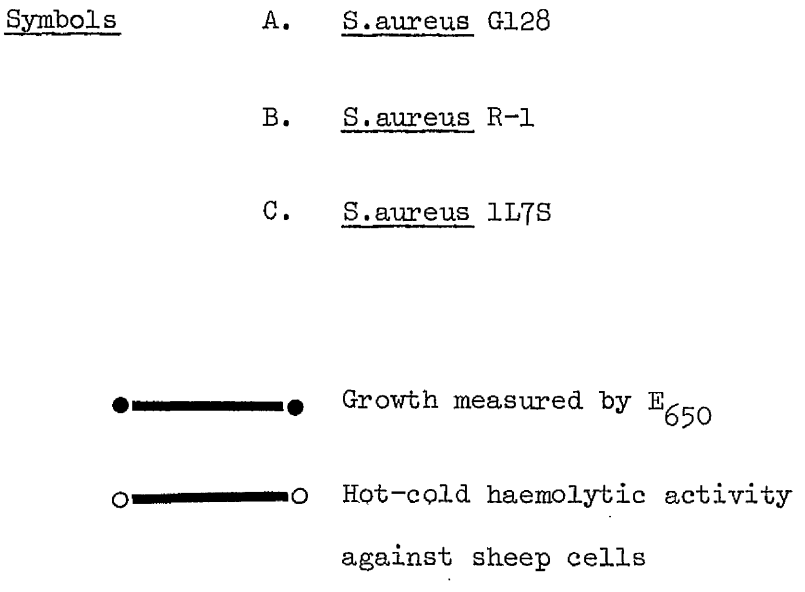
ii. Of the three strains, G128 produced the highest maximum titre of β-lysin (200,000 HU/ml) and 1L7S the lowest maximum titre (4,000 HU/ml).

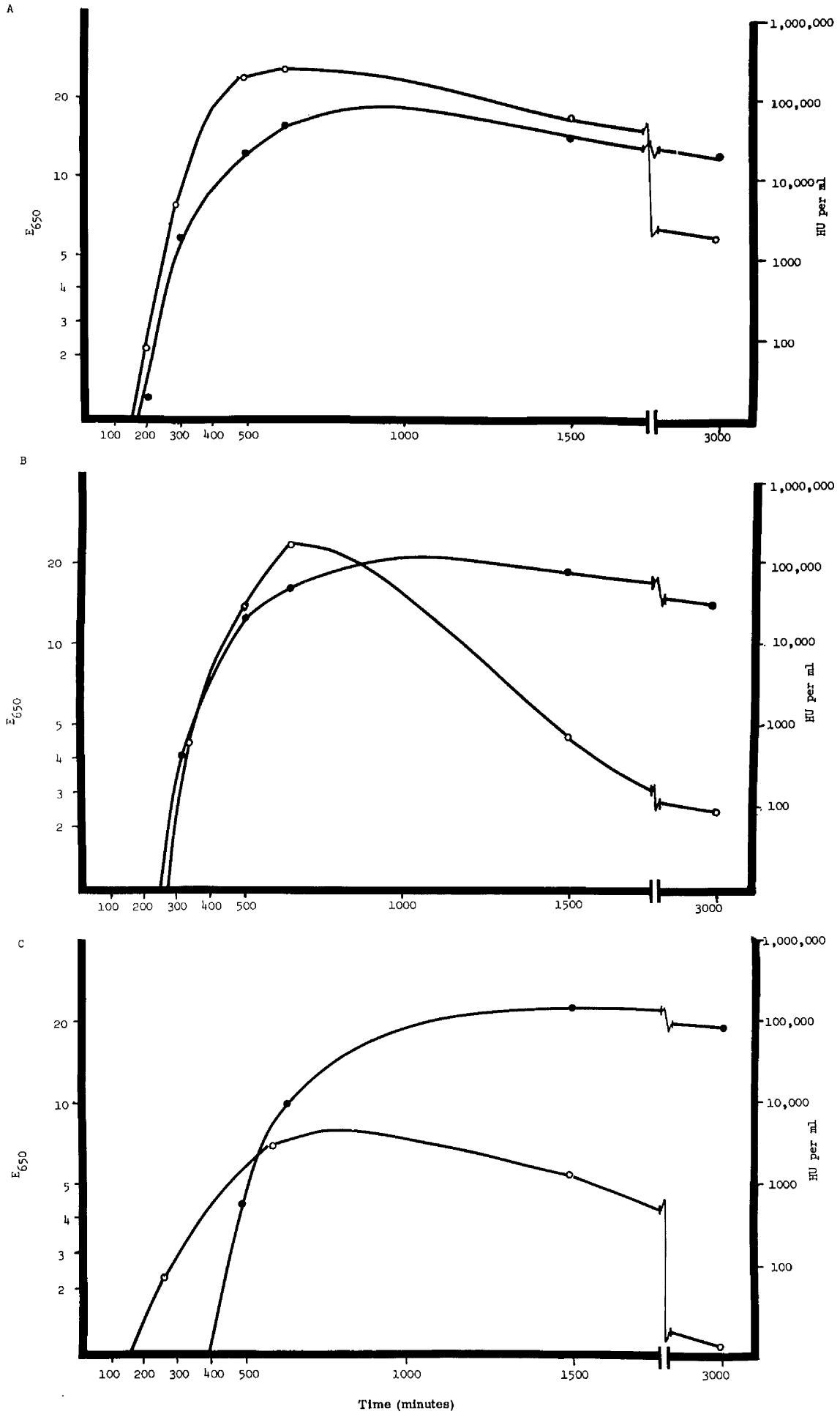
iii. Lysin activity in the culture supernatant declined after prolonged incubation.

iv. The rate of production of β-lysin was approximately the same for both G128 and R-1, but was slower for 1L7S.

Prolonged incubation of the three strains resulted in little further growth once the logarithmic phase passed. β-lysin production also stopped when the stationary phase of growth was reached and it appeared that β-lysin production was a feature of cells in the exponential phase of growth. In addition, both strains G128 and R-1 caused considerable haemolysis of sheep erythrocytes at 37°C, indicating the presence of one or more of the other haemolysins produced by S. aureus.

Figure 5 : Production of β-lysin by S. aureus strains G128, R-1 and 1L7S




A
B
C
E_{650}
HU per ml
1,000,000
100,000
10,000
1000
100
20
10
5
4
3
2
100
200
300
400
500
1000
1500
3000
Time (minutes)

The decline in titre of β-lysin after prolonged (50 h) incubation was greatest in strain 1L7S and least in strain G128.

2. Purification of β-lysin

The method of purification used throughout this work was a modification of the method of Bernheimer, Avigad & Kim (1974). The purification and recovery at each stage of purification in a typical experiment is presented in Table 9. 1 litre of culture supernatant yielded approximately 1 mg of purified lysin with a specific activity of approximately 62 x 10^6 HU/mg protein. The purification obtained was 38,000-fold and the recovery of haemolytic activity was greater than 1000%.

The first stage involved concentration of the supernatant. This was achieved by adding solid $(NH_4)_2SO_4$ at 4°C to give 85% saturation. Care was taken during the addition of the solid since it "salts out" dissolved air from the solution as fine air bubbles. These may denature the lysin since many enzymes are denatured at interfaces. A further loss of material can arise through the tendency for some of the precipitate to "float" even after high speed centrifugation.

In the second stage, Biogel P60 was used for fractionation because of the reported low recoveries of β-lysin from Sephadex G100 and G50 columns (Wadström & Möllby, 1971a).

The elution profile shown in Figure 6 revealed four protein components, three of which could be identified as containing δ-, α- and β-lysin activities by their action on cod, rabbit and sheep erythrocytes.

Table 9 Purification and recovery of β-lysin

Purification step	Volume (ml)	Haemolytic activity (HU/ml)	Protein (mg/ml)	Specific activity (HU/mg)	Total haemolytic activity (HU)	Recovery of activity (%)	Purification factor
Culture supernatant	945	6,400	3.87	1,700	6×10^6	100	1
Ammonium sulphate precipitate	8.75	510,000	75.13	6,800	4.5×10^6	75	4
Biogel P-60 eluate (after concentration)	10.5	260,000	4.16	62,000	2.7×10^6	45	37
CM cellulose eluate (after concentration)	6.75	27,000,000	0.68	38,000,000	170×10^6	2,900	23,000
Isoelectric focusing (after removal of ampholines)	4	20,000,000	0.32	62,500,000	80×10^6	1,300	38,000

Figure 6 : Fractionation of β-lysin on Biogel P-60

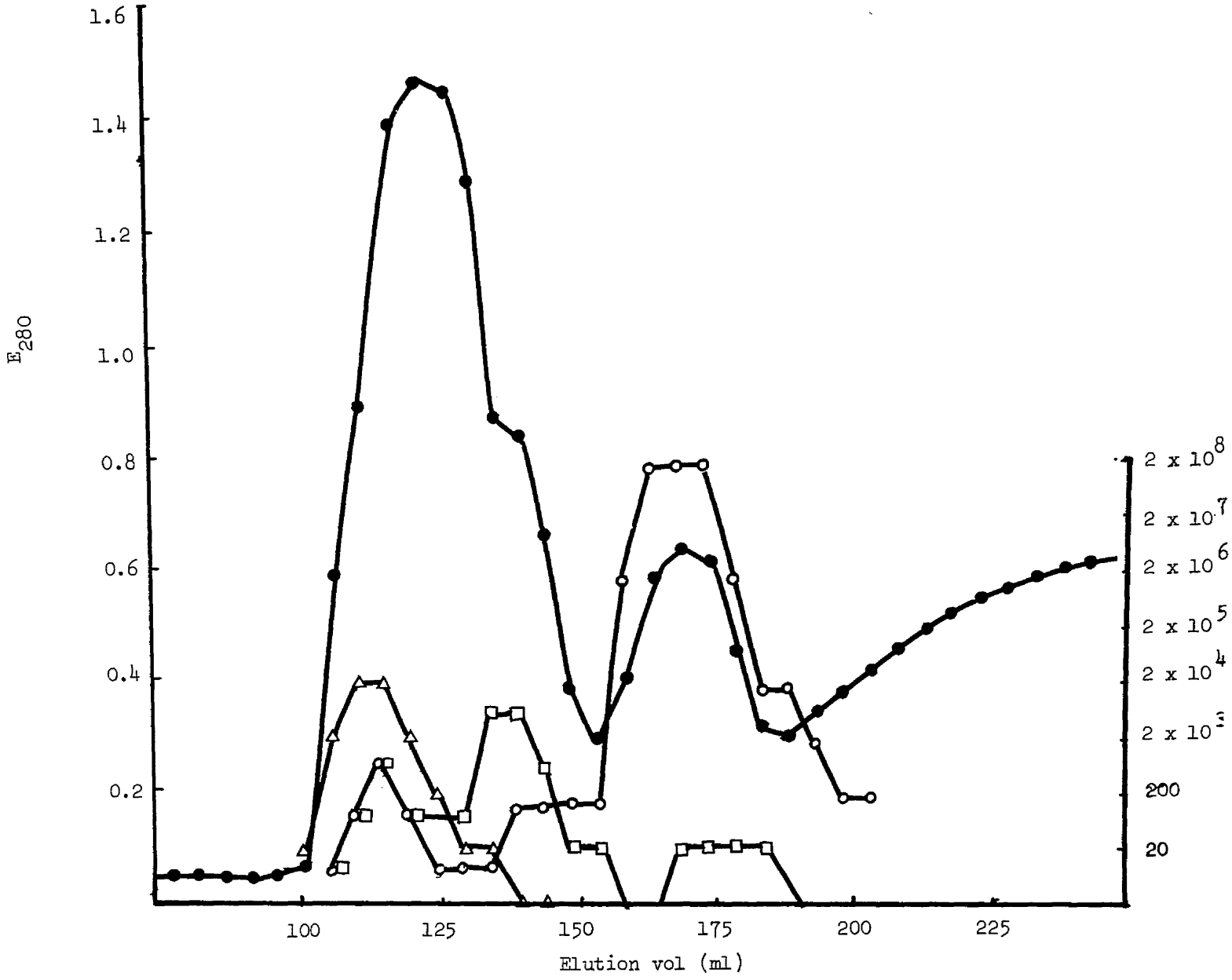
5 - 7 ml dialysate containing approximately 60 mg/ml protein applied to a Biogel P-60 column (2.5 x 90 cm). The column was eluted with 0.05 M phosphate buffer, pH 7.2.

E_{280}

hot-cold haemolytic activity against sheep cells

haemolytic activity against rabbit cells

haemolytic activity against cod cells


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
E_{280}
2 x 10^8
2 x 10^7
2 x 10^6
2 x 10^5
2 x 10^4
2 x 10^3
200
20
100
125
150
175
200
225
Elution vol (ml)

The early fractions from the ascending limb of β-lysin activity were discarded to avoid contamination with α-toxin. During large scale purification procedures the large amount of anionic material still present at this stage tended to form a considerable precipitate at the bottom of isoelectric focusing columns, impairing the resolution of the column and decreasing the amount of protein which could be applied, thus making the process unnecessarily expensive. As a result it was decided to include ion-exchange chromatography prior to electrofocusing.

Fractionation of the product from the Biogel P60 column by CM cellulose column chromatography yielded two main protein peaks which showed differing amounts of β-lysin activity (see Figure 7). There was almost a 3,000% recovery of activity and 23,000-fold purification. Almost all of this activity was recovered in the main cationic peak. Because the activation in this step is most likely to be due to the removal of inhibitors present in the first protein peak, and since the anionic peak contained a certain amount of lytic activity at 37°C, further purification was continued using the main cationic form of β-lysin.

Prior to electrofocusing, β-lysin preparations were dialysed against a 1% (w/v) solution of glycine to ensure the necessary low salt concentration. Isoelectric focusing of β-lysin gave one major peak with a pI of 9.3 and a minor peak with a pI of 9.75 (see Figure 8). Over 90% of the haemolytic activity on the column was recoverable from the major peak, however this corresponded to approximately 60% of the haemolytic activity applied to the column. Despite this decrease in total activity there was an increase in the purity of the preparation.

Figure 7 : Fractionation of β-lysin on carboxymethyl cellulose (CM 32)

10 ml of dialysate containing approximately 4 mg/ml protein were applied to a Whatman CM 32 column (2.0 x 22 cm). The column was eluted with 100 ml of 0.05 M phosphate buffer pH 6.5 followed by 300 ml of a linear gradient of phosphate buffer formed in a 440 ml LKB gradient mixer by mixing 150 ml of 0.05 M phosphate buffer pH 6.5 and 150 ml of 0.2 M phosphate buffer, pH 6.5, containing 0.2 M NaCl.

E_{280}

hot-cold haemolytic activity against sheep cells

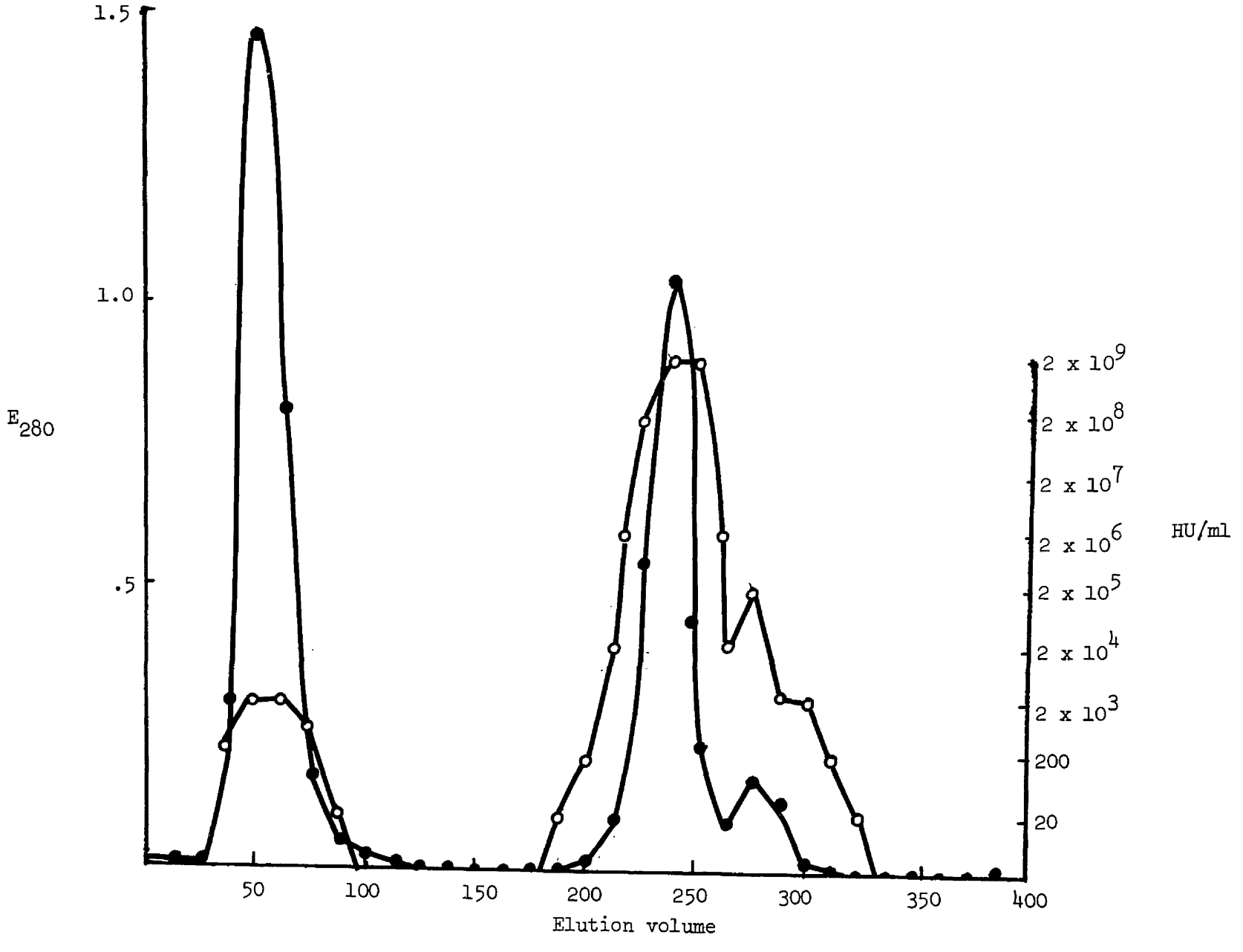
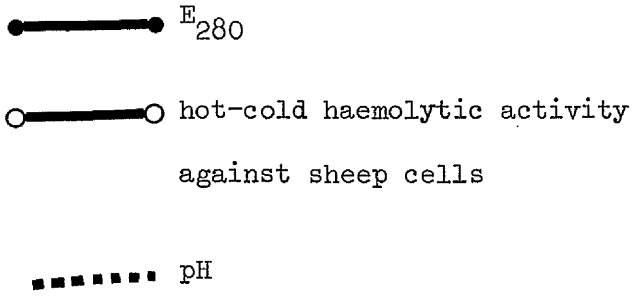
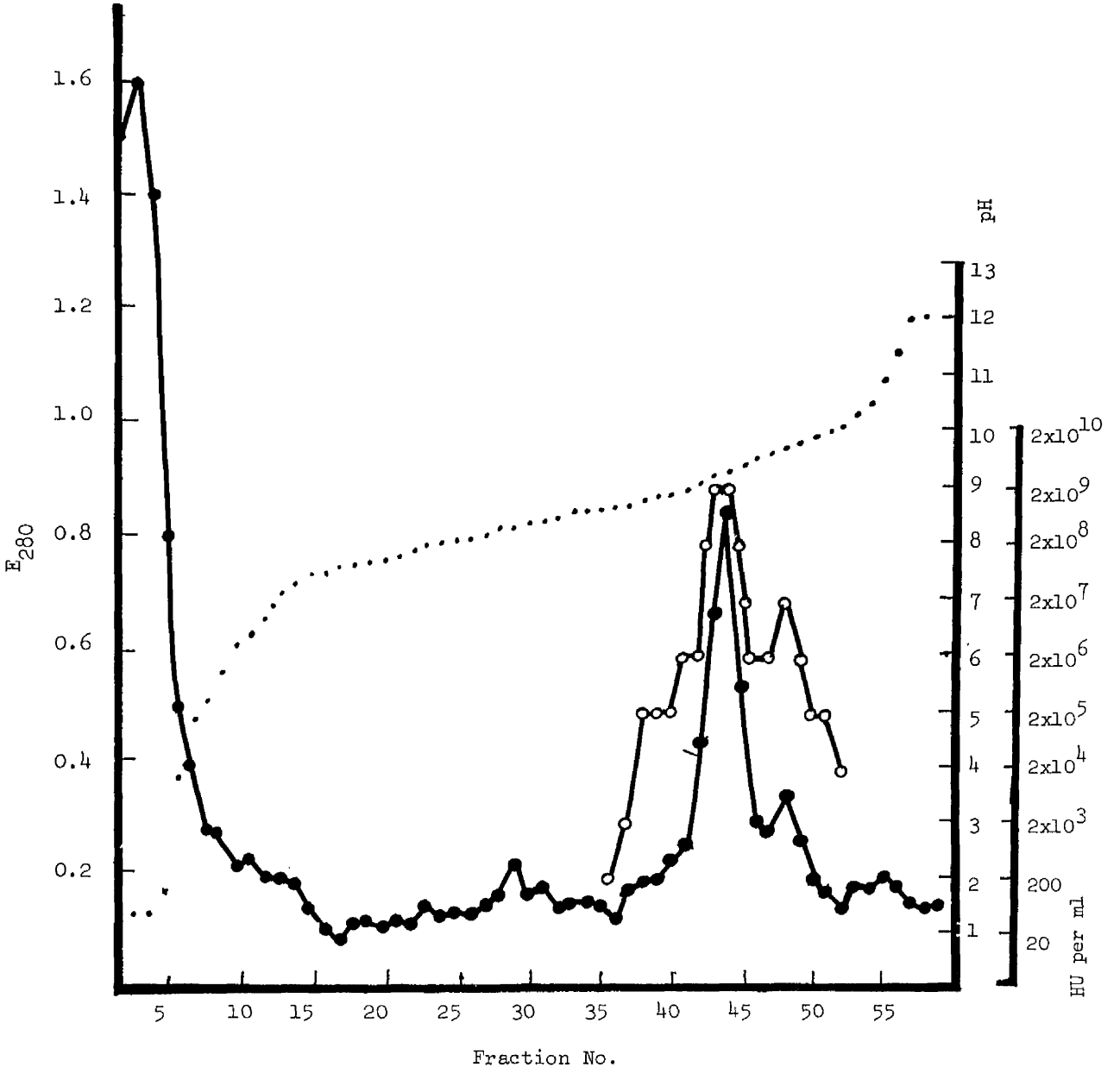

1.5
1.0
.5
E_{280}
2×10^9
2×10^8
2×10^7
2×10^6
2×10^5
2×10^4
2×10^3
200
20
HU/ml
50
100
150
200
250
300
350
400
Elution volume

Figure 8 : Electrofocusing of partially purified β-lysin in a pH 7 - 10.5 gradient

5.0 mg β-lysin applied to an LKB 8101 column.

Fraction size = 2 ml.




1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
E_{280}
pH
13
12
11
10
9
8
7
6
5
4
3
2
1
$2x10^{10}$
$2x10^{9}$
$2x10^{8}$
$2x10^{7}$
$2x10^{6}$
$2x10^{5}$
$2x10^{4}$
$2x10^{3}$
200
20
HU per ml
5
10
15
20
25
30
35
40
45
50
55
Fraction No.

Fractions were analysed for purity by electrofocusing in polyacrylamide gel slabs, and in every experiment the peak fractions (fractions 43 and 44, Figure 8) showed one major band with a pI of approximately 9.3 and a minor band with a pI of 9.7 (see Plate 1). To obtain optimal resolution, only the material from the two peak fractions were retained. These fractions were dialysed overnight against 85% saturated ammonium sulphate and the precipitate collected by centrifugation. The precipitate was either stored in saturated ammonium sulphate, or dialysed against 50% (v/v) glycerol and stored at -20°C.

3. Criteria of purity

i. Polyacrylamide gel electrophoresis

The degree of purity of β-lysin was monitored by SDS-polyacrylamide gel electrophoresis (Plate 2). Purified β-lysin contained 1 major component having a molecular weight of 33,000 (Figure 10). Trace amounts of impurities were visible when high (100 µg) amounts of protein were applied.

ii. Immunodiffusion

Purified β-lysin was analysed by immunodiffusion with antiserum prepared against a crude culture supernatant of S. aureus G128. Plate 3 shows that in Ouchterlony plates a single line of precipitation was observed for highly purified β-lysin. Plate 4 shows that immunodiffusion against crude β-lysin gave numerous lines.

Plate 1. Polyacrylamide gel isoelectric focusing of β-lysin

Fractions 43 and 44 were taken from the experiment shown in Figure 8. The pattern of bands obtained from a solution of haemoglobin obtained by lysing sheep erythrocytes in distilled water is shown alongside for comparison.

Plate 2. SDS-polyacrylamide gel electrophoresis of β-lysin

Increasing amounts of β-lysin (25, 50, 75 and 100 μg) were applied across the gel. The purified proteins are:

B = bovine serum albumin

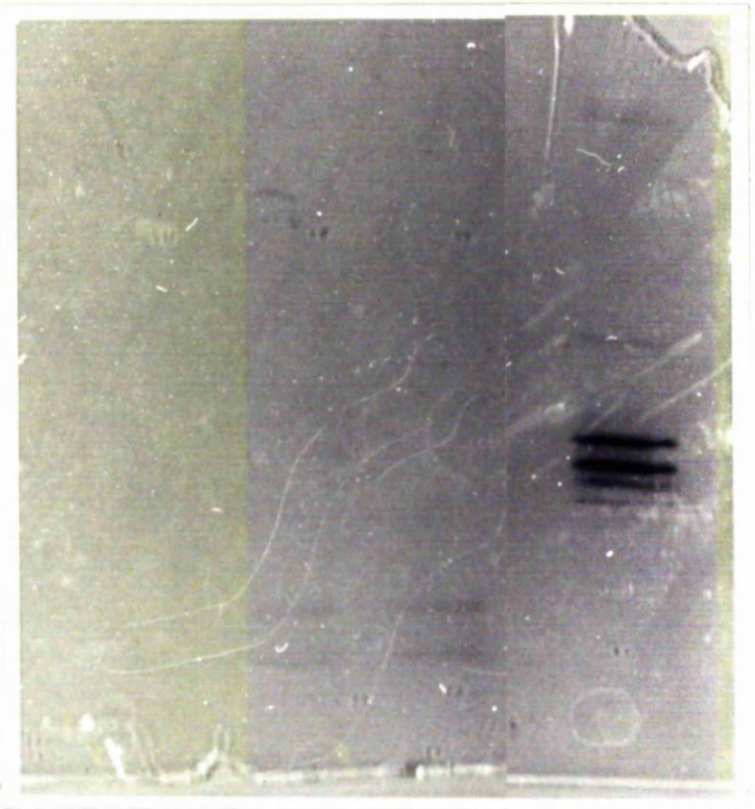
O = ovalbumin

C = chymotrypsinogen

T = trypsin

Y = cytochrome C

L = β-lysin



43 44

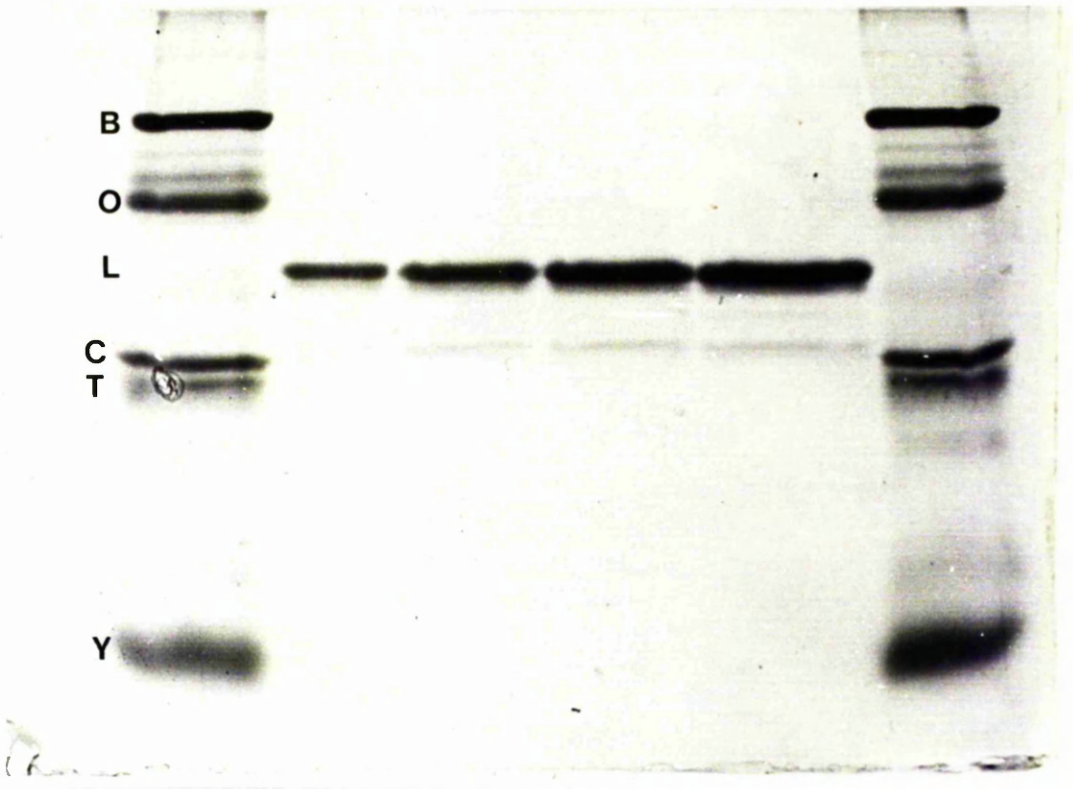


Plate 3. Double gel diffusion analysis of β-lysin

A = antiserum

L = highly purified β-lysin

Plate 4. Agar gel diffusion pattern of antiserum from different rabbits against crude β-lysin

A = antiserum

L = crude β-lysin


A


A
A

iii. Freedom from contaminating proteins

Purified β-lysin was assayed for several of the extracellular proteins produced by S. aureus. Highly purified β-lysin (100 μg) was found to be free from coagulase, lipase, fibrinolysin, hyaluronidase, protease, leukocidin, phosphatase, nuclease and egg yolk factor activities. The threshold of detection and estimated upper level of contamination for some of these activities is shown in Table 10.

iv. Haemolytic activity

The lysin (100 μg) was not haemolytic at 37°C to sheep, ox, human or rabbit erythrocytes, nor was it haemolytic at 22°C to cod erythrocytes. The thresholds of detection and estimated upper levels of contamination by α-toxin and δ-lysin are included in Table 10.

4. Sphingomyelinase activity

The specific activity of β-lysin as a sphingomyelinase C was measured using the substrate decrease assay of Zwaal & Roelofsen (1974). Sphingomyelin hydrolysis was monitored by assaying for lipid-soluble phosphorus. One unit (IU) of sphingomyelinase C is defined as the amount of enzyme which causes the hydrolysis of 1 μmole of sphingomyelin per min, and the specific activity is expressed as units per milligram of enzyme protein. From Figure 9, 2.5 μg of enzyme protein causes the hydrolysis of 0.78 μmoles sphingomyelin per min, so the specific activity is

$$\frac{0.78 \times 1000}{2.5} = 312 \text{ IU/mg}$$

Table 10. Estimated upper limits of contamination of β-lysin by some of the extracellular proteins produced by S. aureus

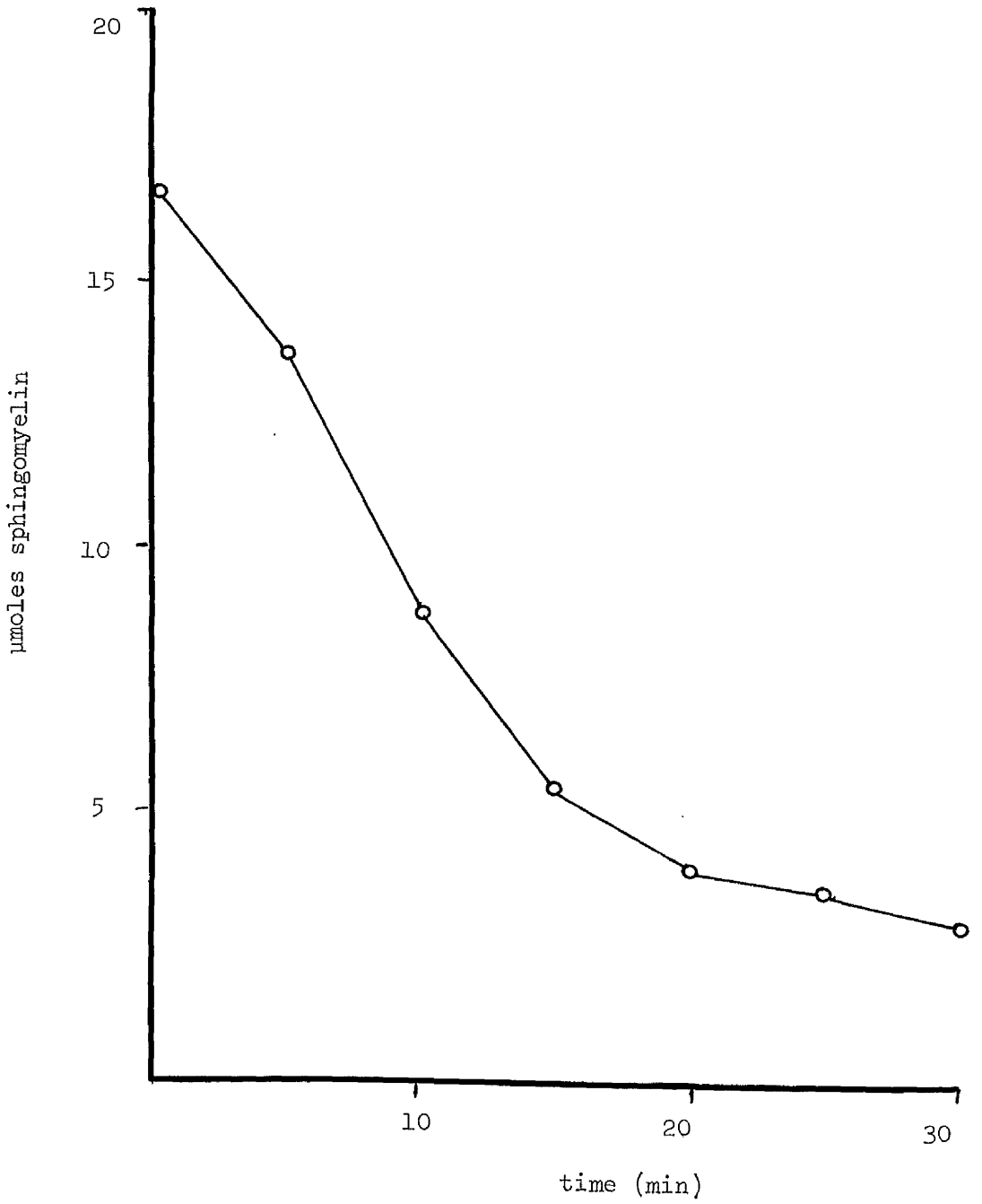
Test	Sensitivity of test (μg detectable)	Amount of β-lysin tested (μg)	Upper limit of contamination μg/mg β-lysin
α-toxin[1]	0.0001 (20 HU)	10	10^{-2}
δ-lysin[2]	1.7 (2 HU)	100	17
Phosphatase	1.0	100	10
Hyaluronidase	<0.01	100	10^{-1}
Protease	<0.01	100	10^{-1}
Nuclease	<0.01	100	10^{-1}
EYF using PLC as standard	<0.01	100	10^{-1}

[1]Prepared by the method of Arbuthnott, Freer & Bernheimer (1967)

[2]Prepared by the method of Birkbeck, Chao & Arbuthnott (1974).

Figure 9 : Hydrolysis of sphingomyelin by β-lysin

The reaction mixture consisted of 15 mg sphingomyelin dissolved in 2 ml chloroform, 1 ml of 0.1 M Tris buffer pH 7.4 containing 1 mM $MgCl_2$ and an aliquot containing 2.5 µg of β-lysin.


20
15
10
5
µmoles sphingomyelin
10
20
30
time (min)

As can be seen from Figure 9, the hydrolysis of sphingomyelin was linear for about 15 min, after which the rate of reaction tended to tail off.

II CHARACTERIZATION OF β-LYSIN

1. Molecular weight estimations

i. Polyacrylamide gel electrophoresis

Highly purified β-lysin was solubilized using the dissociating agent SDS. Proteins are known to bind SDS in proportion to their molecular weights and the resulting complexes lose charge specificity and migrate solely according to molecular size (Reynolds & Tanford, 1970). The distance migrated by purified β-lysin in the presence of SDS was compared with the distances migrated by several other purified proteins (Plate 2). The standard curve used for the molecular weight estimation is shown in Figure 10. A value of 33,000 was obtained for the molecular weight of β-lysin.

ii. Gel filtration

The molecular weight was also estimated by Biogel P-60 gel filtration. In this instance a partially purified preparation of β-lysin was used, since purified staphylococcal β-lysin is unstable. The elution behaviour of β-lysin as compared to several purified proteins is shown in Figure 11. The results give a molecular weight of 32,500 ± 1,500 which is in good agreement with result obtained by SDS polyacrylamide gel electrophoresis.

Figure 10 : Estimation of the molecular weight of β-lysin by SDS-polyacrylamide gel electrophoresis

(see Plate 2)

Plot of the logarithm of the molecular weight of standard proteins against Rf values.

B = bovine serum albumin

O = ovalbumin

C = chymotrypsinogen

T = trypsin

Y = cytochrome C

L = β-lysin

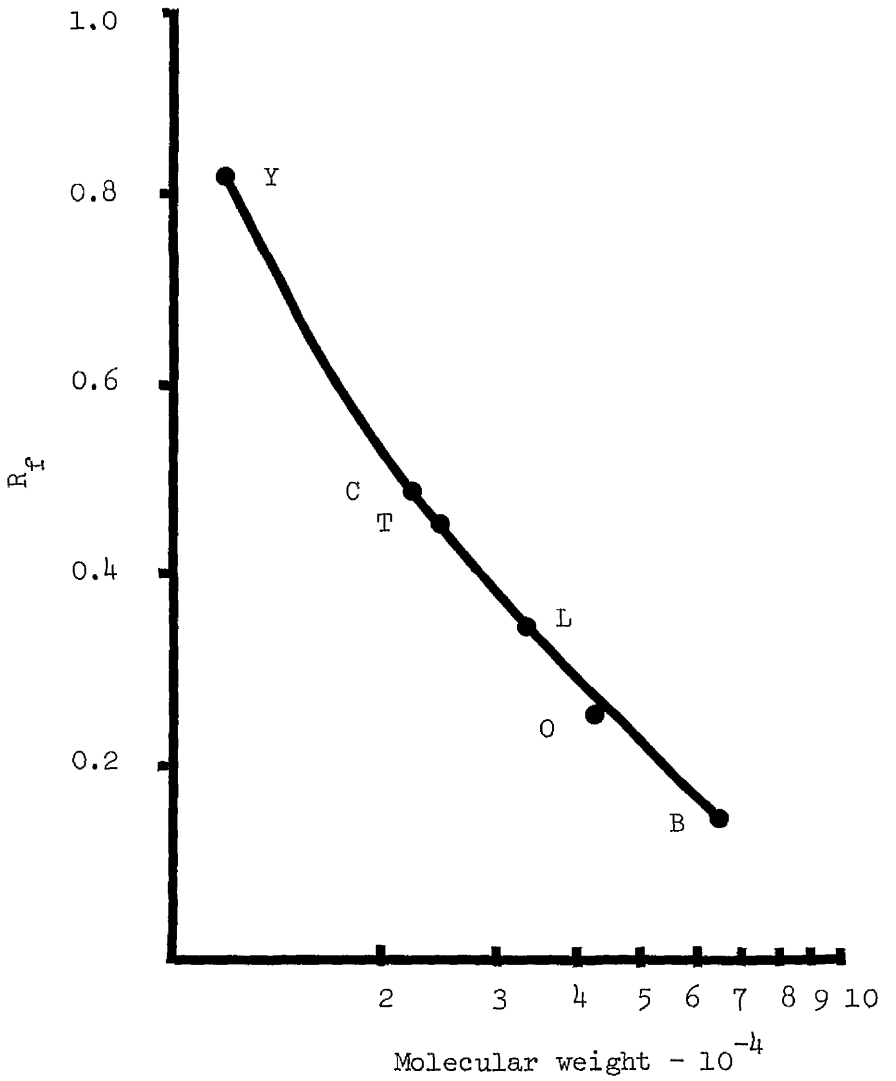

1.0
0.8
0.6
0.4
0.2
R_f
Y
C
T
L
O
B
2
3
4
5
6
7
8
9
10
Molecular weight - 10^{-4}

Figure 11 : Estimation of the molecular weight of β-lysin by chromatography on Biogel P-60

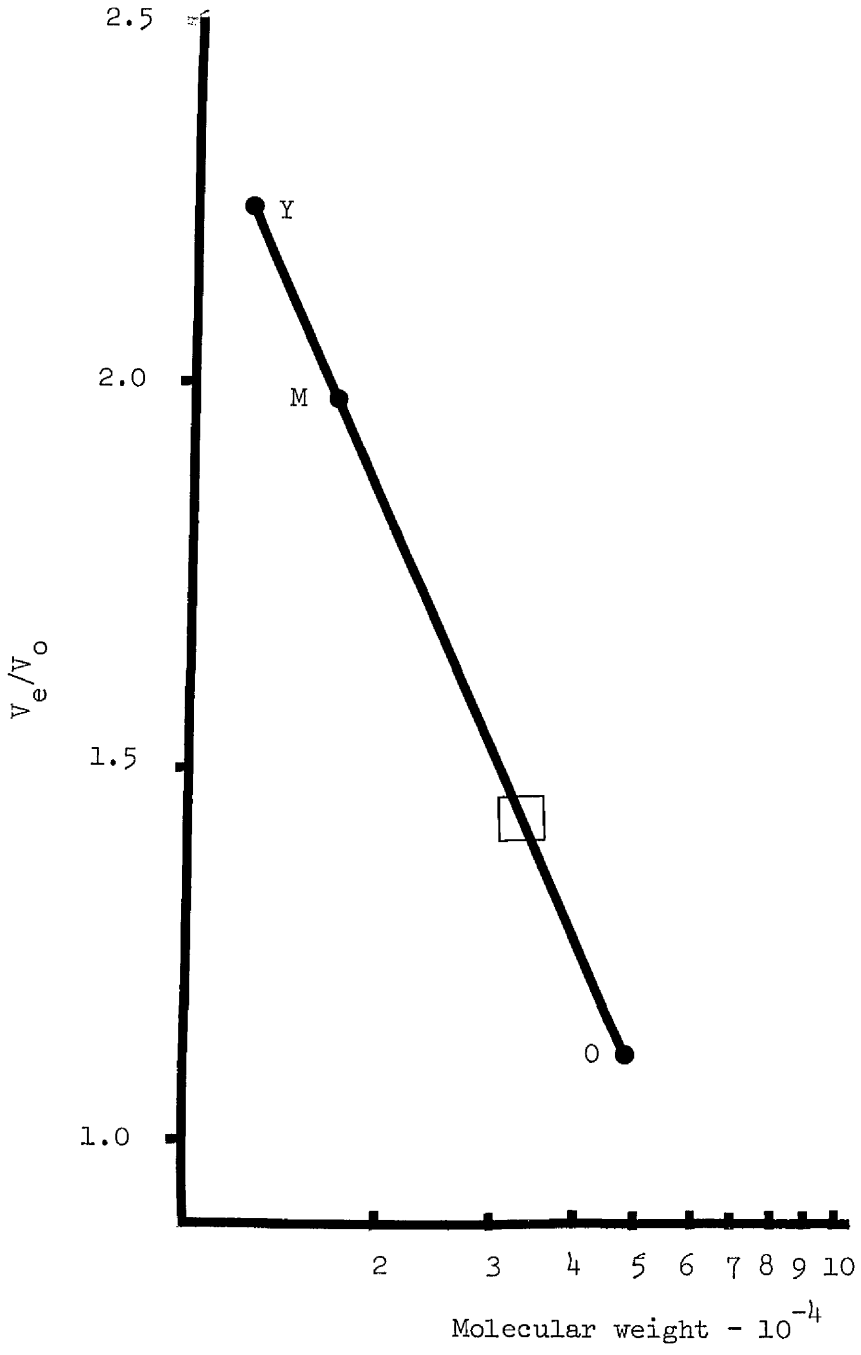
Plot of the logarithm of the molecular weight of standard proteins against the ratio of elution volume (V_e) to void volume (V_o).

O = ovalbumin (M.wt. = 43,000)

M = myoglobin (M.wt. = 17,000)

Y = cytochrome C (M.wt. = 11,700)

□ represents elution behaviour of β-lysin


2.5
2.0
1.5
1.0
Y
M
O
V_e/V_o
2
3
4
5
6
7
8
9
10
Molecular weight - 10^{-4}

2. Estimation of S_{20w}

Purified β-lysin gave a single symmetrical peak in the analytical ultracentrifuge. This peak sedimented with an S_{20w} of 3.1. The peak remained symmetrical after centrifugation for 50 min.

The molecular weight, M, is related to the sedimentation coefficient, s, by the relationship

$$M = \frac{RTs}{D(1 - \bar{\nu}\rho)}$$

where R is the gas constant, T is the temperature (^{o}K), D is the diffusion coefficient, $\bar{\nu}$ is the partial specific volume and ρ is the density of the solute. It is obvious that it is not possible to extrapolate directly from the S_{20w} value to the molecular weight. However, a crude estimation of the molecular weight (MW) can be obtained from the sedimentation constant alone (Schachman, 1959; Martin & Ames, 1961) assuming a spherical shape for the materials

$$\frac{S_{20w_1}}{S_{20w_2}} = \left[\frac{MW_1}{MW_2}\right]^{\frac{2}{3}}$$

and assuming the same value of $\bar{\nu}$. A value of 3.1S may be expected from a globular protein with a molecular weight in the region of 30-35000.

See p. 157, section II.4

III BIOLOGICAL EFFECTS OF β-LYSIN

1. Effects on erythrocyte ghost membranes

i. Analysis of erythrocyte ghost membranes

Freshly prepared ghosts were assayed for phosphorus and

protein content and dialysed against distilled water. The preparation was then freeze-dried and weighed. Protein content was measured by the method of Lowry et al. (1951) after dispersion and clarification with 0.1% (w/v) sodium dodecylsulphate (SDS). Bovine serum albumin in 0.1% (w/v) SDS was used as a standard; this concentration of SDS did not interfere with protein determinations. Phosphorus was assayed by the method of Allen (1940).

As can be seen from Table 10, approximately one half of the dry weight of bovine and human erythrocyte membranes consisted of protein. The total lipid content in each was slightly over 40%; however it can be seen that bovine cells had a lower phospholipid:total lipid ratio than human cells. This is due to bovine cells having a relatively higher cholesterol content (Hanahan, 1969). The remaining unidentified portion represented the carbohydrate content of the ghost membrane.

ii. β-lysin treatment of erythrocyte ghost membranes

Freshly prepared ghosts in 0.01 M Tris-HCl buffer, pH 7.4, made 1 mM with $MgCl_2$, were incubated for one h at 37°C with 100 µg of β-lysin (300,000 HU). Samples were removed at 5 and 60 min for examination by phase contrast microscopy and freeze-etching. The reaction was halted by the addition of 5% (w/v) bovine serum albumin and 10% (v/v) trichloracetic acid. After centrifugation, the supernatant was assayed for phosphorus by the method of Allen (1940).

Purified β-lysin was capable of releasing water-soluble organic phosphorus (phosphorylcholine) from both human and bovine

erythrocyte ghost suspensions. There was no increase in the inorganic phosphate content of the supernatant fractions. From Table 11 it can be seen that 25% of human and 35% of bovine phospholipids are hydrolysed by this treatment.

Thin-layer chromatography of the extracted lipids, shown on Plate 5, suggest that the sphingomyelin in erythrocyte membranes is extensively degraded. The other major phospholipids present (phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and phosphatidylinositol) are not attacked by β-lysin. On the basis of published phospholipid levels in bovine and human erythrocytes, the release of phosphorus from β-lysin treated erythrocyte membranes corresponds to the hydrolysis of nearly 80% of the sphingomyelin in bovine ghosts, and 90% of the sphingomyelin in human ghosts. The results from thin-layer chromatography support this level of degradation and it can be concluded that β-lysin acts as a sphingomyelinase.

iii. Microscopic appearance of β-lysin treated erythrocyte ghost membranes

Samples were removed from the reaction mixtures described in the preceding section. Before taking samples for freeze-etching and phase contrast microscopy it was noted that ghost membrane pellets which had been exposed to the enzyme were more opaque than control (untreated) pellets. The appearances of control and β-lysin-treated bovine erythrocyte ghosts under phase contrast optics are shown in Plates 6 and 7. Treated bovine ghosts appeared degraded and reduced in size, and contained several relatively phase-dense masses. They also appeared to contain a varying number of vesicles.

Table 11 : Composition of human and bovine erythrocyte ghost membranes (% dry weight)

Component	Human ghosts	Bovine ghosts
Protein	49.2	49.5
Total lipid	43.6	44.4
Phospholipid	32.5	25.5
Unidentified	7.2	6.1

Table 12 : Effect of β-lysin (sphingomyelinase) on erythrocyte ghosts

Substrate	Phosphorus release (as % total phospholipid)	Sphingomyelin (as % total phospholipid)	% sphingomyelin degraded
Sphingomyelin	76.5	100	76.5
Bovine ghosts	35.6	46.8	77.0
Human ghosts	24.8	26.9	90.6

Lipid contents from Rouser *et al.* (1968).

Plate 5. Thin-layer chromatography of the lipids extracted by chloroform:methanol from treated and untreated bovine erythrocyte membranes.

Plates were spread with silica gel G. 100 µl (200 µg) lipid was applied as a streak at the origin.

Plates were developed using chloroform:methanol:water 65:35:4 and sprayed with acid molybdate to detect phosphorus.

C = lipids from control (untreated) erythrocyte membranes.

Sph = pure sphingomyelin (2 mg/ml)

T = lipids from β-lysin treated erythrocyte membranes.

Note the decrease in sphingomyelin content and the increase in neutral lipids (due to ceramide accumulating) migrating at the solvent front in treated cells.

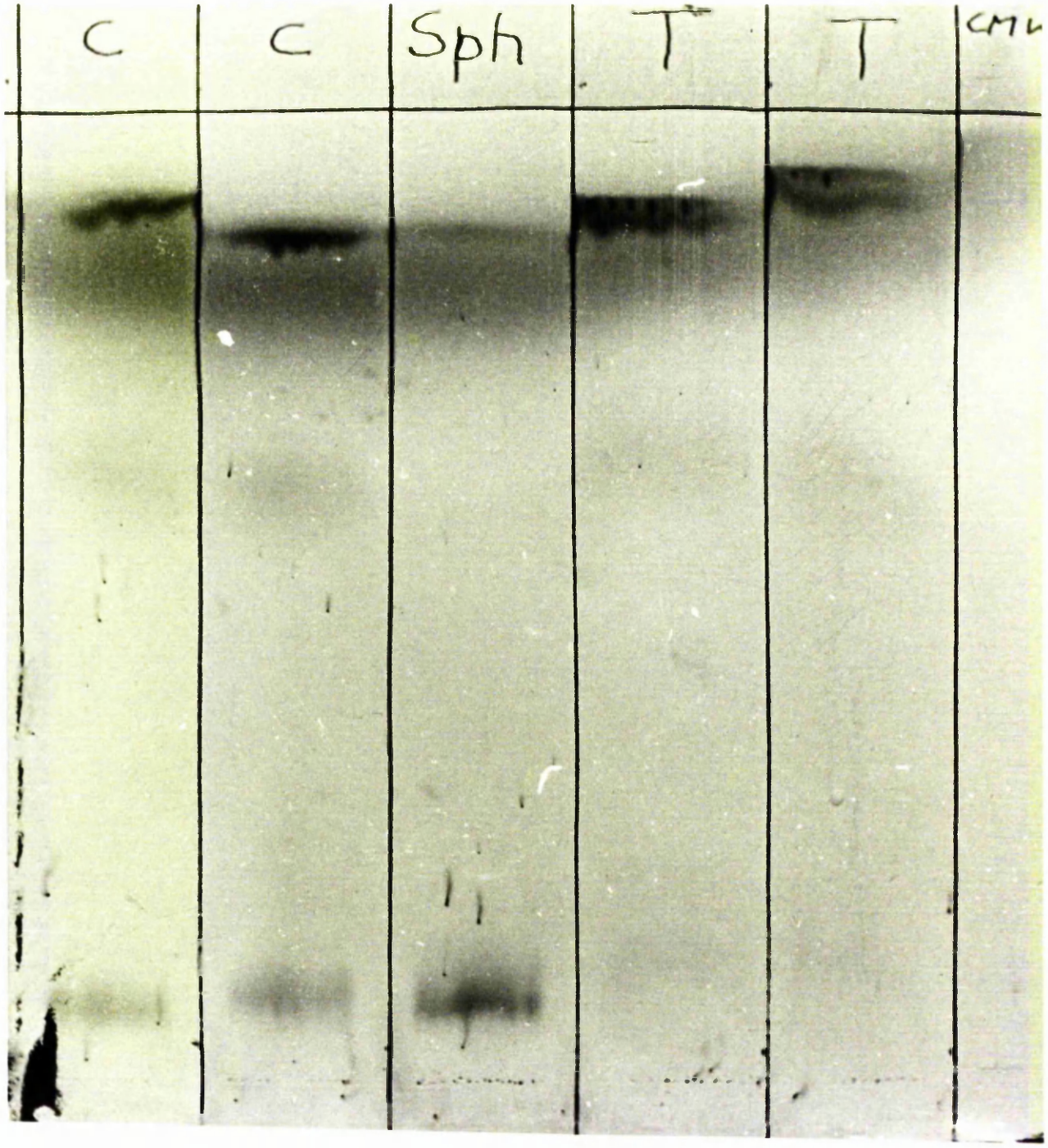

C
C
Sph
T
T
CM

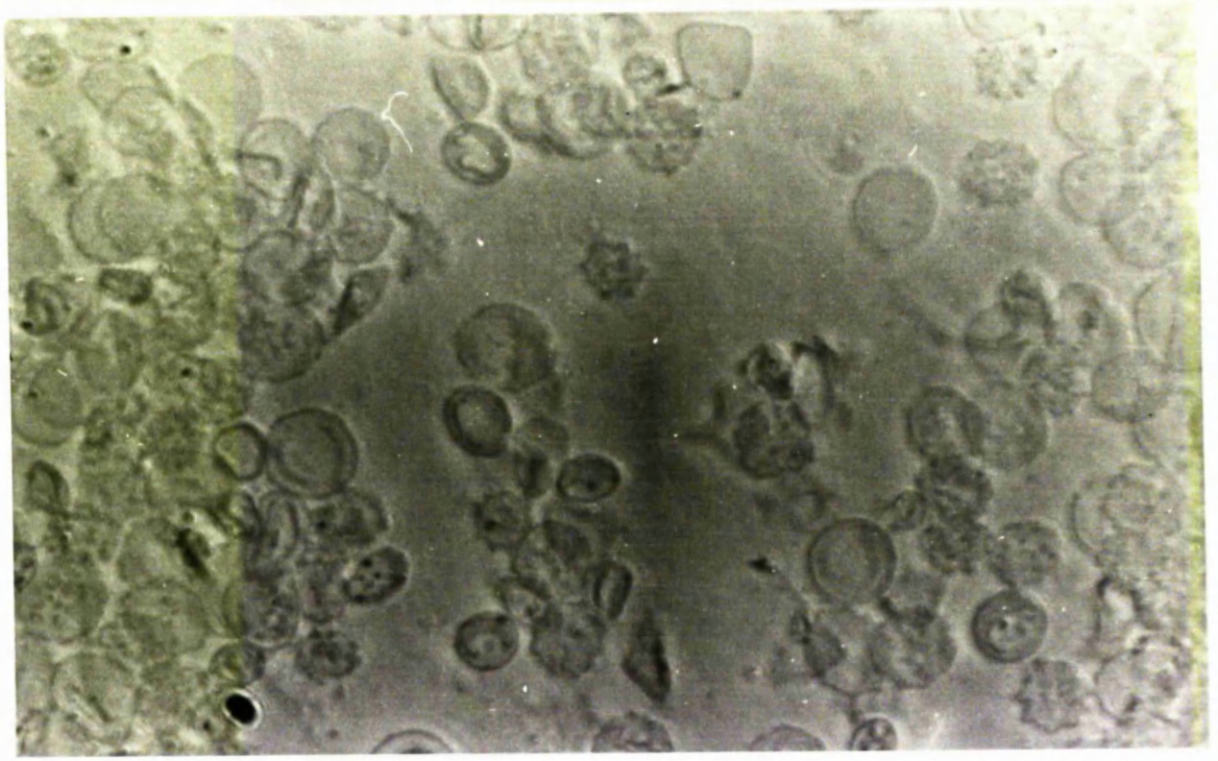
Plate 6. Phase contrast appearance of control bovine erythrocyte ghosts

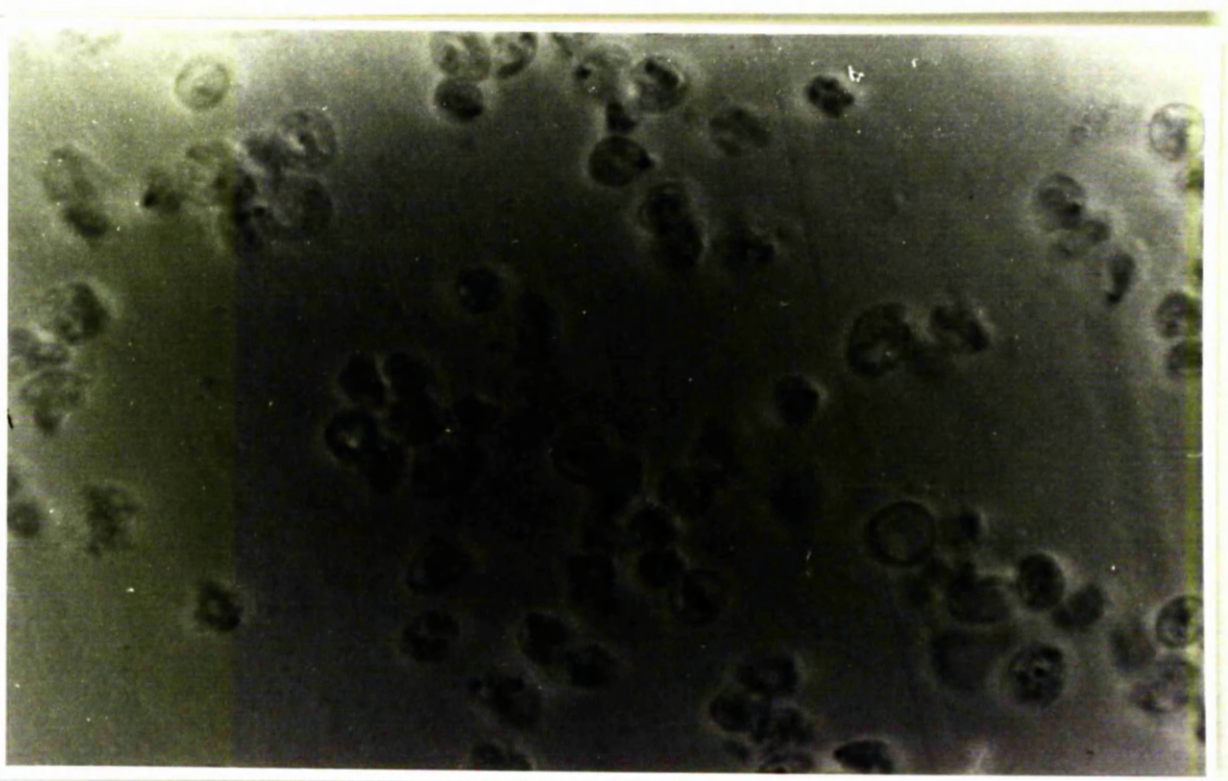
Magnification 3,125 x

Plate 7. Phase contrast appearance of bovine erythrocyte ghosts treated with β-lysin

Note the appearance of black dots in the membrane and the internal vesiculation.

Magnification 3,125 x.





When the replicas of freeze-etched bovine erythrocyte ghosts were examined by electron microscopy, they showed extensive invagination and the appearance of vesicles in ghosts treated with β-lysin (Plate 10), a feature not seen in control membranes (Plates 8 and 9). Large globules of particle-free solid material were frequently seen within ghosts (Plates 10 and 11). These bodies probably correspond to the phase-dense droplets seen under phase-contrast microscopy, and are possible accumulations of the ceramide (N-acyl sphingosine) product of degradation. Treated erythrocyte ghosts also showed raised, particle-free areas in the protoplasmic fracture face (PF) distinct from the endoplasmic surface (ES) of etched erythrocyte ghosts (Plates 12, 13 and 14). There was nothing to suggest that such areas arose as a consequence of particle aggregation.

Micrographs of control and β-lysin treated human erythrocyte ghosts as seen by phase-contrast optics are shown in Plates 15 and 16. Again, treated human ghosts appeared reduced in size, and contained vesicles with phase-dense masses apparent in the bilayer. These effects were, however, less pronounced than with bovine ghosts. Replicas of freeze-etched human erythrocyte ghosts also showed vesicles (Plates 18 and 19), accumulations of solid material and particle-free areas on the protoplasmic fracture face. Plate 17 shows untreated human erythrocyte membranes for comparison. The solid bodies, common in treated bovine erythrocyte membranes, were less abundant in human ghosts, presumably because of their lower sphingomyelin content.

Plate 8. Replica of freeze-etched isolated bovine erythrocyte ghosts

The sample of the control reaction mixture was taken at 60 min.

Note the smooth, regular outline of the cross-fractured ghosts. The encircled arrow head in the bottom left-hand corner of the micrograph indicates the direction of shadow. Etching time was 30 sec.

Magnification = 23,200.

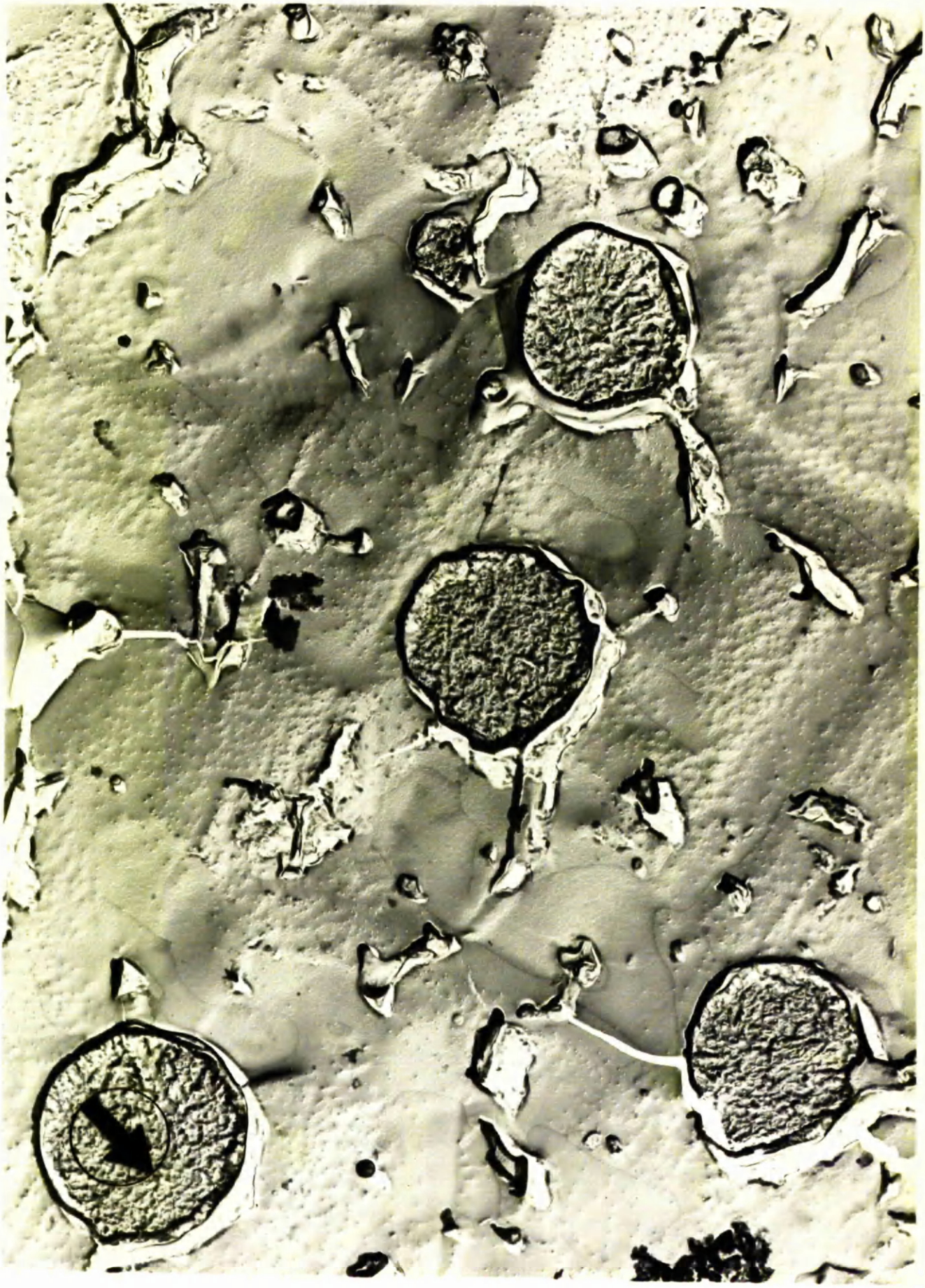


Plate 9. Replica of freeze-etched isolated bovine erythrocyte ghost

The sample of control reaction mixture was taken at 65 min. Note the regular outline of the ghost and the even distribution of intramembrane particles in the fracture plane (PF).

Etching time was 30 sec.

Magnification = 130,000 x.


ES
PF

Plate 10. Replica of freeze-etched isolated bovine erythrocyte ghosts treated with β-lysin

The sample was taken 60 min after the addition of β-lysin. Note the extensive invaginations of the cell membrane in the main picture. These are clearly derived by a process of inward collapse. Note also the presence of apparently solid inclusions (arrowed). The encircled arrow head in the bottom left hand corner of the micrograph indicates the direction of shadow.

Etching time was 30 sec.

Magnification = 22,200.

Plate 11. Replica of freeze-etched isolated bovine erythrocyte ghost treated with β-lysin

The sample was taken 60 min after the addition of β-lysin. Note the large apparently solid inclusion showing different levels in the fracture plane. The encircled arrow head in the bottom left hand corner of the micrograph indicates the direction of shadow.

Etching time was 30 sec.

Magnification = 61,600.

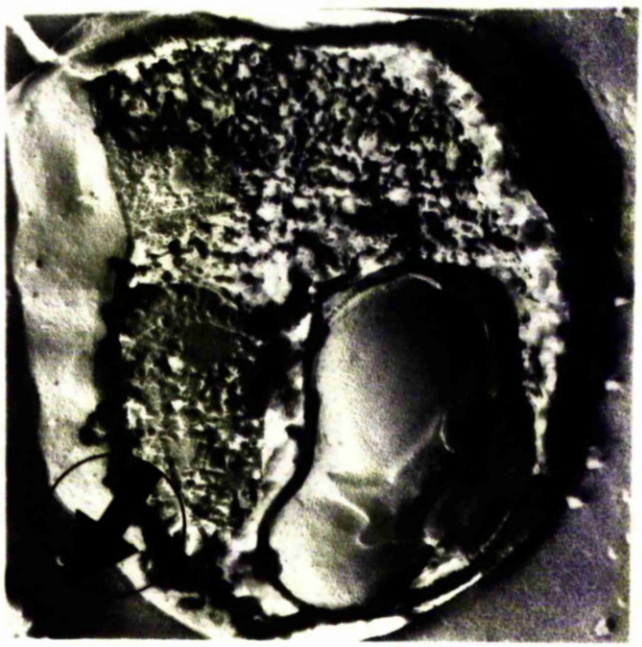


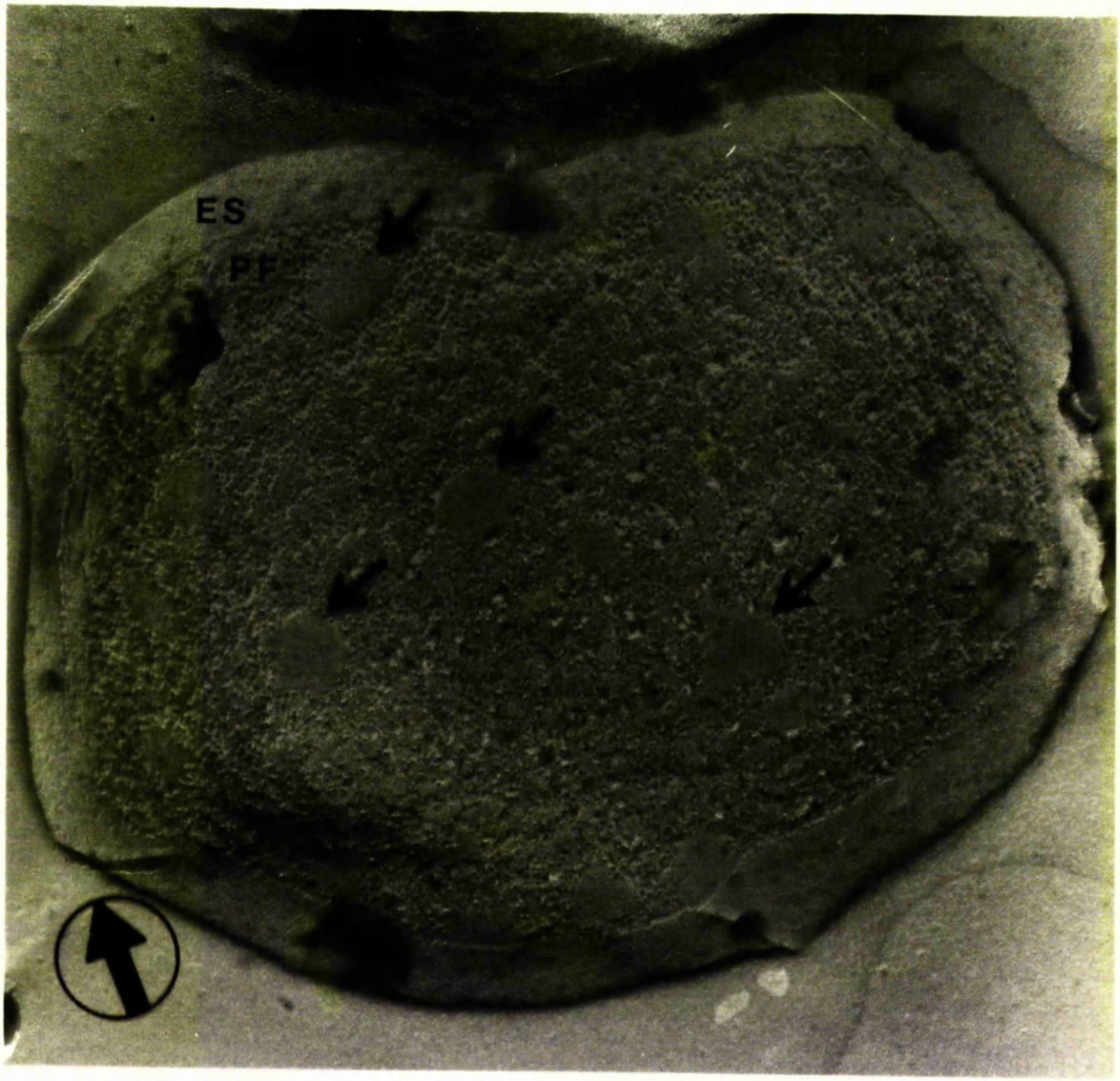
Plate 12. Replica of freeze-etched bovine erythrocyte treated with β-lysin

The sample was taken 5 min after addition of β-lysin. Note the appearance of smooth-raised particle-free areas (arrowed) in the fracture plane (PF) separate from the extracellular surface (ES) of the ghost.

The encircled arrow head in the bottom left hand corner of the micrograph indicates the direction of shadow.

Etching time was 30 sec.

Magnification = 113,750.

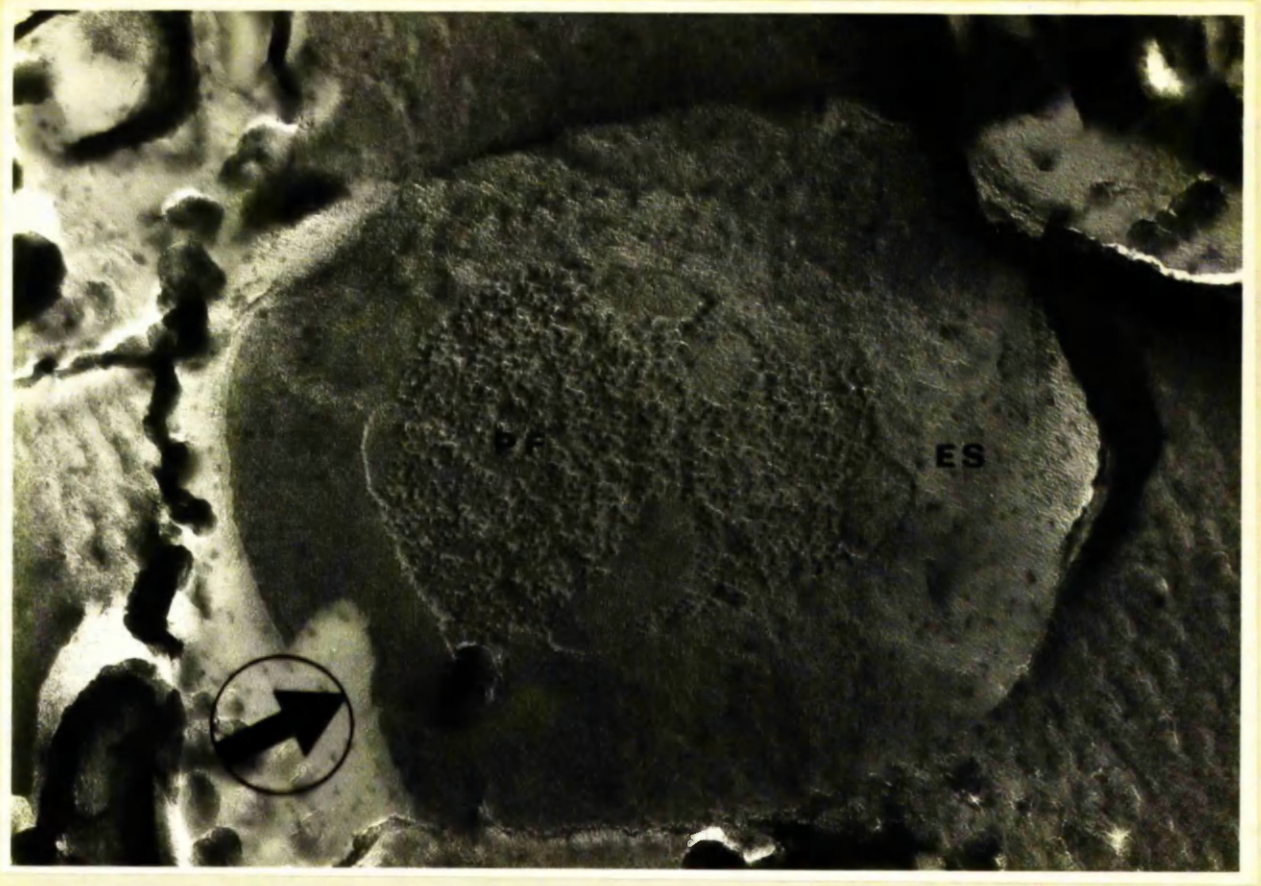

ES
PF

Plates 13 and 14. Replicas of freeze-etched isolated bovine erythrocyte ghosts treated with β-lysin

Samples were taken at 5 min (upper plate) and 65 min (lower plate) after the addition of β-lysin. These pictures clearly show that the particle free areas appearing in the fracture plane (PF) do not result from a process of particle accumulation, nor do they correspond to fragments of the extracellular surface (labelled ES) of the membrane remaining attached to the ghost.

Encircled arrows in the bottom left hand corner of each micrograph indicate the direction of shadowing. Etching time was 30 sec in each case.

Magnification = 113,750.


PF
ES

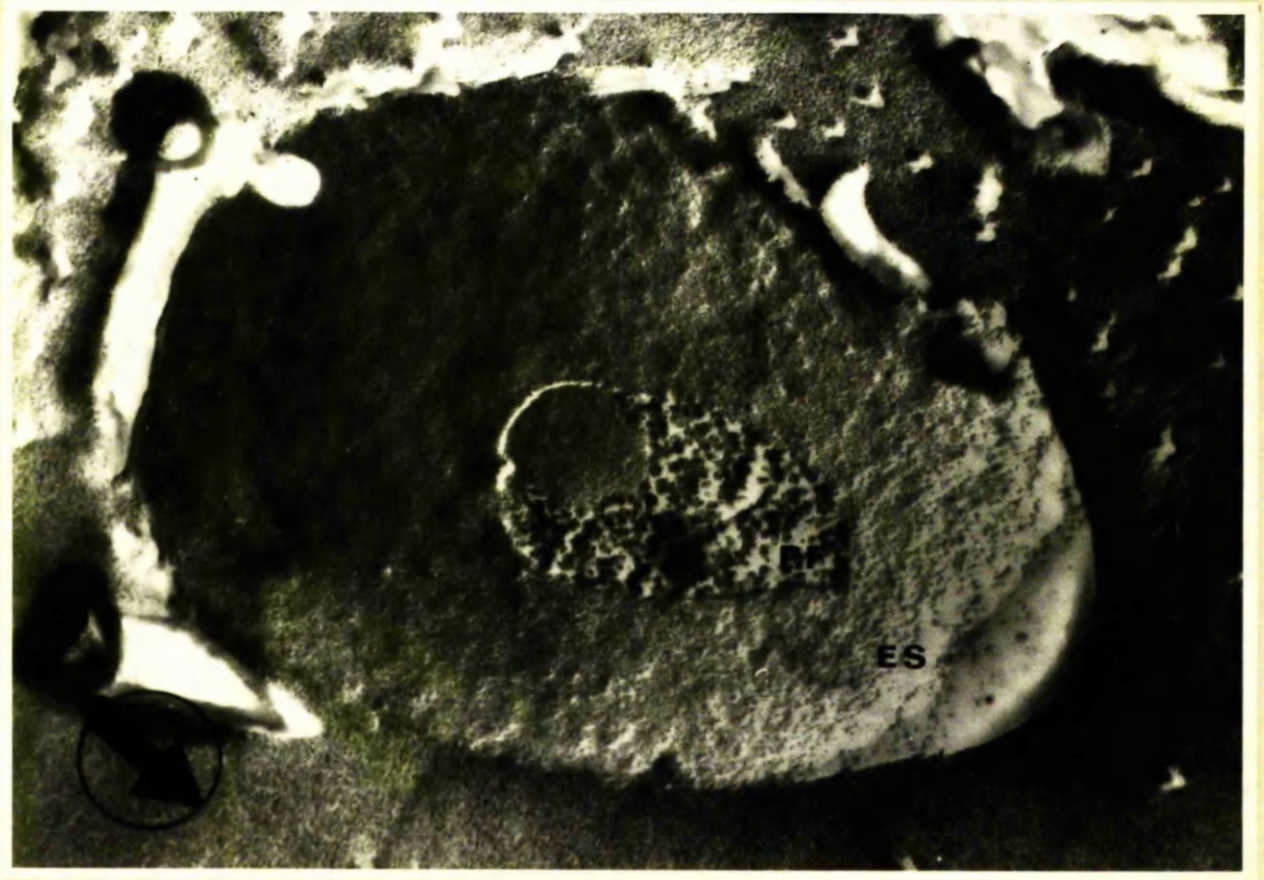

PF
ES

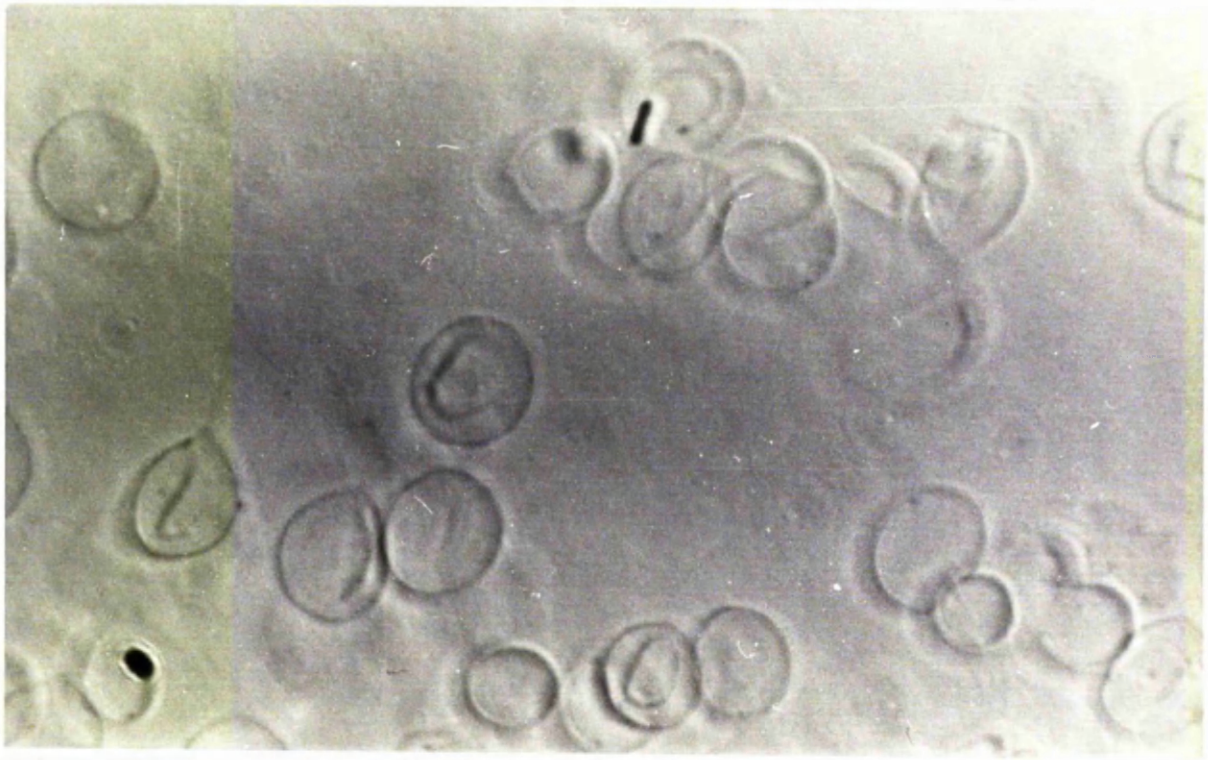
Plate 15. Phase-contrast appearance of control human erythrocyte ghosts

Magnification = 3,125 x.

Plate 16. Phase-contrast appearance of control human erythrocyte ghosts treated with β-lysin

Note that there is comparatively less vesiculation and fewer "black dots" when compared with Plate 7.

Magnification = 3,125 x.



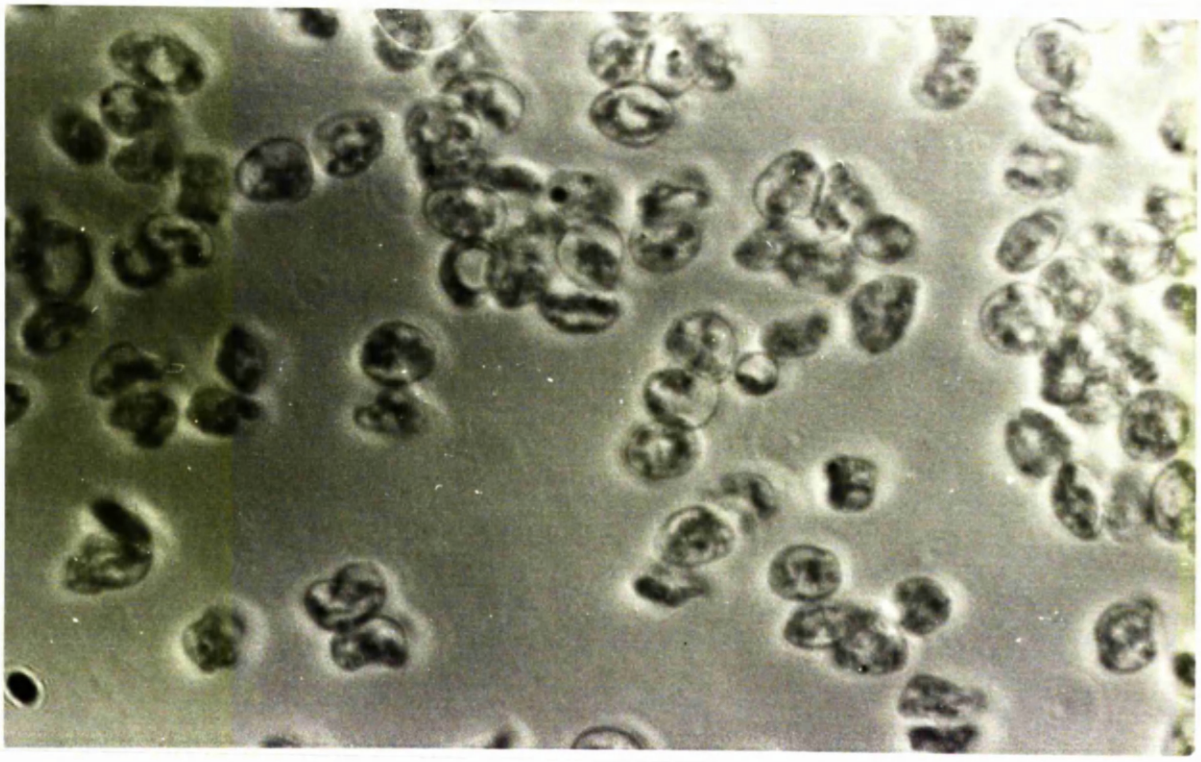


Plate 17. Replica of freeze-etched isolated human erythrocyte ghosts

The sample was taken 60 min after the start of the experiment.

The encircled arrow head in the bottom left hand corner of the micrograph indicates the direction of shadow.

Etching time was 30 sec.

Magnification = 13,500.

Plate 18. Replica of freeze-etched isolated human erythrocyte ghosts treated with β-lysin

The sample was taken 60 min after the addition of β-lysin.

As with crossfractured bovine erythrocyte ghosts, there is extensive vesiculation coupled with the appearance of apparently solid inclusions. The encircled arrow in the bottom left hand corner of the micrograph indicates the direction of shadow.

Etching time was 30 sec.

Magnification = 13,500.

Plate 19. Replica of freeze-etched isolated human erythrocyte ghost treated with β-lysin

The sample was taken 60 min after the start of the experiment. This micrograph shows the appearance of smooth areas in the fracture plane (PF) as well as extensive vesiculation and several apparently solid inclusions (arrowed).

The encircled arrow head in the bottom left hand corner of the micrograph indicates the direction of shadow.

Etching time was 30 sec.

Magnification = 38,800.


ES
PF

2. β-lysin treatment of intact erythrocytes

As a result of the differences in appearance between treated and untreated bovine erythrocyte membranes, it was decided to examine erythrocytes during the process of hot-cold haemolysis after β-lysin treatment to see if there was any similar change in structure. Serial ten-fold dilutions of β-lysin were made as if a normal titration was being performed in 0.005 M TBS + Mg^{2+}, pH 7.4 and 0.7% bovine erythrocytes were added to each tube. After incubation at 37°C for 30 min, a sample of erythrocytes was taken from a tube (containing approximately 10^5 HU/ml) which had not shown haemolysis. The sample was placed on a slide, prewarmed to 37°C, and examined by phase-contrast microscopy.

Some of the treated bovine erythrocytes (Plate 20) appeared smaller and rounded, having lost the characteristic biconcave disc appearance. As time progressed (and the slide cooled), phase-dense droplets appeared close to the surface of the cell and the erythrocytes lost their dark appearance as haemoglobin started to leak out. These droplets increased in number and size until all the haemoglobin had leaked out of the cell. The ghosts produced in this fashion resembled erythrocyte membranes treated with β-lysin after lysis had occurred but there was less vesiculation and many more phase-dense droplets were apparent.

3. Effects of β-lysin in mice

i. Intravenous injection

Four groups of five mice were injected intravenously as follows.

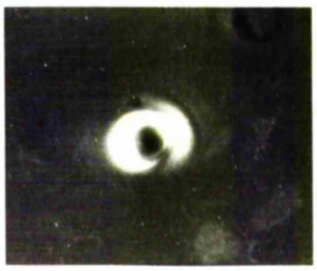
Plate 20. Phase-contrast micrographs of bovine erythrocytes treated with staphylococcal β-lysin

The first 12 micrographs were taken of the same group of erythrocytes after 30 min exposure to β-lysin. It can be seen that as the slide cools, the erythrocyte loses its shape and "black dots" start to appear in the membrane.

The last four micrographs were of a different erythrocyte undergoing haemolysis.

The entire procedure occurs in 3 - 4 min.

Magnification = 3,125 x.



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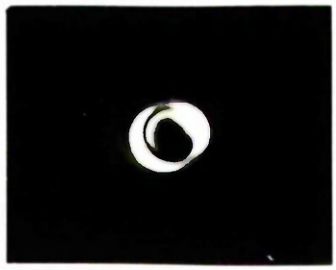
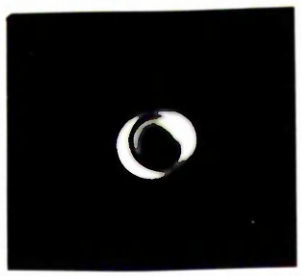
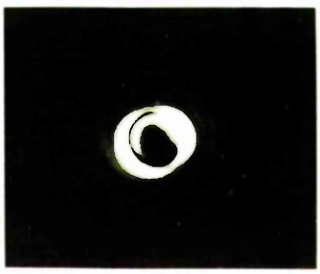


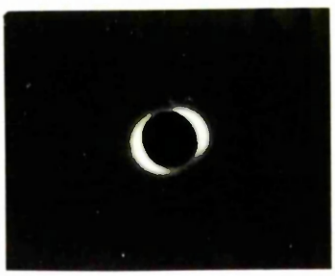
3



4



5



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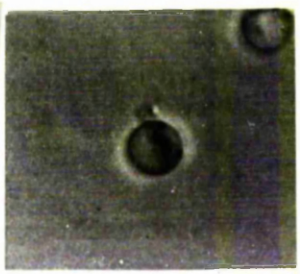

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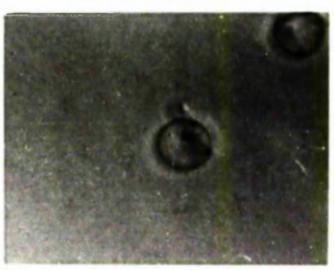

10

11

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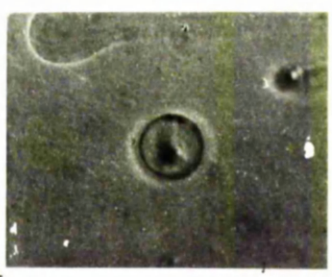
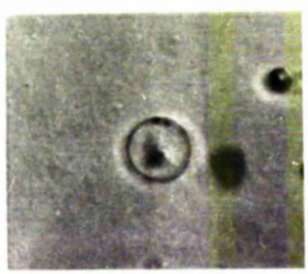
14



15



16

The first group received 0.1 ml of 0.05 M TBS + Mg^{2+} diluent; the second, third and fourth groups were injected with 10 µg, 100 µg and 150 µg of β-lysin respectively. There were no fatalities, and the change in weight of each mouse was recorded every 24 h for 7 days, after which the experiment was terminated. The mean weight and standard deviation was determined for each group and was plotted against time (Figure 12). The injection of highly purified β-lysin in doses ranging from between 10 and 150 µg (0.47 - 7.14 mg/kg mouse tissue) appeared to be non-toxic, and to have no detrimental effects on the increase in weight of mice.

ii. Intradermal injection

Three male hairless mice were each injected intradermally at four sites on the back with 5, 10, 25 and 50 µg of purified β-lysin in 0.05 ml TBS + Mg^{2+}. The mice showed no form of lesion, nor was there any reddening of the skin. Intravenous injection of 0.1 ml of 0.5% (w/v) Evans blue in phosphate buffered saline resulted in the mice colouring a uniform shade of blue. The absence of any localization of the dye round the injection sites suggested that there was no increase in local vascular permeability. These tests serve to prove that not only is the highly purified toxin non-dermonecrotic, but that it is also free from contamination by α-toxin and epidermolytic toxin.

4. Pathogenicity of S. aureus G128 for sheep

A Scottish blackface ewe was injected with 10.75×10^{11} colony forming units (c.f.u.) of S. aureus G128 in 5 ml of 0.05 M PBS, pH 7.5. The sheep died after approximately 5 h. A sample of blood was then

Figure 12 : Effect of β-lysin on the increase in weight of 3 - 4 week old mice

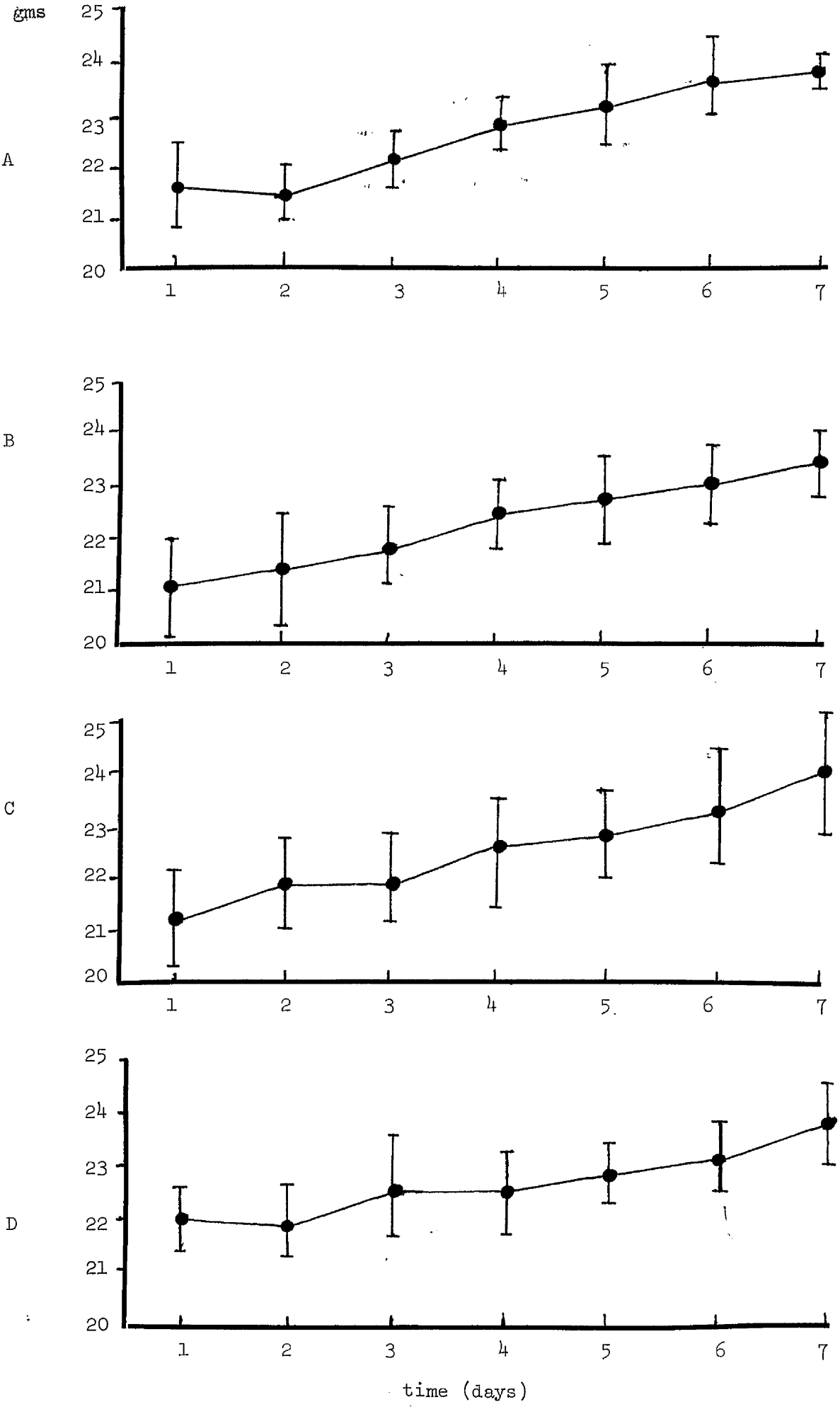
Groups of 5 mice were injected as follows:

Group A : 0.1 ml of 0.05 M TBS + Mg^{2+} (diluent)

Group B : 0.1 ml diluent containing 0.1 mg/ml β-lysin

Group C : 0.1 ml of diluent containing 1 mg/ml β-lysin

Group D : 0.15 ml of diluent containing 1 mg/ml β-lysin


gms
A
B
C
D
25
24
23
22
21
20
1
2
3
4
5
6
7
time (days)

collected from the heart and analysed for lipid content, along with a blood sample taken before injection with S. aureus for comparison. The packed cell volume of the sample taken from the dead sheep was 36% as compared to 45.5% for that from the control sample, and the plasma fraction showed that some haemolysis had occurred. Phase contrast microscopy showed that there were few erythrocyte ghosts present and that these ghosts did not have the appearance of ghosts treated with β-lysin. The lipids from both control blood and blood taken from the infected sheep appeared identical as demonstrated by thin-layer chromatography (Plate 21); notably there was no evidence of extensive sphingomyelin degradation. The blood from the infected sheep had a bacterial count of 16×10^3 c.f.u./ml on mannitol salt agar.

Plate 21. Thin-layer chromatography of the lipids extracted by chloroform:methanol from ovine erythrocytes before and after injection of S. aureus G128

Plates were spread with silica gel G. 20 µl of a 10mg/ml solution of lipid in chloroform was applied as a spot at the origin.

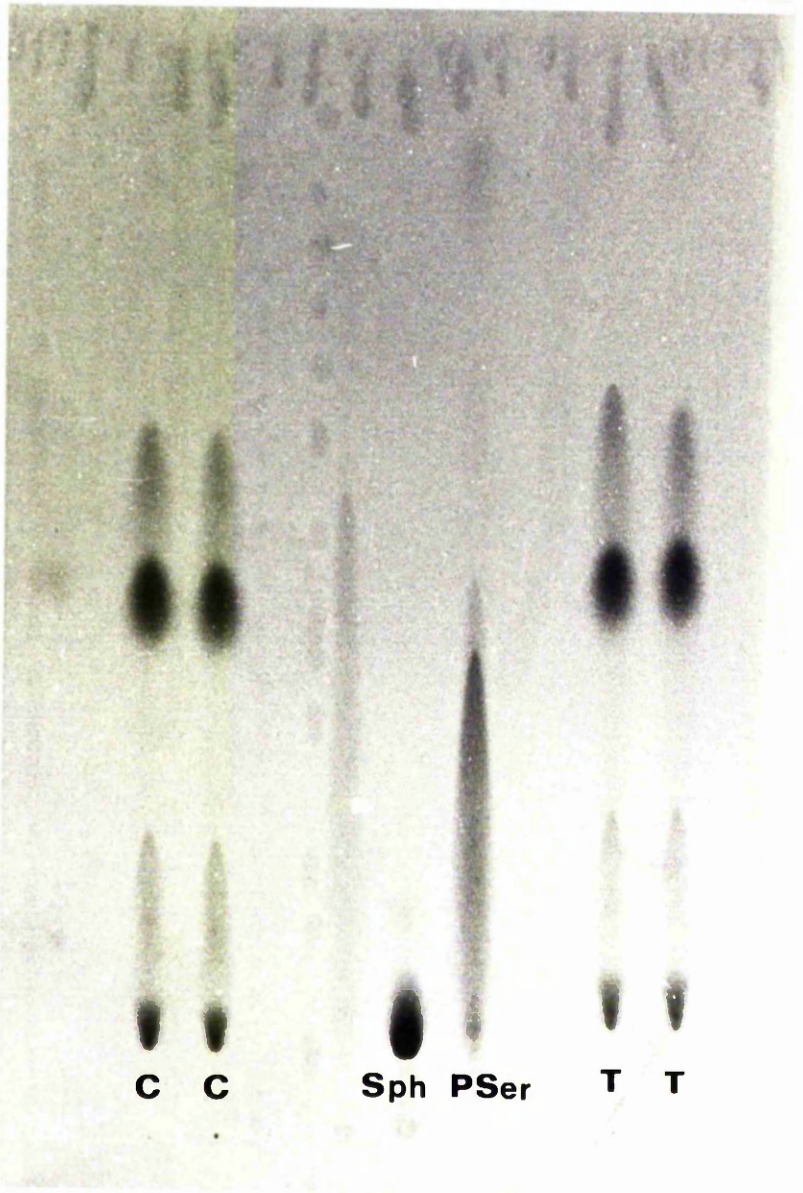
Plates were developed using chloroform:methanol:water 65:35:4 and sprayed with acid molybdate to detect phosphorus.

C = lipids from erythrocytes taken before injection

T = lipids from erythrocytes taken 6 h after injection

Sph = purified sphingomyelin

PSer = purified phosphatidylserine.


C
C
Sph
PSer
T
T

DISCUSSION

I PRODUCTION AND PURIFICATION OF β-LYSIN

Three strains of S. aureus were examined for β-lysin production. Haque & Baldwin (1964) have emphasised the importance of the choice of strain for the production of lysins, since the purification is greatly facilitated by the use of strains which produce large quantities of β-lysin alone. Strain R-1 has been reported to be devoid of α-toxin and δ-lysin activities, although it produces a variety of other extracellular proteins. Strain G128 produces mainly β-lysin although the culture filtrate is haemolytic to human and rabbit erythrocytes.

Strain 1L7s was originally isolated because of its ability to produce coagulase, however it was found that filtrates of this strain did not cause haemolysis of either sheep, human or rabbit erythrocytes at 37°C.

When these three strains were grown on yeast diffusate broth, strain 1L7s was found to be a relatively poor producer of β-lysin, and G128 appeared to have a slight advantage as a source of β-lysin over strain R-1 since the haemolytic titre did not decrease as rapidly after termination of active growth. Thus strain G128 was chosen as the source of β-lysin for these studies. The yeast diffusate broth of Bernheimer, Avigad & Kim (1974) was chosen since the inclusion of yeast diffusate in the culture medium is known to eliminate the requirement of CO_2 for β-lysin production. In order to achieve optimal β-lysin production, cultures were inoculated from colonies which produced a good zone of haemolysis on sheep blood agar plates after incubation at 37°C for 24 h followed by 2 h at 4°C.

It is important in assessing a purification procedure to consider the ease and duration of the process, the purity and yield of the product, the reproducibility and, finally, the economy of the procedure.

Reproducible and consistent results were obtained using the procedure reported here to purify β-lysin. The techniques detailed in this purification procedure allow the preparation of relatively large amounts of purified protein, and in addition, reveal information on the molecular heterogeneity and isoelectric points of β-lysin.

The overall yield was of the order of 1,000%, which points to a considerable increase in hot-cold lytic activity during the process. After isoelectric focusing, the activity was recovered in a fraction of pH 9.3, with minor activity in a fraction of pH 9.75. The degree of purity of the product was assessed by several methods; immunodiffusion, polyacrylamide gel electrophoresis, ultracentrifugation and freedom from contaminating proteins. Although none of these methods is individually conclusive, together they indicate the high level of purity obtained. In several cases it was possible to estimate that the level of contamination by other staphylococcal activities was less than one part in one thousand, and in every case the preparation was free from contaminants to the limits of sensitivity of the tests used. The low specific activity of δ-lysin (1200 HU/mg) meant that amounts of less than 2 μg could not be detected by the assay system used, however the lack of haemolytic activity at 37°C of the preparation against a variety of types of erythrocyte suggest that this figure should be regarded as an upper limit and not taken too literally.

The limitations of isoelectric focusing in a purification procedure are its expense and the problem of removing ampholytes from the purified protein. The use of ampholytes can be kept to a minimum by applying partially purified β-lysin to the column. The focusing of crude culture supernatants is not to be recommended due to the presence of large amounts of extraneous material in the culture filtrate which tend to precipitate in the column, limiting the maximum amount of useful material which can be applied to columns at any time. Removal of ampholytes was achieved at the stage of ammonium sulphate precipitation, a step which also concentrates the protein and provides a method of storage. This method of removal has been recommended (Nilsson, Wadström & Vesterberg, 1970) in preference to dialysis as originally suggested by Vesterberg (1969).

Haque (1974) pointed out that electrophoresis in agar gels is a superior method to isoelectric focusing for the separation of α-, β- and δ-lysins from culture filtrates; however when electrofocusing is used in combination with a method of purification not dependent on charge, such as molecular sieving, there is sufficient resolution to separate the three lytic activities.

The large increase in total haemolytic activity noticed during the procedure is also a feature of the results obtained by Gow & Robinson (1969), Haque & Baldwin (1969) and Wadström & Möllby (1971a). However Bernheimer, Avigad & Kim (1974) were unable to achieve this increase in activity in their purification procedure. This increase always occurs during ion exchange chromatography, and this probably

explains why Bernheimer, Avigad & Kim (1974) have been unable to duplicate this observation because they do not include this step in their procedure.

The activity of β-lysin throughout the purification was monitored by assaying haemolytic activity against sheep erythrocytes, since not only are they highly sensitive indicators of β-lysin activity, but they also serve to indicate the presence of contaminating haemolytic activities by lysis at 37°C. The final product had a specific activity of 312 IU/mg against sphingomyelin. This is greater than the activities of 2 IU/mg quoted by Wadström & Möllby (1971a) or 154 IU/mg quoted by Bernheimer, Avigad & Kim (1974).

Zwaal et al. (1975) recently published a purification procedure for β-lysin using two steps of ion exchange chromatography which yields a product with a specific activity against sphingomyelin of 1900 IU/mg. They claimed that their high specific activity was due to purification being carried out in the presence of glycerol, which stabilised the enzyme as it is extremely prone to denaturation (Wadström & Möllby, 1971a). However, in calculating their specific activity, Zwaal et al. (1975) assumed a value of $E^{1\%}_{280\ nm} = 16$. As the value is in fact 4.24 (Fackrell & Wiseman, 1976b) their specific activity becomes 504 IU/mg.

It should ultimately prove possible to isolate β-lysin by taking advantage of its enzymic properties and purify it by affinity chromatography using sphingomyelin linked to an inert matrix, such as Sepharose. Phospholipase C has been purified by affinity chromatography (Takahashi et al., 1974; Little et al., 1975).

However both staphylococcal δ- and γ-lysins bind to phospholipids (Wiseman, 1975) and the usefulness of the method would be limited should any non-specific absorption to sphingomyelin take place.

II PHYSICOCHEMICAL STUDIES ON β-LYSIN

1. The origins of multiple forms of proteins

The increase in resolving power of many individual fractionation systems, coupled with the variety of physicochemical techniques which are available for the purification of proteins, has revealed the phenomenon of molecular microheterogeneity in proteins. This has been discussed with particular reference to the purification of toxic bacterial proteins by isoelectric focusing by Arbuthnott, McNiven & Smyth (1975).

The principal causes of multiple forms of enzymes as revealed by isoelectric focusing are as follows.

i. Genetic variants of enzymes

These vary only slightly, if at all, in substrate specificity and catalytic activity, but differ in amino acid sequence due to different genes or different alleles of the same gene determining the primary structure of each form.

ii. Non-genetic multiple forms of enzymes

These may be subdivided into

a) Aggregates: These involve protein-protein interactions between

enzymes composed of either identical or non-identical subunits, or interactions between unrelated proteins. The electrophoretic mobility is altered by the blocking or masking of the charges of individual subunits. Klotz (1967) has presented a list of proteins having subunits held together by non-covalent bonds. The presence of aggregation can be readily established by molecular weight determinations.

b) Artifacts: These result from changes in the charge due to

i. Deamidation of asparagine and glutamine residues.

ii. Partial denaturation or cleavage of peptide bonds by proteases.

iii. Attachment of carbohydrate.

iv. Phosphorylation or sulphation.

v. Oxidation of sulphydryl groups.

vi. Oxidation or reduction of prosthetic groups.

vii. Binding of impurities.

viii. Protein-ampholine interactions.

c) Conformers: These have the same amino acid sequence and molecular weight but differ in tertiary structure. Two types of conformational change can be considered

(1) ligand induced conformers

(2) "true" conformational changes.

The former arises by a ligand causing an alteration in protein structure by altering charge and conformation or by being charged itself. True conformational changes are a consequence of a polypeptide

chain being capable of assuming more than one stable conformation.

It is difficult to determine which of possibilities (b) and (c) are taking place, since multiple forms arising by these procedures have closely similar molecular weights and are immunologically identical.

This is particularly the case where insufficient amounts of individual components are available for detailed chemical analysis. However, the presence of conformers is usually suggested when multiple forms can be shown to be interconvertible.

2. Multiple forms of β-lysin

The main objective of this thesis was the isolation of highly purified staphylococcal β-lysin in order to study its interactions with cell membranes. There was no systematic investigation into the relationship of the major peak (pI = 9.3) to any other components which had β-lysin activity. However, it is clear from the results presented that β-lysin displays microheterogeneity.

Chromatography on CM cellulose revealed two major protein peaks containing β-lysin activity, one anionic at pH 6.5 and the other cationic. This is in agreement with the results of Haque & Baldwin (1964, 1969) and Maheswaran, Smith & Lindorfer (1967). There was a third minor protein peak resolved by chromatography on CM cellulose columns which also displayed β-lysin activity. This peak eluted fractionally after the main peak of activity, behaving as a more strongly cationic form of β-lysin.

Isoelectric focusing studies of the main cationic peak from CM cellulose in narrow pH gradients (pH 7.5 - 10.0) indicates the presence of a major form focusing with a pI = 9.3 and a minor form focusing with a pI = 9.75. These appear to correspond to the two forms reported by Bernheimer, Avigad & Kim (1974) focusing with pI's of 9.0 (main form) and 9.5. The discrepancy between these results can probably be explained by the pH values being taken at different temperatures. Electrofocusing studies in broad pH gradients (Wadström & Möllby, 1971a) indicated that β-lysin exists as a single cationic form having a pI value of 9.0 - 9.4. A minor anionic component (pI = 3.0) focused in the acidic region of the pH gradient. They agreed that the cationic peak shows microheterogeneity when refocused in a narrow pH gradient (pH 8 - 10) with peaks appearing at pH 8.8, 9.2, 9.4 and 9.8.

Isoelectric focusing in polyacrylamide gels yields further evidence that the cationic peak appears to show microheterogeneity. The two most active fractions from electrofocusing columns refocused on polyacrylamide gels gave two distinct bands, suggesting not only that two forms were present, but also that they were interconvertible. This is usually taken as evidence that two such forms are conformers of each other. It should be noted that protein taken from CM cellulose fractionation was taken only from the main cationic peak and that it also showed the presence of two components on focusing.

The anionic and cationic forms of β-lysin are possibly comparable to the two antigenic components first reported by Thaysen (1948). Doery *et al.* (1965) found two antigenic components in

their preparations. Wiseman & Caird (1967) reported two antigens in their preparation, although they made the reservation that they did not carry out any other tests of homogeneity. It should be noted that neither author employed CM cellulose chromatography during purification.

Chesbro et al. (1965) reported the presence of two antigenic forms in a procedure lacking CM cellulose chromatography but their preparation was probably contaminated with a staphylococcal lysozyme (Wadström & Hisatsune, 1970). Against their suggestion that there are two antigenic forms is the observation that the recovery of anionic β-lysin decreases on separations in isoelectric focusing columns containing 6 M urea (Möllby & Wadström, 1970) which was suggested to be due to β-lysin aggregating spontaneously with acidic components (Wadström & Möllby, 1971a). Thus it appears that the reason for these two forms is not genetic variation, but is an artifact arising from the strong cationic nature of β-lysin with resulting tendency to bind acidic molecules. The removal of such molecules could explain the activation of β-lysin during ion-exchange chromatography.

3. Molecular weight of β-lysin

The molecular weight of highly purified staphylococcal β-lysin was found to be 33,000 by SDS-polyacrylamide gel electrophoresis. This is in good agreement with the value of 32,500 ± 1,500 obtained by gel filtration of partially purified β-lysin on Biogel P-60.

There is general agreement on the close correlation between migration distance and molecular weight of proteins electrophoresed through polyacrylamide gels in the presence of SDS (Shapiro, Viñuela & Maizel, 1967; Shapiro & Maizel, 1969; Weber & Osborn, 1969). All proteins bind identical amounts of SDS on a weight for weight basis (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970) and so mobility in a gel is a measure of molecular size. SDS is also an effective denaturing and disaggregating agent, and so this method of polyacrylamide electrophoresis determines the molecular weight of proteins as extended polypeptide chains in a monomeric form (Williams & Gratzer, 1971). Thus 33,000 represents the minimum molecular weight of β-lysin. By the same method, Bernheimer, Avigad & Kim (1974) report a value of 30,000. SDS-polyacrylamide gel electrophoresis also has the advantage of indicating the purity of the sample, and the protein studied can be run at the same time as the marker proteins under identical conditions.

Gel filtration is a more laborious and time consuming procedure. The result of 32,500 ± 1,500 is in good agreement with one of the figures obtained by Wadström & Möllby (1971a), which are 38,000 and 33,000, using Sephadex G-100 and the value published by Bernheimer, Avigad & Kim (1974) (30,000). Fackrell & Wiseman (1976b) reported a value of 26,000 by gel filtration. Möllby & Wadström (1973a) found that when using Biogel P-60 for molecular weight determinations, they obtained a molecular weight of 18,000. They suggested that this may be a monomer of the 38,000 protein. This seems unlikely in the light of results obtained from SDS polyacrylamide gel electrophoresis.

Chesbro & Kucic (1971), using both ultracentrifugation and gel filtration, found that the molecular weight of β-lysin purified from a variety of strains had a molecular weight of 11,000 - 17,650. It is possible that their results, and those of Möllby & Wadström (1973a) are in fact values for a fragment of the polypeptide chain, resulting from proteolysis by contaminating enzymes.

Fackrell & Wiseman (1976b) have calculated the minimal molecular weight of β-lysin as 16,110 by amino acid analysis. This figure is approximately half that of Bernheimer, Avigad & Kim (1974) whose weight was 39,900 by summation, assuming the protein contains 4 methionine residues. Fackrell & Wiseman (1976b) were unable to find any methionine in their preparation and it is not valid to compare molecular weights calculated from two preparations with different amino acid contents.

From the results presented in this thesis it can be concluded that cationic β-lysin has a molecular weight in the region of 32,500 and that this represents the minimal molecular weight for the protein.

4. Sedimentation coefficient of β-lysin

The sedimentation coefficient (s) was calculated and corrected to give an $S_{20w} = 3.1$. The molecular weight and amino acid composition (and thus the partial specific volume) of staphylococcal β-lysin are very similar to those of α-toxin, which suggests that the two should have similar values for S_{20w}. The published value for α-toxin is 3.0 (Bernheimer & Schwartz, 1963) which is close to the value reported here for β-lysin.

The published values for the sedimentation coefficient of β-lysin are not in good agreement with the value published in this thesis, as they lie in the region of 1.7 - 1.85 (Gow & Robinson, 1969; Chesbro & Kucic, 1971; Fackrell & Wiseman, 1976b); nor are they consistent with a molecular weight in the region of 32,500. There are several possible explanations for this. Certain particles exhibit a pronounced dependance of sedimentation coefficient on concentration (Schachman, 1959). Sedimentation coefficients usually decrease as the total protein concentration increases so determinations on samples of differing concentration could give different values for the sedimentation coefficient. The molecule may be undergoing fragmentation, by proteolysis, as described earlier, or finally, an incorrect value has been assumed for the partial specific volume ($\bar{\nu}$). Since ρ is usually close to unity in the Svedberg equation, and for proteins $\bar{\nu}$ is about 0.75 a small error in $\bar{\nu}$ is magnified in the term $(1 - \bar{\nu}\rho)$ in the calculation of M. The calculated value for $\bar{\nu}$ for β-lysin is unusually low (0.714), and since s is proportional to $(1 - \bar{\nu}\rho)$, it can be seen that by assuming a higher value for $\bar{\nu}$ the value calculated for S_{20w} will consequently be less than the true value. The situation is probably a combination of these possibilities. The same reasons could also explain the comparatively low molecular weight values of Chesbro & Kucic (1971) calculated using the method of Martin & Ames (1961).

III BIOLOGICAL EFFECTS OF β-LYSIN

1. Effects on erythrocyte membranes

i. Biochemical effects

β-lysin released water soluble organic phosphorus from human and bovine erythrocyte ghosts in amounts indicating extensive sphingomyelin hydrolysis. The extent of sphingomyelin hydrolysis reported here is similar to that found by Colley et al. (1973) and Verkleij et al. (1973) for intact erythrocytes treated with their preparation of β-lysin. It may be noted that although sphingomyelin displays a similar degree of accessibility to the enzyme in bovine and human erythrocyte membranes, erythrocytes of these species differ considerably in their susceptibility to hot-cold haemolysis. This undoubtedly reflects the contribution of sphingomyelin to the stability of the intact erythrocyte from the different species. Verkleij et al. (1973) reported that 100% of sphingomyelin in human erythrocyte ghost-membranes was degraded by β-lysin. Although the figures published here indicate 90% hydrolysis, it is possible that the remaining 10% is significant, since only 77% of sphingomyelin in bovine ghosts is degraded. The remaining sphingomyelin may exist in a state more closely associated with protein and thus less accessible for hydrolysis by the enzyme. Sheep erythrocyte membrane proteins are capable of selectively binding sphingomyelin (Kramer et al., 1972).

Verkleij et al. (1973) purified β-lysin by a method later reported to be unreliable (Zwaal et al., 1975). It is possible that their preparation contained contaminants capable of disrupting the weakened membrane structure, uncovering "buried" sphingomyelin head groups making them available for hydrolysis by β-lysin.

ii. Ultrastructural effects

All the vesicles, invaginations and apparently solid inclusions which characterise the ultrastructural appearance of enzyme-treated membranes from both human and bovine erythrocytes appear in the interior of the ghosts. Since the exchange of phospholipids from one half of the lipid bilayer to the other ("flip-flop") is very slow in vesicle membranes (Kornberg & McConnell, 1971), then a process of invagination with the subsequent formation of internal vesicles may be expected to result from sphingomyelin hydrolysis if a) sphingomyelin is preferentially located in the outer leaflet of the lipid bilayer, and b) if the hydrolysis product, ceramide, migrates laterally in the plane of the membrane and accumulates in pools rather than remaining at the site of hydrolysis. If these assumptions are correct, then an inward collapse of the membrane will occur in order to reduce the surface area occupied by lipids in the depleted outer leaflet of the membrane. Evidence in support of a) has been presented by Casu et al. (1969) and Bretscher (1972a,b) for human and sheep erythrocytes. The ultrastructural evidence presented here is in keeping with suggestion (b). The raised particle-free areas seen in the protoplasmic fracture face of the membrane may represent early stages in the accumulation of ceramide. The particle-free areas appeared as discrete regions raised above the surrounding part of the fracture face, yet lay below the plane of the extracellular surface of the membrane revealed by etching. They differed from particle-free areas of protoplasmic fracture faces which resulted from particle aggregation

due to changes in pH of the suspending medium (Pinto da Silva, 1972) or to exposure of membranes to pronase (Branton, 1971). In these latter cases, the particle-free areas are coplanar with the remainder of the fracture face and there is pronounced aggregation of the membrane particles. The amorphous globules seen in fractured ghosts after β-lysin treatment are probably a later stage in the accumulation of ceramide, but it is difficult to identify ceramide as it is chemically very inert.

Colley *et al.* (1973), in a recent study on the action of phospholipases on erythrocyte membranes, concluded that ceramide, produced by the action of sphingomyelinase C on erythrocyte ghosts, did not accumulate in pools, but remained *in situ* in the membrane. This conclusion was based upon their failure to detect "black dots" by phase contrast optics in human erythrocyte ghost preparations after treatment with sphingomyelinase C. Such "black dots" have been observed in ghost preparations after treatment with phospholipase C from *B. cereus*, and were thought to represent accumulations of diglyceride. Similar phase-dense dots have been seen in sheep erythrocyte ghosts exposed to *Cl. perfringens* phospholipase C (Coleman *et al.*, 1970; Bernheimer, Avigad & Kim, 1974). Bernheimer, Avigad & Kim (1974) also studied the effects of staphylococcal sphingomyelinase C on human and sheep erythrocyte ghosts. Phase contrast microscopy revealed phase dense droplets in enzyme-treated ghosts, and electron microscopy showed the formation of internal vesicles and accumulations of non-osmophilic amorphous material. These findings are in accordance with the phase contrast and freeze-etched appearance of erythrocyte membranes reported here.

In the present study, highly purified sphingomyelinase C was used throughout. This preparation was shown to be homogeneous and relatively free from other staphylococcal activities. By contrast, Colley et al. (1973) using highly purified enzyme in some experiments, but also employed a preparation of sphingomyelinase C of unspecified purity, later shown to be purified by an unreliable method. It must be emphasised that the presence of trace amounts of impurities such as protease or glycosidase activity may influence the action of sphingomyelinase C on cell membranes (Chesbro et al., 1965; Arvidson et al., 1973; Wadström & Möllby, 1971a). For instance, Wadström & Möllby (1971b) found that partly purified sphingomyelinase C was cytotoxic for fibroblasts and HeLa cells, whereas even 10^5 HU of highly purified enzyme was without effect (Thelestam et al., 1973; Wadström, Thelestam & Möllby, 1974). It was shown by following the release of ^{3}H-labelled nucleotides from fibroblasts and HeLa cells that even traces of staphylococcal δ-toxin were sufficient to damage the membrane extensively. Furthermore, whereas the phospholipase from Cl. perfringens seriously affects the rising action potential and input resistance of isolated frog muscle, the β-lysin which has a more restricted substrate specificity had no effect (Boethius et al., 1973).

At the time our work in this area was published (Low et al., 1974, see enclosed publication) we suggested that an aspect of the action of phospholipases on cell membranes, often overlooked, is the possibility that some phospholipases may be surface active, or at least may be potentiated by surface active peptides similar to the direct lytic factors in snake venoms (Klibansky et al., 1968). They

may also induce changes in membrane structure independent of their enzymic activity (Speth et al., 1972). We suggested that the intrinsic surface activity of phospholipases may be important in determining the outcome of enzyme-membrane interactions.

It is known that some phospholipases can hydrolyse the phospholipids of intact erythrocyte membranes, leading to haemolysis of the cell, but other phospholipases cannot hydrolyse the phospholipids of intact cells but can hydrolyse the phospholipids of ghost membranes (Zwaal et al., 1975).

Demel et al. (1975) found that there was a maximal surface pressure below which a phospholipase can not hydrolyse phospholipids in monomolecular films. If this maximal surface pressure is lower than the surface pressure of lipids in the core of erythrocyte membranes, then that particular phospholipase can not hydrolyse phospholipids in that intact erythrocyte. On the other hand, if the maximal surface pressure for that particular phospholipase is above the surface pressure of phospholipids in that erythrocyte membrane, then hydrolysis may proceed. Thus phospholipases must be sufficiently surface active to be able to penetrate the lipid core of membranes if they are to hydrolyse phospholipids. This is in fact the case for staphylococcal β-lysin (Demel et al., 1975).

2. Treatment of intact erythrocyte membranes

Bovine erythrocytes treated with β-lysin appeared slightly shrunken and altered in shape at 37°C. This explains the 'halo' effect of incomplete clearing seen on sheep blood agar plates prior to

incubation at 4°C and is in good agreement with the appearance of freeze-etched human erythrocytes treated with β-lysin (Verkleij et al., 1973), where it appears that ceramide aggregates into small spheres in the bilayer some 7.5 nm - 20.0 nm in diameter.

On cooling, droplets and vesicles start to appear at the surface of the cell, and the cell starts to leak haemoglobin into the environment. The cell begins to take on the appearance of ghosts treated with β-lysin.

If a single lipid system is cooled, it undergoes a phase transition at a particular temperature, this transition being associated with disorder-order transitions where the hydrocarbon chains of lipids go from a liquid-like state to a more regular, ordered conformation (Luzatti, 1968). In mixed lipid systems, lipids tend to "co-crystallize" and result in a phase separation (McConnell et al., 1972). Since biological membranes contain a mixture of lipids it follows that temperature-induced changes in the lipid phase result in phase separations. These also affect the order, mobility and topography of associated proteins, and it has been shown that a drop in temperature below the phase separation temperature results in the clearing of large areas of protein particles from the bilayer of mammalian membranes (Wunderlich et al., 1974). Differential scanning calorimetry and electron microscopy have shown that ceramide does not form a homogenous phase with other phospholipids (Demel et al., 1975). The cohesive forces of the intact erythrocyte must be sufficient to hold ceramide produced in situ by β-lysin, in position in the bilayer at 37°C. Magnesium ions are important in preventing the collapse of

this weakened bilayer (Smyth, Möllby & Wadström, 1975). However, on cooling, a phase separation seems to occur, resulting in the condensation of ceramide into large pools, as visualized by microscopy, and the collapse of the bilayer rapidly follows.

The action of B. cereus phospholipase C on guinea pig erythrocytes might also be expected to result in hot-cold haemolysis by a similar process to that suggested above. However, this enzyme causes haemolysis of guinea pig erythrocytes at 37°C (Bult & Zwaal, 1973). It appears that guinea pig erythrocyte membranes have a lower surface pressure than human or sheep erythrocyte membranes, since B. cereus phospholipase C is capable of causing the hydrolysis of phospholipids in the intact erythrocytes of guinea pigs, but not those of sheep or humans. This surface pressure is probably inadequate to retain diglyceride in situ in the membrane, and thus breakdown of the membrane and haemolysis rapidly follows.

3. Effects in mice

The highly purified preparation of β-lysin described in this thesis was non-toxic and non-dermonecrotic to mice at doses up to 7.14 mg/kg mouse tissue.

It is difficult to explain the discrepancies between reported β-lysin toxicity. Wadström & Möllby (1971b) found β-lysin to be cytotoxic for HeLa cells, human fibroblasts and human thrombocytes, but in more recent studies they reported that human fibroblasts and HeLa cells were resistant. They claimed this was due to including a gel filtration step in the purification which was removing a cytotoxic component from the preparation.

In whole animals, β-lysin was reported as toxic for guinea pigs (only in the presence of Mg^{2+} ions) by Heydrick & Chesbro (1962; non-toxic for rabbits, guinea pigs and mice (with or without Mg^{2+}) by Wiseman (1965); and toxic for rabbits by Gow & Robinson (1969) and for rabbits, guinea pigs and mice by Wadström & Möllby (1971b). It has been reported as being non-dermonecrotic (Maheswaran, Smith & Lindorfer, 1967).

The β-lysin preparations of Wiseman (1965) may be non-toxic due to the presence of an inhibitor, co-purified with lysin as discussed earlier. This would also explain the absence of a massive increase in total enzymic and haemolytic activity after purification. The evidence of Maheswaran, Smith & Lindorfer (1967) does not support this possibility, but they did not perform any test of lethality.

The results presented in this thesis were obtained using a preparation of β-lysin which was purified more extensively than those of Wadström & Möllby (1971a) or Gow & Robinson (1969). This preparation was non-haemolytic against sheep and rabbit erythrocytes at 37°C and cod erythrocytes at 22°C, whilst both the other preparations were haemolytic to sheep and rabbit erythrocytes at 37°C. Neither of these groups titrated their preparations against cod erythrocytes.

From the present data, it appears that β-lysin is non-toxic in the range tested. The toxicity of the preparations of Gow & Robinson (1969) and Wadström & Möllby (1971a) could well be due to the presence of low levels of α-, δ or γ-lysins acting independently or synergistically.

A finding of this nature obviously brings the nomenclature of this extracellular product into question. The term β-toxin is inaccurate, since the protein is not highly toxic. The term β-lysin is confusing, since lysis only occurs when erythrocytes from susceptible species are subjected to the physical stress of being chilled after exposure to the enzyme. The hydrolysis of sphingomyelin in intact erythrocytes is not a lytic effect at 37°C, and it is not likely to be a lytic effect in other types of cell which have lower sphingomyelin contents. The best nomenclature available is to refer to the protein as the enzyme, sphingomyelinase C.

Wiseman (1975) has suggested that β-lysin could offer an advantage to invading staphylococci by permitting a foothold to be gained in a wide variety of tissues containing sphingomyelin. This would result from the disruption of the membranes with the release of metabolites required by the bacteria. He further suggested that since β-lysin production is a characteristic of strains of staphylococci associated with bovine mastitis (Slanetz & Bartley, 1953), the skin and tissues of the teat may be richer in sphingomyelin than those of other body areas. This is not in fact so (White, 1973).

Sphingomyelinase C clearly does not act as a single virulence factor. However, it can hydrolyse sphingomyelin in intact erythrocytes at 37°C, causing physical damage to the cell and making it more susceptible to haemolysis by a variety of agents (temperature shock, pH shock, osmotic shock). It may well have a role in pathogenicity by acting to increase the sensitivity of normally resistant cells to attack by the other extracellular products of staphylococci.

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APPENDIX

Appendix I

Buffers

Tris HCl buffer

Stock solutions:

A. 0.2 M Tris (24.2 g made up to litre)

B. 0.2 M HCl (17.15 ml conc HCl made up to litre)

50 ml A + x ml B diluted to a total of 400 ml gives a 0.05 M solution at the pH shown below:

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

Tris buffered saline (TBS) + Mg^{2+}

Stock solutions:

C. 1 M NaCl 58.5 g/litre

D. 0.1 M $MgCl_2$ (anhydrous) 9.43 g/litre

Tris buffer was made up as before except that 154 ml of 1 M NaCl and 10 ml of 0.1 M $MgCl_2$ were included per litre final volume of buffer. For example, 1 litre of TBS + Mg^{2+} would consist of:

125 ml A 154 ml C

107 ml B 10 ml D giving a final pH of 7.4.

Phosphate buffer

Stock solutions:

A	0.2 M $NaH_2PO_4.2H_2O$	31.2 g/litre
B	0.2 M Na_2HPO_4 (anhydrous)	28.39 g/litre

x ml of A and y ml of B were mixed and diluted to 400 ml to give 0.05 M phosphate buffers at the pH required.

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

Phosphate buffered saline + BSA

Phosphate buffer was made up as before except that 154 ml of 1 M NaCl and 1 gram of bovine serum albumin (BSA) were included per litre final volume of buffer.

Citrate dextrose saline

Glucose	2.05 g
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	0.80 g
NaCl	0.40 g
Distilled water to ..	100.0 ml

Palitsch buffer for coagulase assay

Solution A (0.05 M sodium tetraborate)

$Na_2B_4O_7.10H_2O$	19.069 g
Distilled water to 1,000 ml	

Solution B (0.2 M boric acid salt solution)

H_3BO_3	12.308 g
NaCl	2.925 g
Distilled water to 1,000 ml	

1.2 ml A plus 8.8 ml B gives 10 ml buffer at pH 7.4.

Borate buffer (0.3 M sodium borate, pH 8.3)

Boric acid, H_3BO_3	18.55 g
Sodium tetraborate $Na_2B_4O_7.10H_2O$	28.6 g
Distilled water to 1,000 ml	

Hyaluronidase diluent

0.2 M Na_2HPO_4	5 ml
0.2 M NaH_2PO_4	5 ml
1.0 M NaCl	7.7 ml
Bovine serum albumin fraction V	10 mg

Adjust pH to 6.9 if necessary with 1 M NaOH or 1 M HCl.

Distilled water to 100 ml.

0.3 M Sodium phosphate buffer pH 5.4

Stock solutions:

0.3 M Na_2HPO_4 anhydrous (42.59 g/l)	3.5 ml
0.3 M KH_2PO_4 anhydrous (40.83 g/l)	96.5 ml

Acid-albumin solution for hyaluronidase assay

Glacial acetic acid	4.56 ml
Sodium acetate	3.26 g
Bovine serum albumin (Armour)	1.00 g

Distilled water to 1 litre

Adjust pH to 3.75 with glacial acetic acid.

Appendix II

Isoelectric focusing solutions

Density gradient column — LKB 8101

Anode solution

Sucrose (BDH Analar)	12 g

OR Sorbitol (Merck Lab. Chemicals, Darmstadt)

Distilled water		14 ml
Sulphuric acid		
OR Glacial acetic acid (BDH, Analar)		0.2 ml

Dense solution for gradient

Sucrose or Sorbitol		25 g
Distilled water		32 ml
Ampholine (8% w/v)		8.5 ml
	Total vol	55 ml

Light solution for gradient

Toxin sample		X ml
Distilled water		51-x ml
Ampholines (8% w/v)		4 ml
	Total vol	55 ml

Cathode solution

Distilled water	20 ml
Sodium hydroxide	0.2 g

(The ampholines used were 8% with respect to pH 7-9 and pH 9-11).

Multiphor Gel

Stock solutions:

A. Acrylamide solution 30.0% (w/v)

30.0 g acrylamide (BDH) made up to 100 ml with distilled water.

B. NN' methylenebisacrylamide (Bis) solution 1.0% (w/v)

1.0 g Bis (BDH) made up to 100 ml with distilled water.

C. Riboflavin solution 0.004% (w/v)

4 mg riboflavin (BDH) made up to 100 ml with distilled water.

Solutions were mixed as follows for an experiment in the range pH 3.5 to 9.5. Dissolve 7.5 g sucrose in 36.0 ml distilled water and add the following solutions:

10.0 ml	A
10.0 ml	B
2.8 ml	Ampholine pH 3.5 - 10
0.4 ml	Ampholine pH 9.0 - 11
0.2 ml	Ampholine pH 4.0 - 6
0.2 ml	Ampholine pH 5.0 - 7
0.4 ml	C

Mix the solutions thoroughly and de-aerate by aspirating for one to two min. Pipette into the mounted set of glass plates using a 50 ml pipette. Fill up completely, avoiding air bubbles. Mount the last clamp and polymerise in front of a fluorescent light source.

The removal of the thick glass plate from the polyacrylamide gel is facilitated if the polymerisation set without clamps is stored at $4^{\circ}C$ for about 15 min before taking apart.

Electrode solutions

Anode solution

1 M H_3PO_4

Cathode solution

1 M NaOH.

Staining

2.5 g Coomassie Brilliant Blue R250 is added to 100 ml distilled water at 60°C and stirred vigorously for 10 min. To this add 100 ml 2 N H_2SO_4 at 60°C. After a further 10 min at 60°C the solution is filtered through Whatman No. 1 filter paper (pH approx. 1.2). 10 N KOH is added until a pH of 5 to 5.5 is reached (approx. 22 ml); then trichloracetic acid is added to a concentration of 12 g per 100 ml.

The solution appears blue-green and has a pH of approx. 1.0. Store in a brown bottle and check pH before use. The stain can be filtered if a precipitate appears.

Appendix III

Slab gel electrophoresis

Stock solutions

1. Acrylamide 30 g
   Bis 0.8 g
   in 100 ml H_2O
   Filter and store in brown bottle at 4°C

2. 1 M Tris/HCl buffer pH 8.8

1 M Tris (121 g/1000 ml)	50 ml
1 M HCl	8.1 ml
	dist H_2O to 100 ml

3. 1 M Tris/HCl buffer pH 6.8

1 M Tris	50 ml
1 M HCl	45 ml approx.
	dist H_2O to 100 ml

4. Tris/glycine buffer pH 8.3 (10 x conc)

glycine	144.13 g
Tris	30.28 g
	dist H_2O to 1000 ml

5. 0.8% ammonium persulphate

ammonium persulphate	0.8 g
	dist H_2O to 100 ml

6. Sodium dodecylsulphate (SDS)

Sodium dodecyl sulphate	20 g
	dist H_2O to 100 ml

7. Bromophenol blue

Bromophenol blue	0.1 g
	dist H_2O to 100 ml

Solubilizing buffer

Stock solutions	Amount
Tris/HCl 6.8	2.0 ml
SDS	3.2
β-mercaptoethanol	1.6

Stock solutions	Amount
glycerol	3.2
bromophenol blue	0.32
H_2O	5.68
Total	16.0 ml

Separating gel

Stock solutions	Amount
Acrylamide	14.7 ml
Tris/HCl 8.8	15.0
20% SDS	0.2
N,N,N',N',tetra methyl ethylene diamine (TEMED)	10 µl
Ammonium persulphate	4.0 ml
H_2O	6.1

Pipette approx. 14 ml into gel plates and carefully overlay with 0.375 M Tris/HCl buffer pH 8.8 containing 0.1% w/v SDS. Allow to polymerise, drain and add stacking gel solution.

Stacking gel

Stock solutions	Amount
Acrylamide	1.7 ml
Tris/HCl pH 6.8	1.25
SDS	0.05
TEMED	2.5 µl
Ammonium persulphate	1.0 ml
H_2O	6.0 ml
Total	10 ml

Suspend the sample "comb" approx. 1 cm above the surface of the separating gel and fill the remaining space with stacking gel solution. Allow to polymerise, then remove combs carefully under running buffer.

Fixing and staining solution

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

Destaining solution

Methanol	50 ml
glacial acetic acid	75 ml
distilled water	875 ml

Electrophoresis conditions

Dilute the Tris/glycine stock buffer 1 in 10 and add SDS to give a final concentration of 0.1% (w/v) (add 5 ml per 1000 ml).

Remove the sealing tape from the base of the gel plates and insert into the top of the electrode vessel using a liberal amount of grease around the gaskets to prevent leakage. Fill the vessel with running buffer and then layer samples (25 - 50 µl) into the sample wells.

Run at a constant current of 15 mA/gel for approx. 2 h, and when the tracking dye reaches the end of the gel, switch the power off. Carefully remove the gel from the plates and immerse in fix-stain solution for 90 min. Destain by soaking in several changes of destaining solution.

Appendix IV

Immunological analysis

Double diffusion tests

1% (w/v) Ionagar in borate/sodium chloride buffer (0.03 M sodium borate, 0.14 M sodium chloride, pH 8.3).

Ionagar (Difco, No. 2)	1.0 g
1 M sodium chloride	14.0 ml
0.3 M borate buffer pH 8.3	10.0 ml
1% (w/v) thiomersal	1.0 ml
distilled water	75.0 ml

Dissolve ionagar by boiling gently in saline. Add borate buffer and thiomersal. Dispense in 20 ml amounts. Store at $4^{\circ}C$.

Appendix V

Sphingomyelinase

Sphingomyelin (Sigma Chemical Co. Ltd., St. Louis, Mo.), 15 mg, is dissolved in 2 ml of chloroform; 1 ml of 0.1 M Tris buffer (pH 7.4) containing 1 mM $MgCl_2$ and an aliquot of β-lysin are successively added. The reaction tubes are capped and vigorously shaken (or stirred) at $37^{\circ}C$. At 5 min intervals, samples are taken from the organic phase, dried in 6 x $\frac{5}{8}$ test-tubes, and analysed for phosphorus by the method of Allen (1940) one unit of sphingomyelinase C is defined as the amount of enzyme which causes the hydrolysis of 1 µmole of sphingomyelin per min.

Determination of phosphate by modified Allen method (1940)

All phosphorus is converted to inorganic orthophosphate by digestion with sulphuric acid and hydrogen peroxide. Phosphate then reacts with ammonium molybdate to produce a complex which forms a blue chromogen on reduction with amidol (2:4 diamino phenol hydrochloride). The intensity of the blue colour is measured at 640 nm.

Standard phosphorus solution

The standard used was a solution of 2.193 g KH_2PO_4 (anhydrous) dissolved in 500 ml distilled water. This is equivalent to 1 mg phosphorus per ml.

Reagents

10 N H_2SO_4	280 ml conc H_2SO_4 (BDH Analar) to a final volume of 1000 ml with distilled water.
4.8 N H_2SO_4	48 ml 10 N H_2SO_4 to a final volume of 100 ml with distilled water.
1% Amidol solution in 20% sodium metabisulphate	1 g amidol was dissolved in 100 ml of 20% w/v aqueous sodium metabisulphate and the solution was filtered and transferred to a dark bottle. This solution was prepared fresh daily.

Preparation of phosphorus standards

Reagent	Full Allen	½ Allen	1/5 Allen
Standard P solution volumes containing	20 - 1000 µg P	0 - 150 µg P	0 - 50 µg P
Distilled water to	20.8 ml	10.4 ml	3.9 ml
10 N H_2SO_4	1.2 ml	0.6 ml	-
4.8 N H_2SO_4	-	-	0.5 ml
Amidol reagent	2 ml	1 ml	0.4 ml
8.3% ammonium molybdate	1 ml	0.5 ml	0.2 ml
final volume	25 ml	12.5 ml	5.0 ml

The reagents were added in the order shown and mixed thoroughly. After standing for at least 10 min, but not more than 30 min, $E^{1cm}_{640\ nm}$ was measured against the reagent blank.

Total phosphorus determination

To measure organic phosphorus the samples were added to microkjeldahl flasks. Sulphuric acid was added according to the table and the flask necks were washed down with sulphuric acid. The flasks were heated until all the water evaporated and the sulphuric acid started to fume. Where an appreciable amount of organic material was present the flask contents turned black. In these cases 2 - 3 drops H_2O_2 were added to the contents after cooling and digestion was continued. This procedure was repeated until the contents of the flasks were colourless. Water was then added to bring the volume to 22 ml (full Allen), 11 ml (½ Allen) or 3.9 ml (1/5 Allen). The volumes to be added are 21.65 ml, 10.82 ml and 3.83 ml respectively.

The procedure for the preparation of the curves is then followed. After addition of amidol and ammonium molybdate reagents, the solutions were allowed to stand for 10 - 30 min before the $E^{1cm}_{640\ nm}$ was measured.

Appendix VI

Correction of sedimentation coefficient, S, to S_{20w}

The first correction is one for the viscosity of the solute and this is

$$s_w = s_{obs} \frac{\eta_{solv}}{\eta_w} \qquad 1.$$

where s_{obs} and s_w are the sedimentation coefficients in the solution and in water respectively and η_{solv} and η_w are their respective viscosity coefficients.

The variation of viscosity with temperature will also cause a variation in s.

$$s_{20} = s_{obs} \frac{\eta_t}{\eta_{20}} \qquad 2.$$

where s_{20} is the sedimentation coefficient at 20°C and $\eta_t + \eta_{20}$ are the viscosities at temperatures of t°C and 20°C.

Combining 1 and 2

$$s_{20w}\ (\text{corrected for viscosity}) = s_{obs} \frac{\eta_t}{\eta_{20}} \frac{\eta_{solv}}{\eta_w} \qquad 3.$$

The density of the medium must also be taken into account since if sedimentation is to take place at all then there must be a density difference between the particle and the solvent. The effective mass of

the particle, m_{eff}, is given by

$$m_{eff} = m - m\bar{\nu}\rho = m(1 - \bar{\nu}\rho)$$

where m is the mass of the particle, ρ is the density and $\bar{\nu}$ is the partial specific volume (the increase in volume that occurs when one kilogram of macromolecule is added to an infinite volume of water, i.e. the contribution per kilogram of dissolved material to the total volume of the solution. This figure can be calculated from the amino acid composition (Buzzell & Tanford, 1956).

It follows that

$$s \propto (1 - \bar{\nu}\rho)$$

i.e.

$$\frac{s}{(1 - \bar{\nu}\rho)} = \text{constant}$$

We must relate the density of water at 20°C (ρ_{20}) to the density of the medium (ρ_{obs})

$$\frac{s_{20}}{(1 - \bar{\nu}\rho_{20})} = \frac{s_{obs}}{(1 - \bar{\nu}\rho_{obs})}$$

so that

$$s_{20} = s_{obs} \frac{(1 - \bar{\nu}\rho_{20})}{(1 - \bar{\nu}\rho_{obs})} \qquad 4.$$

The sedimentation coefficient fully corrected for viscosity and density is given by

$$S_{20w} = s_{obs} \frac{\eta_t}{\eta_{20}} \frac{\eta_{solv}}{\eta_w} \cdot \frac{(1 - \bar{\nu}\rho_{20w})}{(1 - \bar{\nu}\rho_{obs})}$$