THE INHIBITION OF STAPHYLOCOCCAL DELTA-HAEMOLYSIN BY HUMAN SERUM

DOUGLAS D. WHITELAW

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Department of Microbiology

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To my parents and my wife, Elspeth

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SUMMARY

It is well established that staphylococcal δ-haemolysin is inhibited by normal mammalian sera and forms a precipitin line on gel diffusion with normal sera. The purpose of this thesis was to quantitate inhibitory activity against δ-haemolysin and to identify the serum factor(s) involved.

The inhibitory titre of normal human serum assayed using purified δ-haemolysin with cod erythrocytes as indicators of haemolysis was 1/500 - 1/1000.

Fractionation of serum by ultrafiltration, gel filtration in Sephadex G-150 and Sepharose 6B and ammonium sulphate precipitation indicated that at least two components of molecular weights approximately 300,000 and > 10^6 were involved. When affinity chromatography was done using δ-haemolysin covalently coupled to Sepharose-4B the selective removal of β-lipoprotein from whole serum was shown by immunoelectrophoresis. High density (α-) and low density (β-) lipoproteins were prepared by zonal ultracentrifugation of serum using potassium bromide density gradients and characterised by polyacrylamide gel electrophoresis, immunoelectrophoresis and gel diffusion using monospecific antisera. Both α- and β-lipoproteins inhibited δ-haemolysin and gave a precipitin line on gel diffusion, while fractions which were shown by immunoelectrophoresis to be devoid of such lipoproteins were non-inhibitory. Proteolytic digestion of lipoproteins with trypsin or papain did not diminish their inhibitory activity, whereas this was diminished by pretreatment with Bacillus cereus phospholipase C. Delta-haemolysin was

inhibited by phospholipids and pretreatment with phospholipase decreased the inhibitory capacity of such phospholipids and also of a serum lipid extract.

The results indicated that binding of δ-haemolysin to serum lipoproteins was via the phospholipid moiety and the implications of such an interaction in terms of *in vivo* activity of the haemolysin are discussed.

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ABBREVIATIONS

BSS	-	basal salt solution
CDS	-	citrate/dextrose/saline
cm	-	centimetre
CM-	-	carboxymethyl-
DEAE	-	diethylaminoethyl
DMSO	-	dimethylsulphoxide
E	-	extinction coefficient
EDTA	-	ethylenediamine tetra acetate
g	-	gram, gravity
hr	-	hour
HIU	-	haemolytic inhibitory unit
HU	-	haemolytic unit
IEF	-	isoelectricfocusing
kg	-	kilogram
M	-	molar
ma	-	milliamp
mg	-	milligram
mm	-	millimetre
min	-	minute
µg	-	microgram
µl	-	microlitre
NCTC	-	National Collection of Type Cultures
PB	-	phosphate buffer
PBS	-	phosphate buffered saline
p.s.i.	-	pounds per square inch
pI	-	isoelectric point
rpm	-	revolutions per minute
SDS	-	sodium dodecyl sulphate

sec	-	second
TBS	-	tris buffered saline
UV	-	ultraviolet
V	-	volt
v/v	-	volume for volume
w/v	-	weight for volume

INTRODUCTION

General remarks

The concept of exotoxins as harmful diffusible products of pathogenic bacteria has long been recognised in microbiology. By the beginning of this century, the potent exotoxins of diphtheria, tetanus and botulism had been demonstrated experimentally and in each case immunity to the toxin conferred immunity against the disease; thus the importance of extracellular toxins in microbial pathogenicity was established.

However, despite the large number of exotoxins recognised, only in a few cases are the roles of individual toxins in pathogenicity fully understood. The main classes and the purification of bacterial toxins have been reviewed by Raynaud and Alouf (1970) and van Heyningen (1970).

In the case of the staphylococci, a wide range of extracellular products with potent biological effects may be formed but only in a few instances have extracellular products been shown to contribute to the pathogenicity of Staphylococcus aureus. This thesis concerns only one of the extracellular products, the δ-haemolysin, but the nature and properties of other extracellular products will also be briefly discussed.

Farrer and McLeod (1960) and Shulman and Nahmias (1972) have dealt with the clinical aspects of staphylococcal infections.

Staphylococcal extracellular products

Following the postulation of Klebs (1872) that there was a

relationship between pathogenicity and toxin production in bacteria, and the demonstration by de Christmas (1888) of toxicity of heated broth cultures of staphylococci recovered from lesions in man, much progress has been made in ascribing roles to the toxins produced by S. aureus. Many attempts have been made to correlate staphylococcal virulence with these factors. However, in spite of the potent biological activity of some toxins there is little clear understanding of virulence in chemical terms. Indeed the existence of an unidentified "virulence factor" produced only in vivo has been postulated by Abramson (1972). However, the potential importance of extracellular products in staphylococcal pathogenicity cannot be disregarded.

Strains of S. aureus release a large number of extracellular enzymes and toxins into their environment during growth. Analytical starch gel electrophoresis studies on potentially pathogenic strains revealed 12 to 14 different proteins while the culture supernatant fluids of non-pathogenic strains contained half as many (Bernheimer and Schwarz, 1961). More recent isoelectric-focusing (IEF) studies by Wadström (1973a) showed that different strains produced quite different protein patterns.

Besides the classical enzymes produced by S. aureus (Table Ia) and cytolytic toxins (vide infra), other entities (succinate oxidase factor, Lominski, Gemmell and Arbuthnott, 1968: lymphocyte mitogens, Kreger, Cuppari and Taranta, 1972: leucocyte chemotactic factors and inhibitors of leucocyte chemotaxis, Russell et al, 1975) have been demonstrated (Table Ib).

Arbuthnott et al (1971) identified a soluble, extracellular

Table 1a Properties of some extracellular products of S. aureus

a) Enzymes

Enzyme	pI	Molecular Wt.	Reference
Nuclease		17,000	Heins et al, 1967
	10.1		" Wadstrom, 1967
Phosphatase	3.8-4.2	58,000	Tirunarayan, 1969
			Malveaux & San Clemente, 1969
Protease	9.4	12,500	Arvidson, Holme & Lindholm, 1973
Esterase	4.5	-	" Wadstrom, 1967
Staphylokinase	5.7,6.3,6.7		Vesterberg & Vesterberg, 1972
Coagulase	5.9-6.1	44,000	Tirunarayan, 1969
			Duthie & Haughton, 1958
Hyaluronidase	7.9	82,000	" " Wadstrom & Mollby, 1972
			Abramson & Rautela, 1971
Lipase	9.5	100,000	Vesterberg et al, 1967
Endo-β-N-acetyl glucosaminidase	9.6	70,000	" Wadstrom & Hisatsune, 1970
Amidase	9.8		" Wadstrom & Vesterberg, 1971
Peptidase	10.4		" "
Cholesteryl de-esterifying enzyme	9.1	25,500, 175,000	Harvie, 1977
			McCartney & Arbuthnott, 1977

Table 1b Properties of some extracellular products of S. aureus

b) Other factors

Factor	pI	Molecular Wt.	Reference
enterotoxin B	8.55,8.25,9.4		Chang and Dickie, 1971 Metzger, Johnson and Collins, 1972
enterotoxin C_1	5.5,5.9,6.7		Chang et al, 1971
enterotoxins A-E	6.8-8.6	28,000-35,000	Bergdoll, 1972
lymphocyte mitogen	5.6-8.8		Kreger et al, 1972
leucocidin	9.0		Wadström (unpublished data quoted by Wadström, 1974)
epidermolytic toxin	7.0	33,000	Melish et al, 1972 Kondo, Sakura and Sarai, 1973 Arbuthnott, Billcliffe and Thompson, 1974

protein devoid of haemolytic activity, and demonstrated *in vivo* its ability to cause extensive splitting of the epidermis in newborn mice similar to that found in Toxic Epidermal Necrolysis (Ritter's syndrome or the scalded skin syndrome: Lyell, 1967: Melish and Glasgow, 1970: Melish, Glasgow and Turner, 1972). This factor (epidermolysin) is the only soluble product of staphylococci (apart from those produced by enterotoxic strains) unequivocally shown to play a role in pathogenicity.

Many *S. aureus* strains also produce *in vitro* various haemolysins (cytolytic toxins) which are believed to play a role in the infected host. It is now established that *S. aureus* produces at least four different haemolysins (designated alpha, beta, gamma and delta). The existence of a fifth (epsilon haemolysin) has been proposed (Elek and Levy, 1950: Elek, 1959), but to date has not been purified. Alpha- and δ-haemolysins are more frequently produced by coagulase positive strains from human origin, while beta-haemolysin is produced principally by strains from animals.

Alpha-haemolysin

Purified α-haemolysin is a protein (Arbuthnott, 1970) devoid of carbohydrate (Watanabe and Kato, 1974) and apparently exists in three physical states: a soluble active form, α3S (Bernheimer and Schwarz, 1963: Lominski, Arbuthnott and Spence, 1963: Cooper, Madoff and Weinstein, 1966: Coulter, 1966); a soluble inactive form, α12S (Bernheimer and Schwarz, 1963: Lominski *et al*, 1963) and an insoluble inactive form (Coulter, 1966: Arbuthnott, Freer and Bernheimer, 1967). The inactive forms were regarded as toxoids since they elicited the formation of

protective antibody and were thought to be aggregates of the active α3S lysin (Arbuthnott et al, 1967). Electron microscopic studies have also demonstrated aggregation (Bernheimer and Schwarz, 1963: Freer, Arbuthnott and Bernheimer, 1968).

The molecular weight of this haemolysin has been estimated as 21,000 to 45,000 daltons by various workers (reviewed by Wiseman, 1975). Madoff (1965) and Coulter (1966) postulated that the lysin associated to give polymeric forms in equilibrium to explain the variation in reported molecular weights and McNiven, Owen and Arbuthnott (1974) suggested that estimates around 30,000 represented mixtures of both a monomer of 22,000 daltons and a dimer of 44,000 daltons. Sucrose density-gradient electrophoresis (Bernheimer and Schwarz, 1963) and IEF (Wadström, 1968: Bernheimer, 1970) have revealed multiple forms of α-haemolysin; McNiven (1973) described four components with isoelectric points of 8.55, 9.15, 7.36 and 6.26 respectively.

Rabbit erythrocytes are particularly susceptible to α-haemolysin, whereas human erythrocytes are only slightly susceptible (Arbuthnott, 1970). Kinetic studies (Lominski and Arbuthnott, 1962) have shown that at low concentration, the rate of haemolysis is dose-dependent, and Bernheimer (1970) proposed an enzymatic action for α-haemolysin from the effect of constant amounts of lysin on varying concentrations of erythrocytes. A three-stage reaction sequence of adsorption, loss of selective cell-membrane permeability, and lysis has been proposed for α-haemolysin (Marucci, 1963: Klainer et al, 1964: Madoff, Cooper and Weinstein, 1964) but as yet its substrate on the erythrocyte membrane has not been identified nor the nature of the interaction been determined.

Wiseman, Caird and Fackrell (1975) have suggested that alpha-haemolysin is produced as a zymogen, which is converted at the cell surface to an active form by an activating protease, acquiring proteolytic activity in the process. Dalen (1976) obtained supporting evidence for proteolytic activity of α-haemolysin but could not rule out the presence of contaminating proteases.

Both cold- and warm-blooded animals are sensitive to the haemolysin and the severity of its effects are dose-dependent. Fackrell and Wiseman (1976b) determined the mean lethal dose (LD_{50}) in mice as 0.68 ± 0.19 µg or 27 to 34 µg/kg of body weight, similar to the value obtained by Arbuthnott (1970). Lominski et al (1963) obtained minimum lethal dose values of 50 µg/kg in mice and 1.3 µg/kg in rabbits injected by the intravenous route. LD_{50} data obtained by others for mice are 600 µg/kg (Goshi, Cluff and Norman, 1963) and about 50 µg/kg intravenously (Watanabe and Kato, 1974).

In an investigation into an outbreak of mastitis in commercially reared rabbits, Adlam et al (1976, 1977) showed that immunisation with purified α-toxoid gave partial protection and reduced the lethal haemorrhagic oedematous form of mastitis lesion to a localised chronic abscess.

In summarising the main pathological findings, Arbuthnott (1970) observed that the haemolysin acted on the peripheral circulation, heart and central nervous system.

For comprehensive reviews on the cytotoxicity of α-haemolysin, see Arbuthnott (1970) and Jeljaszewicz (1972).

Beta-haemolysin

Numerous studies have shown that β-haemolysin is a protein (Haque and Baldwin, 1969; Jackson, 1962; Maheswaran, Smith and Lindorfer, 1967; Wiseman, 1965) and its distinctness from α-haemolysin was first shown by Glenny and Stevens (1935). The various molecular weights ascribed to β-haemolysin have been reviewed by Wiseman (1975) and, like α-haemolysin, multiple forms have been reported (Maheswaran et al, 1967; Haque and Baldwin, 1969; Mollby and Wadstrom, 1970). The recent report by Low and Freer (1977,b) of a molecular weight of 33,000 and isoelectric point of 9.3 for the major form are in close agreement with most published data.

The characteristic reaction of β-haemolysin (which it has in common with the α-toxin of Clostridium perfringens) is that of "hot-cold" haemolysis (Bigger, Bolland and O'Meara, 1927) - haemolytic activity is enhanced if incubation at 37°C is followed by incubation at 4°C - and the erythrocytes of sheep, man and rat, in that order, are decreasingly sensitive to its action. The mechanism is still imperfectly understood, but activity is enhanced by Mg^{2+}, Mn^{2+} and Co^{2+} and inhibited by EDTA and citrate (Jackson and Mayman, 1958; Wiseman, 1965).

Recent studies by Smyth, Mollby and Wadstrom (1975) showed that haemolysis at 37°C could be induced in β-haemolysin pretreated cells when subjected to EDTA, suggesting that exogenously added and/or membrane-bound divalent cations are important for the stability of sphingomyelin-depleted membranes.

The discovery by Doery et al (1963) that sphingomyelin was hydrolysed by the lysin has since been confirmed by many workers (Maheswaran and Lindorfer, 1966, 1967; Wiseman and Caird, 1966, 1967; Fritsche, 1970; Wadström and Mollby, 1971a; Low, Freer and Arbuthnott, 1974), and the high susceptibility of sheep and ox erythrocytes may well be attributable to the high content of sphingomyelin in their cell membranes (Rouser et al, 1968). Despite a report by Chesbro et al (1965) of carbohydrase activity, beta-haemolysin is now agreed to be a sphingomyelinase C, and has found use as a tool in membrane studies (Low et al, 1974; Demel et al, 1975).

The most controversial property of β-haemolysin is that of toxicity, and hindsight suggests that conflicting evidence has arisen through the use of impure preparations. For rabbits, mice and guinea-pigs, β-haemolysin is 10 to 160 times less toxic than α-haemolysin and recent reports by Fackrell and Wiseman (1976,b) and Low and Freer (1977,a) show that 100 μg and 150 μg amounts respectively of purified haemolysin were neither lethal nor dermonecrotic.

Gamma-haemolysin

The first indication of the existence of γ-haemolysin was made by Smith and Price (1938) and its distinctness from α-, β- and δ-haemolysins was confirmed by Marks (1951). It is inhibited by agar (Jackson, 1962; Guyonnet and Plommet, 1970; Mollby and Wadström, 1971; Fackrell, 1973), which precluded its detection by blood agar haemolysis by Elek (1959) who regarded alpha-2, delta- and gamma-haemolysins as being identical. The lysin was inactivated by heating at 55°C for 10 min and by cysteine or ascorbic acid (Jackson, 1962).

The haemolytic activity of the haemolysin is reportedly inhibited by phospholipids (Taylor and Bernheimer, 1974; Fackrell and Wiseman, 1976,b). Gamma-haemolysin acts on human, rabbit and sheep erythrocytes, but not horse erythrocytes (Guyonnet and Plommet, 1970) with rabbit being the most sensitive (Möllby and Wadström, 1971; Fackrell and Wiseman, 1976,b). The toxicity of γ-haemolysin was investigated by Smith and Price (1938) who reported that crude haemolysin killed rabbits, but not mice and guinea pigs, though it was slightly dermonecrotic for the latter. A 100 μg subcutaneous dose of purified haemolysin was innocuous to guinea-pigs and rabbits (Fackrell and Wiseman, 1976,b), as was the same dose intraperitoneally to mice. However, a 50 μg intracardial injection proved fatal to guinea-pigs (Fackrell and Wiseman, 1976,b). The role of γ-haemolysin *in vivo* is unknown but when experimental staphylococcal osteomyelitis was induced in rabbits by Kurek *et al* (1977), enhanced anti-γ-haemolysin antibody titres resulted. The staphylococcal strain used was isolated from a case of human osteomyelitis and Kurek *et al* suggested that γ-haemolysin played a role in pathogenesis of osteomyelitis.

Delta-haemolysin

Purification and physical properties. The discovery of delta-haemolysin as a distinct product of *S. aureus* was made by Williams and Harper (1947) who showed that haemolysis was not suppressed when *S. aureus* was grown in the presence of α- and β-antitoxins on sheep blood agar. They demonstrated its wide spectrum of haemolytic activity and synergistic action with β-haemolysin on sheep erythrocytes. Marks and Vaughan (1950) confirmed these findings and partially purified the haemolysin by extraction of

crude culture supernatants with ethanol, in which delta-lysin was soluble. The haemolysin was insoluble in acetone, non-dialysable, thermostable (100°/2 hours), inactivated by formaldehyde and absorbed by alumina below pH 8.

Jackson and Little (1958) produced delta-lysin from crude culture supernatants adjusted to pH 4 with acetic acid, by precipitation with methanol at -5°C.

Yoshida (1963) crystallised delta-haemolysin from S. aureus strain Foggie by chromatography of concentrated, heated culture supernatants on calcium phosphate gel and DEAE-cellulose. Ultracentrifugation showed the preparation to be highly homogeneous (sedimentation coefficient 6.1S) with a molecular weight of 68,230 calculated from an amino-acid analysis. Lysine, aspartic acid, phenyl-alanine and isoleucine were the amino acids present in greatest amounts, and there were very low levels of cysteine, histidine, arginine, proline and tyrosine. Purified delta-haemolysin was trypsin-sensitive and insoluble in chloroform/methanol (2:1). Subsequent work by Gladstone and Yoshida (1967), using the same preparation, revealed, however, the presence of ribonuclease and β-haemolysin in undefined but significant concentrations.

Hallander (1968) purified the haemolysin by gel filtration, preparative electrophoresis and chloroform/methanol (2:1) extraction. Immunodiffusion against antiserum to δ-haemolysin yielded a single immune precipitation line.

The dialysis membrane technique devised by Birch-Hirschfeld (1934) was used by Marks and Vaughan (1950) for production of delta-haemolysin

Harvesting of the haemolysin was facilitated because the delta-haemolysin was retained on top of the membranes and was separated from the non-dialysable ingredients of the medium. Murphy and Haque (1967), using dialysis membranes over Brain Liver Heart plates under an atmosphere of 10% CO_2, overcame the dilution problems present in broth cultures and thus obtained concentrated preparations. Kleck and Donahue (1968) also employed this cellophane-layer technique, and Murphy and Haque (1974) developed it into a large-scale production process. To eliminate the possibility of β-haemolysin contamination encountered by Yoshida (1963), Murphy and Haque used S. aureus strain 146P, a non-β-haemolysin producer.

Caird and Wiseman (1970) purified the delta-lysin of strain E-delta by isoelectric precipitation (pH 4.0), ammonium sulphate precipitation and DEAE-cellulose chromatography. Purified haemolysin obtained in 16% yield with a 32-fold increase in specific activity, yielded a single precipitation line on immunodiffusion or immunoelectrophoresis against antiserum to δ-haemolysin and the sole N-terminal amino acid was proline. A single protein band was noted on polyacrylamide gel electrophoresis (pH 9.5) but ultracentrifugation revealed two components (2.8S and 9.8S).

The property of solubility in chloroform-methanol (2:1) was exploited by Heatley (1971) using strain 186X. He purified δ-haemolysin by ammonium sulphate precipitation, chloroform-methanol extraction and cyclic transfer between organic and aqueous phases of a two phase system (chloroform/methanol/water) by adjustment of the pH. Purified lysin was

soluble in water, saline or chloroform-methanol (2:1), sparingly soluble in methanol, and insoluble in chloroform, acetone or hexane. A single polydisperse peak (4.9S) was observed on ultracentrifugation, while polyacrylamide gel electrophoresis at pH 4.6 or pH 8.4 showed a main diffuse band with a faint diffuse secondary band.

Kreger et al (1971) studied δ-haemolysin from a mutant strain of S. aureus Wood 46 which produced little α-haemolysin (W46M). The haemolysin was purified by adsorption to hydroxylapatite from crude culture supernatant and elution with phosphate buffers of increasing molarity. A soluble and an insoluble form of the lysin were obtained on dialysis against water. The latter was poorly soluble in water (<0.05 mg/ml) and in a variety of 0.1M buffers unless they contained 8M urea. It was soluble and stable in 0.1M sodium hydroxide and chloroform-methanol (2:1). Soluble haemolysin was soluble in water, various buffer solutions, chloroform-methanol (2:1), but insoluble in chloroform, methanol or acetone. This haemolysin was heterogeneous by gel filtration, sucrose density gradient centrifugation, ion exchange chromatography, polyacrylamide gel electrophoresis and IEF. Two bands were obtained in polyacrylamide gel electrophoresis at pH 4.3 and pH 9.5, and on IEF two protein peaks (pI 9.5 and 5.0) corresponding to the peaks of haemolytic activity were found. Refocusing of the basic haemolysin yielded additional acidic haemolysin but the converse did not apply. Electron microscopy of fractions of purified haemolysin showed two forms, one a fibrous, high molecular weight, 11.9S fraction of pI 5.0 and second a granular, lower molecular weight fraction, 4.9S with a pI of 9.5. The amino acid composition was similar to that reported by Yoshida (1963) and Heatley (1971) but histidine, arginine, proline and tyrosine were not detected (Table 2).

Table 2 Amino acid composition of Staphylococcal δ-haemolysin

Amino acid	Percent of total weight of amino acids				
	Yoshida (1963)	Kreger et al (1971)	Heatley* (1971)	Kantor et al (1972)	Fackrell & Wiseman(1976b)
Lysine	16.55	16.66	13.60	16.20	2.60
Histidine	0.41	-	tr	-	0.40
Arginine	0.94	-	-	-	2.80
Aspartic acid	12.53	13.46	10.90	13.60	16.20
Threonine	6.98	8.02	7.50	9.40	10.60
Serine	5.12	2.46	3.20	4.30	7.50
Glutamic acid	7.99	4.90	5.20	4.60	7.20
Proline	0.45	-	-	-	1.20
Glycine	3.73	3.60	8.10	6.20	6.30
Alanine	4.06	3.09	5.90	4.80	5.00
Half cystine	-	0.09	-	-	-
Valine	4.55	6.64	5.50	6.70	6.60
Methionine	4.88	4.56	4.30	4.00	-
Isoleucine	9.71	18.17	10.50	17.10	9.90
Leucine	7.41	6.04	6.30	4.90	5.90
Tyrosine	1.68	-	-	-	1.20
Phenylalanine	10.40	7.93	7.60	5.50	6.50
Tryptophan	2.61	3.30	4.00*	2.50	10.00**
Ammonia	1.63	1.08	7.40		

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

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

Table 3 Molecular weight estimates and sedimentation coefficients of staphylococcal delta-haemolysin

Method	$S_{20,w}$	Molecular Weight	Reference
Sedimentation velocity	6.1	74,000	Yoshida (1963)
Amino acid analysis		68,230	" "
Sedimentation equilibrium		72,000 - 150,000	" "
Sedimentation velocity	5.5		Kayser and Raynaud (1965)
Sedimentation velocity	1.4	12,000	Kayser and Raynaud (1965)
Gel filtration		> 200,000	Hallander (1968)
Sedimentation velocity	2.8, 9.8		Caird and Wiseman (1970)
Sedimentation velocity	4.9, 11.9 (1.9 in 0.05M NaOH)		Kreger et al (1971)
Sedimentation velocity	4.9		Heatley (1971)
Sedimentation velocity	6.04		Kantor et al (1972)
Sucrose gradient centrifugation (PBS)	6.19	191,000 61,850 (crude) 102,500 (purified)	" "
Sucrose gradient centrifugation (0.1% Tween 80)		21,000	" "
Sucrose gradient centrifugation (1% Tween 80)		< 10,000	" "
SDS gel electrophoresis		< 10,000	" "
Gel filtration (PBS)		195,000	" "
(6M guanidine HCl)		5,200	" "
Amino acid analysis		5,100	" "
Gel filtration		200,000	Fackrell and Wiseman (1976,b)

Using the same strain as Kreger et al (1971), Kantor, Temples and Shaw (1972) purified δ-lysin twenty-fold by adsorption to aluminium hydroxide gel and elution at pH 7.2 with 0.5M phosphate buffer. Polyacrylamide gel electrophoresis, IEF in the presence of 0.1% Tween 80 and gel filtration in 6M guanidine hydrochloride showed a homogeneous preparation, but further studies by gel filtration and of sedimentation velocity in the presence of increasing amounts of Tween 80, led Kantor's group to the conclusion that δ-haemolysin consisted of a basic subunit of molecular weight 21,000 daltons, which was itself a tetramer of individual polypeptide chains of molecular weight 5,100 - 5,200 daltons. Pentameric and decameric association of the basic subunit was assumed to yield polymers of molecular weight consistent with those determined in the absence of Tween 80 by sucrose gradient centrifugation, analytical ultracentrifugation and gel filtration. The amino acid analysis (Table 2) was consistent with a molecular weight of 5,200 daltons, and tryptic digestion yielded 7-8 peptides, also in agreement with the determined seven lysine residues. The model of Kantor et al (1972) provides a reasonable explanation for the conflicting published data on molecular weight, sedimentation coefficient, isoelectric point and heterogeneity of purified δ-haemolysin (Table 3).

Amino acid analyses of δ-haemolysin consistently show lysine, aspartic acid and isoleucine present in high amount, with histidine, arginine, proline, cysteine and tyrosine absent, or present as traces. Caird and Wiseman (1970) however, found the N-terminal amino acid of δ-haemolysin (strain E-delta) to be proline. Although the amino acid composition of delta-haemolysin is disputed there is general agreement that δ-lysin is a simple protein containing little (<1%) lipid, phosphorus and carbohydrate (Yoshida, 1963; Kreger et al, 1971; Heatley, 1971) and is sensitive to proteolytic enzymes (trypsin, pepsin, pronase, chymotrypsin).

The purification procedures of Wiseman and Caird (1968), Kreger et al (1971) and Kantor et al (1972) were compared by Lee et al (1976) who claimed that none of these methods yielded pure preparations of δ-haemolysin with the S. aureus strain 146P.

Biological properties. Delta-haemolysin, in contrast to α-, β- or γ-haemolysins, displays a wide haemolytic spectrum, and different species are more uniformly sensitive (Wiseman, 1970; Jeljaszewicz, 1972). All erythrocyte species are to some degree sensitive to δ-lysin (Gladstone, 1966) and while human red cells have in the past been regarded as the most susceptible indicator of haemolytic activity, recent work in this laboratory (Chao and Birkbeck, 1978) has shown the cod erythrocyte to be four to eight times more sensitive. Synergistic action between β- and δ-haemolysins has been demonstrated with sheep erythrocytes (Williams and Harper, 1947; Marks and Vaughan, 1950; Kreger et al, 1971) and human erythrocytes (Heatley, 1971). Kapral (1972) has also shown synergism between α- and δ-haemolysins on rabbit erythrocytes. It also has an injurious effect on a wide variety of cells in culture (Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967; Thelestam, Möllby and Wadström, 1973), leucocytes (Jackson and Little, 1957; Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967) and bacterial protoplasts and spheroplasts (Bernheimer, Avigad and Grushoff, 1968; Kayser, 1968; Kreger et al, 1971). Cellular organelles (lysosomes and mitochondria) (Bernheimer and Schwarz, 1964; Gladstone and Yoshida, 1967; Evans and Leck, 1969; Kantor et al, 1972; Kreger et al, 1971; Rahal, 1972) and lipid spherules (Freer et al, 1968; Kreger et al, 1971) are also disrupted by δ-haemolysin. The haemolysin also appears unique amongst bacterial

Table 4 Effects of delta-haemolysin in animals

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

Adapted from Wiseman, 1975

toxins in being able to alter water absorption in the ileum while being capable of increasing cAMP levels and being cytotoxic (Kapral et al, 1976; O'Brien and Kapral, 1976). This effect is mediated through a mechanism different from that initiated by cholera toxin (O'Brien and Kapral, 1977).

The question of toxicity of delta-haemolysin must be approached with caution. Numerous figures have been quoted in the past for the effects of the lysin in animals. Marks and Vaughan (1950) injected δ-haemolysin intradermally into man and observed slight erythematous lesions which disappeared within 48 hours. Larger amounts injected into rabbit or guinea-pig skin induced indurated lesions which in the guinea pig became necrotic. Similar findings were reported by Kreger et al (1971), who also found that the minimum lethal doses of δ-haemolysin for mice and guinea pigs were 110 mg/kg and 30 mg/kg respectively. Table 4 shows the effects of delta-haemolysin in animals determined by previous authors.

It is important to bear in mind, however, that any other contaminating haemolysin liable to be present in these preparations will most probably be α-haemolysin, and at the high levels of δ-haemolysin quoted in Table 4, sufficient alpha-haemolysin could be present to cause the pathological changes attributed to δ-haemolysin.

Mode of action. Several investigators have expressed the opinion that surface activity of the haemolysin accounts for lysis of sensitive erythrocytes (Bernheimer, 1970, 1974; Heatley, 1971; Kreger and Bernheimer, 1971; Rahal, 1972). Kreger et al (1971) suggested surface activity for the lysin and Rahal (1972) compared the behaviour of lysin with that of the detergents Triton X-100 and sodium deoxycholate.

Thelestam et al (1973) found kinetics of release of ^{3}H-nucleosides from tissue culture cells by δ-haemolysin to be similar to that caused by Triton X-100, while Bernheimer (1974) has compared the activity to that of the surfactin of Bacillus subtilis. Freer and Arbuthnott (1976) prefer mellitin the principal toxin of bee venom, as a more accurate parallel, but recent surface activity studies by Colacicco et al (1977) showed that unlike mellitin, δ-haemolysin does not resume the α-helix conformation on going into film phase from the extended chain in 6M urea, and was more versatile in structural dynamics and more surface active than δ-toxin. Wiseman and Caird (1968) and Wiseman (1970) have suggested an enzymatic mode of action, due to the release of water-soluble organic phosphorus from phospholipid extracts of mammalian erythrocytes. They found that organic phosphorus was liberated from the erythrocyte in direct proportion to erythrocyte sensitivity. Phosphatidylinositol was postulated as the substrate on the erythrocyte but this remains to be confirmed.

Studies by Low and Finean (1976) described the action of a phosphatidylinositol-specific phospholipase-C produced by S. aureus, distinct from δ-haemolysin which, while of low activity against the purified phospholipid hydrolysed 75% of the phosphatidylinositol in ox, sheep and cat erythrocyte ghosts. More recently Low and Finean (1977) found that in intact erythrocytes phosphatidylinositol was not significantly hydrolysed, suggesting that at least 75-80% of the phosphatidylinositol was located at the inner leaflet of the membrane. Such a location would render it inaccessible for involvement in Wiseman's scheme or, alternatively, Wiseman's findings might be explained by the presence of this enzyme in

small amounts in his δ-haemolysin preparation. Delta-haemolysin coupled to Sepharose retained haemolytic activity suggesting that only superficial contact between haemolysin and erythrocyte is required for lysis (Lee and Haque, 1976).

Inhibitors of δ-haemolysin

Inhibition of delta-haemolysin by mammalian sera

Marks and Vaughan (1950) first reported that the action of delta-haemolysin is inhibited by serum from man, sheep, ox, rabbit and guinea-pig. In addition, a more transient lesion was observed in guinea-pigs given an intradermal injection of delta-haemolysin in horse serum, than with delta-haemolysin in a corresponding amount of saline. Salting out the serum with ammonium sulphate revealed inhibitory activity in both the albumin and globulin fractions. The results of Jackson and Little (1958) suggested a general capacity of serum proteins to inhibit δ-haemolysin, globulins being the most inhibitory of the fractions studied. They noted, however, that the whole globulin fraction, considered to contain all the antibodies, was less inhibitory than two globulin sub-fractions, IV-1 and IV-4 (Cohn et al, 1946), which normally do not contain antibodies. Gladstone and Yoshida (1967) found that all Cohn fractions were inhibitory to delta-haemolysin, especially fractions I, III and IV (fibrinogen, β- and α-globulins respectively), and that sera yielded a precipitin line with δ-haemolysin. On immunoelectrophoresis this occurred in the α-globulin region (Kantor et al, 1972). However, contrary to Marks and Vaughan (1950) and Gladstone and Yoshida (1967), Kapral (1972) found no inhibitory activity with serum albumin.

A study by Maniar, Weslake and Warner (1967) which showed variable amounts of inhibitory activity in the sera of fifty-three individuals prompted Donahue (1969,a) to investigate the phenomenon more closely. The nearly constant levels he found in 105 individuals and its occurrence in foetal calf serum (Donahue, 1969,b) suggested that the inhibitor was not antibody. In view of the report by Kapral (1972) that δ-haemolysin is inhibited by phospholipids, this suggested a role for serum lipid in the inhibition, although a systematic and detailed investigation into the nature of the serum inhibitor has hitherto not been done.

Inhibition of delta-haemolysin by phospholipids

Delta-haemolysin is inhibited by many phospholipids, although conflicting reports are found in the literature. Whereas Gladstone and van Heyningen (1957) and Caird and Wiseman (1970) found cholesterol to be inhibitory, this was not found by Gladstone and Yoshida (1967), Kreger et al (1971) and Kapral (1972). Similarly, lecithin was reported as non-inhibitory by Gladstone and Yoshida (1967) but Kreger et al (1971) and Kapral (1972) found that it inhibited δ-haemolysin. The most detailed study of the action on delta-haemolysin by phospholipids is that of Kapral (1972), following a search for a specific inhibitor of delta-haemolysin in mixtures of δ- and α-haemolysins. A summary of his and other workers' results is shown in Table 5. Only natural phospholipids were inhibitory; synthetic phospholipids and egg lecithin in which the fatty acids were saturated by reduction failed to inhibit delta-haemolysin.

Kapral (1972) concluded that the minimum inhibitory unit was

Table 5 Inhibition of δ-haemolysin by phospholipids

Inhibitory phospholipids	Reference
Cholesterol	Gladstone & van Heyningen, 1957; Caird & Wiseman, 1970.
Phosphatidyl choline (lecithin)	Kreger et al, 1972; Kapral, 1972
Phosphatidic acid (natural)	Kreger et al, 1971; Kapral, 1972
Phosphatidyl serine	" " " "
Phosphatidyl inositol	" " " "
Cardiolipin	" " " "
Sphingomyelin	" " " "
Phosphatidylethanolamine	" " " "
Non-inhibitory agents	
Cholesterol	Gladstone and Yoshida, 1967; Kreger et al, 1971; Kapral, 1972
Myristic acid	Kapral, 1972
Palmitic acid	"
Palmitoleic acid	"
Stearic acid	"
Dipalmitin	"
Tripalmitin	"
Choline	"
Phosphocholine	"
Glycerol	"
Glycerol phosphate	"
Phosphatidyl choline (synthetic)	"
Phosphatidyl choline (egg, catalytically reduced)	"
Phosphatidyl ethanolamine (synthetic)	"
Cardiolipin (synthetic)	"
Albumin (human sera)	"
Inhibitory agents: 21-23 C fatty acids	Kapral, 1976
Stimulatory agents: 13-19 C fatty acids	" "

phosphatidic acid, other components attached to this unit did not significantly augment or diminish efficacy, and he suggested that phospholipids inhibit delta-haemolysin by interfering with its binding to the erythrocyte membrane.

Recently, Kapral (1976) observed that addition of saturated, straight chain fatty acids of thirteen to nineteen carbons increased the activity of delta-lysin, whereas those with twenty-one to twenty-three carbons were inhibitory. Palmitic acid was the most stimulatory of the fatty-acids, but simultaneous addition of egg lecithin competitively antagonised the stimulatory effect.

Other inhibitory systems involving phospholipids and human serum

The literature cites a wide range of inhibitory processes involving human serum in which a role for phospholipids have been implicated. Many viral haemagglutinins are inhibited either by phospholipid or by particular serum lipoproteins. Serum β-lipoprotein has been shown to inhibit haemagglutination by rubella virus (Feldman, 1968; Laufs and Thomasen, 1968; Haukenes and Aasen, 1971; Chang and Weinstein, 1972; Shortridge, Biddle and Pepper, 1972), and Shortridge and Ho (1974) reported that togavirus haemagglutinins were inhibited by β-lipoprotein.

As in the case of delta-haemolysin and its inhibitor, the evidence for the inhibitor of arbovirus haemagglutination is inconsistent. Porterfield and Rowe (1960), Salminen (1962) and Gorman (1970) all claimed serum lipid to be responsible, but Nicoli and Acker (1965) and Bidwell and Mills (1968) implicated β-lipoprotein. Similarly, earlier work on rhabdoviruses such as rabies (Halonen et al, 1968; Kuwert et al, 1968)

and vesicular stomatitis virus (VSV) (Halonen, Nikkari and Toivanen, 1969) suggested the inhibitor of haemagglutination was lipid in nature, and Halonen, Toivanen and Nikkari (1974) attributed the role to lipoproteins. Strains A, C and G of the β-haemolytic Streptococcus pyogenes produce two haemolysins: one oxygen-labile (SLO) and the other oxygen-stable (SLS). Both are inhibited non-specifically by certain serum components, and SLO is inhibited by cholesterol. Streptolysin S closely resembles S. aureus δ-haemolysin in terms of cytotoxicity, biological activity and lack of antigenicity (see below). Stollerman, Bernheimer and MacLeod (1950) found that serum inhibitory titres were not elevated during a streptococcal infection, suggesting a non-antibody system. Since lecithinase abolished inhibitory activity, Stollerman suggested a phospholipoprotein complex to be the inhibitor.

Immunogenicity of delta-haemolysin

The question of the antigenicity of δ-haemolysin has been raised on many occasions. The ability of normal sera to neutralise the haemolytic activity has been observed frequently and since most normal sera have been shown to produce a precipitin line on gel diffusion against δ-haemolysin and to neutralise haemolytic activity, results with sera of immunised animals are difficult to interpret. Rusakova (1970) reported the production of an antigenic toxoid of δ-haemolysin produced by formolisati which induced specific antibodies to the toxin. Elek (1959), McLeod (1963), Kayser and Raynaud (1965), Caird and Wiseman (1970) and Lochmann and Vymola (1974) all reported the production of specific antibodies to δ-haemolysin, although in no instance was there evidence presented that the non-specific

serum inhibitor had been removed. On the other hand, Gladstone and Yoshida (1967), Plommet and Bouillane (1966), Hallander (1968) and Kantor et al (1972) have all been unable to demonstrate an immune response to δ-haemolysin. However, Fackrell and Wiseman (1974) obtained γ-globulin fractions of serum from rabbits immunised intensively with 16 injections of δ-haemolysin which yielded specific quantitative precipitin curves with δ-haemolysin and a single precipitin line in diffusion against purified δ-haemolysin which in blood agar coincided with the edge of the lysis zone. However, Kapral (1976) argued that Fackrell and Wiseman's findings did not prove the antigenicity of delta-lysin. Preimmune sera will precipitate δ-haemolysin (Kantor et al, 1972), and since this reaction appears to result from combination with a substance other than a globulin, Kapral maintains that no proof was given that the precipitate consisted of δ-haemolysin and specific antibody. Recent work by Heatley (1977), however, demonstrated that antibodies to δ-haemolysin could be raised in rabbits. Further observations on the antigenicity of δ-haemolysin are included with this thesis (Birkbeck and Whitelaw, 1976).

Other inhibitory systems in serum

Although not extensively documented, there are factors in serum, some of nonimmunoglobulin nature, involved in host defence, which are capable of precipitation reactions. C-reactive protein (so-called because in the presence of Ca^{2+} it reacts and precipitates with C-substance, a pneumococcal polysaccharide (Tillet and Francis, 1929)) is a protein not normally found in circulating plasma of humans but which appears early in the course of many infections, following inflammatory conditions or where there is tissue damage (Raffel, 1960). Known as an "acute phase protein," its appearance indicates an inflammatory reaction somewhere in the body.

There exists in ruminants the protein conglutinin (Bordet and Streng, 1909), which is antigenically unrelated to the immunoglobulins, and which causes clumping of complement-coated cells. The determinant is a mannose peptide, in bound C_3 which has reacted with C_{3b} inactivator (Lachmann and Müller-Eberhard, 1968) and binding requires Ca^{2+} ions. On the other hand, immunoconglutinins (auto-stimulated antibodies to hidden determinants in the complement molecule exposed on fixation) occur naturally in most mammals, and their reactivity is predominantly directed against bound C_3. They are largely of the IgM class, though immunoconglutinins of other immunoglobulin classes can be detected (Henson, 1968). The mechanism involved in this protection is likely to be the aggregating effect on complement-coated particles, thereby reducing the active invading dose. Another serum protein which could be implicated in non-specific reactions is the initiating factor involved in initiation of the alternative pathway of complement activation (Müller-Eberhard, 1976).

Serum proteins

Since a large proportion of this thesis will be devoted to serum proteins and their separation and identification, the characterisation of serum proteins will be briefly discussed. Blood plasma is the viscous medium through which the erythrocytes and leucocytes are transported and the tissues nourished: serum is the fluid left after the removal of a blood clot, and is similar to plasma except for the absence of proteins of the blood clotting system. As components of a metabolically dynamic system, the plasma proteins fluctuate in disease, and their determination is important in the clinical laboratory. For a general review on plasma proteins see Putnam (1975).

The identification, characterisation and separation of plasma proteins dates from the development of analytical ultracentrifugation by Svedberg and moving-boundary electrophoresis by Tiselius. Tiselius identified four components - albumin, and the three globulin bands termed alpha, beta and gamma, and Tiselius and Kabat (1939) demonstrated that antibodies were γ-globulins. Electrophoretic techniques have been developed to include paper electrophoresis (Cremer and Tiselius, 1950), immunoelectrophoresis (Grabar and Burtin, 1960), crossed-electrophoresis (Laurell, 1965) and the lipoprotein methods of Peeters and Blaton (1968). Two-dimensional electrophoresis of serum in starch gel allowed 22 zones to be resolved (Poulik and Smithies, 1958) and led to the discovery of genetic variants of haptoglobin and transferrin.

Plasma proteins have proved too numerous and varied (Table 6) to be identified and quantitated by any single method and a combination of procedures is required. Precipitation with cold ethanol or other solvents under rigidly-controlled conditions was developed by Cohn et al (1950), and precipitation by ammonium sulphate solutions has been widely exploited (Cohn and Edsall, 1942). The immunoelectrophoretic techniques developed by Grabar and Williams (1953, 1955) and Grabar and Burtin (1960) allowed identification of 30-35 components in serum. A summary of the different patterns obtained by various electrophoretic techniques is presented in Fig 1.

A further major advance in resolution of serum proteins was made by Ornstein (1964) and Davis (1964) with the introduction of polyacrylamide gel electrophoresis. A total of more than 65 proteins can be distinguished by this method.

Figure 1

Schematic representation of the electrophoretic pattern of normal human serum in pH 8.6 buffer as obtained by four methods: (A) Tiselius or free boundary electrophoresis, (B) paper electrophoresis, (C) starch gel electrophoresis and (D) immuno-electrophoresis. The vertical arrow indicates the starting point in each case. IgM remains in the starting slot in starch-gel electrophoresis but moves to the γ- or β-range in other methods.

(Modified from Putnam, 1973)

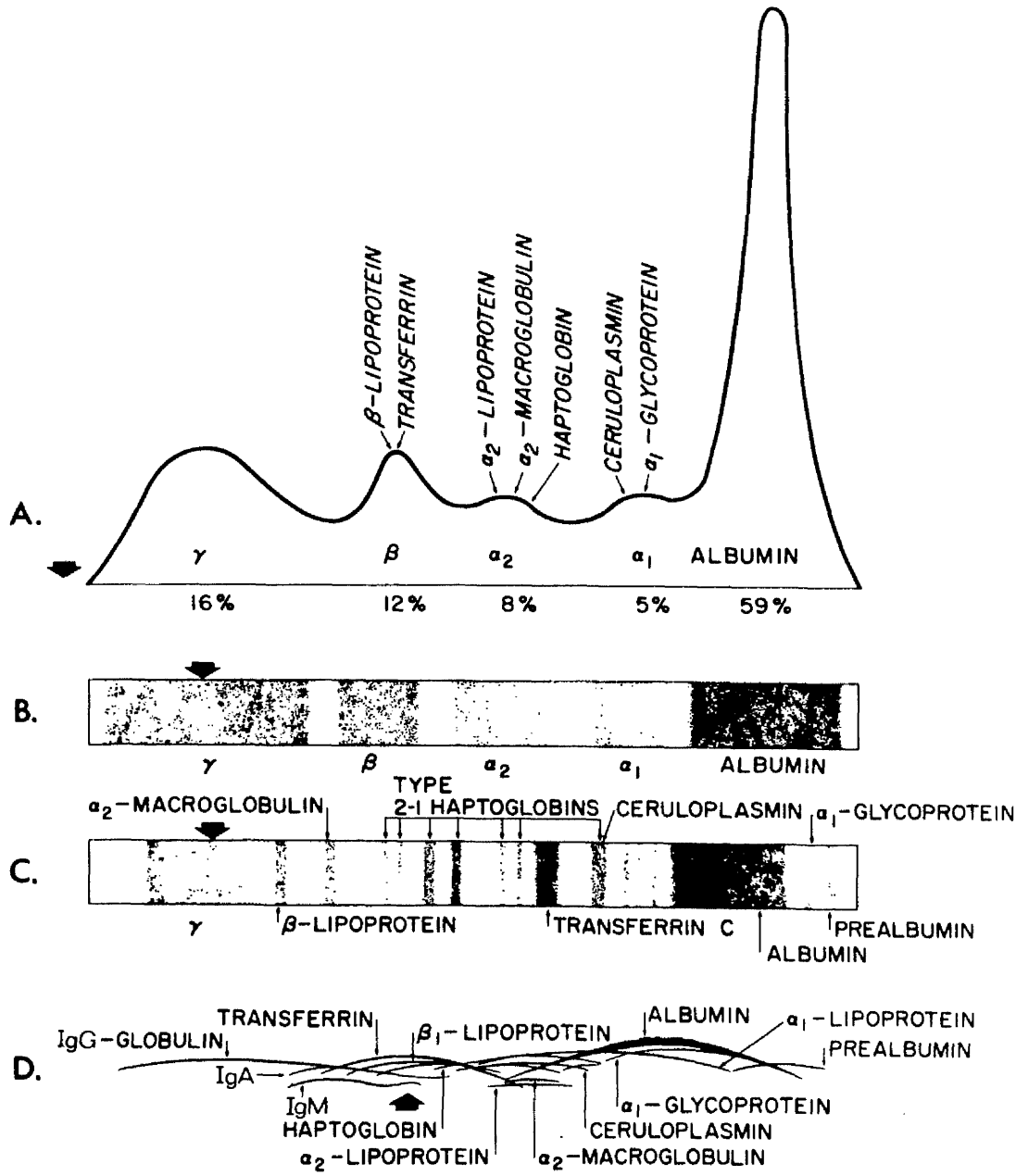

A.
β-LIPOPROTEIN
TRANSFERRIN
α2 - LIPOPROTEIN
α2 - MACROGLOBULIN
HAPTOGLOBIN
CERULOPLASMIN
α1 - GLYCOPROTEIN
γ
β
α2
α1
ALBUMIN
16%
12%
8%
5%
59%
B.
γ
β
α2
α1
ALBUMIN
C.
α2 - MACROGLOBULIN
TYPE
2-1 HAPTOGLOBINS
CERULOPLASMIN
α1 - GLYCOPROTEIN
γ
β-LIPOPROTEIN
TRANSFERRIN C
PREALBUMIN
ALBUMIN
D.
IgG - GLOBULIN
TRANSFERRIN
β1 - LIPOPROTEIN
ALBUMIN
α1 - LIPOPROTEIN
PREALBUMIN
IgA
IgM
HAPTOGLOBIN
α2 - LIPOPROTEIN
α1 - GLYCOPROTEIN
CERULOPLASMIN
α2 - MACROGLOBULIN

Table 6 Molecular weights and normal concentration ranges of human plasma proteins

Protein	Molecular Weight	Normal Range (mg/100 ml plasma)
Prealbumin (thyroxine-binding	61,000	15-35
Albumin	65,000-69,000	3500-5000
$\alpha_1$lipoprotein		
HDL_2	435,000	34-117
HDL_3	195,000	217-270
apoLp-Gln-II-lipoprotein	17,380	
α_1-acid glycoprotein	44,100	60-120
α_1-antitrypsin	45,000-55,000	200-400
Retinol-binding protein	21,400	4-6
Gc-globulin	50,000	30-55
Haptoglobin (Hp 1-1)	100,000	30-190
α_2-macroglobulin	820,000	170-380
Caeruloplasmin	132,000-160,000	27-55
α_2-lipoproteins	5-20 x 10^6	150-230
β-lipoprotein (LDL)	3-20 x 10^6	280-440
Transferrin	76,500	200-300
Hemopexin (β_1B)	80,000	80-100
Complement components		
C1q	400,000	10-20
C1r	150,000	-
C1s	79,000	2-4
C2	117,000	1-3
C3 (β_1C)	185,000	80-140
C4 (β_1E)	240,000	20-50
C5 (β_1F)	175,000	5-15
C6	90,000	-
C7	110,000	-
C8	150,000	< 1
C9	79,000	< 1
C1 esterase inhibitor	104,000	15-35
Coagulation proteins		
Fibrinogen	341,000	200-500
Prothrombin	60,000	(5-10)
Plasminogen	143,000	20-40
Immunoglobulins: IgG	160,000	800-1800
IgA	160,000 + polymers	90-450
IgM	950,000	75-250
IgD	~ 190,000	10
IgE	~ 190,000	0.005-0.5

Serum comprises too complex a starting mixture for many high resolution techniques, and column chromatography is widely used to provide less heterogeneous sub-fractions of whole serum. Sober and Peterson (1954) and Sober et al (1956) developed cellulose derivatives with ionic exchange properties (DEAE-cellulose and CM-cellulose) and Tiselius, Hjerten and Levin (1956) described the use of calcium phosphate (hydroxyl-apatite) gels for fractionation of medium to high molecular weight proteins, elution being achieved in both cases by increasing ionic concentration to displace the adsorbed protein from the gel. Molecular exclusion chromatography with dextran or agar gels (Sephadex, Sepharose) has been widely used in protein separation and has been reviewed by Tiselius, Porath and Albertsson (1963).

The Serum Lipoproteins

The serum lipoproteins solubilise and transport cholesterol, glycerides and phospholipids through the serum from sites of lipid absorption and synthesis to sites of storage and catabolism (Langer, Strober and Levy, 1970). Four lipoprotein classes can be identified in serum by electrophoresis or according to their density: chylomicrons; very low density lipoprotein (VLDL, pre-β- or α_2-lipoprotein); low density lipoprotein (LDL, β-lipoprotein) and high density lipoprotein (HDL, α-lipoprotein). The structure and function of lipoproteins has been reviewed by Scanu and Wisdom (1972) and Morriset, Jackson and Gotto (1975) and their basic properties are shown in Table 7.

Chylomicrons. Chylomicrons transport glycerides of dietary (exogenous) origin from the intestine into the lymph. They are the largest lipo-

Table 7 Physical and chemical nature of human serum lipoproteins

	Density range	Particle size	Sf*	Composition per cent dry weight				
				Protein	Phospholipid	Cholesterol	Cholesterol esters	Triglycerides
α-lipoproteins (high-density lipoproteins)	1.063-1.210	70-100Å	-	45-55	∿ 30	∿ 3	∿ 15	5-8
β-lipoproteins (low-density lipoproteins)	1.006-1.063	100-300Å	0-20	20-25	∿ 22	∿ 8	∿ 35	∿ 10
Pre-β-lipoproteins (very low-density lipoproteins)	0.93-1.006	2000Å	> 20	2-13	10-25	3-8	6-16	50-80
Chylomicrons	< 0.95	> 2000Å	>400	0.5-2.5	3-15	1-5	1-7	79-95

From Masoro (1968).

*Sf refers to the rate of lipoprotein flotation in Svedberg units in a sodium chloride solution of 1.063 g/ml (26°C).

At this density, chylomicrons, VLDL and LDL float, while HDL does not.

proteins and consist predominantly of glycerides with smaller amounts of protein and phospholipid (Lossow et al, 1969).

Chylomicrons are produced in the intestinal mucosa in response to the ingestion of dietary fat and, in man, are normally absent in serum after an overnight fast. They pass to the lymph and enter the bloodstream via the thoracic duct; in the circulation their half-life is only 5-15 min. The site of catabolism is uncertain, but may be the liver. In animals, partial hydrolysis of chylomicron glycerides occurs in the environment of adipose tissue and skeletal muscle, the liberated fatty acids being metabolised or stored as esters (Havel, 1965), while remnants enriched with cholesterol, phospholipids and protein are taken up by the parenchymal cells of the liver (Redgrave, 1970).

Very low-density lipoprotein. This comprises a family of macromolecules with a broad Sf range (Table 7); the density of VLDL is related to the protein content (Gustafson, Alaupovic and Furman, 1965) and the highest density fraction comprises 13% protein (Lossow et al, 1969). Glycerides are the predominant lipids, with smaller amounts of phospholipid and cholesterol.

Synthesis of VLDL occurs predominantly in the liver (Ruderman et al, 1968) and also in the intestinal mucosa (Hatch et al, 1966; Ockner, Hughes and Isselbacher, 1969). The VLDL particles are a major transport vehicle for endogenous triglycerides. Through the action of lipoprotein lipase and subsequent triglyceride hydrolysis, VLDL is converted into smaller lipoprotein particles (LDL) enriched in cholesterol, phospholipid and protein (Levy, Bilheimer and Eisenberg, 1971), but it is

not established whether all VLDL are degraded into LDL and, conversely, whether all circulating LDL are of strictly VLDL derivation: also unresolved is whether liver and intestinal VLDL have the same metabolic fate.

Low density lipoprotein. This can be isolated by preparative ultracentrifugation (density range 1.006 to 1.063 g/ml) and has beta mobility in most electrophoretic media. The molecule is a quasi-spherical particle of molecular weight 2.1 - 2.6 million (Adams and Schumaker, 1969). Cholesterol and cholesterol esters account for 40-45% of LDL by weight with the remainder consisting of approximately 20-25% protein, 25% phospholipid, and 10% glyceride (Bragdon, Havel and Boyle, 1956); one-half to two-thirds of the plasma cholesterol circulates bound to LDL in a normal subject. In man, elevated serum LDL is positively correlated with increased incidence and severity of atherosclerosis (Frederickson and Sloan, 1972). In abetalipoproteinaemia, abnormalities in red blood cells and neural tissues are found suggesting that LDL and cholesterol are required for normal maintenance of some cell membranes (Frederickson, Gotto and Levy, 1972).

Reticuloendothelial cells, smooth muscle cells (Stein and Stein, 1967, 1975) and others take up LDL and inside the cell the cholesterol released by hydrolysis of cholesteryl ester regulates cholesterol metabolism. The main site of LDL catabolism may be the liver.

High density lipoproteins. These are the smallest of the lipoproteins with a density range of 1.063 to 1.21 g/ml (Table 7) and α-mobility on electrophoresis. High density lipoproteins have been subdivided into

two classes, HDL_2 (density 1.085 - 1.12) and HDL_3 (1.12 - 1.21); with increasing density, the relative amount of protein and the ratio of esterified to free cholesterol increases, while the total cholesterol decreases (Glomset et al, 1966). The function of HDL is intimately associated with that of the plasma enzyme, lecithin-cholesterol acyl transferase, which catalyses the esterification of cholesterol (Glomset, 1968); HDL is thought to act as a scavenger for endogenous cholesterol arriving in the bloodstream (Glomset, 1972). Such a role is supported by the observation that subjects with Tangier disease, who have a marked deficiency of normal HDL, accumulate cholesterol esters in the cells of the reticuloendothelial system (Frederickson, Levy and Lees, 1967).

The NMR spectra of the two major lipoprotein subclasses, LDL and HDL, are quite similar, and resemble those of lipoprotein-lipids sonically dispersed in water. The bulk of the lipids are not tightly bound to the apoprotein but are highly mobile, suggesting a micellar structure for LDL and HDL (Hamilton et al, 1974). For the LDL particle, it has been suggested that the surface is occupied by both protein and phospholipid, while neutral lipids are contained in the hydrophobic core (Pollard and Devi, 1971; Deckelbaum, Shipley and Small, 1977).

OBJECT OF RESEARCH

While previous work has shown that there is a fraction in human serum which is inhibitory to δ-haemolysin, conjecture as to its identity has not been supported by detailed experimental evidence.

The first objective of this investigation was to produce a sufficient quantity of purified δ-haemolysin and to develop a reproducible assay for measuring its inhibition by serum and serum fractions.

The second objective was to purify and identify the component(s) in human serum that inhibit δ-haemolysin and to investigate the mechanism of inhibition.

MATERIALS AND METHODS

A. Production of δ-haemolysin

1. Organism

S. aureus strain NCTC 10345 was used throughout this study. The organism was maintained by subculture on horse blood agar.

2. Production of crude staphylococcal δ-haemolysin

Haemolysin was produced by a modification of the method of Kreger et al (1971). The medium consisted of a dialysis diffusate of yeast extract with added casamino acids, glucose and vitamins (Appendix I).

For haemolysin production S. aureus NCTC 10345 was reconstituted from a freeze-dried ampoule and plated on to 10% (v/v) horse blood agar (Oxoid).

After overnight incubation at 37°C a haemolytic colony was seeded into 50 ml of pre-warmed medium contained in a 250 ml flanged Erlenmeyer flask. This was incubated at 37°C in an orbital shaking incubator (Gallenkamp, London) at a shaking speed of 150 r.p.m. After 18 hours the cells were harvested by centrifugation at 17,000 x g for 10 min. The cocci were resuspended in 7.5 ml of fresh medium and 1 ml of this suspension was used to inoculate 500 ml of prewarmed medium in a 2 litre Erlenmeyer flask. One ml of a sterile solution of antifoam (Appendix I) was added to prevent excessive foaming during the subsequent overnight incubation. A total of 6 such 2 litre flasks were processed at one time; this yielded 3 litres of crude haemolysin. Supernatant fluid containing crude staphylococcal δ-haemolysin was separated from the cells by centrifugation at 17,000 x g for 20 min.

3. Purification of staphylococcal δ-haemolysin

This was based on the method of Kreger et al (1971). Hydroxylapatite (Appendix III) was added to crude staphylococcal δ-haemolysin in the ratio 100 g/1500 ml and mixed by stirring for 48 hours at 4°C. The hydroxylapatite was then washed with 500 ml amounts of 0.01M phosphate buffer, pH 6.8 by centrifugation until the supernatant appeared only faintly coloured. The hydroxylapatite was then washed six times with 500 ml 0.4M phosphate buffer, pH 6.8, for 30 to 60 min each time, after which the adsorbed δ-haemolysin was released by washing the hydroxylapatite five times with 400 ml amounts of 1.0M phosphate buffer, pH 7.4, for 45 min each time. The pooled 1.0M phosphate buffer washes were dialysed at 4°C for 48 hours against distilled water, with two changes. The precipitate of insoluble delta-haemolysin which formed at this stage was removed by centrifugation at 47,000 x g for 20 min and the supernatant, containing soluble δ-haemolysin, was lyophilised, redissolved in 0.1M phosphate buffer and solid $(NH_4)_2SO_4$ (B.D.H., AnalaR) added to a final concentration of 70% (w/v) according to the nomogram of Dixon and Webb (1958). After gentle stirring at 4°C for 48 hours, the precipitate was harvested by centrifugation at 17,000 x g for 20 min, resuspended in 0.1M phosphate buffer, and dialysed against distilled water for 48 hours at 4°C. The dialysed solution was lyophilised.

B. Assay of haemolytic activity and haemolysin inhibitors

1. Erythrocytes

Rabbit blood was collected by bleeding from the marginal ear

vein of stock animals housed in this department. Sheep blood was removed by jugular venepuncture from sheep from the grazing stock of the Veterinary School, Glasgow. Cod blood was obtained by bleeding, from the dorsal aorta, freshly caught fish from the Firth of Clyde. In all cases sodium citrate (3.8% w/v) was used as an anticoagulant.

In the early part of the investigation, citrated human blood group "A" obtained from the Haematology Department, Western Infirmary, Glasgow, was used as the source of human erythrocytes. In later stages, for reasons discussed elsewhere, blood freshly withdrawn from the antecubital vein of the author's left arm was used for human cells.

Whole blood samples, with the exception of cod blood, see below, were stored at $4^{\circ}C$ for not more than 5 days. Erythrocytes were washed 6 times with citrate dextrose saline (CDS, Appendix II) and 0.8% (v/v) suspensions in CDS were prepared as follows: 0.9 ml of washed packed erythrocytes was added to 100 ml CDS and the concentration adjusted so that a sample, after haemolysis with saponin powder (B.D.H., Poole, Dorset) gave an E_{545} of 0.8 ± 0.05 in a PYE Unicam SP500 spectrophotometer (Pye Unicam, Cambridge) with glass cells of 5 mm light path.

2. Storage of cod erythrocytes

Whole cod blood was mixed with an equal volume of 20% (v/v) dimethyl sulphoxide (DMSO, B.D.H., Poole, Dorset) in CDS, resulting in a 10% solution with respect to DMSO. After equilibration for 20 min at $0^{\circ}C$ the blood was rapidly frozen by dropwise addition to liquid nitrogen and the pellets stored in aluminium canisters in liquid nitrogen in a British Oxygen Co. CPVS10 liquid nitrogen refrigerator.

When required, pellets were removed, thawed in 10% (v/v) DMSO/CDS and washed in progressively decreasing concentrations of DMSO/CDS (10, 5, 2.5, 1% DMSO/CDS) and finally washed in CDS alone.

3. Doubling dilution titrations

Haemolysin titres were determined by making serial doubling dilutions of haemolysin in 0.4 ml CDS in a WHO tray and adding 0.4 ml of erythrocyte suspension to each well. Wells without haemolysin served as controls. Samples were incubated at room temperature (approx. 20°C) for 30 min when cod cells were used, and at 37°C for 30 min when mammalian cells were used. The 50% haemolysis end point was assessed visually. The dilution causing 50% haemolysis under these conditions was accepted as containing one Haemolytic Unit (HU_{50}). In titrations with sheep erythrocytes, 0.001M Mg^{2+} in Dulbecco's PBSA was used to allow detection of β-haemolysin.

4. Human serum

At the outset of this project, citrated human blood group A obtained from the Haematology Department, Western Infirmary, Glasgow, was used as the source of human plasma. Plasma was separated from the blood by low speed centrifugation (2,000 x g for 20 min), $CaCl_2$ (final concentration 0.005M) was added and the fibrin clot removed after incubation at 37°C for 30 min. Samples of 10 ml were stored at -20°C until required.

Freshly drawn group A blood from the author was used in the latter part of the project as a source of human serum; samples were not frozen but were stored at 4°C for not more than 5 days.

5. Doubling dilution inhibition titration

The inhibitory titre of serum samples was determined by making serial doubling dilutions of sample in 0.2 ml amounts of CDS in a WHO tray, and adding an equal volume of a standard haemolysin preparation containing 4 HU_{50}. After incubation at 37°C for 45 min, the tray was chilled to 4°C, and 0.4 ml of erythrocyte suspension was added to each well. After a further 45 min at room temperature the 50% inhibition end point was assessed visually. The dilution causing 50% inhibition under these conditions was accepted as containing one Haemolytic Inhibitory Unit (HIU). Two wells, one containing no serum sample, and one containing no haemolysin served as controls.

C. Physico-chemical properties of purified δ-haemolysin

1. Protein estimations

Protein estimations were done 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 continual measuring of E_{280} on an LKB Uvicord Ultraviolet Absorptiometer Detector Unit, Type 8303A linked to a control unit and chart recorder. The E_{280}^{1cm} of fractions from isoelectricfocusing troughs was measured manually.

2. Heat stability of δ-haemolysin

A solution of δ-haemolysin in CDS (100 µg/ml) in a 6 x $\frac{5}{8}$" test tube was immersed in a boiling water bath. The tube was stoppered

to minimise evaporation, and 0.2 ml aliquots of lysin were removed at various stages and titrated for haemolytic activity against cod erythrocytes.

3. Spectral analysis

The ultraviolet absorption spectrum was determined with a Pye Unicam SP800 spectrophotometer in 1 cm path length silica cells.

4. Polyacrylamide disc gel electrophoresis

i) Acid gels. This was performed by the acid gel system of Reisfeld, Lewis and Williams (1962) at pH 4.3 in 7.5% polyacrylamide gels. Electrophoresis was done at 6 mA per gel until the tracking dye (Pyronin Y, Difco) reached the final few millimetres, at which time the current was increased to 8 mA per gel. Migration was towards the cathode.

The gels were fixed and stained in 1% (w/v) amido-black in 7% acetic acid for 1 hr and then electrophoretically destained.

ii) Alkaline gels. The 7% gel electrophoresis technique (separation at pH 9.5) described by Davis (1964) was used. Electrophoresis was performed at 2 mA per gel, with migration towards the anode. The fixation and staining were done overnight in 1% (w/v) amido-black in 10% acetic acid and 50% methanol. Gels were rehydrated in 7% acetic acid and destained electrophoretically.

Densitometer traces of gels were made with a Joyce Loebl U.V. Polyfrac Scanner (Joyce Loebl & Co., Durham) and were recorded on a Kipp & Zonen chart recorder (Bryans Southern Instruments, Mitcham, Surrey) set at 5.0 V full scale deflection.

5. Isoelectricfocusing

i) Equipment. A horizontal trough isoelectricfocusing apparatus designed by Talbot and Caie (1975) was used with an LKB 3371 DC power supply.

ii) Procedure. All procedures were carried out in a 4°C cold room and the total power was restricted to a maximum of 0.28 watts. The apparatus was allowed to equilibrate at 4°C, 31.2 ml of 1% ampholines (pH 3.5 - 10; LKB Instruments, London) was added, and a pH gradient established by isoelectricfocusing for 24 hours at a potential of 600 volts. The sample (0.8 ml dialysed against 1% glycine) was introduced into the three centre troughs and focusing was continued at a potential of 800 V for 18 hr and finally at 1,000 V for a further 24 hr. After focusing, fractions, including any precipitated material, were removed by Pasteur pipette and retained for analysis.

iii) Analysis of fractions. The pH of each fraction was measured with a Pye Model 46A Vibret Lab pH Meter with the samples maintained at 4°C in a cooled water bath.

The optical density of each fraction was measured at 280 nm using a Pye Unicam Model SP500 spectrophotometer with silica micro-cells of 1 cm light path. To detect the presence of haemolysin, doubling dilution titrations were performed on each fraction with cod, rabbit and human erythrocytes.

6. Gel filtration

i) Equipment. A glass K70/26 chromatographic column (Pharmacia, Uppsala, Sweden) was used with an LKB Ultrorac 7000 fraction collector and LKB Uvichord ultraviolet spectrophotometer (LKB-produkter, Sweden).

ii) Procedure. All procedures were carried out in a 4°C cold room. Sephadex G-150 columns of 270 ml bed volume were poured and equilibrated with running buffer (Tris-buffered saline, TBS, Appendix II). Samples of δ-haemolysin were chromatographed with upward elution at a flow rate of not greater than 15 ml per hr, and 2.5 ml volume fractions were collected.

iii) Analysis of fractions. The optical density of peak fractions (shown by Uvicord) was measured at 280 nm using a Pye Unicam Model SP500 spectrophotometer with silica cells of 1 cm light path, and doubling dilution titrations were performed on those fractions showing E_{280}-absorbing activity to detect the presence of haemolytic activity against cod cells.

7. Amino-acid analysis

This was carried out by Dr. B. Manson, The Hannah Dairy Research Institute, Ayr. Three samples of δ-haemolysin (0.410, 0.412 and 0.413 mg) were hydrolysed with 6M HCl (4.0 ml in each case) for 24, 48 and 72 hr at 110° in vacuo in sealed Pyrex tubes. Acid was removed by lyophilisation and each residue dissolved in 3 ml citrate buffer pH 2.2. Automatic amino acid analysis was carried out following the procedure of Spackman, Stein and Moore (1958). Threonine and serine content were obtained by extrapolation to zero h hydrolysis and valine and isoleucine by extrapolation to infinity. For other amino acids the average of three values was taken. Excluding tryptophan and ammonia, the recovery from 0.410 mg sample was 0.234 mg. Tryptophan was determined spectrophotometrically by the method of Spies and Chambers (1949).

D. Estimation of other extracellular staphylococcal products

1. β-haemolysin

The procedure was as described for δ-haemolysin except that the diluent (Dulbecco's PBSA) contained 1.0 mM $MgSO_4$; sheep erythrocytes were used instead as indicators of haemolysis and titrations were incubated at 37°C for 1 hour and at 4°C for a further hour.

2. α-haemolysin

This was assayed as described for δ-haemolysin, using rabbit erythrocytes instead of cod erythrocytes, and titrations were incubated at 37°C for 30 min.

3. Lipase

Saline agar (1.5% (w/v) agar (Oxoid), 0.85% (w/v) NaCl) containing 0.01% (v/v) merthiolate 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 the haemolysin sample in saline were layered onto the surface of the agar and the tubes incubated at 37°C for 18 hours. Lipase activity was indicated by clearing due to hydrolysis of tributyrin.

4. Phosphatase

Phenophthalein phosphate (0.5 ml amounts of a 0.1% (v/v) solution) was dispensed in 4" x ½" test tubes; 0.5 ml volumes of lysin sample in 0.05M 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 0.1M 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 SP600 spectrophotometer in cells of 5 mm light path. Alkaline phosphatase from E. coli (Sigma, London) was used as a standard.

5. Coagulase

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

6. 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 haemolysin sample in 0.05M TBS, pH 7.4 were added and the tubes incubated at 37°C overnight. Fibrinolysin activity was indicated by lysis of the clot.

7. Egg yolk factor

An egg yolk extract was made from 20 ml of fresh hen egg yolk by mixing with 10 volumes of 0.05M 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 haemolysin sample in saline were added (0.2 ml) and the tubes incubated at 37°C for 18 hours. The degree of turbidity in the egg yolk extract was measured at 540 nm in an SP600 spectrophotometer in 0.5 mm cells. Phospholipase C prepared by the method of Smyth and Arbuthnott (1974) was used as a standard (to test for sensitivity).

8. 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).

Dilutions of test solution were made in hyaluronidase diluent (see Appendix II). To 0.5 ml volumes of standardised hyaluronic acid solution (see Appendix II), 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^{1cm}_{600} of each solution was read in an SP600 spectrophotometer against an appropriate diluent/acid albumin blank.

9. Protease

Phosphate agar (1.5% (w/v) (Oxoid), 0.05M 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 haemolysin 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, London) was used as a standard (to test for sensitivity).

10. 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 (0.05M phosphate, pH 7.5 with 0.85% NaCl) and was placed on top of a haemolysin sample in saline. The specimen was examined for dead leukocytes, as described by Woodin (1972).

11. Nuclease

Tris-HCl agar (1.5% (w/v) agar (Oxoid) 0.05M 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, London). 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 haemolysin sample: the plates were incubated at 37°C overnight and then flooded with 1.0M 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).

E. Protein separation

1. Ammonium sulphate precipitation of human serum

Human serum was precipitated by $(NH_4)_2SO_4$ in the following stepwise manner: serum in dialysis tubing (Visking, Scientific Instrument Centre, London) was dialysed at 4°C for six hours against 100 x its own volume of 10% saturated $(NH_4)_2SO_4$. The contents of the sac were centrifuged at 10,000 x g for 30 min, an aliquot of supernatant and pellets were removed for inhibitory assay, and the supernatant was returned to dialysis tubing for dialysis against 20% sat. $(NH_4)_2SO_4$. This procedure was repeated for 20%, 30%, 40%, 50%, 60%, 70% saturations of ammonium sulphate respectively, giving a supernatant and pellet sample at each concentration of $(NH_4)_2SO_4$ for determination of inhibitory activity. Such aliquots were dialysed overnight at 4°C against CDS to remove salts prior to titration.

Precipitation of serum at 60% saturation $(NH_4)_2SO_4$ alone was done by dialysis as above, except that after centrifugation the supernatant was dialysed for a second time at 60% saturation to facilitate maximal protein precipitation.

2. Ammonium sulphate precipitation at pH 5

This was based on the method of Freeman and Smith (1970). Serum was diluted with two volumes of 0.9% NaCl and adjusted to pH 5.0 by the addition of glacial acetic acid. Solid $(NH_4)_2SO_4$ was added to bring the concentration to 1.6M (40% saturation) and stirred overnight at 4°C; the solution was then centrifuged at 10,000 g for 1 hr. The supernatant was retained, and the deposit was dissolved in a known volume of water and subjected to a second precipitation with 40% saturated $(NH_4)_2SO_4$. The supernatants from two such treatments were pooled,

concentrated by ultrafiltration (UM05 membrane, exclusion limit 500 daltons; Amicon, U.S.A.) and adjusted to 50% saturation by the addition of solid $(NH_4)_2SO_4$. Fractionation at this concentration continued in the same manner.

The pellet material and supernatants at both concentrations were dialysed against CDS to remove salt prior to inhibitory titrations, and aliquots required for immunoelectrophoretic analysis were dialysed against barbitone buffer (Appendix II).

3. Ultrafiltration of human serum

A ten-fold dilution of human serum was subjected to ultrafiltration in a 50 ml cell (Amicon Corporation, U.S.A.) at 15 p.s.i. positive pressure of nitrogen. Aliquots were collected from material that passed through a particular membrane, prior to filtration of that material through a filter of greater pore size. Thus serum protein solutions comprising components of greater than approximately 500, 1000, 5,000, 10,000, 30,000, 100,000 and 300,000 daltons respectively were collected for analysis for inhibitory activity.

4. Gel filtration of human serum in Sephadex G-150

The apparatus and procedure were similar to those already described (page 43). The optical density of peak fractions (2.5 ml) was measured at 280 nm using a Pye Unicam Model SP500 spectrophotometer with silica cells of 1 cm light path, and doubling dilution titrations were performed on those fractions with E_{280}-absorbing activity to detect the presence of haemolytic inhibitory activity against cod cells.

Fractions for immunoelectrophoretic analysis were dialysed against barbitone buffer, pH 8.6.

5. Gel filtration of human serum in Sepharose

Pouring and equilibration of Sepharose 6-B chromatographic columns was done in the same way as for Sephadex G-150 columns, using the same accessory equipment. Separation was done with upward elution; the flow rate at no time exceeded 15 ml/hr, and 2.5 ml fractions were collected. From the chart record of the LKB Uvichord spectrophotometer peak fractions were identified and the optical density of peak fractions was measured at 280 nm using a Pye Unicam Model SP500 spectrophotometer with silica cells of 1 cm light path, and doubling dilution inhibition titrations were performed on fractions with E_{280}-absorbing activity. Peak fractions from the separation were dialysed against barbitone buffer, pH 8.6, at $4^{\circ}C$ for 12 hr prior to immunoelectrophoretic analysis.

6. Affinity chromatography of δ-haemolysin inhibitor

One gram of freeze-dried, CNBr-activated Sepharose 4-B (Pharmacia, Uppsala, Sweden) was swollen in, and washed with 200 ml 0.001M HCl on a sintered glass filter to remove the stabilising additives (dextran and lactose) present in the freeze-dried powder. The ligand (δ-haemolysin : 10 mg) was dissolved in coupling buffer (borate buffered saline : Appendix II) and mixed with the gel in a 6" x $\frac{5}{8}$" test tube for 18 hours at $4^{\circ}C$. Unbound material was removed by washing with three 50 ml volumes of coupling buffer and remaining active groups were reacted with 1.0M ethanolamine, pH 8 (Appendix II) for two hours at room temperature. Three washing cycles were used to remove non-covalently

absorbed protein and excess blocking reagent, each cycle consisting of a wash at pH 4 (acetate buffered saline; Appendix II) followed by a wash at pH 8 (borate buffered saline). The haemolysin-Sepharose conjugate was stored at 4°C until required.

1 ml of a 1/5 dilution of whole human serum was mixed with conjugate for 2 hr at 37°C with gentle stirring. After the slurry had separated on standing into two phases, a sample was removed from the supernatant (liquid phase) for immunoelectrophoretic analysis.

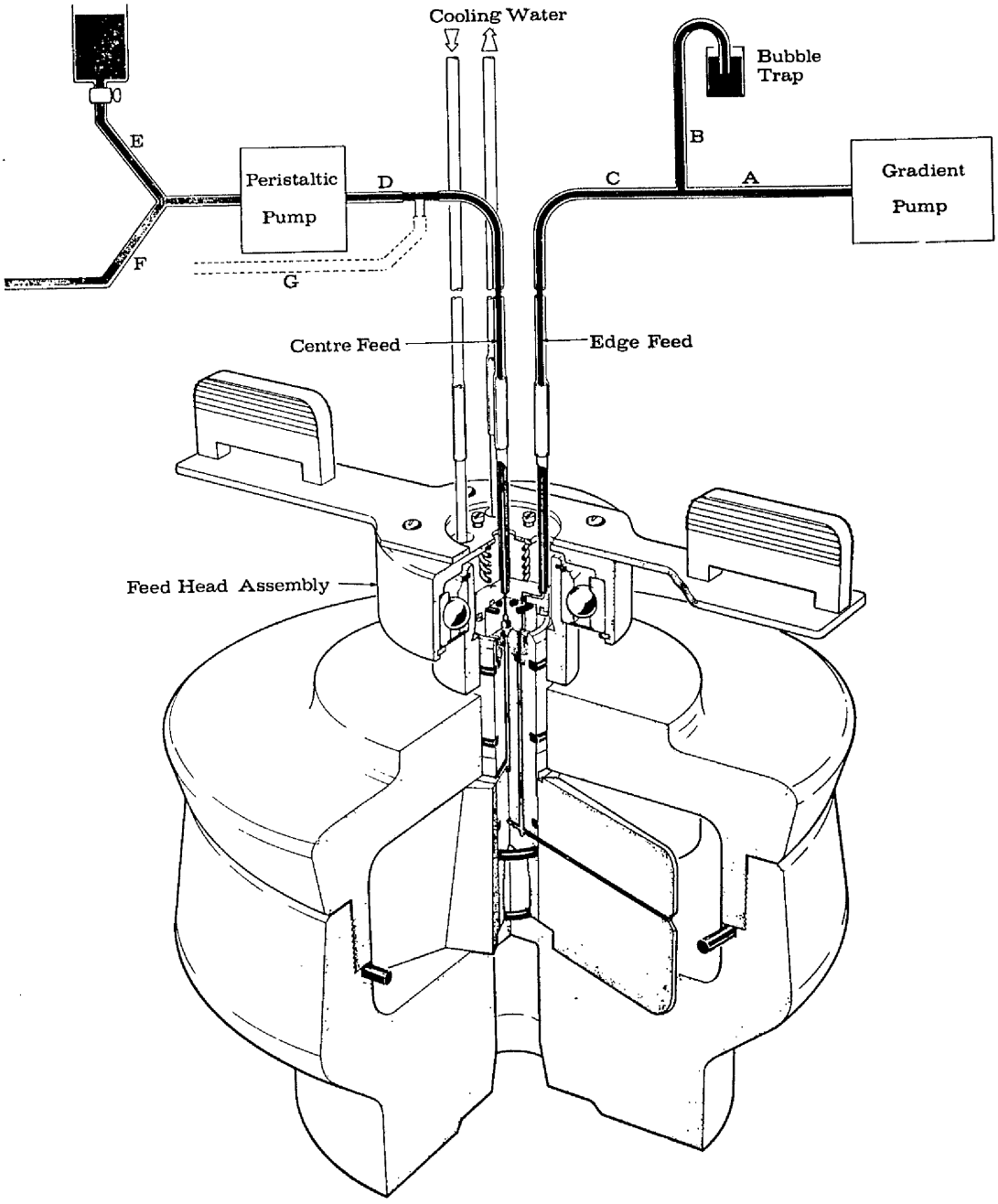
7. Zonal ultracentrifugation

This was done in an MSE BXIV titanium rotor in an MSE 65 superspeed centrifuge by the method of Wilcox, Davis and Heimberg (1971).

Loading procedure. The rotor was loaded with a KBr gradient (containing 350 μM EDTA) from density 1.00 to 1.28 using an MSE gradient mixer. Heavy solution (285 ml of 40% w/v KBr) and 365 ml light solution (350 μM EDTA) were mixed to form the gradient which was loaded into the rotor using a peristaltic pump via the edge feed (flow rate 10 ml/min) at a loading speed of 4,000 rpm. An exploded diagram of the loading assembly is shown in Fig 2. A fresh serum sample (25 ml) was adjusted to 40% (w/v) KBr and applied to the dense end of the gradient, via the edge feed, with a hypodermic syringe. Heavy solution was then applied to the edge of the rotor until light solution emerged from the central feed, ensuring no air was present to disturb the gradient. The centrifuge was then closed, evacuated and run at 44,000 rpm (147,000 x g) for 24 hr at 12°C.

Figure 2. Cut-away diagram of the BXIV titanium rotor

From MSE Technical Publication No. 49


Cooling Water
Bubble Trap
E
Peristaltic Pump
D
C
B
A
Gradient Pump
F
G
Centre Feed
Edge Feed
Feed Head Assembly

Unloading procedure. After decelerating the centrifuge to loading speed and releasing the vacuum, the loading assembly was re-fitted to the rotor. Using the same apparatus as before, heavy solution was applied to the edge of the rotor to displace the gradient at a rate of 10 ml/min out through the centre feed. The first 50 ml was collected in 5 ml aliquots, and thereafter in 10 ml amounts until the entire gradient was displaced from the rotor. Protein content of the eluate was monitored using an LKB Uvichord scanner as a preliminary screening measure. Individual samples were thereafter tested for a) protein content: E_{280} measurements were carried out on each sample; b) density: using a 1 ml volumetric pipette, aliquots of each sample were accurately weighed to determine the density gradient.

Pooling of samples. Because of the very large total volume of the centrifuged material (650 ml) samples were pooled according to the protein profile and concentrated by ultrafiltration in an Amicon cell through a PM 30 membrane. Such concentrated samples were then dialysed first against water to remove salt and then against barbitone buffer to facilitate electrophoretic analysis.

F. Immunological analysis

1. Antisera

The antisera used in this study and their sources are listed in Appendix VI.

2. Double diffusion tests

Double diffusion tests were based on the technique of

Ouchterlony (1958). Wells, 3 mm in diameter, were cut in 3 mm deep 1% Ionagar (Difco) in barbitone buffer, pH 8.6 (Appendix II) in a 5 cm diameter plastic petri-dish. A uniform depth of agar was ensured by pouring the plates on a levelling table (Shandon Southern Instruments, Camberley, Surrey). After introduction of test sample and antiserum to the wells, the plates were left at room temperature for up to seven days in a moist atmosphere (in a plastic container lined with moistened blotting paper).

3. Immunoelectrophoresis

Immunoelectrophoresis was carried out in 1% (w/v) agarose in barbitone buffer. A glass microscope slide (75 mm x 22 mm) was covered to a depth of 1.5 mm with 2.5 ml molten agarose and allowed to set. Two 3 mm diameter wells were cut 1 cm apart at a distance of 15 mm from the end of the slide. The wells were filled with sample containing bromophenol blue tracking dye and electrophoresis was performed at 80V until the dye was 15 mm from the anodal end of the slide.

For development of antigen-antibody precipitin arcs, a 60 mm x 3 mm broad trough was cut parallel to the direction of migration, between the wells, and filled with antiserum (Appendix VI).

4. Two-dimensional immunoelectrophoresis

This was based on the technique of Laurell (1965). The procedure for the first-dimension separation was as for immunoelectrophoresis (see above) except that only one well was cut in the agarose, and tracking dye was omitted from the test sample. After primary electrophoresis, the

gel was carefully removed from the slide with a scalpel, and transferred to one edge of a square glass plate (80 mm x 80 mm). The transferred gel was trimmed with a scalpel until the inward-facing edge was only 2 mm from the sample well and the electrophoresed proteins. The second dimension was prepared by adding 1 ml of antiserum to 9 ml agarose (5 ml of 2% (w/v) agarose plus 4 ml double strength barbitone buffer) at 48°C; after rapid mixing, the molten solution was pipetted onto the plate to a depth of 1.5 mm up to the primary separation gel, ensuring no overlap. When set, the separated proteins from the first dimension were electrophoresed at 30V into the antiserum-containing gel for 18 hours to form precipitin lines.

5. Washing and staining of gels

Gels were washed for two days in 1% (w/v) saline and dried under Whatman's No. 1 chromatography paper. Staining solutions are listed in Appendix VII.

6. Production of rabbit antiserum to an inhibitory fraction

The inhibitory material was prepared as an emulsion with Freund's complete adjuvant. This was done by dropwise addition of adjuvant to antigen, with homogenisation between successive additions, until the resulting emulsion was composed of equal volumes of the two components. The stability of the emulsion was deemed satisfactory if a droplet remained coherent when injected into a beaker of water. The rabbit was given four intramuscular injections, 0.2 ml, one each on days 1, 3, 5 and 11; this was followed by a course of intravenous injections, 0.2 ml being administered on days 50, 52 and 58, and 0.5 ml on days 72 and 75. The animal was exsanguinated 80 days after the start of the treatment.

7. Concentration of rabbit antiserum

The rabbit yielded 60 ml serum; 30 ml saturated $(NH_4)_2SO_4$ was added, bringing the serum to 33% saturation. After 2 hours' stirring at 4°C, the solution was centrifuged at 5000 x g for 30 min, and the supernatant decanted. The pellet was taken up in 20 ml Dulbecco's saline (Appendix II), adjusted to 33% saturation $(NH_4)_2SO_4$, and the precipitation procedure repeated as above. In all, three such cycles were performed. The third such pellet was dissolved in 10 ml Dulbecco's saline, dialysed overnight at 4°C against the same solution, and stored in 1 ml aliquots at -20°C until required.

G. Lipids and Lipoproteins

1. Polyacrylamide disc gel electrophoresis of serum lipoproteins

This was performed by the system of Naito et al (1973) at pH 8.9 in 3.6% polyacrylamide gels. Electrophoresis was done at 3mA per tube for about 35 min or until the fastest moving stained band (α-lipoprotein) was approximately 1 cm from the anodal end of the gel tube. The procedure is outlined in Appendix V.

2. Immunocore electrophoresis

Immunocore electrophoresis was done in the same polyacrylamide system as described by Naito et al (1973), with an immunocore electrophoresis kit (Linton Instrumentation, Harlow, Essex) and electrophoresis was performed under similar conditions of constant current, but for a longer period because of the longer electrophoresis tubes (Appendix V).

The central core of the gel was filled either with a 2 mg/ml solution of δ-haemolysin in agarose, or a 1:10 solution of antiserum (Appendix VI) in agarose. Gels were examined daily for the appearance of precipitation bands. A prestained human serum control served as an indicator of the relative positions of the lipoprotein species.

3. Gradient centrifugation of β-lipoprotein

This was done by the method of Havel, Eder and Bragdon (1955), by flotation at raised densities. The lowest density fractions from zonal ultracentrifugation were mixed with a stock solution of sodium chloride and potassium bromide to give a final density of 1.28 g/ml, and were centrifuged at 45,000 rpm at 8°C for 20 hr in an MSE swing-out rotor. Fractions of 1 ml were collected with the aid of a fraction collector (Ultrorac, LKB) through punctures at the bottom of 25 ml polycarbonate centrifuge tubes.

4. Delipidation of serum with organic solvents

Serum (2 ml) was extracted twice for 12 hr at 4°C with 50 ml diethyl-ether ethanol (3:1, v/v) under magnetic stirring. The lipid extract was isolated by centrifugation and purified by the method of Folch, Lees and Sloane-Stanley (1957), after evaporation under nitrogen to reduce the volume. The pooled extracts after washing were dried down in a rotary evaporator, the water bath temperature at no time exceeding 45°C, and after weighing were taken up in a known volume of chloroform.

5. 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 to a depth of 0.4 mm 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 containing conc. H_2SO_4 and were activated prior to use by heating in a drying oven at 110°C for 60 min. Plates were developed with chloroform:methanol:water (65:25:4 by vol) and the spots of the separated lipids were visualised by exposure to iodine vapour.

The lipids, and their sources, used in TLC and other experiments are listed in Appendix VI. Before use, a known amount of sample (50 µg) was evaporated to dryness under N_2, and resuspended in chloroform. Streak applications were then made on the plate.

6. Inhibition of δ-haemolysin by phospholipids

Each phospholipid was tested as a 0.5 ml volume containing 1 mg/ml. This was evaporated to dryness under nitrogen, and the lipid dissolved in 0.025 ml ethanol, and brought up to 0.2 ml with Dulbecco's saline. This solution contains 500µg phospholipid at a concentration of 12.5% ethanol. Inhibition titration was performed in the usual way with cod erythrocytes as an indicator of lysis.

7. PLC pretreatment of inhibitory phospholipids

B. cereus phospholipase C (E.C.3.1.4.3; Boehringer, Mannheim, Appendix VI) was used. Specific activity of the preparation was 400 U/mg (1 unit hydrolyses 1 µM lecithin/min at 37°C).

The enzyme was used in ten-fold excess calculated from the phospholipid content of normal human serum (Documenta Geigy, 2.09 mg/ml). An equal volume of serum (or phospholipid) was mixed with PBS or enzyme (1 U/ml) and incubated at 37°C for 30 min. Serial (0.2 ml) doubling dilutions were made in PBS, 6 HU50 δ-haemolysin (human erythrocytes) in 0.2 ml was added to each well, and mixtures incubated at 37°C for 30 min. Washed human erythrocytes (0.4 ml; 0.8% v/v in PBS) were then added, and the 50% endpoint assessed visually after 30 min at 37°C.

8. Proteolytic degradation of lipoproteins

An equal volume of enzyme (trypsin, 1 mg/ml or 62.5 μg/ml; papain, 1 mg/ml, Appendix VI) was added to substrate or PBS and incubated at 37°C for 30 min. Serial (0.2 ml) doubling dilutions were made in PBS, 6 HU50 δ-haemolysin (cod erythrocytes) in 0.2 ml was added to each well, and mixtures incubated at 37°C for 30 min. The trays were chilled to 4°C, washed cod erythrocytes (0.4 ml; 0.8% v/v in PBS) were added, and the 50% endpoint assessed visually after 30 min at 37°C.

RESULTS

Preparation of purified S. aureus δ-haemolysin

Selection of strain

Chao (1976) monitored haemolytic activity of several staphylococcal strains which had been observed to produce high levels of haemolysin not neutralisable by anti δ-haemolysin antiserum. By measuring the ratio of haemolytic titre against human and rabbit erythrocytes in culture supernatants, the relative amount of delta- and alpha-haemolysin produced was assessed. These observations showed strain NCTC 10345 to yield both the highest titre against human erythrocytes and the highest titre ratio, and this strain was therefore selected for production of delta-haemolysin.

Preparation and characterisation of S. aureus δ-haemolysin

Delta-haemolysin was purified from 2.5 litres of crude culture supernatant according to the method of Kreger et al (1971) using hydroxylapatite. The supernatants from each washing step were titrated for haemolytic activity against rabbit and cod erythrocytes (Table 8). Addition of hydroxylapatite to the culture supernatant removed virtually all haemolytic activity against cod erythrocytes, and all activity against rabbit erythrocytes. By washing the hydroxylapatite with 1.0M phosphate buffer 52.5% of δ-haemolysin was recovered (Fig 3). On dialysis against distilled water, 60 mg insoluble delta-haemolysin of specific activity 640 HU50/mg was removed. A further loss in activity occurred in the concentration steps prior to ammonium sulphate precipitation and lyophilisation (Table 8 and Fig 3). Concomitant with this loss was the elimination of haemolysin active on rabbit erythrocytes.

Figure 3 Summary of Purification Procedure

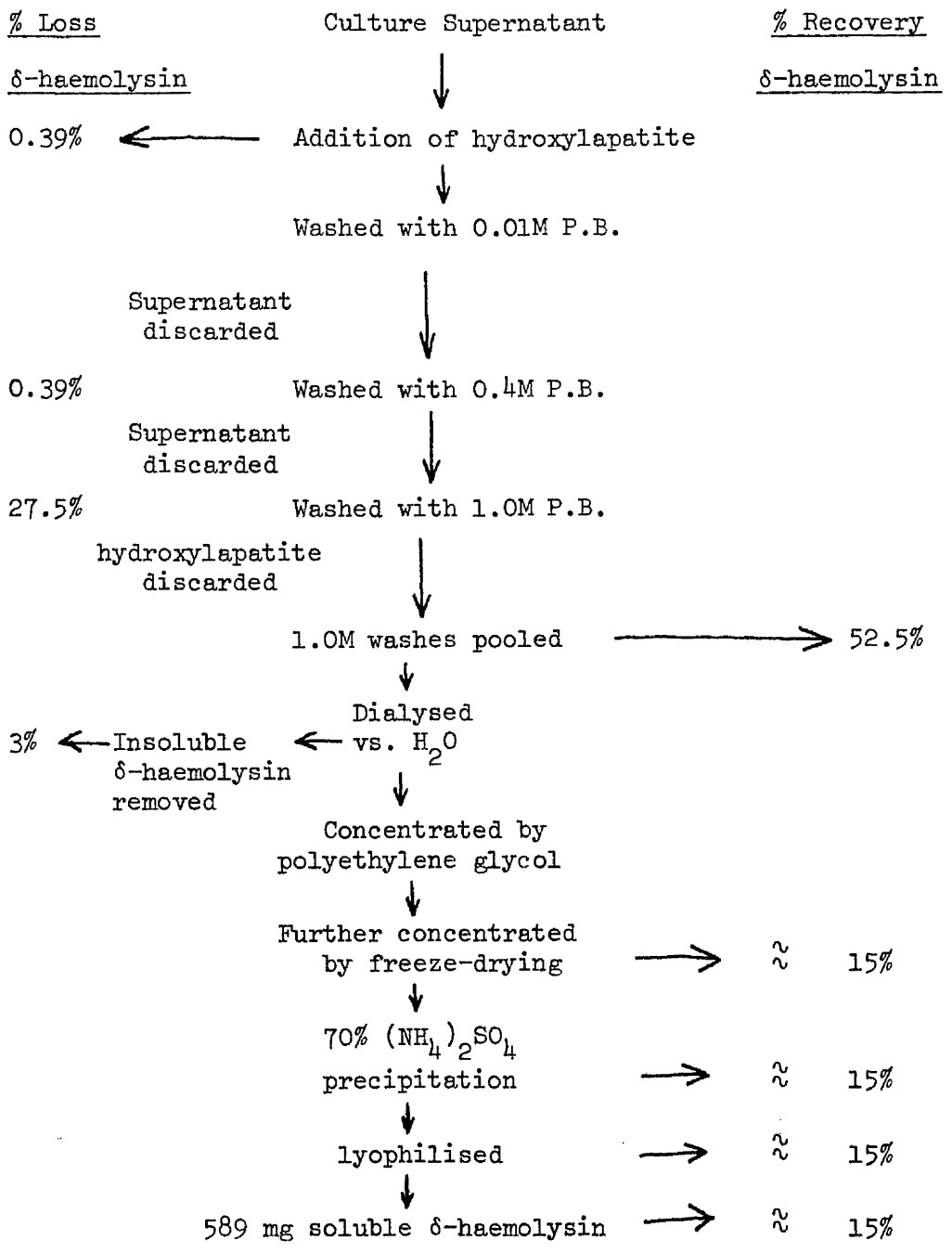


Table 8 Haemolysin activity in supernatant liquids from the purification of δ-haemolysin with hydroxylapatite

Fluid	Volume	Haemolysin titre (HU/ml) Rabbit erythrocytes	Cod erythrocytes	Total Activity (HU50) vs. cod RBC
Culture supernatant	2,500	64	512	1,280,000
Supernatant after addition of hydroxylapatite	2,500	0	2	5,000
0.01M PB* washings (bulked)	2,500	0	2	5,000
0.4M PB* washings				
Supernatant 1	500	8	256	128,000
" 2	500	8	256	128,000
" 3	500	8	128	64,000
" 4	500	2	32	16,000
" 5	500	2	32	16,000
1.0M PB* washings				
Supernatant 1	500	64	1024	512,000
" 2	500	32	128	64,000
" 3	500	16	128	64,000
" 4	500	16	64	32,000
		Total (1M washes)		672,000
Fluid prior to $(NH_4)_2SO_4$ precipitation	1250	0	128	160,000
Protein solution prior to freeze-drying	90	0	2048	184,000
Final lyophilised product:	589 mg	@ 320 HU50/mg		188,000

*P.B. ; phosphate buffer

A final lyophilised preparation of 589 mg soluble delta-lysin with a specific activity of 320 HU50/mg was obtained. This material was used in all subsequent experiments.

Ultraviolet spectrum of purified δ-haemolysin

The UV spectrum of δ-haemolysin (Fig 4) with absorption maxima at 278 nm and 291 nm and with a minimum at 250 nm was similar to that of Kayser and Raynaud (1965), Kreger et al (1971) and Chao (1976).

Disc-gel electrophoresis of purified δ-haemolysin

Electrophoresis of purified δ-haemolysin in polyacrylamide gels revealed a single broad diffuse band in acidic gels (pH 4.3) (Plate 1) and two bands in alkaline (pH 9.5) gels. Densitometer traces of gels of both systems are shown in Fig 5.

Sephadex gel filtration of purified δ-haemolysin

Upward elution in a G-150 Sephadex column at constant flow rate of 15 ml/hour yielded a single peak with molecular weight corresponding to approximately 200,000 daltons.

Isoelectricfocusing of purified δ-haemolysin

When δ-haemolysin was subjected to IEF in a broad pH gradient (pH 3.5 - 10.0), all haemolytic activity was found in a single peak (pI 4.5), which corresponded to the major peak of E_{280}-absorbing material (Fig 6). The haemolytic activity of this peak fraction was eight times greater versus cod erythrocytes than against human. It had no activity

Figure 4 Ultraviolet absorption spectrum of δ-haemolysin

The absorption spectrum of purified soluble δ-haemolysin (500 μg/ml) was measured in the Pye Unicam SP800 spectrophotometer in 1 cm path length silica cells.

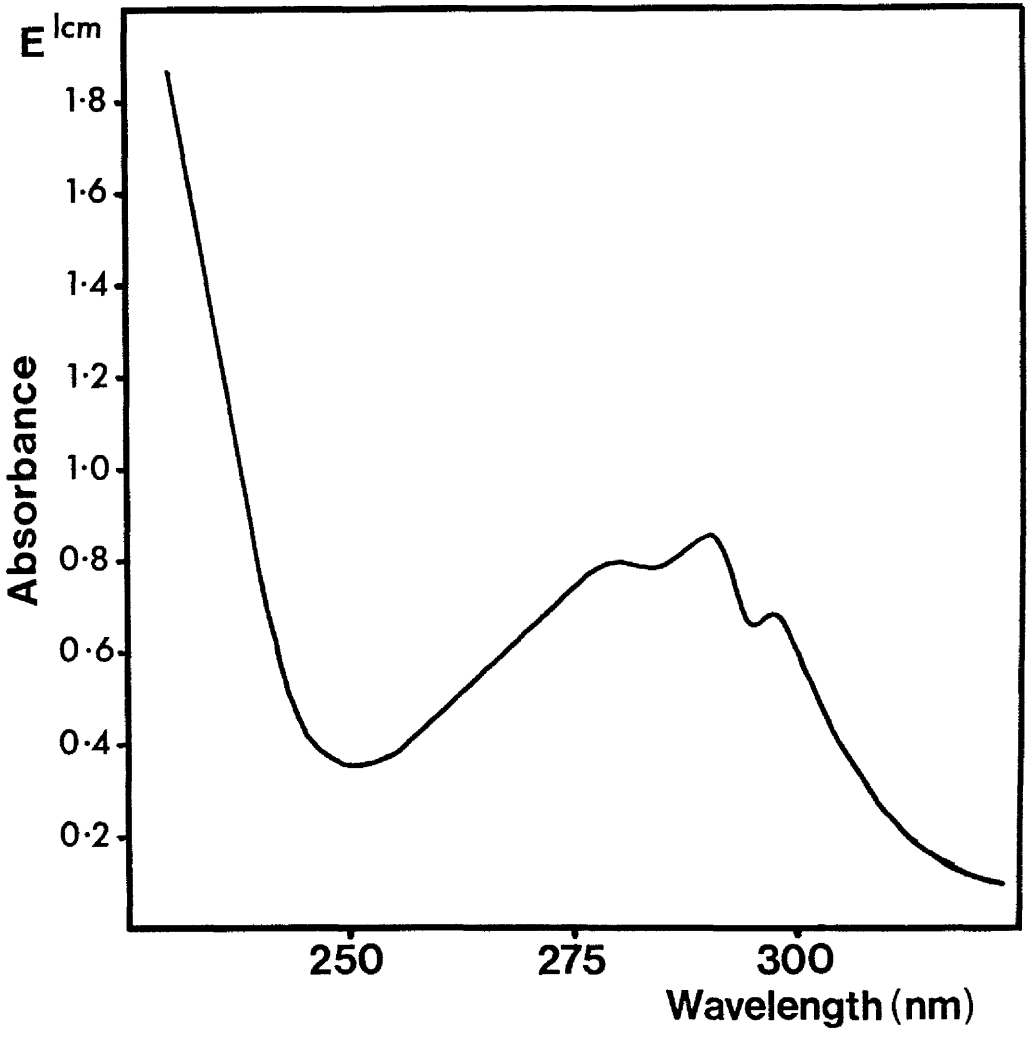

E 1cm
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Absorbance
250
275
300
Wavelength (nm)

Figure 5 Disc gel electrophoresis of purified delta-haemolysin

Densitometer traces

1. 100 µg soluble δ-haemolysin in the pH 4.3, 7.5% gel system of Reisfeld *et al* (1962).
   Migration is towards the cathode.

2. 100 µg soluble δ-haemolysin in the basic (pH 9.5) 7% gel system of Ornstein (1964) and Davis (1964).
   Migration is towards the anode.

SG : stacking gel

TD : tracker dye.

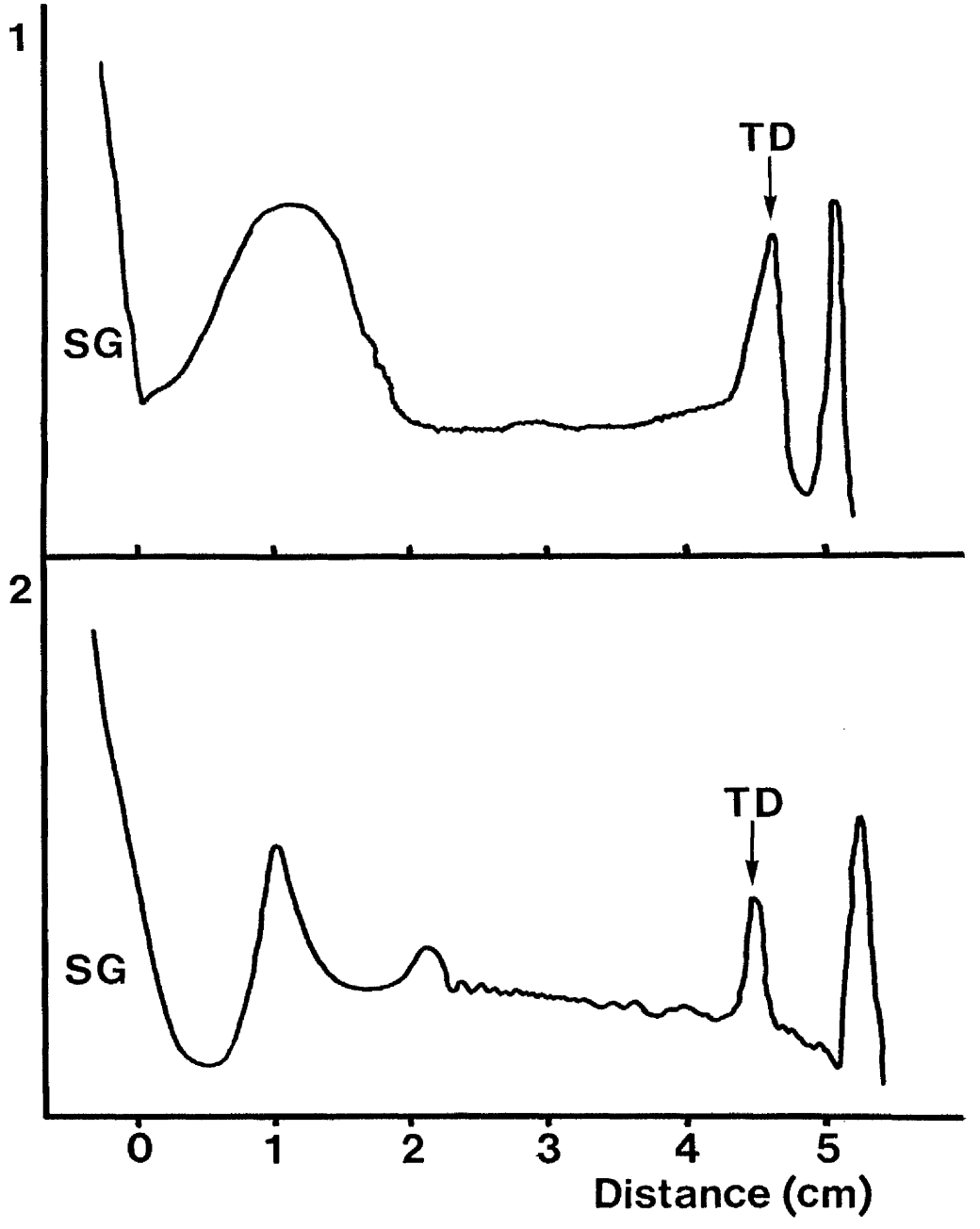
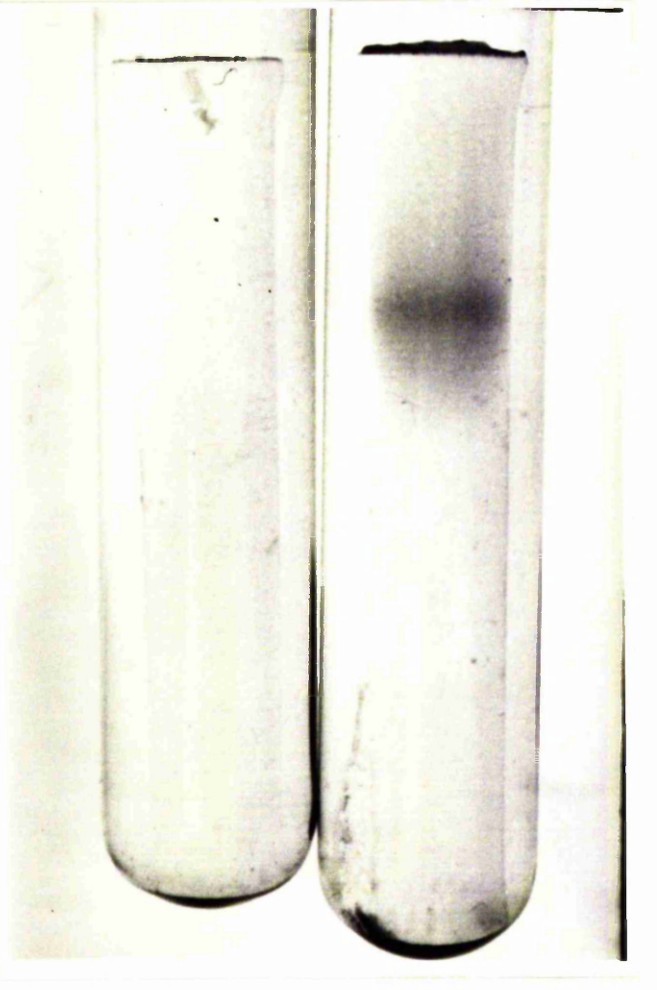

1
TD
SG
2
TD
SG
0
1
2
3
4
5
Distance (cm)

Plate 1 Acid polyacrylamide gel electrophoresis of δ-haemolysin

50 µg of δ-haemolysin were electrophoresed at pH 4.3 by the method of Reisfeld et al (1962).

Left hand gel : blank

Right hand gel : δ-haemolysin



against rabbit erythrocytes. Fractions with a pI greater than 6.0 had no haemolytic activity against rabbit erythrocytes nor any "hot-cold" haemolytic activity against sheep erythrocytes, indicating the absence of detectable quantities of α- or β-haemolysins.

Examination for other staphylococcal products

Purified δ-haemolysin was free from α- and β-haemolysin activity, and devoid of protease, coagulase, lipase, nuclease, leukocidin, hyaluronidase, phosphatase, fibrinolysin and egg-yolk activity at a concentration of 100 μg freeze-dried material/ml.

Amino acid composition of purified delta-haemolysin

Amino acid analysis was carried out by Dr. B. Manson; lysine, isoleucine, and aspartic acid were the predominant amino acids and histidine, arginine, proline, tyrosine and cysteine were absent (Table 9). Ammonia was not determined.

Heat stability of purified δ-haemolysin

The haemolysin proved very resistant to prolonged high temperature, and after $4\frac{1}{2}$ hr at 100°C, half of the original activity remained.

Assay of δ-haemolysin inhibitors

Inhibition of delta-haemolysin by human serum

In the early part of this study, sera used for inhibition analyses were obtained from the blood bank of the Western Infirmary,

Figure 6 Isoelectricfocusing of δ-haemolysin

Purified δ-haemolysin (4 mg) was analysed by isoelectric-focusing, using the apparatus of Talbot and Caie (1975), and the E_{280}, pH and haemolytic activity of each fraction assayed.

□- - -□ E_{280}

●·····● pH

▲------▲ haemolytic activity; 0.8% cod erythrocytes

■——■ 0.8% human "

◇——◇ 0.8% rabbit "

▽——▽ 0.8% sheep "

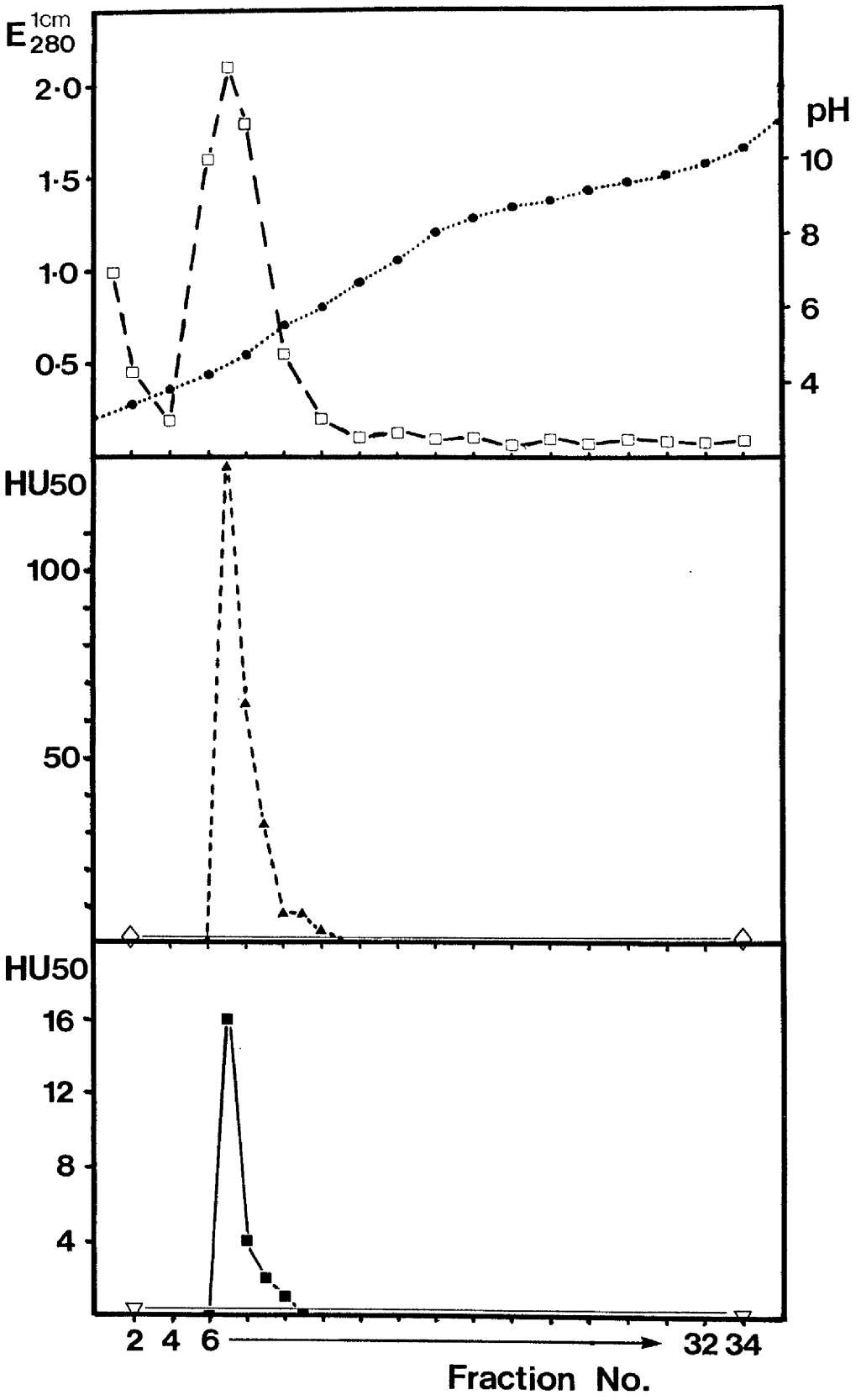

E_{280}^{1cm}
2·0
1·5
1·0
0·5
pH
10
8
6
4
HU50
100
50
HU50
16
12
8
4
2 4 6
32 34
Fraction No.

Table 9 Amino acid composition of staphylococcal δ-haemolysin

Amino acid	Percent of total weight of amino acids
Lysine	16.6
Histidine	-
Arginine	-
Aspartic acid	17.9
Threonine	10.9
Serine	2.9
Glutamic acid	4.8
Proline	-
Glycine	2.3
Alanine	2.4
Half cystine	-
Valine	7.1
Methionine	2.2
Isoleucine	19.6
Leucine	4.3
Tyrosine	-
Phenylalanine	5.2
Tryptophan	3.7

Glasgow. While all samples were inhibitory, with an inhibitory titre of between 256 and 1,000, in order to obtain some degree of uniformity of sample and eliminate individual variation, all later samples were obtained from the author after a 12-hour fast. Such samples invariably gave an inhibitory titre of approximately 1,000 in a doubling dilution titration.

Standardisation of the inhibitory assay

The conditions required for reproducible results in an inhibitory assay had to be such that the amount of δ-haemolysin added to the system was neither too great to be neutralised by an inhibitory sample nor insufficient to cause lysis of the cod red cell indicator. From the result of an experiment using a range of haemolysin concentrations against normal serum a concentration of 6 HU50/ml δ-haemolysin was chosen, and used in all subsequent titrations. At a lower concentration, there was insufficient haemolysin to cause lysis; on the other hand, too high a concentration of haemolysin might mask the activity of samples of low inhibitory titre. At 6 HU50/ml, the inhibitory titre of whole serum was between 512 and 1024 in a doubling dilution titration.

Preliminary studies on the nature of the inhibitor

Ammonium sulphate precipitation

To ascertain the concentration of $(NH_4)_2SO_4$ that would precipitate the inhibitory component of serum, a range of concentrations of $(NH_4)_2SO_4$ were tested, from 20% to 80% saturation. The results are shown in Table 10, and indicate that at a concentration of 70% saturation

Plate 2 Laurell immunoelectrophoresis of whole human serum

10 µl of whole human serum were electrophoresed against anti-whole human serum antiserum (Wellcome) by the method of Laurell (1965).

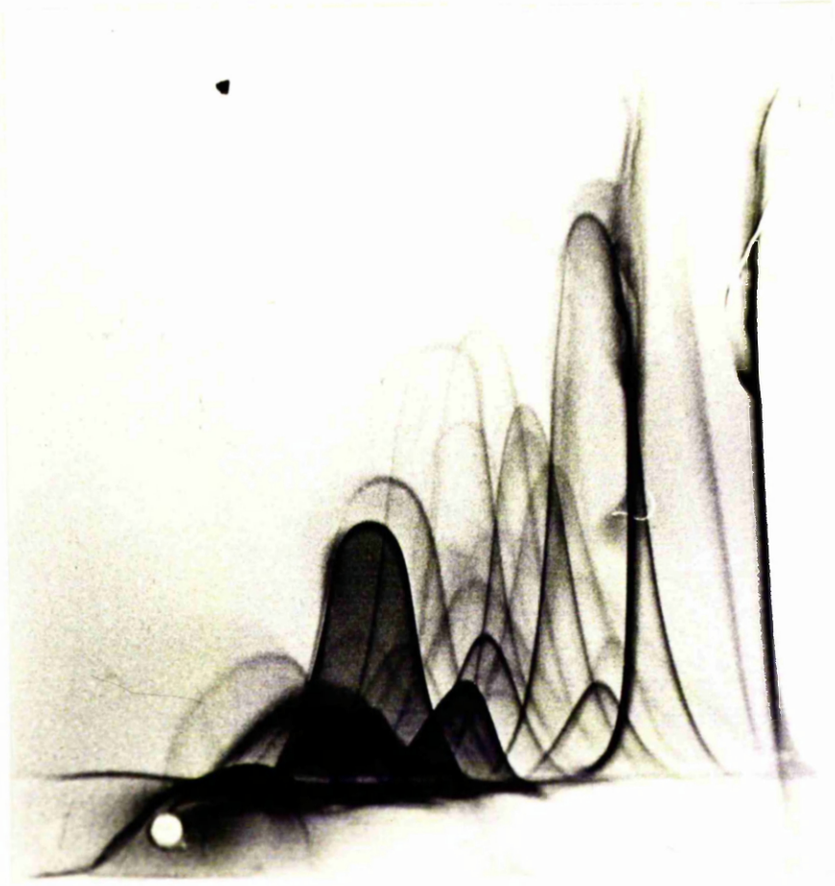


Table 10 Precipitation of serum inhibitor by $(NH_4)_2SO_4$

Serum was dialysed against increasing concentrations of $(NH_4)_2SO_4$ and the supernatants at each stage tested for inhibitory activity against δ-haemolysin.

$(NH_4)_2SO_4$ Fluid	Inhibitory titre (HU_{50}/0.2 ml)
20%* supernatant	1000
30% "	1000
40% "	1000
50% "	1000
60% "	1000
70% "	50
80% "	50
serum control	1000

*percentage saturation at 4°C.

of $(NH_4)_2SO_4$, the inhibitory component was precipitated.

Effect of heat on inhibitor

The inhibitory effect of serum was not abolished by heating at 56°C for 30 min (indicating that the inhibition of δ-haemolysin is not dependent on an intact complement system). However, inhibition was totally abolished by 30 min at 80°C.

Ultrafiltration of human serum

An approximate estimation of the molecular weight of the inhibitor was also made by ultrafiltration of the serum through a range of membranes of increasing pore size (Table 11). No inhibitory material passed through the membrane until a 300,000 M.Wt. membrane was used. Inhibitory activity was then found in the filtrate.

When the filtration cell was flushed with a large volume of buffer, still under positive pressure through the membrane, and the material retained by the membrane was resuspended to the original volume of serum, inhibitory activity was still present.

Gel filtration of human serum

Since the inhibitory activity of serum was present in 60% saturated $(NH_4)_2SO_4$ supernatants, this was taken as a convenient starting material for further experiments, since many serum proteins had been removed by precipitation (Plate 3). Accordingly, a 5 ml sample of 60% $(NH_4)_2SO_4$ supernatant was applied to a Sephadex G-150 column, and fractions assayed for inhibitory activity. A result typical of several separations is shown in Fig 7.

A peak of inhibitory activity corresponded to the void volume of the column, indicating a component in excess of 400,000 M.Wt., and a broader band of activity followed immediately behind. No inhibitory activity was found in the third protein peak, shown by immunoelectrophoresis and Laurell immunoelectrophoresis to be predominantly serum albumin.

Gel filtration in Sepharose 6-B

Since almost all inhibitory activity was eluted at the void volume using Sephadex G150 the same starting material, a 60% $(NH_4)_2SO_4$ supernatant of human serum, was applied to a Sepharose 6-B column, and eluted as with G150. In addition to monitoring E_{280} and inhibitory activity of all fractions, peak fractions were subjected to Laurell immunoelectrophoresis. The results are shown in Fig 8 and Plate 4. Two distinct peaks of inhibitory activity were obtained.

Production of anti-inhibitor antiserum

A 2 ml serum sample which had been concentrated by 60% $(NH_4)_2SO_4$ precipitation and ultrafiltration through a PM30 membrane, was separated by gel filtration through a Sephadex G-150 column. The separation profile is shown in Fig 9. A peak of inhibitory activity with a trailing "shoulder" was found on inhibitory assay of the fractions, and those fractions which comprised the leading edge of the peak were injected into a rabbit to raise anti-inhibitor antiserum.

The antiserum was processed by 33% $(NH_4)_2SO_4$ precipitation to separate immune gamma-globulin from the δ-haemolysin inhibitor present

Table 11 Ultrafiltration of human serum with Amicon (c) membranes

Membrane	Exclusion limit (daltons)	Inhibitory titre (HIU50) retentate	filtrate
UM5	500	640	0
UM10	1,000	640	0
PM10	10,000	640	0
PM30	30,000	640	0
XM100	100,000	640	0
XM300	300,000	160	320

A 1:2 dilution of human serum was used throughout as the filtration solution.

All solutions filtered to dryness, and retentate resuspended to original volume.

Plate 3 Laurell immunoelectrophoresis of a 60% $(NH_4)_2SO_4$ supernatant of human serum

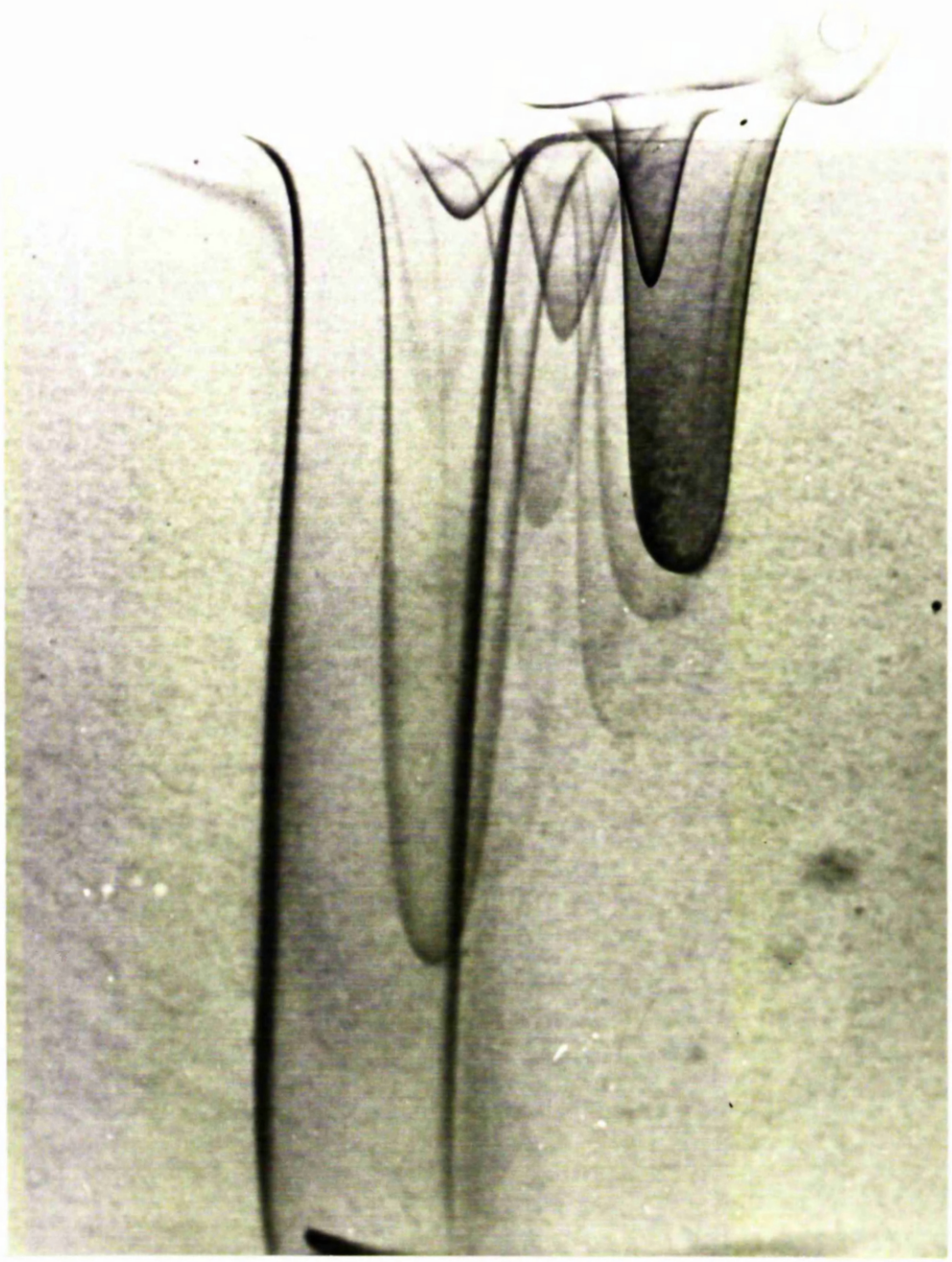


Figure 7 Sephadex G-150 gel filtration of 60% $(NH_4)_2SO_4$ supernatant of human serum

An LKB Uvichord profile is shown.

Fractions were assayed for inhibitory activity and peak fractions for E_{280}.

●——● - transmission at 280 nm.

o----o - inhibitory activity.

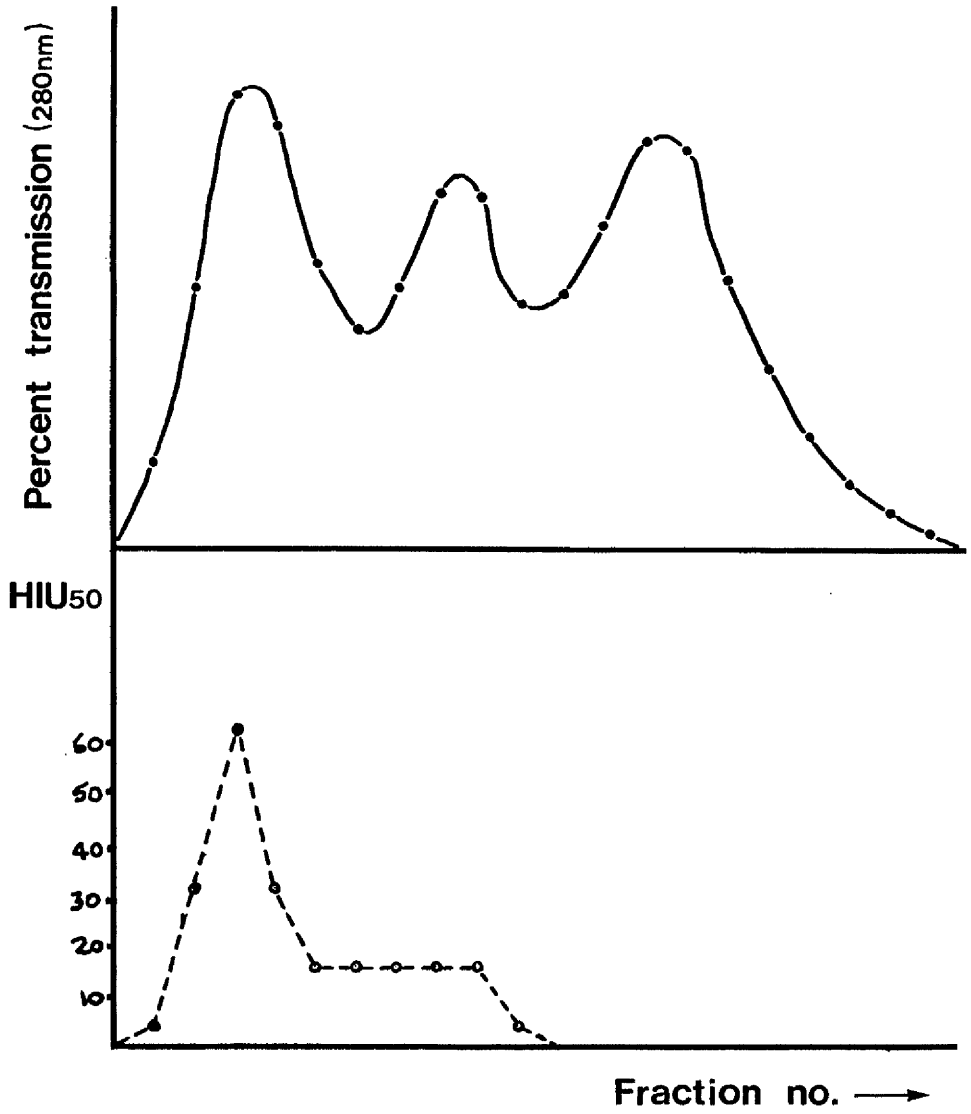

Percent transmission (280nm)
HIU50
60
50
40
30
20
10
Fraction no. ⟶

Figure 8 Sepharose 6-B gel filtration of 60% $(NH_4)_2SO_4$ supernatant of human serum

An LKB Uvichord profile is shown.

Fractions were assayed for inhibitory activity and peak fractions for E_{280}.

●——● - transmission at 280 nm.

o- - -o - inhibitory activity.

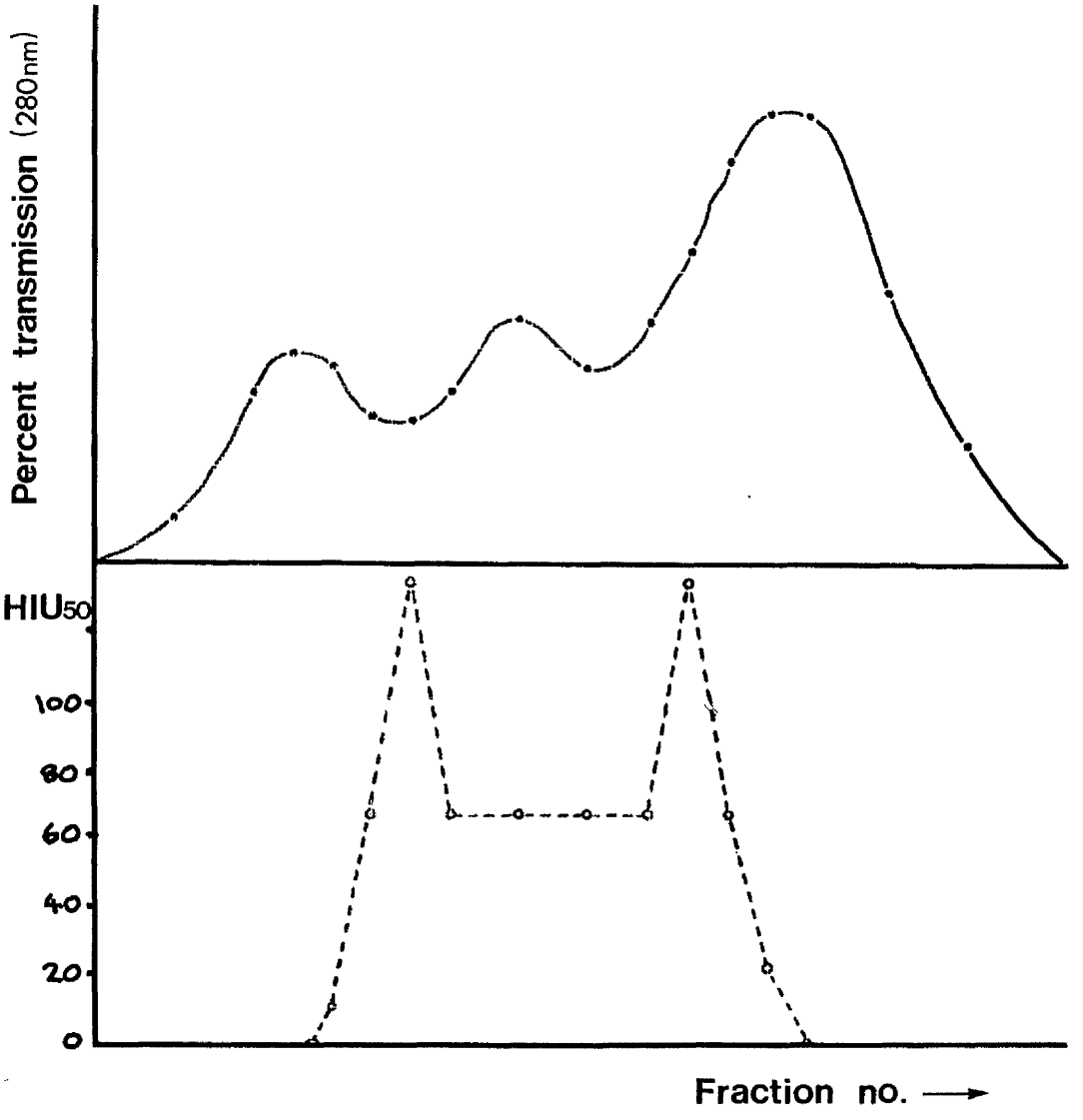
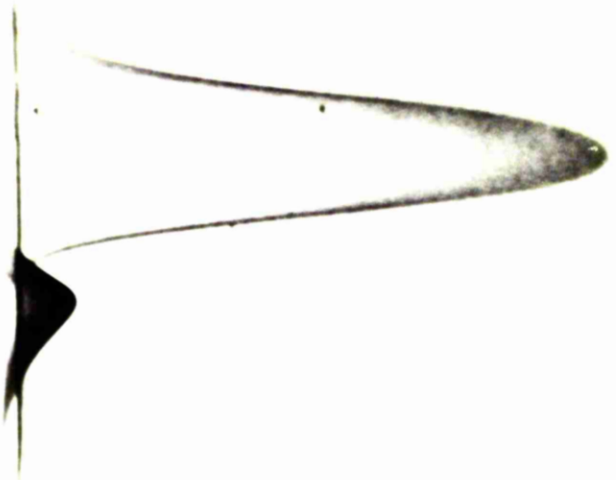

Percent transmission (280nm)
HIU50
100
80
60
40
20
0
Fraction no. ⟶

Plate 4 Laurell immunoelectrophoresis of peak fractions from Sepharose 6-B gel filtration

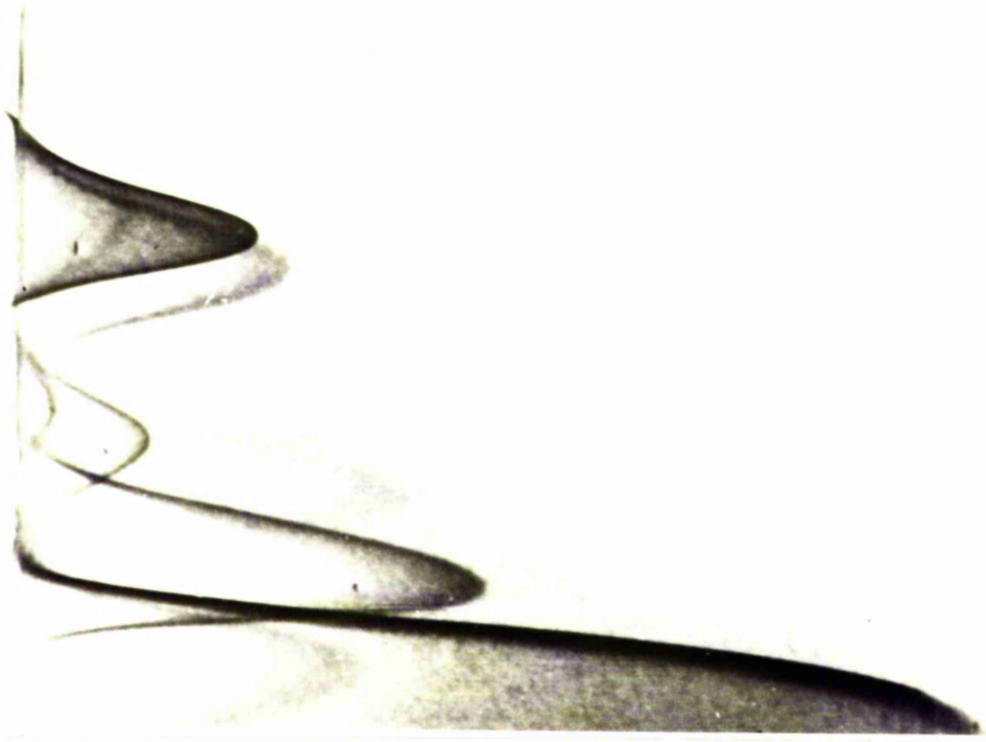
a) peak 2

b) peak 3.

a)



b)



in the rabbit serum (Table 12). Incubation of the antiserum with whole human serum for 60 min decreased the δ-haemolysin inhibitory activity of the human serum by 40%.

Two dimensional immunoelectrophoresis of the antiserum showed the presence of antibodies to five components of human serum (Plate 5). Three of these could again be tentatively identified, on the basis of their profiles and electrophoretic mobilities, as haptoglobin, β-lipoprotein and α-macroglobulin.

Ammonium sulphate precipitation of serum at pH 5.0

The precipitation of serum proteins at low pH had previously been documented (Freeman and Smith, 1970), and by their techniques, the behaviour of the inhibitor of δ-haemolysin under such conditions was studied.

Serum adjusted to pH 5.0 with acetic acid was precipitated at 40% and 50% saturation with $(NH_4)_2SO_4$. Immunoelectrophoretic analysis showed that all serum proteins remaining in a 40% supernatant were precipitated at 50% concentration (Plate 6), while inhibitory titrations on each fraction indicated that inhibitory activity present in a 40% supernatant was precipitated when the solution was raised to 50% saturation with $(NH_4)_2SO_4$.

Isoelectricfocusing of serum

A 60% $(NH_4)_2SO_4$ supernatant of human serum dialysed against 1% glycine was focused in a broad pH gradient (pH 3.5 - 10.0) and all inhibitory activity was found in a single peak (pI 5.5) corresponding to

Figure 9 Sephadex G-150 separation of 60% $(NH_4)_2SO_4$ supernatant containing serum proteins of M. Wt. > 30,000.

N.B. Dotted portion of graph showing inhibitory activity represents fractions chosen to raise anti-inhibitor antiserum.

o- - -o Inhibitory activity

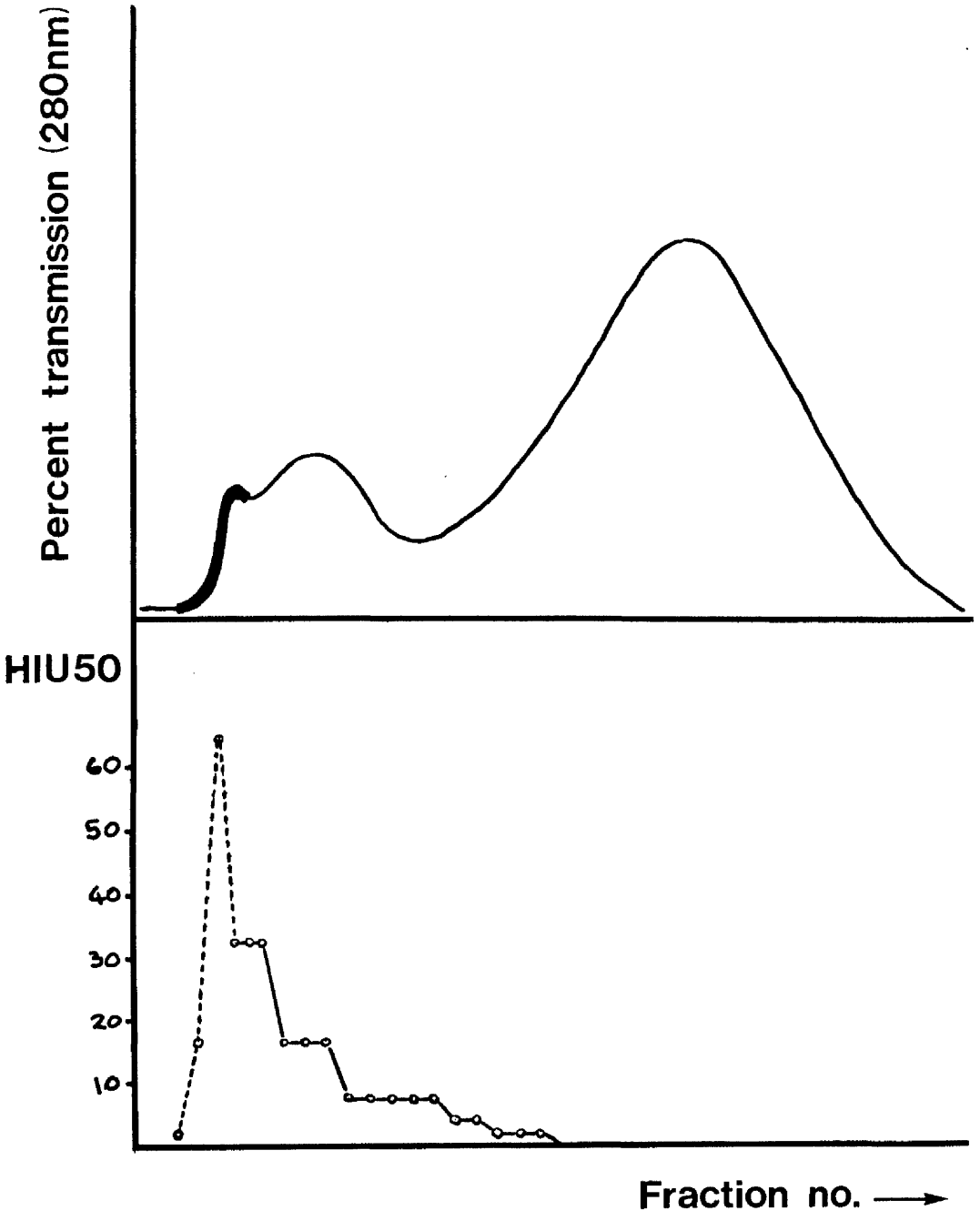
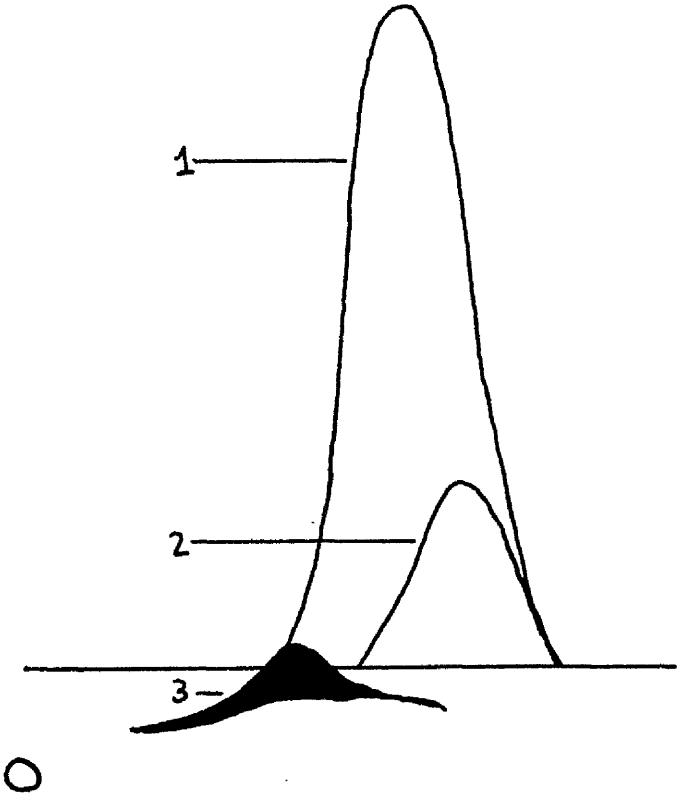

Percent transmission (280nm)
HIU50
60
50
40
30
20
10
Fraction no.

Plate 5 Laurell immunoelectrophoresis of human serum against partially purified antiserum to the inhibitor

Anti-serum was raised in a rabbit against material from the breakthrough peak obtained on separation of human serum by Sephadex G-150 gel filtration. After concentration by 33% saturated $(NH_4)_2SO_4$ precipitation, whole serum was electrophoresed against this anti-serum.

1. - haptoglobin
2. - α_2-macroglobulin
3. - β-lipoprotein



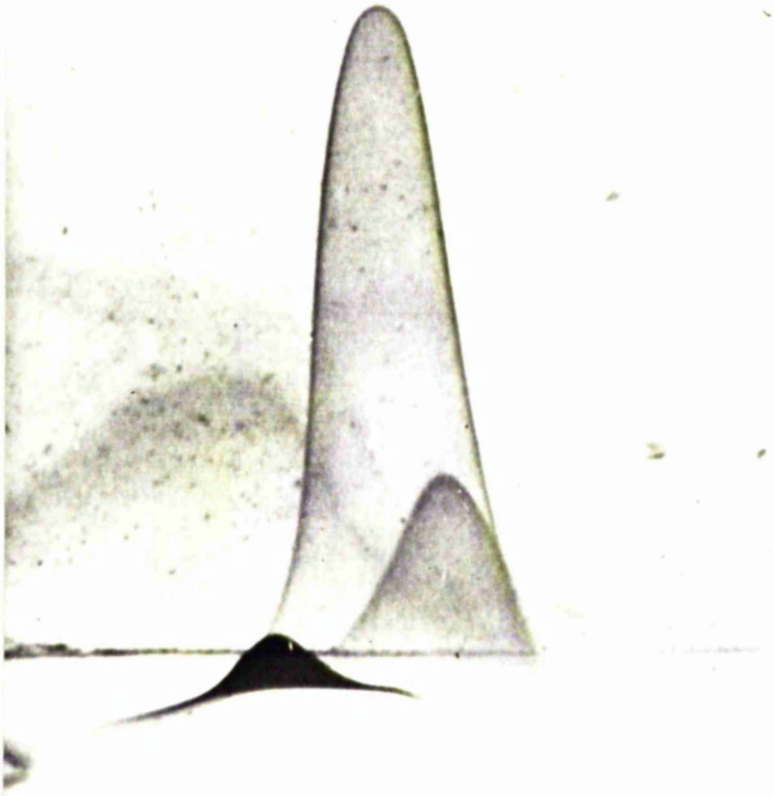


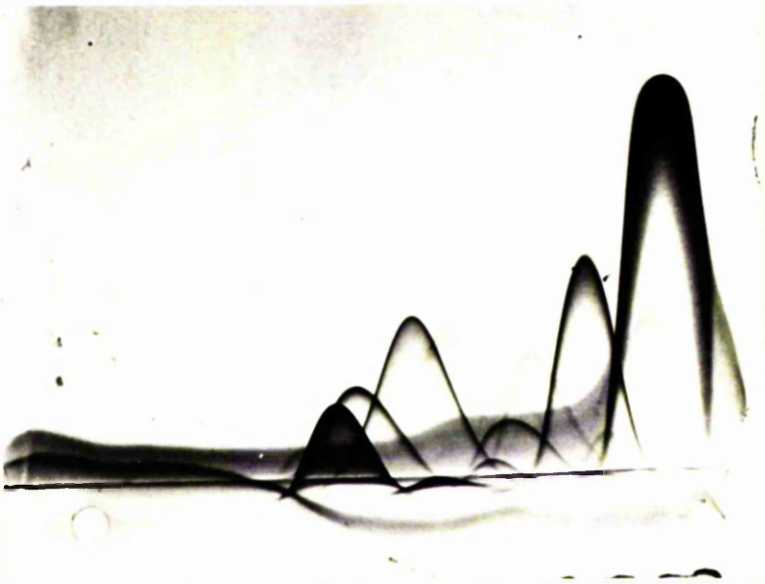
Plate 6 Laurell immunoelectrophoresis of fractions from $(NH_4)_2SO_4$ precipitation at pH 5

a) 40% supernatant

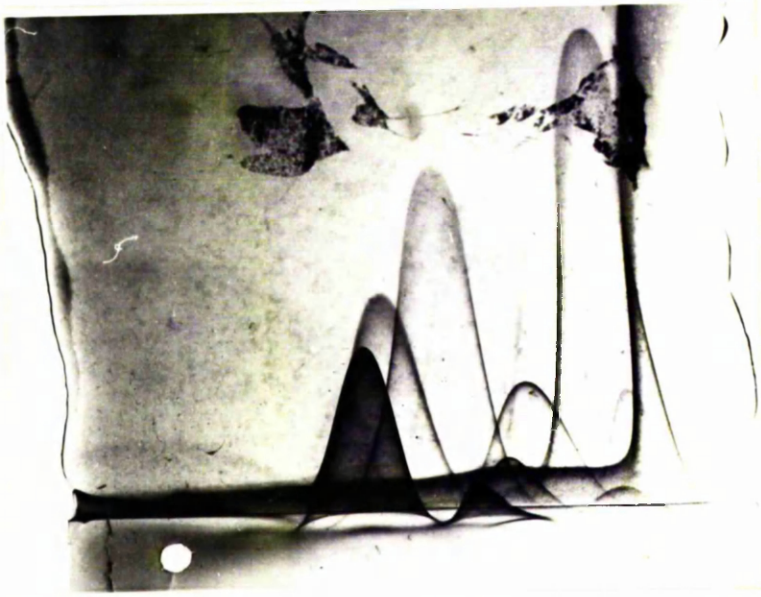
b) 50% pellet

c) 50% supernatant

a)



b)



c)

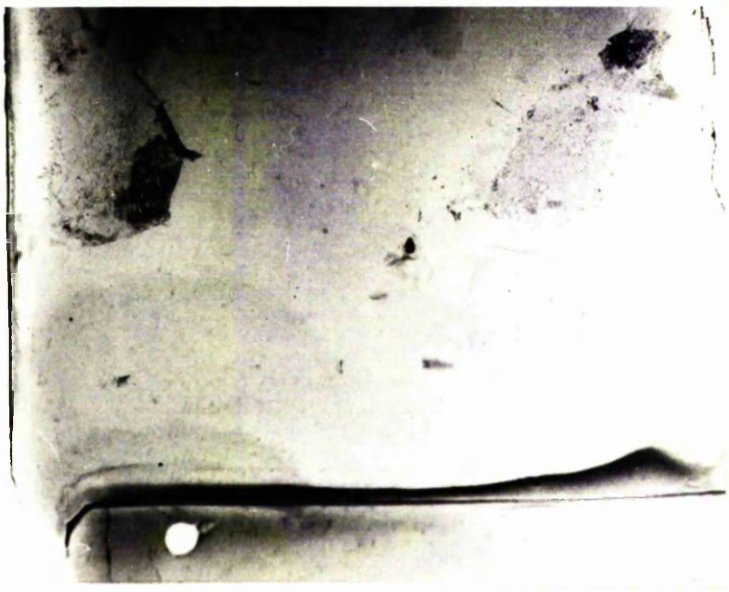


Table 12 Concentration of rabbit antiserum

The rabbit antiserum which was raised against the high molecular weight peak from Sephadex G-150 chromatography was precipitated three times with 33% saturated $(NH_4)_2SO_4$ to separate immune gamma-globulins from the non-specific δ-haemolysin inhibitors in the rabbit serum.

Sample	Titre (HIU50)
1st 33% supernatant	64
2nd " "	2
3rd " "	0
33% precipitate	0
whole human serum	1024

the major peak of E_{280}-absorbing material (Fig 10) which by Laurell immunoelectrophoresis was shown to comprise a large number of serum proteins.

The above preliminary studies indicated that the amount of inhibitor in human serum samples obtained under uniform conditions (page 71) is relatively constant. It was stable at temperatures that inactivated complement, and remained in solution at a level of saturation of $(NH_4)_2SO_4$ which precipitated immunoglobulins, albumin and a large percentage of other serum proteins. The results from gel filtration in the two support media and ultrafiltration suggested that more than one component of serum was responsible for the inhibition; ultrafiltration indicated one inhibitory component of between 100,000 and 300,000 daltons, which correlated with the slower-moving inhibitory peaks from both gel filtrations. Sephadex G-150 chromatography and ultrafiltration suggested a M.Wt. of more than 400,000 daltons for the faster moving inhibitory peak and in Sepharose a value in excess of 1,000,000 was obtained, but these results cannot determine whether several serum components are involved, or aggregates of one basic moiety. Immuno-electrophoretic studies, however, showed that the high M.Wt. inhibitory fractions from gel filtration comprised only a few serum proteins, which could be identified as α_2-macroglobulin, haptoglobin and β-lipoprotein by comparison with the published studies of Freeman and Smith (1970).

Affinity chromatography of δ-haemolysin inhibitor

Purified δ-haemolysin was covalently coupled to CNBr-activated Sepharose-4B by a modification of the technique of Axen, Porath and

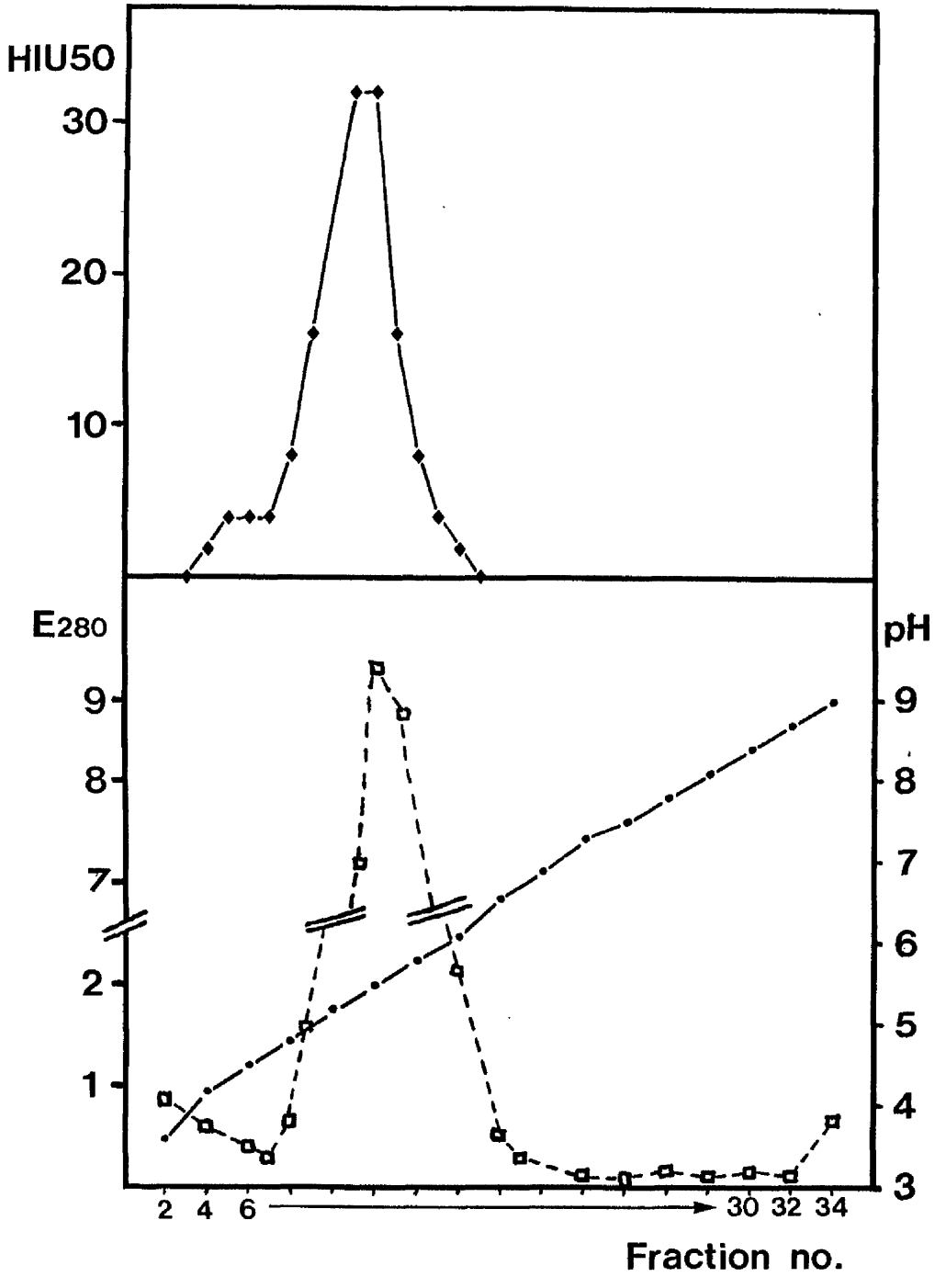
Figure 10 Isoelectricfocusing of serum

A 60% $(NH_4)_2SO_4$ supernatant of human serum (0.5 ml) was analysed by isoelectricfocusing using the apparatus of Talbot and Caie (1975) and the E_{280}, pH and inhibitory activity of each fraction assayed.

□- - -□ E_{280}

●——● pH

◆——◆ inhibitory activity


HIU50
30
20
10
E280
9
8
7
2
1
pH
9
8
7
6
5
4
3
2 4 6
30 32 34
Fraction no.

Ernback (1967), developed by Pharmacia Fine Chemicals. After incubation of dilute human serum with the matrix-immobilised δ-haemolysin, immuno-electrophoretic analysis of the eluant showed a marked decrease in intensity of the β-lipoprotein band (Plate 7).

Control CNBr-Sepharose gel was prepared in the same manner as the experimental gel, except that δ-haemolysin was not coupled to it, resulting in total hydrolysis of all available active groups on treatment with ethanolamine. No decrease in intensity of the β-lipoprotein arc occurred in this gel after mixing with human serum, indicating that there was no non-specific removal of β-lipoprotein in the experimental system.

Serum lipoproteins

Zonal ultracentrifugation of human serum

When human serum was ultracentrifuged in a KBr gradient in a Titanium BXIV rotor by the method of Wilcox *et al* (1971) three separate peaks of ultraviolet-absorbing activity were obtained (Fig 11). Because of the very large volume of fluid to be tested (650 ml), and the very low levels of inhibitory activity in small-volume individual fractions, the total fluid was bulked into 7 large fractions, each of which was concentrated by ultrafiltration through a 30,000 M.Wt. membrane. Titration of these seven pooled fractions (Table 13) revealed inhibitory activity in the first two UV absorbing peaks, and minimal inhibitory activity in the major, third peak.

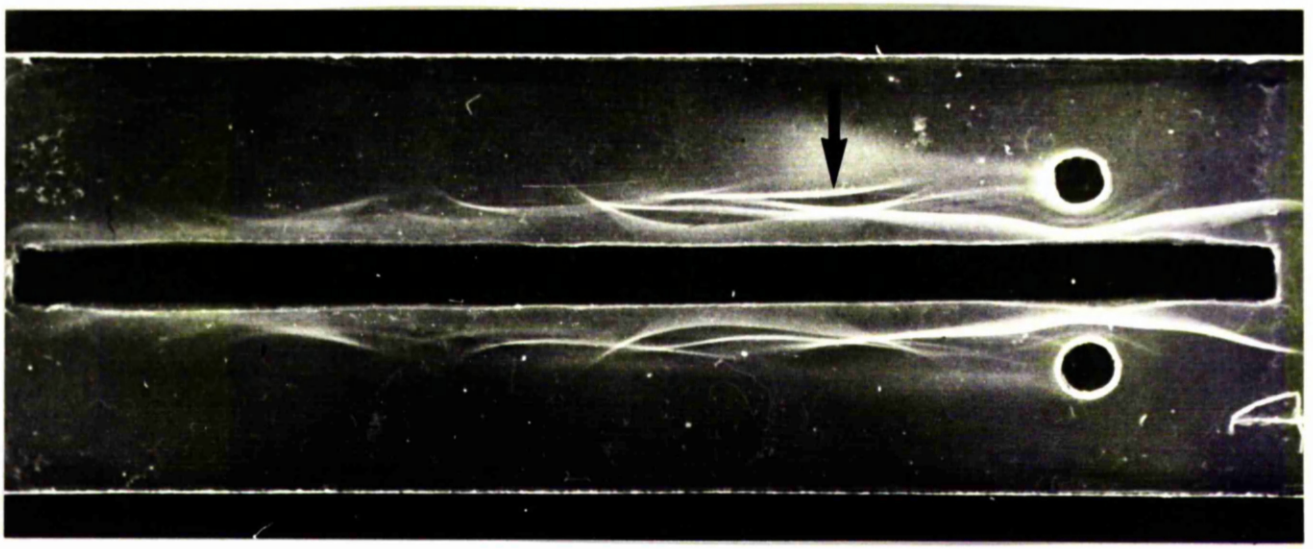
Plate 7 Immunoelectrophoresis of human serum after affinity chromatography with δ-haemolysin

Serum was electrophoresed against anti-whole human serum antiserum before and after exposure to δ-haemolysin-linked CNBr-Sepharose.

Top : Whole human serum

Bottom : Serum after affinity chromatography

Arrow indicates β-lipoprotein arc.



(+) Anode

Cathode (-)

Two dimensional immunoelectrophoresis of zonal fractions

The bulked fractions (i.e. fractions 1, 4, 5, 7) from zonal centrifugation corresponding to peak UV absorbance were analysed by Laurell immunoelectrophoresis against anti-whole human serum antiserum (Plate 8).

The first peak (fraction 1; density, 1.06) yielded only one precipitation line on electrophoresis. This line had the mobility of a β-globulin and on staining with Sudan black (a stain specific for lipoproteins) was identified as β-lipoprotein. Against monospecific anti-β-lipoprotein serum, the same precipitation peak resulted, confirming its identity as β-lipoprotein (Plate 9). The second peak (fractions 4, 5 ; density 1.11 - 1.18) also yielded only one precipitation line on immuno-electrophoresis, with an electrophoretic mobility characteristic of an α-globulin. This line was not stained by Sudan black, but did stain with Oil Red, another lipoprotein-specific stain. Similarly, against monospecific anti-α-lipoprotein serum, the same single peak resulted, confirming its identity as α-lipoprotein (Plate 9).

The third peak, corresponding to a density of 1.21 - 1.28 (fraction 7) yielded many lines on immunoelectrophoresis, none of which stained by Sudan Black, the overall appearance of the precipitation arcs being that of whole serum minus the lipoprotein components (Plate 8c).

Polyacrylamide disc-gel electrophoresis of zonal fractions

Disc gel electrophoresis was performed on zonal ultra-centrifugation fractions by the method of Naito et al (1973) using 3.5%

Figure 11 Zonal Ultracentrifugation of serum

25 ml serum adjusted to 40% (w/v) KBr was separated on a KBr gradient of density 1.0 - 1.28, for 24 hours at 120,000 x g* and the density, E_{280} and inhibitory activity of selected fractions assayed.

●——● density

——— E_{280}

*by the method of Wilcox et al (1971).

The inhibitory titres of the seven pooled fractions are shown by the dotted histograms.

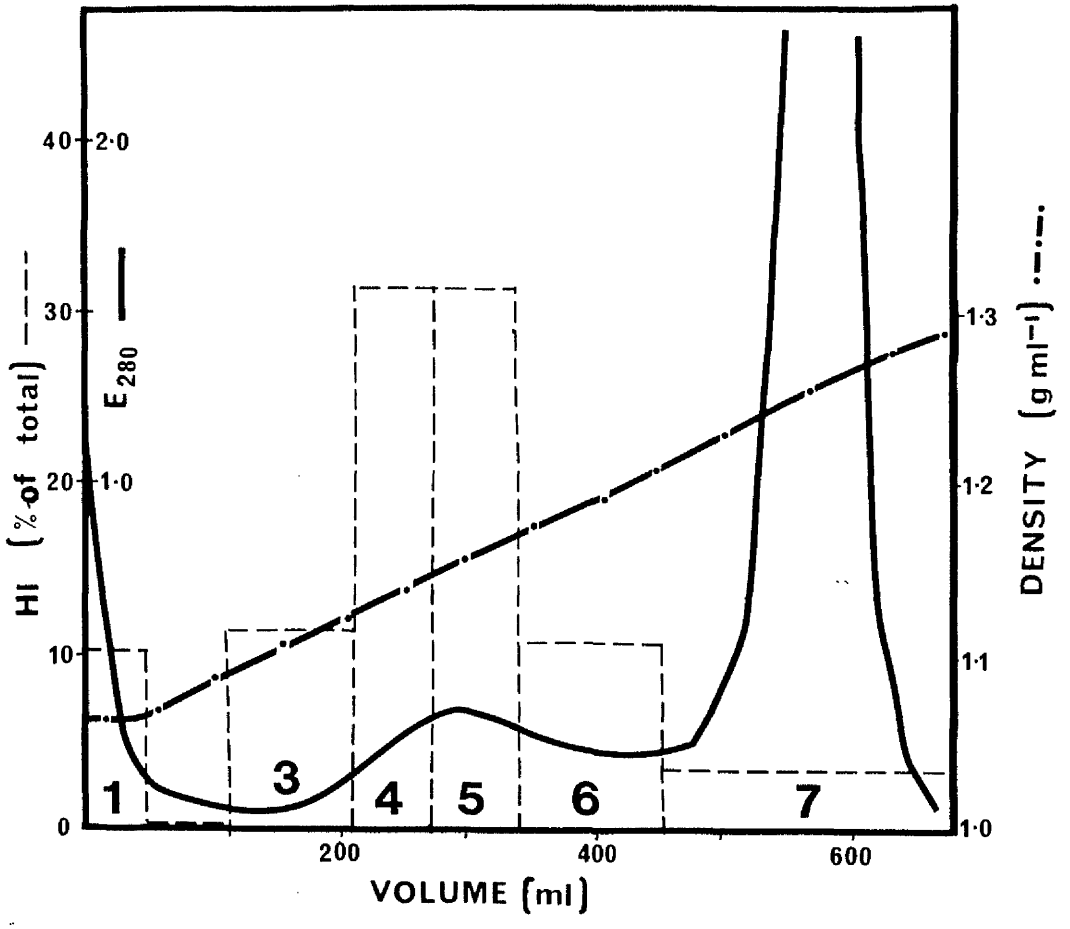

HI (% of total)
E_{280}
DENSITY (g ml^{-1})
40
30
20
10
0
2·0
1·0
1·3
1·2
1·1
1·0
200
400
600
VOLUME (ml)
1
3
4
5
6
7

Table 13 Inhibition titres of zonal ultracentrifugation pooled fractions

Sample	Original Volume (ml)	Final Volume (ml)	Concentration factor	Inhibitory titre (HIU50/0.2 ml)
1	40	2.7	14.8	16
2	80	3.7	21.6	0
3	125	6.0	20.8	8
4	85	4.0	21.3	32
5	80	4.0	20.0	32
6	120	5.5	21.8	8
7	150	7.5	20.0	2
Control				64

Plate 8 Laurell immunoelectrophoresis of fractions from zonal ultracentrifugation of human serum

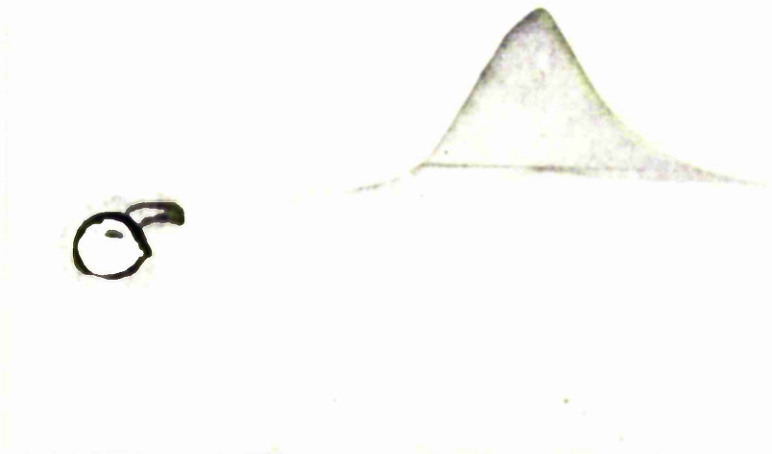
a) Fraction 1 (Fig 11, Table 14)

b) Pooled fractions 4 and 5

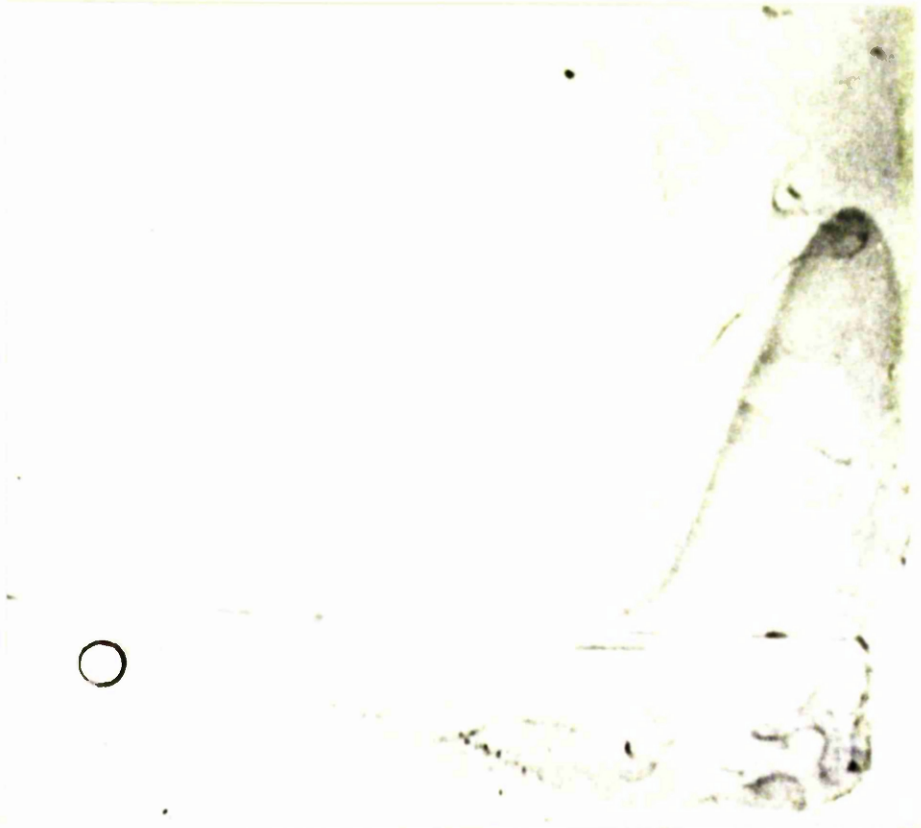
c) Fraction 7.

Electrophoresis against anti-whole human serum antiserum.

a)



b)



c)

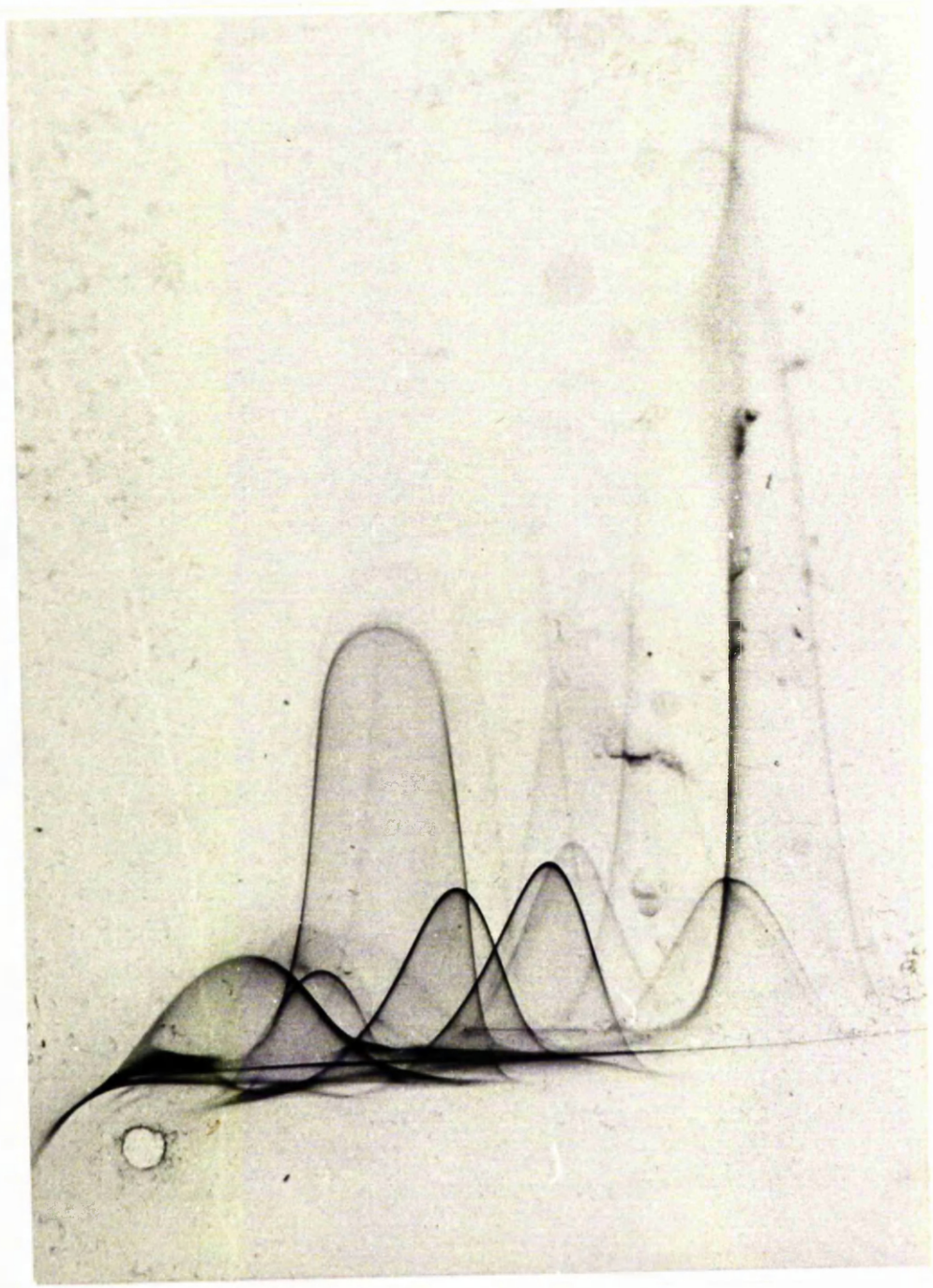
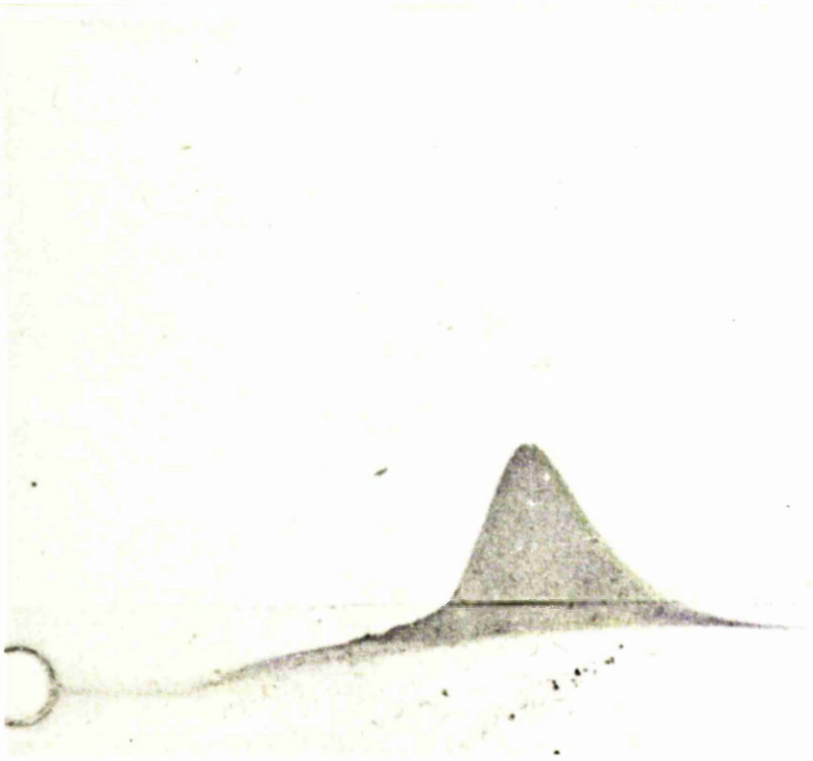


Plate 9 Laurell immunoelectrophoresis of fractions from zonal ultracentrifugation of human serum

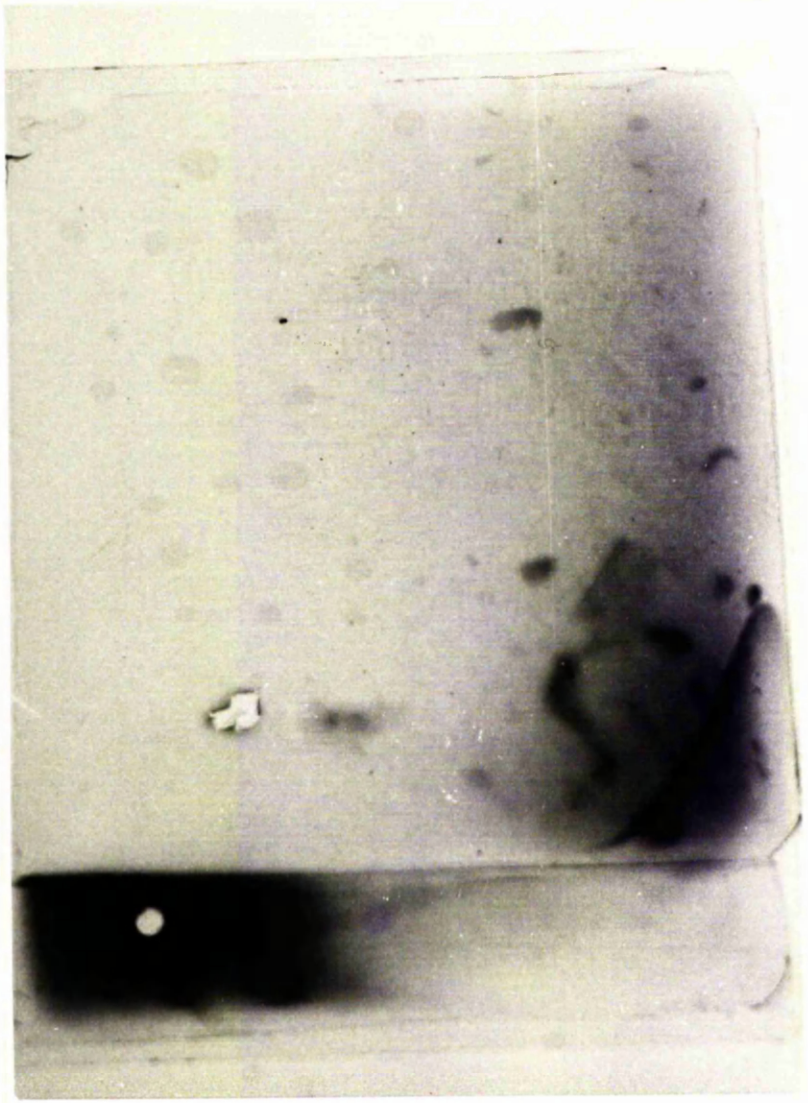
a) Fraction 1 vs monospecific anti-β-lipoprotein antiserum

b) Combined fractions 4 & 5 vs monospecific anti-α-lipoprotein antiserum

a)



b)



polyacrylamide with samples pre-stained by Sudan Black (Plate 10). Under these conditions, human serum yielded two darkly staining bands, one of rapid mobility (shown by Naito et al to be α-lipoprotein) and the other a slow-moving band which barely penetrated the separating gel (β-lipoprotein). Fraction 1 material from zonal ultracentrifugation stained with the lipoprotein-specific stain and migrated as a β-lipoprotein; similarly, electrophoresis showed fraction 4 to be α-lipoprotein. Fraction 7 contained a trace of α-lipoprotein, thus explaining the low level of inhibitory activity detected in this sample (Table 13).

As already mentioned (page 31) several components comprise the β-lipoprotein fraction of human serum. In an attempt to detect these, the first four small fractions (10 ml each) which together comprised the bulked fraction 1, were individually screened by disc-gel electrophoresis (Plate 11). This showed that the fraction of lowest density contained three separate components indistinguishable in the bulk fraction.

Attempts to separate these three components by the flotation technique of Havel, Eder and Bragdon (1955) at raised densities in a swing-out rotor were unsuccessful. Electrophoresis of samples from such a flotation indicated an identical β-lipoprotein composition throughout the gradient.

Immunocore electrophoresis of serum and zonal ultracentrifugation fractions

The "immunocore" method of electrophoresis was used in two different ways, firstly by the classic method of immunoelectrophoresis, and secondly by exploiting the ability of δ-haemolysin and its inhibitor to form an immune-like precipitate in gels. In both cases, samples were

Plate 10 Polyacrylamide disc-gel electrophoresis of fractions from zonal ultracentrifugation

Peak E_{280}-absorbing fractions from zonal ultracentrifugation were electrophoresed by the method of Naito et al (1973).

Left to right : whole human serum

peak 1 (β-lipoprotein)

peak 3

peak 2 (α-lipoprotein)

blank

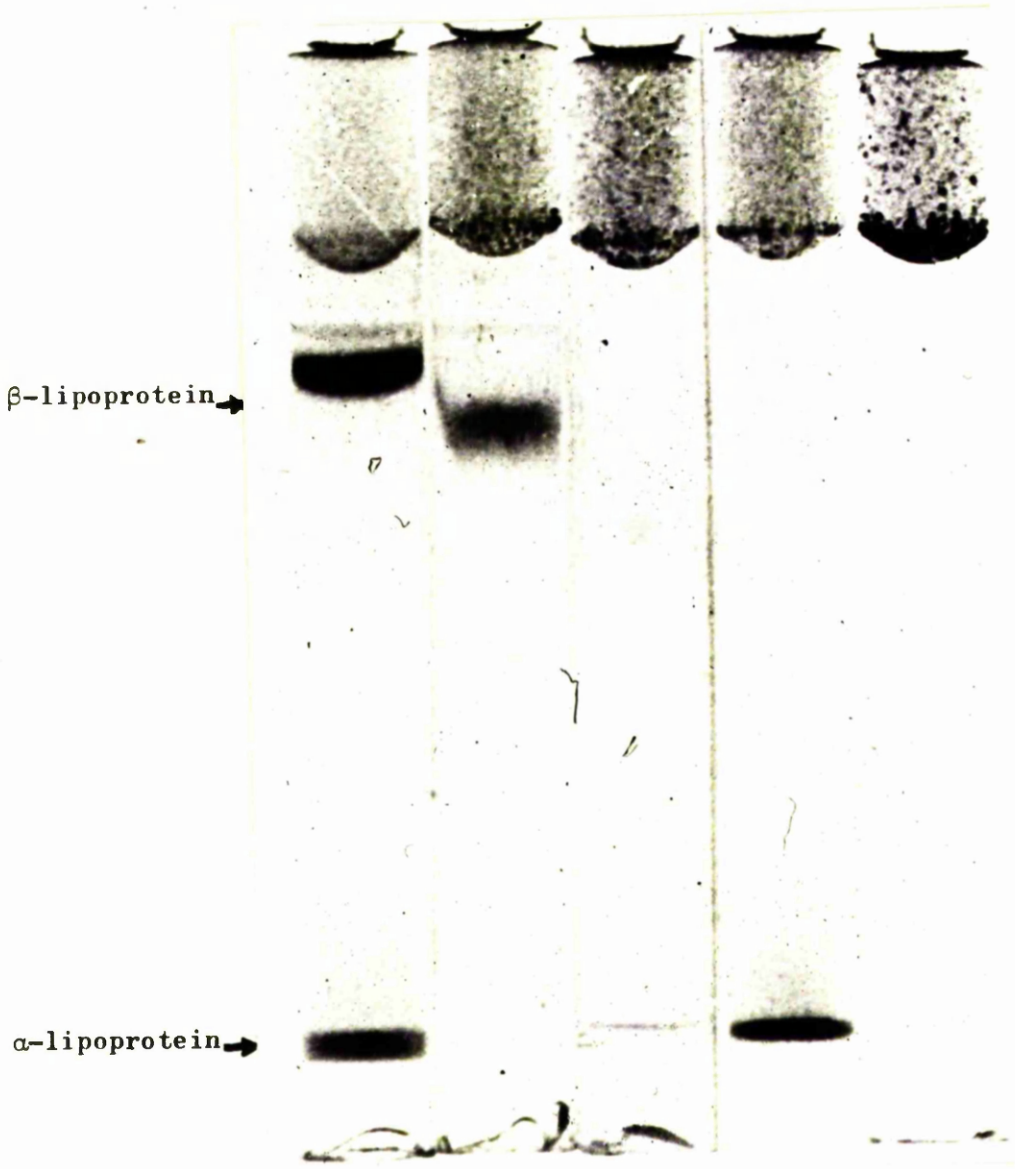

β-lipoprotein
α-lipoprotein

Plate 11 Polyacrylamide disc-gel electrophoresis of subfractions of β-lipoprotein

Fraction 1 from zonal ultracentrifugation was screened for lipoprotein content prior to bulking (see text) by the method of Naito et al (1973).

Left to right : whole human serum

Fraction 3

Fraction 2

Fraction 1_4

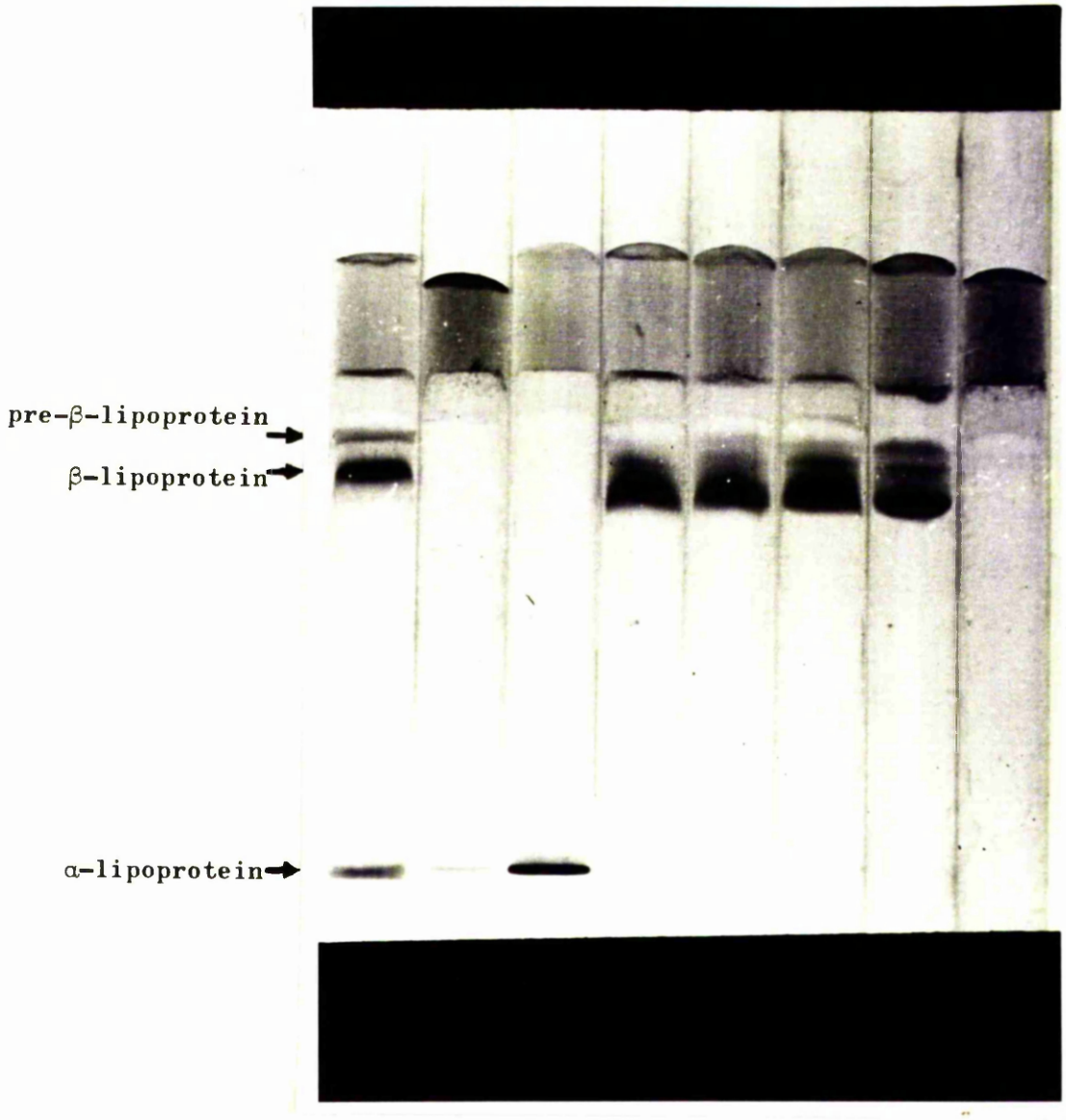
Fraction 1_3

Fraction 1_2

Fraction 1_1

Blank

↓ decreasing density


pre-β-lipoprotein
β-lipoprotein
α-lipoprotein

separated by polyacrylamide gel electrophoresis (Naito et al, 1973) except that only one reference sample was prestained with Sudan Black (Plate 12a). By the first method a whole serum sample, developed with anti-whole human serum yielded a number of immune precipitation lines (Plate 12b). Comparison with complementary prestained serum samples showed the position of the β- and α-lipoprotein bands. These positions were confirmed by immunocore electrophoresis in the same system, of β- and α-lipoprotein purified by zonal ultracentrifugation with development of the precipitin lines using homologous antisera (Plate 12c). When, however, a 2 mg/ml solution of δ-haemolysin was incorporated in the core gel instead of an antiserum, bands of precipitation developed in the gel at positions corresponding to the immune precipitates formed by purified β- and α-lipoproteins with their homologous antisera (Plates 12 and 13).

Effect of rabbit anti-inhibitor serum on zonal α-lipoprotein and β-lipoprotein

Zonal fractions of β-lipoprotein and α-lipoprotein, pure by the criteria of disc gel and immunoelectrophoresis, were incubated for 60 minutes at 37°C with rabbit anti-inhibitor serum prior to titration for inhibitory activity. The inhibitory titre of α-lipoprotein was unaffected, but that for β-lipoprotein was totally abolished by pretreatment with anti-inhibitor serum.

Laurell immunoelectrophoresis of whole human serum against anti-inhibitor anti-serum (Plate 5) indicated the presence of antibodies to 5 serum proteins, one of which was β-lipoprotein (page 83). The

Plate 12 Immunocore electrophoresis of whole serum

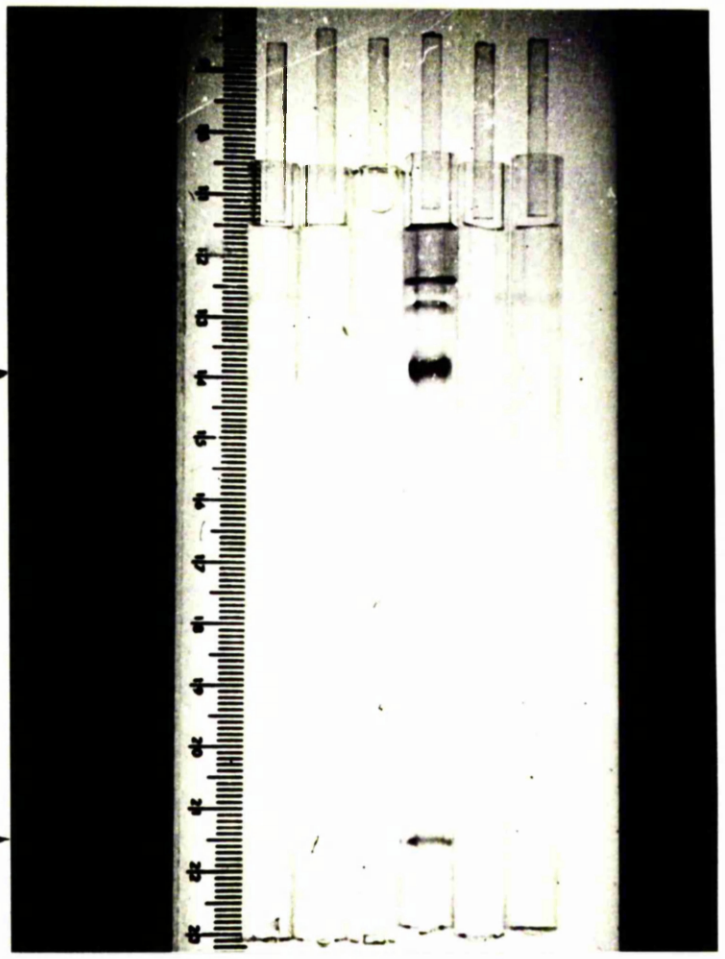
a) Appearance of gels after electrophoretic separation. Gel number 4 from the left contains prestained serum and shows the relative positions of β-(top) and α-lipoproteins (bottom). The other gels were not prestained.

b) Typical appearance of a section of gel containing electrophoresed human serum proteins developed against anti-whole human serum anti-serum, showing Ag-Ab precipitin arcs.

β-lipoprotein

a)

α-lipoprotein



b)

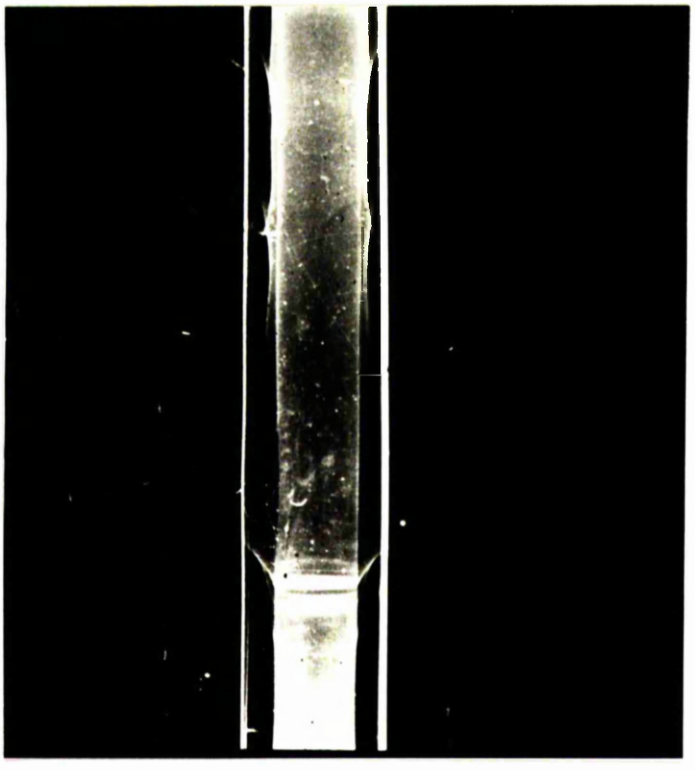


Plate 12c Appearance of gels from figure a) after 18 hours' development

The central core of the left hand gel was filled with agar containing anti-whole human serum antiserum. Arrows indicate positions of the prestained bands which are still visible.

The other 4 gels were filled with δ-haemolysin in agar at concentrations of 2, 1.75, 1.5 and 1 mg/ml respectively.

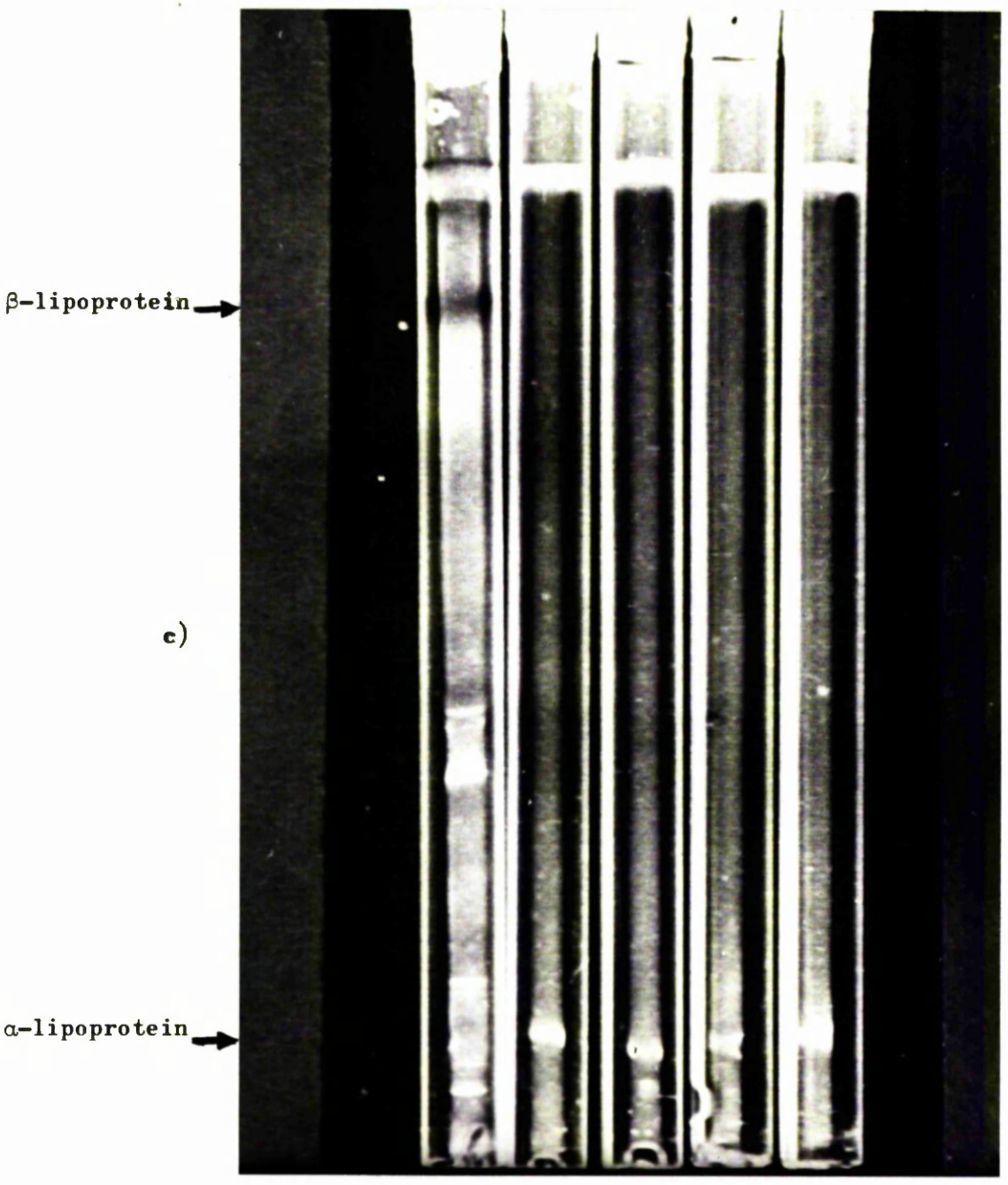

β-lipoprotein
c)
α-lipoprotein

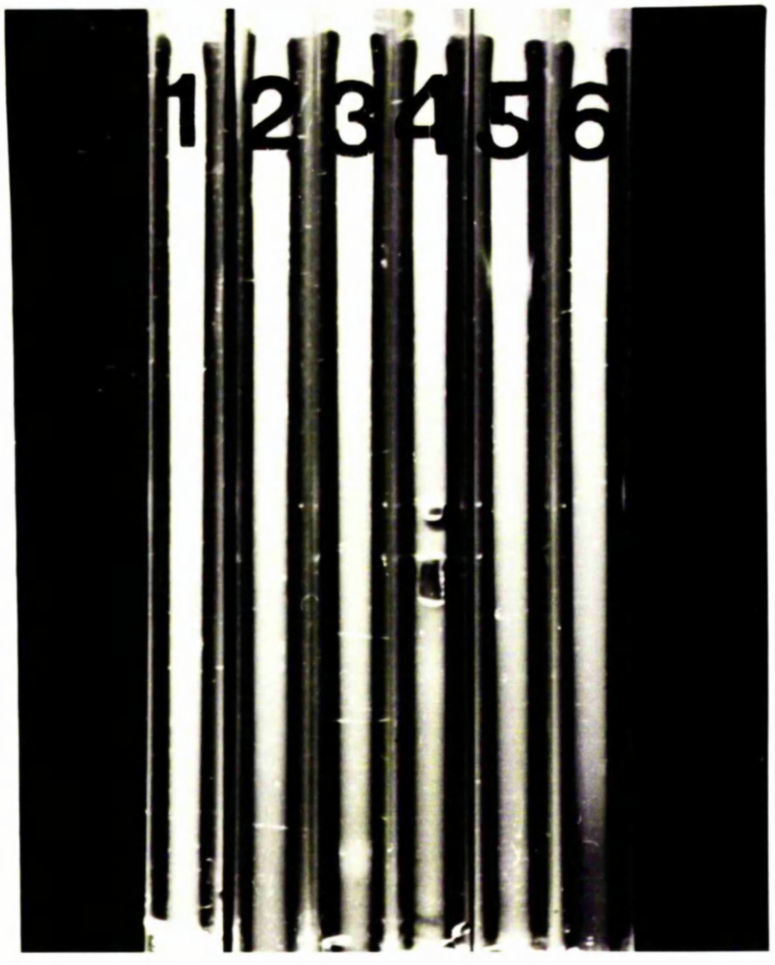
Plate 13. Immunocore electrophoresis of α- and β-lipoproteins

α- and β-lipoproteins were electrophoresed in the immunocore system and developed against their respective monospecific anti-sera or δ-haemolysin at a concentration of 2 mg/ml.

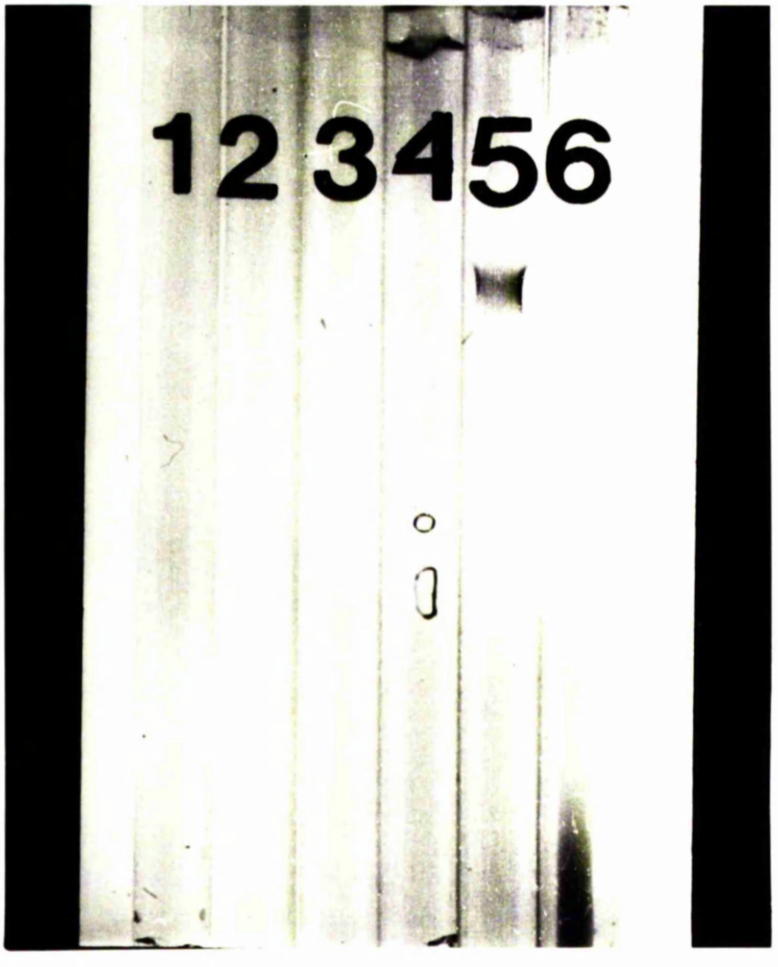
Gels 1 and 4	:	Blank		
Gel 2	:	β-lipoprotein	vs	δ-haemolysin
Gel 3	:	α-lipoprotein	vs	δ-haemolysin
Gel 5	:	β-lipoprotein	vs	anti-β-lipoprotein antiserum
Gel 6	:	α-lipoprotein	vs	anti-α-lipoprotein antiserum

a) Negative illumination. Hazy β-lipoprotein:haemolysin precipitation very difficult to visualise by photography.

b) Positive illumination.


1 2 3 4 5 6
β-lipoprotein
α-lipoprotein

a)


1 2 3 4 5 6
β-lipoprotein
α-lipoprotein

b)

above result confirmed the inhibitory role of β-lipoprotein in the protein mixture from which the antiserum was derived.

From the above experiments it was concluded that the factors in human serum responsible for the inhibition of S. aureus δ-haemolysin were the lipoproteins. However, as discussed previously (page 31), the serum lipoproteins are a complex group of molecules, composed of varying amounts of protein, triglycerides, cholesterol and phospholipid, and the investigations thus far did not indicate whether the entire lipoprotein complex or a particular moiety interacted with δ-haemolysin. Although Kapral (1972) found that some natural phospholipids inhibited the action of the haemolysin, the possible involvement of protein could not be ruled out.

The approach adopted was to determine whether enzymic disruption of a particular part of the lipoprotein complex affected its behaviour towards δ-haemolysin.

Effect of trypsin and papain on the serum inhibitors of δ-haemolysin

a) Trypsin

The effect of trypsin on serum, β-lipoprotein and α-lipoprotein inhibition was tested with and without the presence of trypsin inhibitor (Table 14). In control titrations neither enzyme nor its inhibitor affected cod erythrocytes, and the enzyme above 125 µg/ml inactivated δ-haemolysin; this effect was abolished by trypsin inhibitor. The inhibition of δ-haemolysin by serum or α- and β-lipoprotein was not affected by trypsin treatment.

Table 14 The effect of trypsin on the inhibition by human serum of δ-haemolysin

	Inhibitory titre to δ-haemolysin	
	(a) *	(b)
Serum	32	32
Serum + trypsin	32	32
Serum + trypsin + trypsin inhibitor	32	32
Serum + trypsin inhibitor	32	32
Trypsin	32	0
Trypsin + trypsin inhibitor	0	0
Trypsin inhibitor	0	0

*a) 1 mg/ml trypsin, and equivalent trypsin inhibitor.

b) 62.5 µg/ml " " " " "

b) Papain

In a similar fashion, papain was found to have no effect on cod erythrocytes nor any effect on δ-haemolysin per se. The inhibitory effect of α- and β-lipoproteins and whole serum on δ-haemolysin was not abolished by treatment with papain.

Inhibition of δ-haemolysin by phospholipids

Preparation of serum lipids

As a preliminary step to investigate the role of the phospholipid moiety of lipoproteins in the inhibitory process, a serum lipid extract was obtained by the method of Folch et al (1957). The phospholipid content of this extract was determined by thin layer chromatography with commercial lipid standards (Fig 12). Phosphatidyl-ethanolamine, phosphatidylcholine and sphingomyelin were identifiable after staining under iodine vapour.

Inhibition of δ-haemolysin by phospholipids and serum lipids

Inhibitory activity of phospholipids and serum lipids was measured using a slight modification of the standard assay procedure to compensate for the insolubility of lipids in aqueous solution (page 59). All phospholipids tested were inhibitory to δ-haemolysin (Table 15) the highest activity being shown by phosphatidyl glycerol, and the least by phosphatidylethanolamine. The serum lipid extract was also inhibitory, but the apoprotein residue from the extraction was inactive against the haemolysin.

Figure 12 Thin layer chromatography of commercial phospholipids and serum lipid extract

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PA	phosphatidic acid
C	cardiolipin
S	sphingomyelin
SL	serum lipids

solvent	65	:	25	: 4
	chloroform		methanol	water

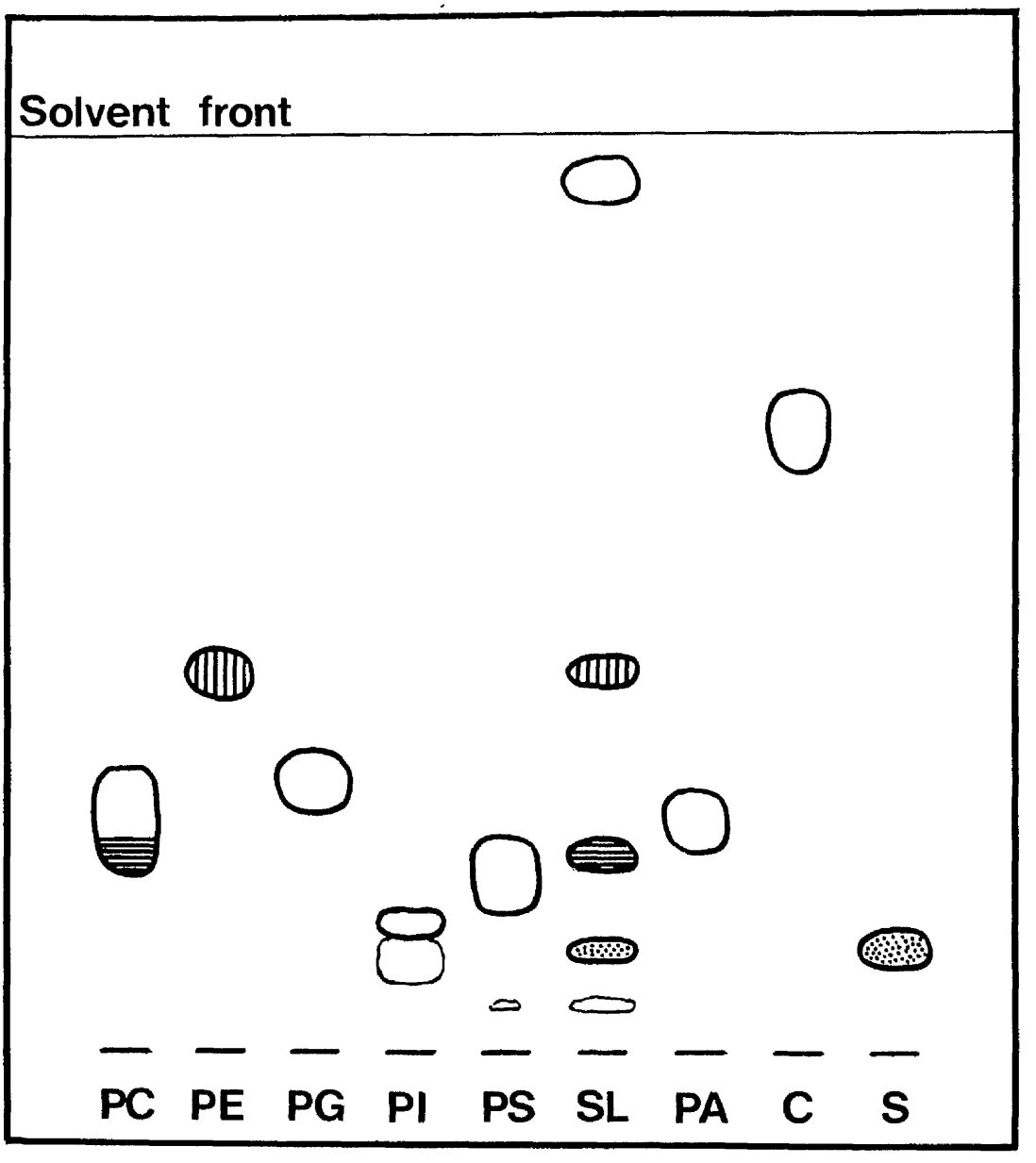

Solvent front
PC
PE
PG
PI
PS
SL
PA
C
S

Table 15 Inhibition of δ-haemolysin by phospholipids and a serum lipid extract

Inhibitory Sample	Inhibitory titre* (HIU50/0.2 ml)
Sphingomyelin	64
Phosphatidic acid	128
Phosphatidylcholine	512
Phosphatidylethanolamine	8
Phosphatidylglycerol	1024
Phosphatidylinositol	512
Phosphatidylserine	512
Cardiolipin	128
Serum Lipids	64

*Using cod erythrocytes as indicators of haemolysis.

Preliminary experiments on enzymic degradation of the phospholipid inhibitors were complicated by the observation that B. cereus phospholipase C (Fig 13), was very haemolytic towards the cod erythrocytes employed in the inhibitory assays (25000 HU/mg). It had no effect, however, on human red cells which were substituted for cod cells in the haemolysin assay.

Determination of conditions for inhibitory assays using human erythrocytes in place of cod

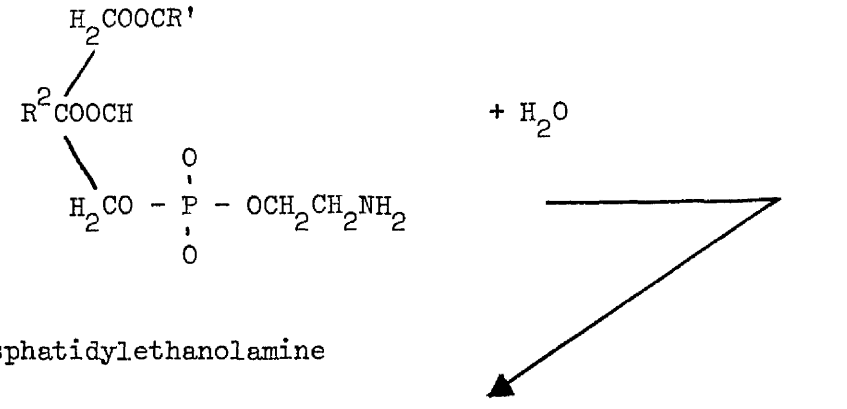
Human erythrocytes were four times less sensitive than cod erythrocytes to the action of δ-haemolysin. As a result, with the same concentration of δ-haemolysin in the inhibitory assay, a different inhibitory titre was obtained with the same serum sample when titrated against both species. However, by increasing the concentration of δ-haemolysin in the assay four-fold, the human erythrocyte system yielded titres of a level comparable to those obtained with cod erythrocytes.

Effect of B. cereus phospholipase C on inhibition of δ-haemolysin by phospholipids and zonal ultracentrifugation fractions

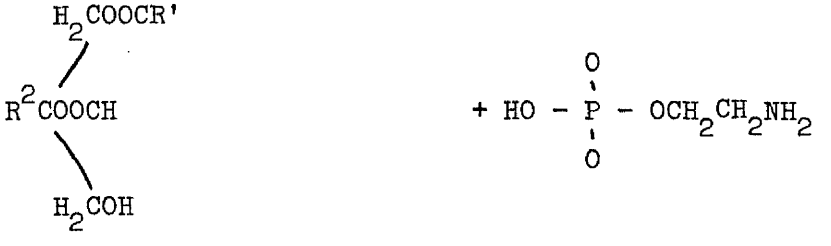
B. cereus phospholipase C (E.C.3.1.4.3) under the conditions of the assay system employed completely abolished the inhibitory capacity of phosphatidylethanolamine and phosphatidylinositol and diminished the effects of phosphatidic acid, phosphatidylcholine and phosphatidylglycerol and phosphatidylserine. The serum lipid extract was reduced four-fold in its inhibitory capacity against δ-haemolysin (Table 16). Sphingomyelin and cardiolipin were unaffected by pre-treatment with the enzyme. Serum

Figure 13 Mode of action of B. cereus phospholipase C

(phosphatidylcholine cholinephosphohydrolase EC 3.1.4.3)



phosphatidylethanolamine



1,2-Diacylglycerol O-phosphorylethanolamine

The hydrolysis of phosphoglycerides by phospholipase C results in the production of a diacylglycerol and a phosphorylic base (Zwaal et al, 1971).

α- and β-lipoproteins, purified by zonal ultracentrifugation, both had diminished inhibitory activity after enzyme treatment (Table 16).

Table 16 Reduction of inhibitory activity by pre-treatment with B. cereus phospholipase C (E.C.3.1.4.3.)

Inhibitor	Reduction by enzyme
Sphingomyelin	0
Phosphatidic acid	x 2
Phosphatidylcholine	x 4
Phosphatidylethanolamine	> x 8
Phosphatidylglycerol	x 8
Phosphatidylinositol	> x 512
Phosphatidylserine	x 16
Cardiolipin	0
Serum Lipids	x 4
β-lipoprotein	x 8
α-lipoprotein	x 8

DISCUSSION

The principal objective of this investigation was to identify the factors in human serum involved in the neutralisation of staphylococcal δ-haemolysin. Many previous studies have recorded this property of human serum, and suggested which factors might be responsible. But no definitive investigations to identify the inhibitory components have been undertaken.

One of the major difficulties envisaged in this study was to be able to identify the serum proteins present in any sub-fraction, and a large part of the investigation was therefore devoted to the use of various immunoelectrophoretic techniques in conjunction with different serum fractionation methods.

The inhibitory assay system required purified δ-haemolysin and the production, purification and characterisation of δ-haemolysin was also a major part of the work. However, determination of the properties of purified δ-haemolysin was regarded as secondary to the prime objective of investigating the inhibitory serum component.

Preparation of delta-haemolysin

The staphylococcal strain used for the production of δ-haemolysin, NCTC 10345, was chosen on the basis of its high human/rabbit erythrocyte titre ratio which reflected production of a large amount of δ-haemolysin and only a small quantity of α-haemolysin (Chao, 1976).

When δ-haemolysin was purified from crude culture supernatants of strain NCTC 10345 using hydroxylapatite, virtually all haemolytic activity against cod and rabbit erythrocytes was removed by hydroxylapatite

(Fig 3). Of the original haemolytic activity, 52.5% was recovered on elution with 1M phosphate buffer and the final yield of δ-haemolysin was approximately 236 mg per litre of culture supernatant. The yield of purified δ-haemolysin was similar to that obtained by Kreger et al (1971) but the total recovered activity (∿10,000 HU/litre supernatant tested using human erythrocytes) was about one-third of that found by these authors.

Properties of purified δ-haemolysin

Sufficient δ-haemolysin was prepared in one batch to be used throughout the study, and for this reason the small amount of insoluble δ-haemolysin produced on dialysis prior to concentrating and freeze-drying of the pooled 1.0M phosphate washes was discarded. This material, 60 mg, constituted 3% of the original activity, and the final lyophilised product of soluble δ-haemolysin comprised 15% of the culture supernatant activity. Fig 3 also shows that a considerable loss of activity occurred on dialysis, concentration by polyethylene glycol and batch freeze-drying but thereafter recovery was constant.

Throughout the study, freeze-dried δ-haemolysin was stored at 4°C in a desiccator in the dark as a fluffy, white powder, and over a period of 18 months, neither its appearance nor its haemolytic activity altered. Adsorption to glass or plastic surfaces was not evident (Heatley, 1971). The specific activity of the δ-haemolysin preparation used for the experiments described in this thesis was 320 HU/mg protein (0.8% cod erythrocytes) and 40 HU/mg (0.8% human erythrocytes) which was low compared to the preparations of Kreger et al (1971) using a similar strain of S. aureus and similar purification procedure. However, it had virtually

no haemolytic effect against rabbit and sheep erythrocytes (Marks and Vaughan, 1950) unlike the preparations of Kreger.

Other preparations made in this laboratory (Chao, 1976) and a sample provided by Dr. A. Kreger had specific activities comparable to that of Kreger et al (1971) with a more typical haemolytic spectrum. The reasons for the lower specific activity and narrower haemolytic spectrum were not investigated.

The ultraviolet absorption spectrum (Fig 4) showing a maximum at 271 nm with a small shoulder at 291 nm was very similar to those reported by Kayser (1968), Kreger et al (1971), Heatley (1971) and Chao (1976). The shoulder at 291 nm was consistently found and the ultraviolet spectrum was virtually identical to that of the amino acid tryptophan which would, in the absence of tyrosine, be expected to dominate the ultraviolet spectrum. Amino acid analysis of the preparation showed the absence of cysteine, histidine, arginine, proline and tyrosine explaining the ultraviolet profile and agreeing with the observations of Heatley (1971), Kreger et al (1971) and Kantor et al (1972). Fackrell and Wiseman (1976,b), however, reported proline one of the amino acids not present in this preparation, as the N-terminus of δ-haemolysin. Their haemolysin also varied in having trace amounts of histidine, tyrosine and arginine, but no methionine. Analysis of δ-haemolysin by polyacrylamide gel electrophoresis in acidic or alkaline buffers (Fig 5, Plate 1), yielded results comparable to those found by Kreger et al (1971), Heatley (1971) and Chao (1976), in that two bands were found in alkaline gels and a single diffuse band in acidic gels.

The secondary band seen in alkaline gels was faint in comparison to the principal band and may have been due to a contaminating protein. This seems unlikely, due to the amino acid composition, which would necessitate a contaminant devoid of the same amino acids, and since tests confirmed the absence of the other S. aureus extracellular products protease, coagulase, lipase, nuclease, gelatinase, phosphatase, fibrinolysin and egg-yolk activity. A second explanation would adopt the model of Kantor et al (1972) which postulates low molecular weight subunits of δ-haemolysin which might account for the secondary component.

The preparation was sensitive to trypsin (Kreger et al, 1971) and very thermostable (Marks, 1951; Gladstone and van Heyningen, 1957; Hallander, 1968). Indeed, on one occasion, brief heating at 100°C showed an increase in haemolytic activity, suggesting disaggregation of a complex, which might explain the overall low specific activity of the preparation. Like Yoshida's (1963), the preparation was poorly soluble at neutral pH and low ionic strength, and it precipitated at its pI, unlike Kreger's preparation. Mg^{++} and Ca^{++} ions had no effect on activity. Delta-haemolysin eluted from a Sephadex G-150 column as a single homogeneous peak with a molecular weight of approx. 200,000, which agrees closely with the value reported by Hallander (1968) and Fackrell and Wiseman (1976,b) using the same separation technique.

Purified δ-haemolysin of strain NCTC 10345 was focused as a single peak (pI 4.5) which contained all of the 280 nm-absorbing material and haemolytic activity. No cationic δ-haemolysin was detected. Several authors have reported an isoelectric point of around 4.5 for purified δ-haemolysin (Wadström, 1968; Kreger et al, 1971; Kantor et al,

1972). However, in contrast to the reports of Kreger et al (1971), Kantor et al (1972), McNiven et al (1974) and Fackrell and Wiseman (1976,b), no cationic δ-haemolysin was found in the present study. However, since refocusing of the cationic component yielded both anionic and cationic forms of δ-haemolysin (Kreger et al, 1971) and isoelectric-focusing in Tween 80 yielded only the anionic form (Kantor et al, 1972) it is likely that either total conversion of any cationic form to the anionic form had occurred in the present study or that the different isoelectric focusing apparatus caused the difference.

Therefore the preparation of δ-haemolysin possessed properties broadly similar to those described by Kreger et al (1971), Heatley (1971) and Kantor et al (1972), and the distribution of protein and haemolytic activity on isoelectricfocusing showed the preparation to be homogeneous, with no evidence for contamination with the cationic α-, β- and γ-haemolysins

Storage of human serum

Initially, whole blood from hospital blood banks was used as a source of human serum, but this was deemed unsuitable after immuno-electrophoretic studies on a freshly-drawn sample and the same sample after refrigerated storage showed the disappearance of some serum proteins with time. Thus fresh serum, drawn after 12 hours fasting and stored at $4^{o}C$ for no longer than 72 hours, was taken as the most uniformly constant material available. The reasons for the disappearance of these proteins were not pursued, although the appearance of a fine precipitate in the serum after prolonged storage or freezing and thawing was thought to be a possible contributory factor.

Doubling dilution inhibitory assay of human serum

Some variation in inhibitory activity of different serum samples was observed. However, since the haemolytic activity of the δ-haemolysin preparation remained unchanged throughout the course of the investigation, and a constant amount of δ-haemolysin (6 HU_{50}) was used in the inhibitory assay, and since duplicate estimations on any one sample yielded identical titres, the variation was judged not to be a weakness of the assay itself, but rather a reflection of varying levels of inhibitory factor present in different sera. The fluctuation observed was similar to that seen by Donahue (1969,a) but much less than that of Maniar et al (1967), thus underlining the desirability of standardising the test material with regard to source as described above.

Preliminary studies on the nature of the inhibitor

While suggestions as to the identity of the serum factors which inhibit δ-haemolysin have been made (Donahue, 1969,a,b; Kreger et al, 1971; Kantor et al, 1972), none have been accompanied by detailed experimental evidence, nor has a specific investigation been done. For this reason, an experimental approach was adopted which was not influenced by previous unsubstantiated reports, and with no preconceived ideas as to the identity of the inhibitor. In this way, it was hoped that the discovery of one factor would not preclude the discovery of any other, should more than one serum factor be involved.

Blood clotting factors are not involved in the inhibition, there being no difference in activity between plasma and serum, nor is an intact complement system from the evidence of heat inactivation studies.

The involvement of an acute phase protein, i.e. C-reactive protein, was also eliminated since inhibitory activity was unaffected by the presence or absence of Ca^{++}. Gel filtration and ultrafiltration indicated that the inhibitor was of M.Wt. greater than 100,000 daltons. These results, in conjunction with those of Laurell electrophoresis on inhibitory and non-inhibitory fractions, enabled γ-globulin and albumin to be eliminated as possible inhibitors. This confirmed the observation of Kapral (1972) regarding the inhibitory effect of albumin, a previous study having found albumin inhibitory (Gladstone and Yoshida, 1967).

The elution profile observed on Sephadex G-150 chromatography suggested that more than one molecular weight species was involved in inhibiting δ-haemolysin; one in excess of 400,000 M.Wt., the exclusion limit for Sephadex G-150, and one in the region intermediate between this value and the M.Wt. of albumin. This was confirmed by chromatography on the larger pore gel Sepharose 6-B, which yielded two distinct peaks of inhibitory activity, although whether distinct proteins or aggregates of the same protein were involved was unclear. A detailed study of the behaviour of serum proteins under ammonium sulphate precipitation at pH 5, and their chromatographic properties on gel filtration has previously been done (Freeman and Smith, 1970). This method precipitated at 50% saturation of ammonium sulphate all proteins which had not been precipitated in a 40% saturated solution. Further, Laurell immuno-electrophoresis of the breakthrough peak in Sephadex G-150 gel filtration gave a result similar to that reported here; three proteins were present which could be positively identified as haptoglobin, α_2-macroglobulin and β-lipoprotein. Since the fraction containing these serum proteins

inhibited δ-haemolysin, it was concluded that one of these proteins was inhibitory (assuming the inhibitor to be antigenic).

The first results implicating β-lipoprotein as inhibitory to δ-haemolysin were obtained by affinity chromatography. Prior to this, Laurell immunoelectrophoresis of inhibitory fractions from gel filtration, ammonium sulphate precipitation and ultrafiltration had shown β-lipoprotein in inhibitory fractions, though always in the presence of other serum proteins. Delta-haemolysin immobilised on an inert matrix of CNBr-Sepharose specifically removed β-lipoprotein from diluted serum (Plate 7). Kapral (1972) showed that many phospholipids (as well as human serum) were inhibitors of δ-haemolysin and since β-lipoprotein is only one of a complex group of serum proteins whose role in blood is the transport of triglycerides and phospholipids, the possibility that all these proteins were capable of inhibiting δ-haemolysin through their phospholipid moiety required more detailed investigation.

Zonal ultracentrifugation of human serum

In this study, purified fractions of human serum lipoproteins were obtained by the zonal ultracentrifugation method of Wilcox et al (1971) by exploiting the lower density of lipoproteins compared to other serum proteins. The resolution obtained by this method was similar to that of Wilcox et al (1971); β-lipoprotein migrated to a point in the gradient equivalent to density 1.06 g ml^{-1}, and α-lipoprotein to a point corresponding to 1.21 g ml^{-1}. These values agreed closely to the published values of 1.063 and 1.21 respectively (Langer et al, 1970). The peaks of inhibitory activity against δ-haemolysin corresponded to the two peaks of E_{280}-

absorbing material which corresponded in density to the expected values for the two lipoprotein species, with only a residual amount of inhibition being present in the major E_{280}-absorbing high density peak (Fig 11).

That the low density peak was β-lipoprotein was confirmed by polyacrylamide gel electrophoresis (Naito et al, 1973) and by the presence of a single Sudan Black-staining peak on Laurell immunoelectrophoresis with anti-whole human serum and monospecific anti-β-lipoprotein antiserum. The identity and homogeneity of the α-lipoprotein fraction was similarly confirmed. Perhaps most important of all, however, was the demonstration that all the inhibitory activity against δ-haemolysin in human serum resided in these two protein classes, and that the small amount of activity present in the zonal ultracentrifugation fraction containing the remainder of the serum proteins could be attributed to the trace amount of α-lipoprotein remaining in the dense fraction, as shown up by polyacrylamide gel electrophoresis (Plate 10; Table 13).

As previously discussed (page 31) β- and α-lipoproteins are simplified terms referring to a family of lipoproteins: "beta" encompassing the chylomicrons, very-low-density lipoproteins (VLDL) and low density lipoproteins (LDL); "alpha" has two subclasses designated high-density lipoproteins (HDL_2 and HDL_3) and a third called the very-high-density lipoproteins (VHDL). Limited experiments were attempted to separate, in particular, the β-lipoprotein into chylomicrons, VLDL and LDL, but with little success. This was due mainly to the very narrow gradient range over which these families are distributed, $p < 1.006 - 1.063$, and in the time available, the part of the molecule responsible for the inhibition of δ-haemolysin was at this stage regarded as of prime importance and the major aim of the thesis.

Precipitation studies

Immunoelectrophoresis of the high molecular weight inhibitory peak from Sephadex G-150 electrophoresis indicated that only four or five proteins were present in that fraction (Plate 5). A rabbit antiserum prepared against this mixture gave a precipitation reaction on radial gel diffusion with the mixture, and also abolished the inhibitory effect when incubated at 37°C with the fraction prior to titration. That β-lipoprotein was present in this sample (Freeman and Smith, 1970) was confirmed by gel diffusion of zonal-centrifugation-purified β-lipoprotein against the rabbit antiserum and also diffusion of the fraction against commercial monospecific anti-β-lipoprotein antiserum, a single line resulting in each case. Similarly, gel diffusion with monospecific anti-α-lipoprotein, anti-human serum, and anti-human serum albumin confirmed that there was no cross-contamination in purified α- and β-lipoprotein samples or contamination with other human serum proteins. Preincubation of inhibitory samples with these antisera confirmed that α- and β-lipoproteins were both inhibitory and also eliminated albumin as an inhibitor of δ-haemolysin (Gladstone and Yoshida, 1967). In the same way, preincubation of the rabbit antiserum abolished the inhibitory effect only of β-lipoprotein on delta: α-lipoprotein remained inhibitory.

Alpha- and β-lipoproteins were further confirmed as the sole serum inhibitors of δ-haemolysin by the immunocore electrophoretic technique (Zeineh et al, 1972). Purified zonal ultracentrifugation preparations of α- and β-lipoproteins gave single immune precipitates on electrophoresis against whole human serum antiserum, employing the lipoprotein disc-gel

electrophoretic technique of Naito et al (1973) in the first dimension. Control gels stained specifically for lipoproteins with Sudan Black confirmed the relative positions of the two lipoprotein species (Plate 10) and their identities were confirmed by development against monospecific antisera (Plate 13). When the antisera in the central cores were replaced in subsequent tests with δ-haemolysin, precipitates again formed in the gel corresponding to the positions of α- and β-lipoproteins.

Inactivation studies on δ-haemolysin inhibitors

The findings of Kapral (1972), that chromatographically pure preparations of phospholipids (Fig 12) were inhibitory to the action of δ-haemolysin were confirmed. Phosphatidyl choline, phosphatidyl glycerol, phosphatidyl inositol and phosphatidyl serine were all inhibitory at levels below 0.2 μg/HD50 (cf. Kapral's 1.5 μg/HD50), sphingomyelin, phosphatidic acid and cardiolipin were of similar activity to those found by Kapral, and phosphatidyl ethanolamine was the least active of all those phospholipids tested. In addition, a serum lipid extract chromatographically shown to contain phosphatidyl choline, sphingomyelin and phosphatidyl ethanolamine, completely inhibited the haemolytic activity of δ-haemolysin at a level of 1.2 μg dry weight/HD50. In drawing comparisons to Kapral's results, however, it must be born in mind that his indicator red cell was the human erythrocyte which has been shown (Chao, 1976) to be 4 to 8 times less sensitive to the action of δ-haemolysin than cod erythrocytes, and his δ-lysin was of higher activity than that reported here. Taking this into account, the correlation between those inhibitory levels reported here and those of Kapral (1972) may be much closer, assuming a similar inhibitory mechanism with both species of erythrocyte. This need not

necessarily be the case, however, since results of Kapral suggest that phospholipids inhibit δ-haemolysin by interfering with its binding to the erythrocyte membrane.

Enzyme studies with protease, trypsin and papain, on purified α- and β-lipoproteins eliminated the apoprotein moiety of the phospholipoprotein complex as the anti-δ-haemolysin component. Trypsin itself is inhibitory to delta-haemolysin confirming Kreger's et al (1971) findings, papain is not, and at the concentrations used in the enzyme assays, neither had an effect on the indicator red cell. In addition, the protein residue from a serum lipid extraction failed to inhibit the action of δ-haemolysin, but while this might indicate that apoprotein is non-inhibitory, one must consider the possibility that structural conformation changes or denaturation of the protein may have occurred during the lipid extraction. However, pretreatment of whole serum, α- and β-lipoprotein with trypsin and papain did not alter the inhibitory properties of the preparation, which suggests that apoprotein does not play a role.

The non-protein constituents of serum lipoproteins, as discussed earlier (pages 31-35), are phospholipids, neutral fats, triglycerides and cholesterol, but from the findings with serum lipid extracts, and those of Kapral (1972), phospholipids seemed most likely to be responsible for the inhibition of δ-haemolysin. Throughout the study, α-lipoprotein was more inhibitory than β-lipoprotein, and in terms of dry weight, the largest non-protein constituent of α-lipoprotein is phospholipid, approx. 60% of the total lipid component (Table 7); similarly, β-lipoproteins have a non-protein component predominantly composed of phospholipid and cholesterol esters, the latter having been shown to be non-inhibitory

(Kapral, 1972). Thus, these data when considered together indicate the phospholipid moiety as the mediator of inhibition. Enzyme degradation by B. cereus phospholipase C confirmed this hypothesis (Table 16). The high haemolytic activity of B. cereus phospholipase C on cod erythrocytes is compatible with the finding of Addison and Ackman (1971) that lecithin is the predominant phospholipid in cod erythrocytes. Bacillus cereus phospholipase C cannot degrade phospholipids in intact cells, but produces complete degradation of the main phospholipid classes in erythrocyte ghosts with the exception of sphingomyelin (Zwaal et al, 1971); in mixed monolayers of lecithin and sphingomyelin, spread at an initial surface pressure above 31 dynes/cm, B. cereus phospholipase C cannot act unless sphingomyelin is first degraded (Demel et al, 1975). In this study, pretreatment of all phospholipids tested with B. cereus phospholipase C decreased to some extent the inhibitory action against δ-haemolysin, except for sphingomyelin. In the case of phosphatidyl inositol and phosphatidyl ethanolamine inhibitory activity was totally abolished. With the serum lipid extracts, shown chromatographically to contain three phospholipids, the reduction was not so dramatic (two-fold) but since the predominant phospholipid detected in the extract was sphingomyelin, this was not unexpected. Most significant of all, however, was the observation that both α-lipoprotein and β-lipoprotein were reduced in inhibitory activity against δ-haemolysin when pretreated with the phospholipas

The results presented in this thesis, therefore, show that α- and β-lipoproteins are the components of human serum which inhibit the activity of δ-haemolysin, and that an intact phospholipid moiety of the lipoprotein is essential for activity. However, the mechanism by which

this operates is unclear. Kapral (1972) concluded that the minimal inhibitory unit was phosphatidic acid, and later (1976) found that straight chain fatty acids of twenty-one to twenty-three carbons also inhibited δ-haemolysin. This may well explain the inability seen here to totally abolish the inhibitory effect of some phospholipids, since cleavage by phospholipase of, e.g. lecithin to phosphoryl choline and diacylglycerol would still leave intact on the latter molecule a long-chain fatty acid of required length to inhibit δ-haemolysin (assuming such a fatty-acid residue was present).

Kapral (1972) also suggested that phospholipids inhibit δ-haemolysin by interfering with its binding to the erythrocyte membrane, although whether this would be sterically possible when the phospholipid was in a very high molecular weight complex as it is with serum lipoproteins is again uncertain. Streptolysin S (SLS) from Streptococcus pyogenes is a haemolysin which closely resembles δ-haemolysin in terms of cytotoxicity, biological activity and difficulty of proving antigenicity, and has also been found to be inhibited by a serum phospholipoprotein complex (Stollerman et al, 1950). As is reported here, lecithinase abolished inhibitory activity against SLS which suggests that activity against SLS also resides within the phospholipid moiety. Streptolysin O (SLO) is inhibited by cholesterol, in this case the mechanism appearing to involve exposed cholesterol molecules on peptide fragments of the whole molecule (Watson, personal communication), and perhaps a similar reaction involving phospholipid may be in operation with δ-haemolysin.

Identification of α- and β-lipoproteins as the inhibitors of

δ-haemolysin explains some of the inconclusive results obtained during the early stage of the investigation. Thus for Sephadex G-150 chromatography the two M.Wt. species involved in inhibition were respectively above and below the exclusion limit for the gel. Similarly, the fact that despite exhaustive filtration inhibitory activity was retarded by a 300,000 M.Wt. membrane, can also be explained by the presence of two M.Wt. species. Isoelectricfocusing also was of limited value, since α- and β-lipoproteins focus at pI 5.5 and 5.4 respectively, which coincides with a large majority of other serum proteins.

Pathogenic importance of δ-haemolysin

In view of the above results, it is interesting to speculate on the pathogenic importance of δ-haemolysin, and a possible role for it in S. aureus infections. While it is apparent that δ-haemolysin is neutralised in vitro by human serum, such a situation in vivo need not necessarily follow. It is possible that although inhibition by lipoprotein might appear to be an efficient substitute for an immune response, with lipoprotein binding with the haemolysin to give a product similar to an immune complex, such an interaction may be weak, and the haemolysin may be released intact from its lipoprotein carrier at sites of lipoprotein catabolism. Alternatively, the fate of a lipoprotein-haemolysin complex may be phagocytosis by macrophages, or removal by the liver or spleen. Under such conditions, δ-lysin may be able to exhibit its diverse activity against tissue cells and cause damage within these organs.

There is a veterinary problem which in some respects is not dissimilar to the phenomenon involving δ-haemolysin. A current working hypothesis adopted in schistosomiasis research ('bilharziasis' in man) is that there are antigens of host origin that serve to protect the

parasite from immune attack. This is achieved by attachment of the host protein to the surface of the parasite, completing the immunological disguise of the adult worm and protecting it from immune attack (Terry and Smithers, 1975). In this way the parasite remains viable. The situation with δ-haemolysin may perhaps be viewed in a similar way: a disguising association with serum lipoprotein may in effect make it "not foreign" to the host's immune mechanisms, and leave its biological potential undiminished. Thus from the point of view of the host, a true immune response to δ-haemolysin may be a desirable event.

Elucidation of the factors in serum responsible for the neutralisation of δ-haemolysin made it possible to stimulate an antibody response to the haemolysin (see appended paper). The reported non-antigenicity of delta-haemolysin (Gladstone and Yoshida, 1967; Hallander, 1968; Kantor et al, 1972) can now be ascribed to the removal of the antigen by lipoproteins before induction of the immune response. Further, the presence of non-specific haemolysin inhibitors in sera from animals in which an immune response had been elicited have hindered detection of immune precipitin lines in immunochemical and serological tests. We have shown that the presence of antibodies to δ-haemolysin can be more easily demonstrated by the removal of lipoproteins from immune sera prior to immunochemical analysis.

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APPENDIX

Appendix I

Media

Yeast diffusate medium (Bernheimer and Schwarz, 1963)

Yeast extract diffusate	3200 ml
Bacto Casamino Acids (Difco)	64 g
Glucose	8 g
Nicotinic Acid	3.7 mg
Aneurine hydrochloride	0.4 mg

The pH was adjusted to 7.1 with 1N NaOH and the medium autoclaved at 15 lb/in^2 for 15 min.

Yeast extract diffusate

Yeast extract (Difco)	200 g
Distilled water	500 ml

Yeast extract was dissolved in distilled water by steaming and after cooling was poured into a 50 cm length of 2.3/4" Visking dialysis tubing (Scientific Instrument Centre, London) previously soaked in 70% ethanol to minimise contamination. The dialysis sac was immersed in 1600 ml distilled water in a 5 l beaker and stirred for 48 hours at $4^{\circ}C$. The dialysis sac and contents were discarded and the diffusate was made up to 1600 ml with distilled water. Duplicate batches of diffusate were normally prepared to yield 3200 ml of diffusate.

"Antifoam" (1 ml of a 1/20 dilution/500 ml culture media; Silcolapse 5000, I.C.I. Stevenson, Ayrshire) was added to prevent frothing.

Appendix II

Buffers and Diluents

Citrate/Dextrose/Sodium chloride solution (CDS, Hodgkins and Ridgway, 1964)

Dextose (Glucose)	2.05 g
Trisodium citrate	0.80 g
Sodium chloride	0.40 g
Distilled water to	100.00 ml

The solution was sterilized by membrane filtration.

Dulbecco's A phosphate buffered saline (Dulbecco and Vogt, 1954)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water to	1000 ml

The pH was adjusted to 7.3 if necessary and the buffer autoclaved at 15 lb/in^2 for 15 min.

Potassium phosphate buffers

i) 1.0M phosphate (pH 7.4) buffer.

Stock solns:

A: 1.0M solution of potassium dihydrogen orthophosphate (13.6 g KH_2PO_4 in 100 ml distilled water).

B: 1.0M solution of potassium hydrogen orthophosphate (17.4 g K_2HPO_4 in 100 ml distilled water).

Nineteen ml of solution A and 81 ml of solution B were mixed and the pH adjusted by adding either stock solution until a pH of 7.4 was obtained.

ii) 0.4M phosphate (pH 6.8) buffer.

Stock solutions were obtained by dilution of the above 1.0M stock solutions.

Approximately 45 ml of solution A and 55 ml of solution B were mixed and the pH adjusted to 6.8 by adding either stock solution.

iii) 0.01M phosphate (pH 6.8) was obtained by 1/40 dilution of the 0.4M phosphate buffer.

Sodium phosphate buffers, 0.1M, 0.01M and 0.001M, pH 6.8 (Sørensen, 1909)

A: 0.1M sodium dihydrogen orthophosphate (13.9 g Na_2HPO_4/l in distilled water).

B: 0.1M disodium hydrogen orthophosphate (35.8 g $Na_2HPO_4.12H_2O$/l in distilled water).

Five hundred and ten ml of solution A and 490 ml solution B were mixed and the pH adjusted to 6.8.

The 0.01M and 0.001M buffers were obtained by dilution of the above buffer in distilled water.

Tris HCl buffer

Stock solutions

A: 0.2M Tris (24.2 g made up to 1 litre)

B: 0.2M HCl (17.15 ml conc HCl made up to 1 litre)

50 ml A + x ml B diluted to a total of 400 ml gives a 0.05M 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), pH 8.0

To the appropriate solution above, 67.5 g NaCl was added, and made up to 1 litre with buffered solution.

Palitsch buffer for coagulase assay

Solution A: (0.05M sodium tetraborate)

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

Solution B: (0.2M basic 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.

Hyaluronidase diluent

0.2M Na_2HPO_4	5 ml
0.2M NaH_2PO_4	5 ml
1.0M NaCl	7.7 ml
Bovine serum albumin fraction V	10 mg
Adjust pH to 6.9 if necessary with 1M NaOH or 1M HCl	
Distilled water to 100 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.	

Borate buffer

Solution A:	boric acid, H_3BO_3	12.4 g
	Distilled water to 1,000 ml	
Solution B:	borax, $Na_2B_4O_7.10H_2O$	19.05 g
	Distilled water to 1,000 ml	

For 0.1M buffer, pH 8.0, containing 1.0M NaCl, 58.4 g NaCl is added to 250 ml of solution A plus 24.5 ml of solution B, and made up to 1,000 ml with dist. H_2O.

For 0.1M Buffer, pH 8.3, containing 0.5M NaCl, 29.2 g NaCl is added to 250 ml of solution A plus 50 ml of solution B, and made up to 1,000 ml with dist. H_2O.

Acetate buffer

0.1M acetate buffer, pH 4.0.

Solution A:	glacial acetic acid	11.55 ml
	Distilled water to 1,000 ml	
Solution B:	sodium acetate, CH_3COONa	16.4 g
	Distilled water to 1,000 ml	

For 0.1M buffer, pH 4.0, add 410 ml of solution A to 90 ml of solution B and add dist. H_2O to 1,000 ml.

Acetate buffer plus sodium chloride

0.1M acetate, pH 4.0 containing 1.0M NaCl.

NaCl was added to the above buffer at a concentration of 58.4 g/l.

Barbitone buffer, pH 8.6

Barbitone (diethylbarbituric acid)	1.84 g
Barbitone sodium	10.31 g
Distilled water to 1,000 ml.	

Appendix III

Preparation of hydroxylapatite (Tiselius et al, 1956)

Solutions

i)	$CaCl_2$ 0.5M	2 litres
ii)	Na_2HPO_4 0.5M	2 litres
iii)	Phosphate buffers, pH 6.8 - see appendix II	
iv)	NaOH (40% w/w)	100 ml

Procedure

Calcium phosphate has been shown to exist in several crystalline forms including brushite and hydroxylapatite. Tiselius et al (1956) showed that hydroxylapatite could be prepared from brushite. Brushite was prepared by allowing 2 litres of aqueous solutions of $CaCl_2$ and Na_2HPO_4 to run at an equal flow rate (120 drops/min) into a glass beaker under stirring. The supernatant was removed by decantation and the precipitate washed four times by decantation with distilled water, resuspended to 4 l with distilled water and 100 ml freshly prepared 40% (w/w) NaOH was added. The brushite was boiled under stirring for one hour and very fine material was removed by decantation. The precipitate was washed by decantation four times with distilled water, resuspended in 3 litres of 0.01M phosphate buffer (pH 6.8) and heated just to boiling. The supernatant was decanted and fresh phosphate buffer added and the suspension boiled for 5 min. The supernatant was again decanted and the suspension boiled for 15 min, in fresh 0.01M buffer and then boiled twice in 0.001M phosphate buffer for 15 min to convert the brushite to hydroxylapatite. The suspension was stored in 0.001M phosphate buffer at $4^{o}C$.

Appendix IV

Reagents for Protein Estimation (Lowry et al, 1951)

Reagent A: 2% sodium carbonate (Na_2CO_3) in 0.1N sodium hydroxide.

Reagent B: 0.5% copper sulphate ($CuSO_4.5H_2O$) in distilled water.

Reagent C: 1% aqueous solution of potassium sodium tartrate.

Reagent D: Equal volumes of reagents B and C were mixed and 1 ml added to 50 ml of reagent A. The solution was discarded after one day.

Reagent E: Folin-Ciocalteau reagent (B.D.H., Poole, Dorset). The Folin reagent was standardised by titration against 1N NaOH using phenolphthalein indicator and diluted to give a 1N solution.

Appendix V

Disc Gel Electrophoresis

Abbreviations

BIS N,N'-methylenebisacrylamide (B.D.H., Poole, Dorset)

Tris Tris(hydroxymethyl)aminomethane (Puriss, A.R., Koch-Light, Colnbrook, Bucks.)

TEMED N,N,N',N'-tetramethylethylenediamine (B.D.H., Poole, Dorset)

SDS sodium dodecylsulphate (Koch-Light, Colnbrook, Bucks.)

i) Acid system (pH 4.3 - 7.5% acrylamide)

I Stock solutions

A)	1N Potassium hydroxide	48 ml
	Acetic acid (glacial)	17.2 ml
	TEMED	4.0 ml
	Distilled water to 100 ml	
B)	1N Potassium hydroxide	48 ml
	Acetic acid (glacial)	2.87 ml
	TEMED	0.46 ml
	Distilled water to 100 ml	
C)	Acrylamide	30 g
	BIS	0.8 g
	Distilled water to 100 ml	
D)	Acrylamide	10 g
	BIS	2.5 g
E)	Riboflavin	4 mg
	Distilled water to 100 ml	
F)	Ammonium persulphate	0.28 g
	Distilled water to 100 ml	
G)	Sucrose (B.D.H., Analar)	40 g
	Distilled water to 100 ml	

Preparation of separating gel

Solution A	2 ml
Solution C	4 ml
Distilled water	2 ml
Solution F	8 ml

The solution was mixed well and 1.0 ml volumes pipetted into 7.5 x 0.5 cm (internal diameter) disc gel tubes sealed at one end with parafilm. The solution was carefully overlaid with 0.1 ml distilled water and the gels allowed to polymerise for 30 min in the dark.

II Preparation of large pore solution (pH 6.8)

Solution B	0.5 ml
Solution D	1.0 ml
Solution E	0.5 ml
Distilled water	2 ml

Water was removed from the gel tubes by inversion onto absorbant paper and each tube was rinsed with large pore solution and carefully overlaid with 0.15 ml large pore solution and 0.1 ml distilled water. Large pore gels were photopolymerised for 15 min.

III Tray buffer (pH 4.5)

L-alanine	31.2 g
acetic acid (glacial)	8 ml
Distilled water to 1000 ml	

The buffer was diluted 10 fold for use.

Staining solution

Amido black (1%) in 7% acetic acid.

Destaining gel

Solution A	1 ml
Solution C	2 ml
Distilled water	1 ml
Solution F	4 ml

Destaining solution

Acetic acid (7%) in distilled water.

ii) Alkaline system (pH 8.9 - 7% acrylamide)

I. Stock solutions

A)	1N HCl	48 ml
	Tris	36.6 g
	TEMED	0.23 ml
	Distilled water to 100 ml	
B)	1N HCl	48 ml
	Tris	5.98 g
	TEMED	0.46 ml
	Distilled water to 100 ml	
C)	Acrylamide	28 g
	Bis	0.735 g
	Distilled water to 100 ml	
D)	Acrylamide	10 g
	Bis	2.5 g
	Distilled water to 100 ml	
E)	Riboflavin	4.0 mg
	Distilled water to 100 ml	
F)	Ammonium persulphate	0.14 g
	Distilled water to 100 ml	

G) Sucrose 40 g

Distilled water to 100 ml

II Preparation of separating gel and large pore solution

The procedure was exactly as for the acid gel system except that the solutions specified above were used.

III Tray buffer (pH 8.3)

Tris	6 g
Glycine	28.8 g
Distilled water to 1000 ml	

The buffer was diluted 10 fold for use.

Staining solution, destaining gel and destaining solution

These were as described for the acid gel system.

Disc gel electrophoresis of serum lipoproteins

Alkaline system (pH 8.9 - 3.6% acrylamide)

I Stock solutions

A)	Tris	36.6 g
	TEMED	0.46 ml
	dilute HCl (1 mol/l)	48 ml
	Distilled water to	98 ml
	pH to 8.9 with 1M HCl	
	Distilled water to 100 ml	

B)	Tris	5.98 g
	TEMED	2.3 ml
	Dilute HCl (1 mol/l)	48 ml
	Distilled water to	98 ml
	pH to 6.6 with 1M HCl	
	Distilled water to 100 ml	
C)	Acrylamide	9.6 g
	Bis	0.252 g
	Distilled water to 100 ml	
D)	Acrylamide	10.0 g
	Bis	2.5 g
	Distilled water to 100 ml	
E)	Riboflavin	4.0 mg
	Distilled water to 2,000 ml	
F)	Riboflavin	15.38 mg
	Distilled water to 1,000 ml	
G)	Ammonium persulphate	0.14 g
	Distilled water to 100 ml	
H)	Sudan Black B	10 mg
	Ethylene glycol	10 ml

Prepare two weeks before use and store in the dark.

Reservoir buffer (pH 8.4)

Tris	4.0 g
glycine	19.2 g
Distilled water to 1,000 ml	

Preparation of separating gel

Solution A	2 ml
Solution C	6 ml
Solution G	8 ml

The solution was mixed well and 1.0 ml volumes pipetted into 8 x 0.6 cm (internal diameter) disc gel tubes sealed at one end with parafilm. The solution was carefully overlaid with 0.2 ml distilled water and the gels allowed to polymerise for 30 min.

Preparation of spacer gel

Solution B	1 ml
Solution D	2 ml
Solution E	5 ml

Water was removed from the gel tubes by inversion onto absorbant paper, and each tube overlaid with 0.1 ml spacer gel. Water was again carefully applied to prevent a meniscus, and the gel photopolymerised under a fluorescent lamp for about 30 min.

Preparation of sample gel

Solution B	1 ml
Solution D	2 ml
Solution F	0.65 ml
* Working Sudan Black B Solution	2.35 ml

*Made by mixing 1.2 ml Solution H with 8.8 ml ethylene glycol.

The water overlay was removed as before, and 0.2 ml sample gel added to each tube, followed by 20 µl of sample. The tubes were covered with parafilm, and inverted about ten times to mix sample and gel. After overlaying with reservoir buffer, the gels were photopolymerised for about 45 min.

Immunocore electrophoresis

Alkaline system (pH 8.9 - 3.6% acrylamide)

Stock solutions : as for electrophoresis of serum lipoproteins.

Disc gel tubes 125 mm x 6 mm (internal diameter) were filled with the same quantities of separating, spacer and sample gel as described above, the presence of the central rod (185 mm x 3 mm) resulting in a longer column of gel.

Appendix VI

Phosphatidic acid (Na salt)	Batch no. 62057
(ex egg lecithin, lyophilised)	Koch-Light
Phosphatidyl ethanolamine	Batch no. 62510
(ex *E. coli*)	Koch-Light
L-α-phosphatidyl-DL-glycerol ($CHCl_3$/MeOH)	Lot no. 15C-8130
(ex egg lecithin, ammonium salt)	Sigma, London
L-α-phosphatidyl inositol ($CHCl_3$)	Lot no. 124C-8160
(ex soybean, ammonium salt)	Sigma, London
phosphatidyl-L-serine ($CHCl_3$)	Lot no. 114C-8260
(ex bovine brain)	Sigma, London
cardiolipin (diphosphatidylglycerol)	Lot no. 43C-1840
(ex bovine heart, Na salt) (EtOH)	Sigma, London
sphingomyelin	Lot no. 88B-0320
(ex bovine brain)	Sigma, London
lecithin ($CHCl_3$)	
(ex egg yolk)	
Sheep anti-whole human serum	Lots K0034, K8152
	Wellcome Reagents, England
Rabbit anti-α-lipoprotein	
	Boehringer, Mannheim
Rabbit anti-β-lipoprotein	
	Boehringer, Mannheim

Trypsin	Type 1: 2 x crystallised from bovine pancreas. Ethanol precipitate. Activity: 10,000 BAEE units/mg. Sigma, London
Trypsin inhibitor	Type 1-5: lyophilised from soybean. Chromatographically prepared. 1 mg inhibits 1-3 mg trypsin, activity of 10,000 BAEE units/mg protein. Sigma, London.
Papain	2 x crystallised from papaya latex, sodium acetate suspension. Activity 16-40 BAEE units/mg. Sigma, London.
B. cereus phospholipase C (E.C.3.1.4.3)	In 3.2M ammonium sulphate 400 U/mg: lecithin substrate, 37°C. Boehringer, Mannheim.

Appendix VII

Stains

a) Protein Stains

i) Azocarmine

Azocarmine	1 g
1M Acetic acid	425 ml
0.1M Sodium acetate	425 ml
Glycerol	150 ml

Plates were washed in 2% acetic acid containing 15% glycerol to remove excess stain.

ii) Amido black

Amido black	1 g

Solutions as for azocarmine.

b) Lipoprotein Stains

i) Oil Red O

Oil red O	1 g
60% ethanol	1000 ml

Saturation was accomplished at 37°C with occasional stirring over 16-24 hours. After cooling to room temperature, the solution was filtered and stored in dark bottles.

ii) Sudan Black

Sudan black (1 g) was suspended in the same way as for Oil Red O.

c) Transferrin stain

Bathophenanthroline	2 mg
Deionised water	2 ml
0.02M sodium acetate	18 ml

After immersion of plates in the bathophenanthroline reagent for 2 hours, 0.1 ml thioglycolic acid was added. After a further 5 min, plates were washed in 2% acetic acid. Transferrin was stained red.

d) Caeruloplasmin stain

0.2M Sodium acetate	50 ml
0.02M acetic acid	50 ml
p-phenylenediamine	21.6 mg

After warming to 37°C, plates were immersed for 2-3 hours and subsequently washed in 2% acetic acid. Caeruloplasmin-antibody precipitates were stained brown.

Pyronin Y

Pyronin Y	2 g
Dist. H_2O	100 ml

Stored in dark bottles.

Bromophenol blue

Bromophenol blue	10 mg
Dist. H_2O	100 ml

Neutralisation of Staphylococcal Delta-haemolysin by Human Plasma Lipoproteins.

By D.D. Whitelaw and T.H. Birkbeck (Department of Microbiology, University of Glasgow, Garscube Estate, Bearsden, Glasgow G61 1QH)

Staphylococcal δ-haemolysin is neutralised by normal mammalian sera (1) and forms a precipitin line on gel diffusion with normal sera (2) but the serum factors involved have not hitherto been identified.

The inhibitory titre of normal human plasma assayed using purified δ-haemolysin with cod erythrocytes as indicators of haemolysis (3) was 1/500-1/1000. Fractionation of normal human plasma by Sephadex G150 chromatography, ammonium sulphate precipitation and isoelectric focusing indicated that plasma lipoproteins were the inhibitors of δ-haemolysin. Purified high-density and low-density plasma lipoproteins were obtained by zonal ultracentrifugation (4) using potassium bromide density gradients in the MSE BXIV titanium zonal rotor, and characterised by ultracentrifugation, polyacrylamide gel electrophoresis (5), immunoelectrophoresis and gel diffusion using monospecific antisera. Both high-density and low-density lipoproteins neutralised δ-haemolysin and gave a precipitin line on gel diffusion but the fractions containing the remainder of the plasma proteins were non-inhibitory.

The inhibitory activity of lipoproteins was not diminished by treatment with papain or trypsin whereas Bacillus cereus phospholipase C (EC 3.1.4.3) destroyed inhibitory activity. In the latter case, δ-haemolysin was assayed using human erythrocytes since B. cereus phospholipase C was highly haemolytic for cod erythrocytes (25000 HU/mg) but inactive with

human erythrocytes. Phospholipase C also abolished the inhibitory activity of phosphatidyl choline and phosphatidyl glycerol providing further evidence that binding of δ-haemolysin to plasma lipoproteins was via the phospholipid moiety (cf. 6).

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Immunogenicity of Staphylococcal Delta-Haemolysin

By T.H. Birkbeck and D.D. Whitelaw (Department of Microbiology, University of Glasgow, Garscube Estate, Bearsden, Glasgow G61 1QH).

The binding of serum lipoproteins to staphylococcal δ-haemolysin to neutralise cytolytic activity or to form precipitin lines on gel diffusion (1) has undoubtedly contributed to the difficulty in demonstrating the immunogenicity of δ-haemolysin although precipitinogens have been demonstrated in IgG fractions of hyperimmune rabbit sera (2).

We have immunised rabbits with purified soluble Staphylococcus aureus (strain NCTC 10345) δ-haemolysin by subcutaneous (with or without Freund's complete adjuvant) or intravenous (without adjuvant) routes, using a schedule similar to that of Fackrell and Wiseman (2). On immunodiffusion, immune sera gave two precipitin lines with purified δ-haemolysin, only one of which was present in pre-immune sera. After zonal centrifugation in potassium bromide density gradients lipoprotein-free pre-immune sera contained no precipitating or neutralising activity against δ-haemolysin but all immune sera gave a single precipitin line on immunodiffusion and neutralised 6 HU_{50} δ-haemolysin at dilutions of up to 1/320. Highest neutralising titres were obtained in rabbits immunised subcutaneously with δ-haemolysin emulsified in Freund's complete adjuvant (titres 1/160 and 1/320) and lowest titres were obtained by intravenous immunisation (1/10-1/20).

A single precipitin line with a reaction of complete identity was formed on immunodiffusion between lipoprotein-free immune serum and δ-haemolysin purified by isoelectrofocussing (pI 10.0 strain Wood 46),

hydroxylapatite chromatography (strain NCTC 10345) and concentrated crude culture supernatants of strain Wood 46. The neutralising and precipitating activities of lipoprotein-free immune sera were present in IgG fractions prepared by precipitation with ammonium sulphate (33% saturation) and DEAE-cellulose chromatography. Other serum proteins were absent from the IgG fractions as judged by polyacrylamide gel electrophoresis or immunoelectrophoresis and it was concluded that staphylococcal δ-haemolysin was immunogenic in rabbits.

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