The Immunogenicity of Staphylococcal Delta-Haemolysin

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To my husband, David,

and my parents for so

many things.

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SUMMARY

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Native δ-haemolysin of Staphylococcus aureus was poorly immunogenic in the mouse, inducing low anti-δ-haemolysin antibody titres in only 50% of immunised animals. Treatment of δ-haemolysin with formaldehyde at pH 5 or pH 7.5 caused a rapid loss of haemolytic activity and, on treatment for 7 days at pH 5, gave a product of higher immunogenicity than the native material.

Treatment of δ-haemolysin with formaldehyde at pH 9.5 for 7 days reduced the haemolytic activity by 97% but failed to enhance the immunogenicity.

Antisera to formaldehyde-treated δ-haemolysin contained antibodies directed against antigenic sites not present on the native molecule.

Formaldehyde-treated δ-haemolysin had an increased electrophoretic mobility and a reduced isoelectric point but there was no evidence of polymerisation. The enhanced immunogenicity may be due to a reduced affinity for phospholipids, resistance to enzymic digestion, increased rigidity and/or the masking of a suppressor determinant on the molecule.

Treatment of δ-haemolysin with glutaraldehyde under acid, neutral or alkaline conditions produced a rapid loss of haemolytic and immunogenic activities.

High doses of δ-haemolysin (62.5 - 1000 μg) gave enhanced vascular permeability (EVP) when injected intradermally in the rabbit. The response was obtained at a critical time of 1 h between injection of sample intradermally and of Pontamine Sky Blue Dye intravenously. The EVP activity was resistant to heating at 100°C. Treatment of the haemolysin with lecithin or formaldehyde reduced, but did not eliminate,

this activity. Tryptic digestion studies indicated that the EVP activity was associated with fragments which lacked both haemolytic and antigenic activities, and suggested that the various biological activities of the molecule may not be mediated by a common site or mechanism.

Several strains of S. aureus were screened for haemolysin production. The majority of human and animal strains produced high titres of α- and δ-haemolysins but little or no β-haemolysin. The antigenic reactivity of the crude culture supernates with rabbit anti-δ-haemolysin antisera indicated that all animal strains and all but one of the human strains produced a haemolysin which was antigenically identical to purified δ-haemolysin from strain NCTC 10345. The human strain, LS26, produced a δ-haemolysin which precipitated with antibodies against purified δ-haemolysin and which carried specificities directed against determinants not exposed on the native molecule. These results suggested that "hidden" antigenic determinants may be "exposed" during immunisation with δ-haemolysin which induce specific antibody formation. Production of δ-haemolysin by strain LS26 may expose these "hidden" antigenic determinants which would therefore be free to precipitate with the specific antibody.

Several of the crude delta-haemolysin preparations had very similar isoelectric points to that of purified δ-haemolysin. Only two out of seven crude preparations induced the production of anti-δ-haemolysin antibodies.

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ABBREVIATIONS

A	-	amps
Ab	-	antibody
BIS	-	NN'-Methylenebisacrylamide
BSA	-	bovine serum albumin
CCY	-	casein(acid-hydrolysed)-casein(trypsin-hydrolysed)-yeast extract medium
CDS	-	citrate/dextrose/saline
CIE	-	counter-immunoelectrophoresis
cAMP	-	cyclic adenosine monophosphate
DMSO	-	dimethylsulphoxide
E	-	extinction coefficient
E^{xmm}_{ynm}	-	extinction value of a solution in a cell of x mm light path at a wavelength of y nm
EDTA	-	ethylene diamine tetra acetate
EVP	-	enhanced vascular permeability
F5	-	pH5 formaldehyde-treated δ-haemolysin
F7.5	-	pH7.5 formaldehyde-treated δ-haemolysin
F9.5	-	pH9.5 formaldehyde-treated δ-haemolysin
FCA	-	Freund's Complete Adjuvant
g	-	gram
g	-	force of gravity
G5	-	pH5 glutaraldehyde-treated δ-haemolysin
G7.5	-	pH7.5 glutaraldehyde-treated δ-haemolysin
G9.5	-	pH9.5 glutaraldehyde-treated δ-haemolysin
HI	-	haemolysin inhibitory unit
HSA	-	human serum albumin
HU	-	haemolysin unit
I	-	ionic strength

IgG - immunoglobulin G

LD_{50} - median lethal dose

mA - milliamp

MMD - 3,6-Bis(acetoxymercurimethyl)dioxan

NAHTL - N-acetyl-DL-homocysteinethiolactone

NCTC - National Collection of Type Cultures

PAGE - Polyacrylamide Gel Electrophoresis

PB - phosphate buffer

PBSA - phosphate buffered saline A } Appendix II

PBS_2 - phosphate buffered saline 2

PHA - passive haemagglutination

pI - isoelectric point

rbc - red blood cell (erythrocyte)

rpm - revolutions per minute

RIE - rocket immunoelectrophoresis

S - sedimentation coefficient

S_{20w} - sedimentation coefficient corrected to $20^{\circ}C$ in distilled water

SDS - sodium dodecyl sulphate

-SH - thiol group

TEMED - N,N,N',N' tetramethylethylenediamine

TEN - toxic epidermal necrolysis

UTI - urinary tract infection

UV - ultraviolet

V - volt

W - watt

INTRODUCTION

Staphylococcal Infections

The occurrence of staphylococci in human pus was first described by Robert Koch in 1878. Pasteur (1880) successfully cultivated the organism in liquid media and Ogston (1882) later showed it to be pathogenic for mice and guinea pigs.

Staphylococcus aureus, as well as occurring as a harmless commensal in the nose and on the skin of many healthy persons, is also the cause of a diverse array of both superficial and systemic infections (Table 1).

Although strains of high virulence can occasionally cause outbreaks of infection in certain human populations, S. aureus is more frequently an opportunistic pathogen in the sense that it causes infection most commonly in tissues and sites of lowered host resistance. Table 2 lists the many factors and conditions which may lead to the establishment of a staphylococcal infection.

Staphylococcal infections have been reviewed by Shulman and Nahmias (1972) and Musher and McKenzie (1977). The skin is the most common site of infection and, once virulent staphylococci have gained a foothold in deeper tissues of the body, their multiplication causes necrosis and eventual abscess formation.

In severe infections the organisms may break through the localised barrier of the lesion(s) and invade the lymphatics and bloodstream. If bacteraemia becomes established, metastatic foci frequently develop (Table 1).

The gastrointestinal tract is also a frequent site of infection. The most commonly found staphylococcal enteritis is food poisoning mediated by an enterotoxin formed by certain staphylococci during

Table 1 Spectrum of Staphylococcal Infections

Local Infections

Site	Infection
skin	carbuncles, boils, abscesses, impetigo, toxic epidermal necrolysis
eye	conjunctivitis
nose and throat	pharyngitis, sinusitis
lung	primary pneumonia
gastrointestinal tract	entercolitis
vagina	cervicitis, pelvic abscesses

Metastatic Infections

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

Adapted from Shulman and Nahmias (1972).

Table 2. Factors predisposing to S. aureus infection

Predisposing Factor		Examples	Possible resulting infection
1. Injury to normal skin	-	traumatic abrasions and wounds, surgical incisions, burns, 1° skin diseases	Initially local with consequent bacteraemia and metastasis.
2. Prior viral infections	-	influenza, measles	Secondary pneumonia.
3. Leucocyte defects	-	decreased number, defects in chemotaxis, phagocytosis or intracellular killing	Local and metastatic abscesses, pneumonia, recurrent deep skin abscesses.
4. Humoral immunity defects	-	immunoglobulin deficiency, imbalanced or unsteady humoral specific immunity	Local and metastatic
5. Presence of foreign bodies	-	intravenous catheters, sutures	Local and metastatic
6. Prophylactic and therapeutic use of antibiotics	-	inhibition of normal bacterial flora and proliferation of resistant staphylococci	Superficial infections including pneumonia and enterocolitis
7. Miscellaneous	-	diabetes mellitus, alcoholism, coronary artery disease, malignant tumours	Local and metastatic

Adapted from Shulman and Nahmias (1972)

multiplication in foodstuffs. A quite different entity is staphylococcal enterocolitis in which the organisms multiply in the lumen of the gut. This most commonly occurs in patients who receive antibiotic therapy and may have their normal bowel flora suppressed; the ensuing overgrowth of antibiotic-resistant staphylococci may result in enterocolitis.

This review shall be primarily confined to discussing the extracellular products of S. aureus, in particular the haemolysins, and their role in virulence. However, initially, the immunity to staphylococcal infections will be reviewed, and the factors affecting the immune response generally will be outlined in a later section.

Immunity to staphylococcal infections

Since all species of animals used for experimentation show a high degree of resistance to staphylococcal infection and yet also show very little resistance to superficial colonisation by these organisms, the study of immunity to staphylococci has proved very difficult.

The interaction of staphylococci with phagocytic cells plays an extremely important role in the host's "immune" response to staphylococcal infection (Koenig, 1972). This non-specific cellular immunity is primarily a function of polymorphonuclear leukocytes and macrophages and defects in these systems can lead to increased susceptibility to staphylococcal infections. When chemotaxis is impaired or when leukocytes are incapable of responding to chemotactic and phagocytic stimuli, recurrent staphylococcal infections may occur (Mandell, 1972; Clark et al., 1973). Efficient phagocytosis of staphylococci by neutrophils is dependent on bacterial opsonisation by serum factors including specific antibody (Wheat, Humphreys and White, 1974) as well as components of the classical (Forsgren and Quie, 1974) and alternative (Jason, 1972) complement pathways.

However, different strains can exert a heterogeneity in opsonic requirements possibly due to cell wall protein A (Verhoef et al., 1977; Verhoef, Peterson and Verbrugh, 1979).

Virulent staphylococci generally resist phagocytosis or intracellular killing by phagocytes. Immunisation with surface components which have antiphagocytic activity protected mice against challenge with staphylococci (Morse, 1962; Fisher, Devlin and Erlandson, 1963; Ekstedt, 1966; Easmon and Glynn, 1976). Recently, two surface components, an acid polysaccharide and a strain-specific mucopeptide complex, have been purified and implicated as inhibitors of in vitro opsonisation of organisms by leukocytes (Karakawa, Young and Kane, 1978).

Specific cellular immunity (cell-mediated immunity) does not appear to be of prime importance in protecting against staphylococcal infections since individuals with defective cell-mediated immunity do not normally suffer from severe staphylococcal infection. Indeed, cell-mediated immunity can be harmful rather than beneficial (Easmon and Glynn, 1975b) and there is evidence that it is an important contributing factor in recurrent staphylococcal infections (Mudd, Taubler and Baker, 1970).

Since patients with deficiencies in humoral immunity are more susceptible than normal subjects to infection by staphylococci, it is probable that humoral immunity is important. The role of both antitoxic and antibacterial humoral immunity to staphylococcal infections has been reviewed by Ekstedt (1972). Immunisation with α-haemolysin toxoids (Goshi et al., 1961, 1963a,b; Taubler, Kapral and Mudd, 1963), leucocidin (Souckova-Stepanova, Gladstone and Vanecek, 1965; Ward, 1979), whole cells (Brodie, Guthrie and Sommerville, 1958; Agarwal, 1967a,b,c; Yoshida, Ichiman and Ohtomo, 1975; Hasegawa and San Clemente, 1978; Yoshida and Ichiman, 1978) as well as surface antigens (Morse, 1962;

Fisher et al., 1963; Ekstedt, 1966; Yoshida, Ichiman and Ohtomo, 1976) has given protection against subsequent challenge with viable staphylococci.

Antibacterial antibodies, as opposed to antitoxin antibodies, appear to act by promotion of phagocytosis and therefore may be useful in preventing establishment of staphylococcal infection (Ekstedt, 1972). Since α-haemolysin, as well as other extracellular products, are produced late in the growth cycle of staphylococci (Elek, 1959; Gladstone and Glencross, 1960; Coleman and Abbas-Ali, 1977) it is probable that antitoxic immunity is only effective once an infection is established. Indeed, it has been suggested that antitoxic immunity can only alter the development of infection (Ekstedt, 1972) and may only prevent more acute symptoms and have little effect on established or chronic infections (Adlam et al., 1977; Anderson, 1978).

The main defence mechanism against staphylococcal infections, however, appears to be an inflammatory response (Easmon and Glynn, 1975a; Anderson, 1978). Anti-inflammatory substances of staphylococci have contributed to an increased infectivity (Hill, 1968, 1969; Easmon, Hamilton and Glynn, 1973). Easmon and Glynn (1975a) concluded that α-antitoxin immunity was protective against the dermonecrotic effects of staphylococci not only because of its specific neutralising power, but also because the antigen/antibody reaction, taking place locally in the tissue, elicited a brisk inflammatory response which proved protective. Non-specific acute inflammation also protected against dermonecrosis.

Extracellular products of S. aureus

Staphylococci produce a large number of extracellular toxins and enzymes during growth (Table 3), although the number and quantity may vary. Bernheimer and Schwartz (1961) detected 12-14 different proteins

Table 3 Some Extracellular Products of S. aureus - Excluding the Haemolysins

Enzymes	Reference
Nuclease	Heins et al., 1967; Wadstrom, 1967.
Phosphatase	Tirunarayanan, 1969; Malveaux and San Clemente, 1969.
Protease	Arvidson, Holme and Lundholm, 1973; Wadstrom and Vesterberg, 1971.
Esterase	Wadstrom, 1967.
Staphylokinase	Vesterberg and Vesterberg, 1972.
Coagulase	Tirunarayanan, 1969; Duthie and Haughton, 1958.
Hyaluronidase	Wadstrom and Mollby, 1972; Abramson and Rautela, 1971.
Lipase	Vesterberg et al., 1967.
Endo-β-N-acetyl glucosaminidase	Wadstrom and Hisatsune, 1970.
Amidase	Wadstrom and Vesterberg, 1971.
Cholesteryl de-esterifying enzyme	Harvie, 1977; McCartney, Beastall and Arbuthnott, 1977.
Phospholipase C	Low and Finean, 1976, 1977.
Other factors	
Enterotoxins A-E	Bergdoll, 1972.
Lymphocyte mitogen	Kreger, Cuppari and Taranta, 1972.
Leucocidin	Wadstrom et al., 1974.
Epidermolytic toxin	Melish, Glasgow and Turner, 1972; Kondo, Sakurai and Sarai, 1973; Arbuthnott, Billcliffe and Thompson, 1974.
Pyrogenic exotoxin	Schlievert, Schoettle and Watson, 1979.

by analytical starch gel electrophoresis; more recent studies by isoelectric focusing in polyacrylamide gels have shown that certain strains of S. aureus give as many as 25 to 30 bands while others give only 4 to 10 bands (Wadstrom, Thelestam and Mollby, 1974). Excellent reviews on the extracellular products of S. aureus are by Bernheimer (1965, 1968, 1970), Gladstone (1966), Jeljaszewicz (1967, 1972), Bergdoll (1967, 1970, 1972), Arbuthnott (1970), Wiseman (1970, 1975), Woodin (1970, 1972), Abramson (1972) and Kwarecki et al. (1973).

1. Staphylococcal haemolysins

The effect of diffusible products of staphylococci on mammalian tissues, notably red blood cells and leukocytes, was first detected nearly 100 years ago by several workers (Von Leber, 1888; De Christmas, 1888; Brieger and Fraenkel, 1890; Van de Velde, 1894; Neisser and Wechsberg, 1901). Work on these "toxic" substances was greatly stimulated in 1928 by a tragic accident in Bundaberg, Australia. Sixteen out of twenty-one children died after being inoculated with diphtheria toxin-antitoxin from a single rubber capped vial which had been kept at room temperature in subtropical heat for several days. Burnet (1929, 1930) concluded that the deaths were due to a soluble toxin elaborated by the staphylococci cultured from the vial.

Since then it has been established that S. aureus produces several distinct toxins. From the haemolytic spectra and serological specificities four haemolysins (alpha, α-; beta, β-; gamma, γ- and delta, δ-) have been resolved. The existence of a further haemolysin (Epsilon, ε-) has been postulated (Elek and Levy, 1950) but not definitely identified because of the complexities created by the multiple molecular forms of α-, β- and δ-haemolysins and absence of a purified preparation. Indeed, a postulated ε-haemolysin was shown to be identical to δ-haemolysin

by Kleck and Donahue (1968). A further haemolysin, which lyses horse erythrocytes, has recently been reported by Turner and Pickard (1979).

The purification and properties of staphylococcal haemolysins have been reviewed by Wiseman (1975).

(i) Alpha-haemolysin

Rabbit erythrocytes are highly susceptible to α-haemolysin while the erythrocytes of other species are less so (Bernheimer, 1968; Chao and Birkbeck, 1978b). The production of α-haemolysin is frequently found in strains from human origin (Elek and Levy, 1950; Fraser, 1964; Jeljaszewicz, 1972) whereas strains of canine origin produce very little of this haemolysin (Fraser, 1964).

Alpha-haemolysin has been separated into four different forms by sucrose gradient electrophoresis (Bernheimer and Schwartz, 1963), isoelectric focusing (Wadstrom, 1968; McNiven, Owen and Arbuthnott, 1972) or hydroxylapatite chromatography (Dalen, 1975) (Table 4). In contrast, Six and Harshman (1973a,b) only found two molecular forms of α-haemolysin on ion exchange chromatography while Watanabe and Kato (1974) found only one component on isoelectric focusing. Recent studies by Cassidy and Harshman (1976b) and Phimister (1979) revealed only a single form of α-haemolysin after purification by glass bead chromatography. The different forms of α-haemolysin, although varying in charge properties, possess similar biological activities (Wadstrom, 1968; Bernheimer, 1970; McNiven et al., 1972).

Alpha-haemolysin occurs as a soluble inactive aggregated form (12S) as well as the active monomeric (3S) form (Arbuthnott, Freer and Bernheimer, 1967). Dissociation of this 12S component in 8M urea yielded the active 3S form supporting the contention that 12S was an inactive form of 3S. The aggregated form of the haemolysin also has similar

Table 4. Molecular forms of Staphylococcal α-haemolysin*

Separation Method	Number of forms	Main component			Reference
		Designation	pI	Mol.Wt.	
Sucrose gradient electrophoresis	4				Bernheimer & Schwartz (1963)
Ion exchange chromatography	2	A	7.2	28,000	Six and Harshman (1973a,b)
		B	8.4	28,000	
Isoelectric focusing	4	αIa	8.6	41,000	Wadstrom (1968)
	4	αA	8.55	36,000	McNiven et al. (1972)
	1	-	7.98	51,000	Watanabe & Kato (1974)
	4	-	8.6	-	Dalen (1975)
Isoelectric focusing in urea	4				Dalen (1975)
Hydroxylapatite chromatography	4				Dalen (1975)

*Adapted from Wadstrom (1978)

immunological characteristics to the biologically active form and has therefore been regarded as a toxoid.

As well as being lytic towards erythrocytes, α-haemolysin is reported to be toxic towards cultured cells (Nogrady and Burton, 1961; Madoff, Artenstein and Weinstein, 1963; Korbecki and Jeljaszewicz, 1964, 1965; Hallander and Bengtsson, 1967; Galanti et al., 1968; Thelestam, Mollby and Wadstrom, 1973; Paradisi et al., 1976). However, release of ^{3}H-nucleotides from human diploid embryonic lung fibroblasts only occurred when high concentrations of α-haemolysin were incubated for several hours with the culture cells (Thelestam et al., 1973; Thelestam, 1976). On the other hand, δ-haemolysin caused rapid and massive release of these markers (Thelestam et al., 1973) and it has therefore been suggested that the earlier reports of cytotoxicity of α-haemolysin for cells in tissue culture may be due to contamination by δ-haemolysin since, in most cases, only crude or partially purified preparations were used (Thelestam, 1976).

In vivo, α-haemolysin causes necrosis when injected subcutaneously into rabbits (Watanabe and Kato, 1974) or injected into the lactating mammary tissue of rabbits (Ward et al., 1979). Alpha-haemolysin is also lethal in both rabbits and mice in small intravenous (Lominski, Arbuthnott and Spence, 1963) and intraperitoneal (Fackrell and Wiseman, 1976b) doses and it inhibits the infiltration of leucocytes (Takeuchi et al., 1978). The pharmacological effects of α-haemolysin in vivo have been reviewed by Jeljaszewicz, Szmigielski and Hryniewicz (1978); the peripheral circulation, heart and central nervous system are the main areas affected (Arbuthnott, 1970).

The mode of action of α-haemolysin on cell membranes is still unclear. Bernheimer (1974), Wiseman (1975), Freer and Arbuthnott (1976)

and McCartney and Arbuthnott (1978) have reviewed this topic and two mechanisms have been postulated. These are that α-haemolysin causes membrane damage due to its surface active properties or secondly by enzymic activity. The surface activity of α-haemolysin has been investigated in detail by Buckelew and Colacicco (1971), Colacicco and Buckelew (1971), Bernheimer et al. (1972), Arbuthnott, Freer and McNiven (1973) and Freer, Arbuthnott and Billcliffe (1973) and it has been suggested that the mode of action might involve penetration and disorganisation of the hydrophobic region of the cell membrane (McCartney and Arbuthnott, 1978). However, experimental evidence has not been produced to explain the high specificity of α-haemolysin for rabbit erythrocytes (Freer and Arbuthnott, 1976).

Wiseman (1975) has reviewed the evidence supporting the possible enzymic activity of α-haemolysin. It has been postulated that α-haemolysin is activated by a membrane-bound protease to become itself a proteolytic enzyme (Wiseman and Caird, 1970; Wiseman and Caird, 1972; Wiseman, Caird and Fackrell, 1975). This has not been confirmed by others (Freer et al., 1973). The finding that α-haemolysin activity was associated with cholesteryl esterase has suggested a different enzymic mechanism (Harvey, 1974). However, these two entities have now been separated by isoelectric focusing (McCartney et al., 1977; Harvey, 1977). More recent studies have attempted to find a specific receptor for α-haemolysin on erythrocyte membranes (Cassidy and Harshman, 1976a), however, the involvement of specific high affinity membrane receptors in the lytic mechanism of α-haemolysin has been disputed (Phimister and Freer, 1979).

(ii) Beta-haemolysin

Glenny and Stevens (1935) first showed β-haemolysin to be

serologically distinct from α-haemolysin. A characteristic phenomenon of this haemolysin is its "hot-cold" haemolytic action on sheep erythrocytes (Walbum, 1921; Bigger, Boland and O'Meara, 1927). That is, haemolysis is greatly enhanced if incubation at 37°C is followed by a period of incubation below 10°C. Although various hypotheses have been offered, the nature of the reaction is still not fully understood (Wiseman, 1970; Meduski and Hochstein, 1972; Bernheimer, 1974; Mollby et al., 1974; Mollby, 1976). However, Smyth, Mollby and Wadstrom (1975) have shown that divalent cations are important for the stability of sphingomyelin-depleted membranes and have suggested that "hot-cold" haemolysis may be a consequence of the temperature dependence of divalent ion stabilization.

It is now well established that β-haemolysin is a sphingomyelinase C having a substrate range confined to sphingomyelin and lysophosphatidylcholine (Doery et al., 1963, 1965; Maheswaran and Lindorfer, 1967; Wiseman and Caird, 1967; Wadstrom and Mollby, 1971a,b). Erythrocytes of the sheep, goat and cow are highly sensitive to β-haemolysin and correlation between the sphingomyelin content of erythrocytes of different species and their sensitivity to β-haemolysin has been shown by Wiseman and Caird (1967). The substrate specificity of β-haemolysin has made it a particularly useful agent in the study of biological membranes (Low et al., 1974; Mollby, 1976; Avigad, 1976).

Beta-haemolysin is a protein (Jackson, 1963; Wiseman, 1965; Maheswaran, Smith and Lindorfer, 1967; Haque and Baldwin, 1969) with a disputed molecular weight. This topic has been reviewed by Wiseman (1975) and a molecular weight of 33,000 and isoelectric point of 9.3 for the major form of β-haemolysin has recently been reported by Low and Freer (1977b).

The toxicity of β-haemolysin both *in vivo* and *in vitro* has also been disputed (Elek, 1959; Wiseman, 1965, 1970; Wadstrom and Mollby, 1972). However, the cytopathic effect of partially purified β-haemolysin reported for various cell lines (Korbecki and Jeljaszewicz, 1965; Wiseman, 1968) has been confirmed using highly purified preparations (Wadstrom and Mollby, 1972; Mollby and Wadstrom, 1973; Kwarecki *et al.*, 1973; Thelestam *et al.*, 1973; Thelestam and Mollby, 1975b; Szmigielski *et al.*, 1976). As well as tissue culture cells, β-haemolysin damages leukocytes (Gladstone and Van Heyningen, 1957; Szmigielski *et al.*, 1976), macrophages (Chesbro *et al.*, 1965) and blood platelets (Wadstrom and Mollby, 1972).

Mollby and Wadstrom (1973) reported that intraperitoneal or intravenous injection of highly purified β-haemolysin into mice or rabbits was lethal within a few minutes. Similarly, Gow and Robinson (1969) showed β-haemolysin to be toxic but this has not been confirmed in other investigations (Wiseman, 1965; Fackrell and Wiseman, 1976b; Low and Freer, 1977a). Similarly, the previously reported dermonecrotic activity of β-haemolysin (Wiseman, 1965; Wadstrom and Mollby, 1972) has not been confirmed (Fackrell and Wiseman, 1976b; Low and Freer, 1977a).

(iii) Gamma-haemolysin

The existence of γ-haemolysin was first described by Smith and Price (1938). Later Smith (1956) isolated a "rough" variant known as strain 5R which produced large amounts of this haemolysin. Purified γ-haemolysin (strain 5R) consists of two factors of pI 9.8 and 9.9 (Taylor and Bernheimer, 1974) which act synergistically (Guyonnet, Plommet and Bouillanne, 1968; Guyonnet and Plommet, 1970). Separation of these proteins was obtained using hydroxylapatite chromatography but

not by isoelectric focusing (Taylor and Bernheimer, 1974), which may account for the single component found by other workers (Mollby and Wadstrom, 1971; Fackrell and Wiseman, 1976a).

Rabbit, human and sheep erythrocytes are sensitive to the haemolytic action of γ-haemolysin while those of the horse are resistant (Guyonnet and Plommet, 1970). Very little is known about the mode of action of γ-haemolysin. Haemolytic activity is inhibited by EDTA (Fackrell and Wiseman, 1976b), phospholipids and sterols (Taylor and Bernheimer, 1974; Fackrell and Wiseman, 1976b) and also by sulphated polymers such as agar, dextran sulphate and heparin (Mollby and Wadstrom, 1971). This inhibition by agar precluded its detection by blood agar haemolysis by Elek (1959) who regarded alpha-2, delta- and gamma-haemolysins as being identical. Since sodium ions are required for lysis the action may be enzymic (Wiseman, 1975).

In vitro, γ-haemolysin was reported to be cytotoxic for human leucocytes and lymphoblast cells (Fackrell and Wiseman, 1976b) and also for rabbit peritoneal granulocytes (Szmigielski et al., 1976). Other workers failed to detect liberation of labelled nucleotides after incubation of the haemolysin with human fibroblast and HeLa cell cultures (Thelestam et al., 1973).

In vivo, γ-haemolysin was not lethal for mice although intracardial injection of 50 μg was fatal for guinea pigs (Fackrell and Wiseman, 1976b).

(iv) Delta-haemolysin

In 1947 Williams and Harper first observed that various strains of S. aureus, grown on sheep blood agar, produced a haemolytic agent which was not neutralised by either α- or β-antitoxins. This agent was

termed δ-haemolysin and has provided an interesting area of research for more than 30 years.

a) Production: The production of δ-haemolysin was first described by Marks and Vaughan (1950) who used the dialysis membrane technique devised by Birch-Hirschfeld (1934). The δ-haemolysin produced was retained on top of these membranes, separating it from the non-dialysable components of the medium. Since then many workers have employed this technique with some variations in the medium (Jackson and Little, 1958; Hoffman and Streitfeld, 1965; Murphy and Haque, 1967; Wiseman and Caird, 1968; Kleck and Donahue, 1968; Murphy and Haque, 1974).

More recently, a semi-defined fluid medium (Gladstone and Van Heyningen, 1957; Bernheimer and Schwartz, 1963) comprising protein hydrolysate and diffusate of yeast extract has been employed (Yoshida, 1963; Heatley, 1971; Kreger et al., 1971; Kapral and Miller, 1971; Kantor, Temples and Shaw, 1972) for production of large volumes of high potency crude toxin. Williams and Harper (1947) reported that δ-haemolysin production did not occur in fluid medium, but the importance of adequate aeration may have been overlooked (Yoshida, 1963; Turner, 1978b). Other factors which are important for maximum δ-haemolysin production in liquid media are 1) the concentration of yeast diffusate, 2) the volume of the medium and 3) the strain of S. aureus (Turner, 1978b).

b) Purification: Marks and Vaughan (1950) partially purified δ-haemolysin by extraction of the crude supernate with ethanol in which δ-haemolysin was soluble. Jackson and Little (1958) recovered partially purified δ-haemolysin from heated ($60^{\circ}C$/10-15 min) crude culture supernates by precipitation with ethanol at pH 4 and $-5^{\circ}C$.

More recently, Heatley (1971) purified δ-haemolysin by ammonium

sulphate precipitation of heated (60°C/1 h) crude culture fluids of strain 186X, followed by chloroform-methanol extraction and cyclic transfer between organic and aqueous phases of a two phase system (chloroform/methanol/water) by adjustment of the pH. A single polydisperse peak (4-9S) was observed on ultracentrifugation of the purified haemolysin while on PAGE at pH 4.6 or pH 8.4 a main diffuse band with a faint diffuse secondary band was shown.

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

The use of ion exchange chromatography as a step in purification of δ-haemolysin has been employed by several workers. Yoshida (1963) obtained a crystalline preparation of δ-haemolysin produced by strain Foggie by chromatography of concentrated, heated culture supernates on calcium phosphate gel and DEAE-cellulose. The haemolytic fraction was lyophilised and recrystallised 2-3 times by dissolution in Tris buffer and dialysis against water. A sedimentation coefficient of 6.1S was obtained by ultracentrifugation. However, Gladstone and Yoshida (1967) subsequently showed this preparation to be contaminated with significant quantities of ribonuclease and β-haemolysin.

Caird and Wiseman (1970) purified δ-haemolysin of strain E-delta by isoelectric precipitation (pH 4), ammonium sulphate precipitation and DEAE-cellulose chromatography. Purified haemolysin, obtained in a 16% yield with a 32-fold increase in specific activity, yielded a single precipitin line on immunodiffusion or immunoelectrophoresis against antiserum to δ-haemolysin. A single protein band was noted on PAGE (pH 9.5) but ultracentrifugation revealed two components (2.8S and 9.8S).

More recently, Kreger et al. (1971), using a mutant strain of Wood 46 which produced very little α-haemolysin (W46M), purified δ-haemolysin by adsorption to hydroxylapatite and elution with phosphate buffers of increasing molarity. After dialysis against water both soluble and insoluble forms were recovered. The soluble haemolysin was heterogeneous by gel filtration, sucrose density gradient centrifugation, ion exchange chromatography, PAGE and isoelectric focusing. Two bands were obtained in PAGE at pH 4.3 and pH 9.5 and, on isoelectric focusing, two protein bands (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 a second granular lower molecular weight fraction (4.9S) with a pI of 9.5. Unlike the preparation of Yoshida (1963), Kreger et al. (1971) stated that their preparation was free of ribonuclease and β-haemolysin as well as other haemolysins and enzymes. However Fackrell and Wiseman (1974) found the preparation of Kreger et al. (1971) to be contaminated with α- and γ-haemolysins and concluded that this method of purification was no more satisfactory than that of Yoshida (1963).

Kantor et al. (1972) achieved a 20-fold purification of δ-haemolysin (strain W46M) by adsorption to aluminium hydroxide gel and elution with 0.5M phosphate buffer pH 7.2. Delta-haemolysin was homogeneous by PAGE, isoelectric focusing in the presence of 0.1% Tween 80 (pI 5.2) and gel filtration in 6M guanidine hydrochloride.

Recently the efficacy of three of these procedures (Wiseman and Caird, 1968; Kreger et al., 1971; Kantor et al., 1972) as purification techniques for δ-haemolysin was disputed by Lee et al. (1976). They

claimed that none of these methods yielded pure preparations of δ-haemolysin of S. aureus strain 146P.

c) Physiochemical properties: Table 5 summarises the findings on the isoelectric point of δ-haemolysin. There are also various conflicting reports of the molecular weight of δ-haemolysin (Table 6). However, by gel filtration and sedimentation velocity centrifugation in increasing concentrations of Tween 80, Kantor et al. (1972) proposed a model to explain these variations in molecular weight, sedimentation coefficient and isoelectric points. They suggested that δ-haemolysin consists of 4 individual polypeptide chains of molecular weight 5100 to 5200 daltons which form a basic subunit of 21,000 daltons. Association of five or ten of these basic subunits would then yield polymers of molecular weights consistent with those determined in the absence of Tween 80 by sucrose gradient centrifugation, analytical ultracentrifugation and gel filtration.

Amino acid analyses of δ-haemolysin consistently show lysine, aspartic acid and isoleucine present in high amounts, with histidine, arginine, proline, cysteine and tyrosine absent or present as traces (Table 7). Caird and Wiseman (1970), however, found the N-terminal amino acid of δ-haemolysin (strain E-delta) to be proline. It is nevertheless agreed that δ-haemolysin is a simple protein containing less than 1.5% lipid, phosphorus or carbohydrate (Yoshida, 1963; Kreger et al., 1971; Heatley, 1971; Turner, 1978a) which is sensitive to various proteolytic enzymes (trypsin, chymotrypsin, pronase, papain and pepsin) (Kreger et al., 1971; Kantor et al., 1972).

Delta-haemolysin differs from the other haemolysins of S. aureus in being relatively thermostable (no loss of activity after 80°C for 1 h; Kreger et al., 1971), highly surface active (Colacicco et al., 1977) and

Table 5. Isoelectric point of Staphylococcal δ-haemolysin

pI			Reference
3.32;	3.75;	8.45	Maheswaran and Lindorfer, 1970.
		9.6	Mollby and Wadstrom, 1970.
	5.0;	9.5	Kreger et al., 1971.
		10.01	McNiven et al., 1972.
	5.2		Kantor et al., 1972.
	4.45		Chao, 1976.
		9.6	Fackrell and Wiseman, 1976b.
	4.5		Whitelaw, 1978.
	4.5;	9.5	Turner, 1978a.

Table 6 Molecular Weight Estimates and Sedimentation Coefficients of Staphylococcal δ-haemolysin

Method	S_{20w}	Molecular Weight (daltons)	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 & Raynaud (1965)
Sedimentation velocity	1.4	12,000	"
Gel filtration		> 200,000	Hallander (1968)
Sedimentation velocity	2.8 ; 9.8		Caird & 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	6.19	191,000 61,850 (crude)	"
Centrifugation (PBS)		102,500 (purified)	"
Sucrose gradient		21,000	"
Centrifugation (0.1% Tween 80) sucrose gradient		< 10,000	"
Centrifugation (1% Tween 80) SDS gel electrophoresis		< 10,000	"
Gel filtration (PBS)		195,000	"
6M guanidine		5,200	"
aa analysis		5,100	"
Gel filtration		200,000	Fackrell & Wiseman (1976b)

Table 7 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)	Whitelaw (1978)	Turner† (1978a)
Lysine	16.55	16.66	13.60	16.20	2.60	16.60	18.40 ; 12.92
Histidine	0.41	-	trace	-	0.40	-	0.97 ; 1.68
Arginine	0.94	-	-	-	2.80	-	1.75 ; 3.35
Aspartic acid	12.53	13.46	10.90	13.60	16.20	17.90	14.85 ; 10.25
Threonine	6.98	8.02	7.50	9.40	10.60	10.90	6.94 ; 4.59
Serine	5.12	2.46	3.20	4.30	7.50	2.90	4.33 ; 3.22
Glutamic acid	7.99	4.90	5.20	4.60	7.20	4.80	6.06 ; 13.10
Proline	0.45	-	-	-	1.20	-	1.04 ; 2.22
Glycine	3.73	3.60	8.10	6.20	6.30	2.30	3.47 ; 4.06
Alanine	4.06	3.09	5.90	4.80	5.00	2.40	3.72 ; 5.25
Half cysteine	-	0.09	-	-	-	-	0.26 ; 0.31
Valine	4.55	6.64	5.50	6.70	6.60	7.10	6.12 ; 6.90
Methionine	4.88	4.56	4.30	4.00	-	2.20	0.26 ; 0.19
Isoleucine	9.71	18.17	10.50	17.10	9.90	19.60	12.99 ; 10.97
Leucine	7.41	6.04	6.30	4.90	5.90	4.30	7.25 ; 8.92
Tyrosine	1.68	-	-	-	1.20	-	3.37 ; 2.18
Phenylalanine	10.40	7.93	7.60	5.50	6.50	5.20	6.64 ; 8.25
Tryptophan	2.61	3.30	4.00	2.50	10.00	3.70	
Ammonia	1.63	1.08	7.40				1.57 ; 1.66

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

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

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

adsorbing quickly and strongly to glass and polypropylene (Heatley, 1971). In addition, δ-haemolysin is amphipathic, from its ability to dissolve in both organic (Marks and Vaughan, 1950; Jackson and Little, 1958; Heatley, 1971; Kreger et al., 1971; Colacicco et al., 1977) and aqueous (Heatley, 1971; Kreger et al., 1971; Colacicco, 1972) media.

d) Biological properties: Delta-haemolysin has a wide haemolytic spectrum (Wiseman, 1970; Jeljaszewicz, 1972). Gladstone (1966) stated that all erythrocytes tested were susceptible to δ-haemolysin. Human erythrocytes have generally been used to detect haemolytic activity, but recent work in this laboratory (Chao and Birkbeck, 1978b) has shown that cod erythrocytes are more sensitive than mammalian erythrocytes to the action of δ-haemolysin (Table 8). Synergistic action between β- and δ-haemolysins has been shown with both sheep (Williams and Harper, 1947; Marks and Vaughan, 1950; Kreger et al., 1971) and human (Heatley, 1971) but not fish (Chao and Birkbeck, 1978b) erythrocytes.

In vitro, δ-haemolysin exerts a strong cytopathic effect on various cell lines (Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967; Thelestam et al., 1973), leucocytes (Jackson and Little, 1957; Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967) and bacterial protoplasts and spheroplasts (Bernheimer, Avigad and Grushoff, 1968; Kayser, 1968; Kreger et al., 1971). Cellular organelles such as lysosomes and mitochondria (Bernheimer and Schwartz, 1964; Gladstone and Yoshida, 1967; Evans and Lack, 1969; Kreger et al., 1971; Kantor et al., 1972; Rahal, 1972) and lipid spherules (Freer, Arbuthnott and Bernheimer, 1968; Kreger et al., 1971) are also disrupted by δ-haemolysin. Delta-haemolysin also prevented growth of certain bacterial species for up to 4 days (Kreger et al., 1971). In addition to being cytotoxic, δ-haemolysin can also inhibit water absorption (Kapral et al., 1976) and increase cyclic

Table 8 Relative Sensitivity fo δ-haemolysin of Erythrocytes of Different Species

Species	Haemolytic activity compared to that of human (%)				
	Wiseman (1970) Strain Neman	Wiseman (1970) Strain Edelta	Kreger et al. (1971) W46M	Kantor et al. (1972) W46M	Chao & Birkbeck (1978b) NCTC10345
Human	100	100	100	100	100
Rabbit	25	25	200	50	60
Sheep	25	12.5	40	50	20
Horse	3	6			100
Guinea Pig	6	12.5	50		
Pig			40		
Calf/Bovine	12.5	12.5	40		
Goat			20		
Cat			20		
Chicken			20		
Monkey		50			
Cod					400

AMP (cAMP) levels (O'Brien and Kapral, 1976) in the guinea pig ileum. The haemolysin also increases the vascular permeability in guinea pig skin (O'Brien and Kapral, 1976). The mechanism by which δ-haemolysin produces these effects is different from that initiated by cholera toxin (O'Brien and Kapral, 1977) but may be due to its ability to augment intercellular movement of ions across the mucosa rather than the stimulation of transcellular processes (O'Brien, McClung and Kapral, 1978).

The toxicity of delta-haemolysin *in vivo* has been studied by several workers (Table 9). For example, Marks and Vaughan (1950) injected δ-haemolysin intradermally in man and observed slight erythematous lesions which disappeared within 48 h. 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). It must, however, be emphasised that the lethal and dermonecrotic effects observed with the large doses shown in Table 9 could well be due to contamination with α-haemolysin which is considerably more toxic than δ-haemolysin.

e) *Mode of action:* Wiseman and Caird (1968) proposed that δ-haemolysin possessed phospholipase C activity since the purified haemolysin was shown to release acid-soluble phosphorus from phospholipid extracts of erythrocytes in direct proportion to their haemolytic sensitivity. However, other workers failed to detect phospholipase C activity in their preparations (Kreger *et al.*, 1971; Heatley, 1971; Kapral, 1972; Rahal, 1972) and the kinetic studies on δ-haemolysin were not consistent with an enzymic mode of action (Marks and Vaughan, 1950; Jackson and Little, 1958; Bernheimer, 1970; Kapral, 1976).

Recently, Low and Finean (1976, 1977) purified a phosphatidyl inositol-specific phospholipase C from *S. aureus* which had no detectable

Table 9 Effects of δ-haemolysin in animals

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

haemolytic activity. The phosphatidyl inositol in erythrocyte ghosts was readily hydrolysed by this phospholipase C but was not hydrolysed in intact erythrocytes. This suggested that at least 75-80% of the phosphatidyl inositol was located in the inner leaflet of the membrane and therefore would render it inaccessible for the hydrolytic action of Wiseman's preparation. Thus, the activity of Wiseman's preparation may be due to contamination by this enzyme.

The high surface activity (Colacicco et al., 1977) and the high content of hydrophobic amino acids (Table 7) of δ-haemolysin have led to the suggestion that δ-haemolysin affects membranes by a detergent-like action (Kreger et al., 1971; Bernheimer, 1974; Alouf, 1977). Evidence for this detergent-like action has been shown in kinetic studies in which lysis of cells occurred rapidly without a lag period (Wadstrom and Mollby, 1972; Thelestam et al., 1973; Kapral, 1976) although the temperature dependence of the reaction has been disputed (Thelestam et al., 1973; Kapral, 1976). The lytic activity (Thelestam and Mollby, 1975a,b) and the metabolic changes in rabbit liver mitochondria (Rahal, 1972) caused by δ-haemolysin have been compared to the non-ionic detergent Triton X-100. The principal toxin of bee venom, melittin, has similarly been compared with δ-haemolysin (Thelestam and Mollby, 1975a,b; Freer and Arbuthnott, 1976). Lee and Haque (1976) have recently suggested that only superficial contact between δ-haemolysin and erythrocytes is required for lysis.

f) Inhibition by normal sera and by phospholipids: Neutralisation of δ-haemolysin by normal serum from various animals was first reported by Marks and Vaughan (1950). Formation of a precipitin line has also been found between normal serum and δ-haemolysin on immunodiffusion (Gladstone and Yoshida, 1967) and immunoelectrophoresis (Kantor et al., 1972). Identification of the inhibitory components by serum fractionation has

been attempted by several workers (Marks and Vaughan, 1950; Jackson and Little, 1958; Gladstone and Yoshida, 1967; Kantor et al., 1972) and recent work has identified α- and β-lipoproteins as the inhibitory factors to δ-haemolysin present in normal serum (Whitelaw and Birkbeck, 1978). The lipoproteins were purified by zonal ultracentrifugation of serum in potassium bromide density gradients and were shown to inhibit δ-haemolysin and give a precipitin line on gel diffusion. Fractions, which, by polyacrylamide gel electrophoresis were shown to be devoid of such lipoproteins, were non-inhibitory (Whitelaw, 1978).

In addition to neutralisation by normal serum, δ-haemolysin is neutralised by many phospholipids, although conflicting reports are found in the literature (Table 10). Recently, Kapral (1976) found that C_{13} to C_{19} saturated fatty acids potentiated the haemolytic activity of δ-haemolysin and that fatty acids with longer chain lengths inhibited haemolytic activity. This action was thought to occur by influencing the binding of δ-haemolysin to the erythrocyte membrane.

g) Immunogenicity: The production of specific antibodies to δ-haemolysin has been reported by McLeod (1963), Kayser and Raynaud (1965), Caird and Wiseman (1970) and Lochman and Vymola (1974). Also, Rusakova (1970) reported the production of an antigenic toxoid of δ-haemolysin by formolisation which induced a specific antibody to the haemolysin. However, there was no evidence that non-specific lipoprotein inhibitors had been removed from the sera. The failure to detect an elevated neutralising capacity or additional precipitin lines on gel diffusion of sera after immunisation with δ-haemolysin led to the view that δ-haemolysin was not antigenic (Gladstone and Yoshida, 1967; Kleck and Donahue, 1968; Hallander, 1968; Kantor et al., 1972).

Table 10 Inhibition of δ-haemolysin by phospholipids

Inhibitory phospholipids	Reference
Cholesterol	Gladstone and van Heyningen, 1957; Caird and Wiseman, 1970.
Phosphatidyl choline (lecithin)	Kreger et al., 1972; Kapral, 1972; Whitelaw and Birkbeck, 1978.
Phosphatidic acid (natural)	Kreger et al., 1971; Kapral, 1972; Whitelaw and Birkbeck, 1978.
Phosphatidyl serine	" " " "
Phosphatidyl inositol	" " " "
Cardiolipin	" " " "
Sphingomyelin	" " " "
Phosphatidyl ethanolamine	" " " "
Phosphatidyl glycerol	Whitelaw and Birkbeck (1978)
Serum lipids	"
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 : 21-23C fatty acids	Kapral, 1976
Stimulatory agents : 13-19C fatty acids	" "

Fackrell and Wiseman (1974) obtained specific anti-δ-haemolysin antibodies in sera of rabbits immunised intensively with increasing concentrations (200 μg - 2 mg) of δ-haemolysin. Precipitinogenic activity resided in an IgG fraction prepared by ammonium sulphate fractionation and ion-exchange chromatography. The purified anti-δ-haemolysin gave a single line on gel diffusion and immunoelectrophoresis with purified δ-haemolysin and, unlike Gladstone and Yoshida (1967), the line of precipitation also coincided with the zone of haemolysis caused by δ-haemolysin in blood-agarose gel diffusion tests. Kapral (1976) argued that Fackrell and Wiseman's findings did not prove the antigenicity of δ-haemolysin. Preimmune sera will precipitate δ-haemolysin (Kantor et al., 1972) and, since this reaction results from the combination with a substance other than globulin, Kapral maintains that no proof was given that the precipitate consisted of δ-haemolysin and specific antibody.

Further work has, however, also shown δ-haemolysin to be immunogenic in rabbits (Birkbeck and Whitelaw, 1976, 1979). Rabbits were immunised subcutaneously with δ-haemolysin emulsified in Freund's Complete Adjuvant (FCA). High neutralising titres were found in these immune sera and, on immunodiffusion, they gave two precipitin lines with purified δ-haemolysin while only one precipitin line was present with pre-immune sera. After removal of lipoproteins by zonal centrifugation in potassium bromide density gradient, the immune sera, unlike the pre-immune sera, retained their precipitinogenic and neutralising activities.

The immunogenicity of δ-haemolysin has also been shown by Heatley (1977). Immunoglobulin G fractions from the sera of rabbits immunised with either "insoluble" or "solvent-transferred" δ-haemolysin precipitated with and neutralised both forms of the haemolysin. This did not occur with pre-immune sera. By an affinity chromatography technique the

antigenicity of δ-haemolysin was confirmed (Heatley and Gladstone, 1977).

Comparative studies of δ-haemolysins produced by different strains of S. aureus have recently indicated that antigenic differences may occur between these haemolysins (Turner, 1978a).

2. Role of Extracellular Products in Virulence

Studies of the pathogenesis of S. aureus and the role of various factors in virulence has been greatly hampered by the lack of suitable experimental models. Laboratory animals are very resistant to infection by staphylococci and staphylococcal abscesses can be produced in mice only by subcutaneous injection of large numbers ($>10^6$) of organisms (James and MacLeod, 1961; Fisher and Robson, 1963; Takeuchi and Suto, 1976; Musher and McKenzie, 1977; Takeuchi et al., 1978). However, in the presence of a foreign body such as a suture (James and MacLeod, 1961) or cotton-dust (Noble, 1965; Hill, 1968, 1969) subcutaneous abscesses can be produced with as few as 100 viable organisms. Table 11 reviews various animal models which have been developed to study staphylococcal infections.

Three main approaches have been used to study the possible virulence factors in staphylococcal infections:

1) Purification of extracellular products and investigation of their effects in vitro and in vivo.
2) Investigation of the protective effect of active or passive immunisation with extracellular products.
3) Comparison of mutants of a strain of known virulence.

The role of several staphylococcal extracellular products shall be individually discussed.

(i) Alpha-haemolysin: The majority of strains of S. aureus excrete α-haemolysin (Elek and Levy, 1950; Jeljaszewicz, 1960a,b) and production

Table 11 Experimental models for the study of staphylococcal diseases of man

Infection	Experimental Animal	Reference
Toxic Epidermal Necrolysis	Mouse	Melish & Glasgow, 1970 Arbuthnott et al., 1972 Melish et al., 1972
Impetigo	Syrian hamster	Dajani & Wannamaker, 1970, 1971, 1972
Mastitis	Mouse	Anderson, 1971, 1972, 1974 Anderson & Mason, 1974
	Rabbit	Adlam et al., 1976
Osteomyelitis	Rabbit	Andride, Nagel & Southwich, 1974 Kurek et al., 1977
Endocarditis	Rabbit	Garrison & Freedman, 1970 Linnemann, Watanakunakorn and Bakie, 1973
	Dog	Hamburger et al., 1967
Superficial lesions	Mouse	Noble, 1965 Agarwal, 1967a,b,c Hill, 1968, 1969 Weksler and Hill, 1969 Easmon and Glynn, 1975a,b McKay and Arbuthnott, 1974a,b, 1979

of α-haemolysin *in vivo* has been shown in rabbits, mice and guinea pigs (Gladstone and Glencross, 1960; Kapral, Keogh and Taubler, 1965; Foster, 1967; Bartell *et al.*, 1968; Takeuchi and Suto, 1974; Anderson, 1974; Takeuchi *et al.*, 1978). Tissue necrosis has been linked directly and indirectly with α-haemolysin production *in vivo* (Foster, 1967; Goshi *et al.*, 1961, 1963a,b) as has increased invasiveness (Kimura, 1971; Takeuchi and Suto, 1974).

Alpha-haemolysin deficient mutants had either a completely (Van der Vijver, Van Es-Boon and Michel, 1975a,b) or partially (Taubler *et al.*, 1963) reduced ability to cause dermonecrosis in mice and had a reduced ability to multiply in the kidneys of mice challenged intravenously (Van der Vijver *et al.*, 1975b). Also, the lethal dose for mice of such mutants on intraperitoneal injection was higher than that of the wild type (Kapral, 1965).

(ii) Beta-haemolysin: Beta-haemolysin is produced mainly by strains of *S. aureus* of animal origin (Elek and Levy, 1950; Fraser, 1964). Kimura (1971) showed that mutant strains of *S. aureus* producing β-haemolysin, but not α-haemolysin, were lethal but less invasive in mice challenged intraperitoneally than mutants producing α-haemolysin. Production of β-haemolysin during experimental infection in mouse skin was shown by Takeuchi and Suto (1974) and a β-haemolysin producing strain of *S. aureus* caused cutaneous changes in mouse skin (Takeuchi *et al.*, 1978). However, a strain of *S. aureus* that produced β-haemolysin but not α-haemolysin failed to cause clinical mastitis when injected into the udder of cows (Slanetz and Bartley, 1953) and anti-β-haemolysin did not protect rabbits against mastitis after intramammary challenge with staphylococci (Adlam *et al.*, 1977).

(iii) Delta-haemolysin: The production of δ-haemolysin correlates as

well with potential pathogenicity of strains of S. aureus as does α-haemolysin (Elek and Levy, 1950; Jeljaszewicz, 1960a,b). Kapral (1974) reported that lethal doses of δ-haemolysin (200 HD_{50}) were produced in the peritoneal cavity of mice and rabbits within 2 h of inoculation if the inoculum was large enough (2×10^8 or more) and δ-haemolysin was of prime importance in infection if α-haemolysin was not produced. Mutant strains of S. aureus lacking δ-haemolysin had reduced dermonecrotic activity in local lesions which developed after subcutaneous injection (Van der Vijver et al., 1975a,b).

A role for δ-haemolysin in staphylococcal enteritis has been postulated by O'Brien and Kapral (1976). This is based on the evidence that δ-haemolysin inhibits water absorption in the guinea pig and rabbit ileum (Kapral et al., 1976), causes increased cAMP levels in the guinea pig ileum and increased vascular permeability in guinea pig skin (O'Brien and Kapral, 1976). These results are comparable with the enterotoxin of V. cholerae which is solely responsible for clinical cholera (Hendrix, 1971). However, unlike cholera enterotoxin, δ-haemolysin did not cause spindling of Chinese hamster ovary cells, rounding of Y-1 adrenal cells nor promote steroid production by these cells (O'Brien and Kapral, 1977) all of which are related to an elevation of intracellular cAMP content. Delta-haemolysin did, however, lyse both Chinese hamster ovary and Y-1 adrenal cells but the increase in cAMP levels caused by δ-haemolysin may be mediated through a mechanism different from that initiated by cholera toxin (O'Brien et al., 1978).

(iv) Gamma-haemolysin: Studies on antibodies induced during staphylococcal osteomyelitis in humans indicate that γ-haemolysin is produced in vivo since elevated titres of specific antibodies have been found in nearly all osteomyelitic subjects (Taylor and Plommet, 1973; Taylor et al., 1975). Similarly Kurek et al. (1977) found synthesis of antibodies against γ-haemolysin in rabbits with experimental staphylococcal osteo-

myelitis and they have proposed a role for γ-haemolysin in the pathogenesis of osteomyelitis.

(v) Epidermolytic toxin: Toxic epidermal necrolysis (TEN) or Staphylococcal Scalded Skin Syndrome is a disease of infants and children characterised by intra-epidermal splitting and extensive exfoliation of the epidermis. The epidermolytic toxin of S. aureus has been unequivocally shown to be responsible for these symptoms. On subcutaneous injection of purified toxin or of strains producing this toxin into neonatal and adult mice, the symptoms mimic those found in humans (Melish and Glasgow, 1970; Melish et al., 1972; Arbuthnott et al., 1972; Kapral and Miller, 1971; Elias et al., 1974a,b; Elias, Fritsch and Epstein, 1977). Because of the role of epidermolytic toxin in TEN it can be recognised as a major virulence determinant in this syndrome.

(vi) Enterotoxins: Five serologically distinct enterotoxins (A, B, C, D and E) have so far been recognised (Bergdoll, 1972). The enterotoxins are produced in some foods by staphylococci (mainly phage group III strains) and the ingestion of toxin leads to staphylococcal food poisoning with classical symptoms of diarrhoea and vomiting 1-6 h after ingestion (Dack, 1956). Various animals have been used to study the effects of the enterotoxin, including rhesus monkeys, cats, dogs, rabbits, rats and mice (Bergdoll, 1972) but they are less sensitive to the enterotoxin than man. Besides their known role in food poisoning, enterotoxins may also figure in cases of enterocolitis. However, enterocolitis cannot be connected with the consumption of food since it occurs sporadically, has a more prolonged course (Bergdoll, 1972) and usually occurs in patients on antibiotic therapy in which resistant staphylococci develop (Matthais, Shooter and Williams, 1957). Although there is indirect evidence that staphylococci produce enterotoxin while growing in the intestinal tract (Bergdoll, 1972) there is no direct proof that the toxin is involved in

entercolitis. Indeed, as stated earlier, O'Brien and Kapral (1976) have suggested that δ-haemolysin may play a role in entercolitis.

(vii) Coagulase: The production of coagulase has been considered as a protective mechanism for staphylococci against phagocytosis (Hale and Smith, 1945) but this has not been confirmed by other workers (Cowdery et al., 1969; Foster, 1962a,b; Anderson and Chandler, 1975). An alternative role for coagulase has been proposed by Ekstedt (1956) who suggested that coagulase may protect the organism against the antibacterial activity of human serum since coagulase negative strains, suspended in partially purified coagulase and injected intracerebrally, caused significant mortality (Ekstedt and Yotis, 1960). Injection of the organisms or coagulase alone at the same dose level was innocuous.

Coagulase negative mutant strains were as virulent in experimental animals as parent strains (Kapral and Li, 1960). Also, coagulase negative strains have been implicated in various infections including endocarditis (Brandt and Swahn, 1960), wound infections (Wilson and Stuart, 1965) and urinary tract infections (Mitchell, 1964; Mortensen, 1969). This is therefore an indication that coagulase production may not be necessary for virulence of staphylococci. Gemmel, Thelestam and Wadstrom (1976) suggest that the possession of multiple toxins and enzymes by staphylococci rather than the mere possession of coagulase determines pathogenicity.

(viii) Leucocidin: Panton-Valentine leucocidin comprises two components known as F (fast) and S (slow) components which act synergistically to produce their cytotoxic effect. The leucocidin specifically kills polymorphonuclear leucocytes and macrophages but no other cell type (Woodin, 1972).

Gladstone and Glencross (1960) showed that leucocidin was produced *in vivo* in experimental infection and, in humans, raised levels of anti-leucocidin were associated with infection (Gladstone *et al.*, 1962). Large doses of leucocidin injected into rabbits caused a marked but brief granulocytosis (Gladstone, 1966) and, measured in this way, leucocidin was relatively non-toxic. Also, mutants lacking leucocidin did not differ in virulence from the wild type when injected subcutaneously or intravenously into mice (Van der Vijver *et al.*, 1975b). However, it has recently been shown that the leucocidin can produce dermonecrosis in rabbits and pre-immunisation with the F component or both F and S components inhibited this dermonecrosis (Ward, 1979).

A role in pathogenicity has also been suggested for many of the extracellular enzymes produced by staphylococci (Abramson, 1972) but, as concluded by Anderson (1976), they may only be secondary factors in host damage and may be more important in facilitating the growth of the organism *in vivo*. Indeed, all of the products discussed above may favour multiplication of the organism which, in turn, may explain its pathogenicity (Anderson, 1976). However, the existence of an unidentified "virulence factor" produced only *in vivo* cannot be dismissed (Abramson, 1972).

The Immune Response

The ability of a protein to elicit an immune response reflects a complex series of factors such as the structural properties of the immunogen, the immunisation schedule and the genetic features of the species and strains to which the studied animals belong. Excellent reviews on all aspects of the immune response are by Abramoff and La Via (1970), Sela (1974) and Hobart and McConnell (1975).

1. Factors affecting the immune response

(i) Structural properties of the immunogen

The study of synthetic polypeptides has led to some understanding of the role which amino acid composition, disposition of groups, conformation of the molecule, charge and other factors may play in determining immunogenicity (Crumpton, 1974; de Weck, 1974).

a) Physical state: Particulate antigens as a class, have been regarded as being more immunogenic than soluble antigens (Nossal, Ada and Austin, 1964; Draper and Hirata, 1968; Katsura, 1972) possibly due to a more efficient uptake of the particulate antigens by macrophages (Draper and Hirata, 1968). However, Rabin and Rose (1973) found no evidence for different processing mechanisms by macrophages dependent on the physical state of the antigen.

b) Foreignness: In order to be immunogenic, molecules must, in general, be foreign to an organism and the more foreign, the more immunogenic the molecule is likely to be. Burnet and Fenner (1949) based their discussion on the mechanism of antibody formation on the ability of the antibody producing mechanisms to distinguish 'self' from 'non-self'. That is, antibodies are formed against only those areas of a foreign protein which are not shared by the animal's own proteins. For example, the N-terminal, non helical segments of collagen $\alpha 1$ chains from different vertebrates show a closer similarity in amino acid sequence than $\alpha 2$ chains and are less immunogenic than $\alpha 2$ chains in rabbits (Lindsley, Mannik and Bornstein, 1971). The apparent relationship between immunogenicity and the degree of foreignness may, in part, explain why proteins of similar size, shape and overall amino acid composition differ so markedly in their immunogenicities (Prager and Wilson, 1971).

c) Molecular size: The importance of the physical state of a protein in controlling the induction of an immune response has been shown in various studies in which there was a progressive rise in protein immunogenicity with increasing size and aggregation (Dresser, 1962; Nossal et al., 1964; Reichlin, Nisonoff and Margoliash, 1970; Parish and Ada, 1972; Warren, Spero and Metzger, 1973).

Although the reason for the enhancement of the immunogenicity of proteins by polymerisation is not known several plausible hypotheses have been suggested. Dresser (1962) and Frei, Benacerraf and Thorbecke (1965) have suggested that antigens in an aggregated form are required for phagocytosis by macrophages which facilitate a more efficient antigen presentation to lymphocytes (Unanue, 1970, 1972, 1978). Reichlin et al. (1970) postulated that retardation of renal excretion may increase the immunogenicity of polymers. Differences in the quaternary structure and/or conformational alterations that occur during polymerisation have also been proposed to explain the increase in immunogenicity (Crumpton, 1974), such changes influencing the T- and B-cell co-operation. However, Feldman and Basten (1971) have suggested that it is the lack of a requirement for T helper cells by the polymers which gives them an enhanced immunogenicity due to the possession of multiple and identical antigenic sites available to interact with B cells.

A number of attempts have been made to determine the minimum molecular size that is conducive with good immunogenicity. However, recent studies (Dintzis, Dintzis and Vogelstein, 1976) have suggested that a minimum number of antigenic receptors, probably 10-20, must be connected together in a spacially continuous cluster before an immunogenic signal is delivered and an immune response only occurs when a sufficient number of such signals per cell has been delivered.

d) Molecular Rigidity: A rigid structure of the determinant groups has been suggested as a prerequisite for immunogenicity (Haurowitz, 1952; de Weck, 1974). For example, gelatin is feebly immunogenic and, due to its amino acid composition, free rotation occurs around the longitudinal axis and therefore the molecule has no fixed conformation. However, attachment of tyrosine, tryptophan or phenylalanine converted gelatin into a relatively powerful immunogen (Haurowitz, 1950; Sela, 1966) by increasing its rigidity.

(ii) Immunisation procedure

As well as the physical state of the antigen, the mode of administration to the animal can affect the resulting immune response.

a) Route of Injection: Several workers have found that intravenous, intraperitoneal and subcutaneous injections were in descending order of efficiency for immunisation of rabbits (Webster, 1965, 1968) or mice (Katsura, 1972). Similarly, the intra-lymph node route of injection was reported to be superior to other routes (Newbold, 1965) but this claim was rejected by other workers (Horne and White, 1968; Birkbeck and Stephen, 1971).

b) Dose of immunogen: The dose of antigen injected into an animal may be extremely important in the induction of antibody production. For example, Mitchison (1968) found that mice given either high or low doses of BSA failed to respond to a later challenge dose of the antigen. However, injection of intermediate doses induced a high antibody response after injection of the challenge dose. Such unresponsiveness or "immunological tolerance" may occur at either the T- or B-cell although the dose of antigen required for induction as well as the duration of the tolerant state differs in these two types of lymphocyte (Weigle, 1973;

Howard and Mitchison, 1975). The mechanism of immunological tolerance is at present unresolved although several proposals have been suggested (Humphrey, 1976). These include, the blockage of B-cell receptors by antigen (Borel, 1976) or the irreversible modulation of these receptors (Klaus and Abbas, 1977; Klaus, 1979). Suppression by T-cells has also been proposed as a mechanism for B-cell tolerance (Taylor and Basten, 1976) although Parks and Weigle (1979) failed to show a major role for suppressor cells in inducing or maintaining a tolerant state. However, recent reviews by Fernandez et al. (1979), Kettman et al. (1979) and Marshall-Clarke and Playfair (1979) have further discussed the mechanisms of B lymphocyte tolerance.

c) Antigenic competition: The term antigenic competition has been used to describe the phenomenon whereby an animal, immunised with two antigens either simultaneously or in close sequence manifests a decreased response to one or both antigens (Adler, 1964). Liacopoulos and Ben-Efraim (1975) proposed that competition may be due to 1) obliteration of the 'handling' of antigens by macrophages through some defect of antigen 'processing' which interferes with the various functional properties of the cells or 2) inhibition of antigen-specific T- or B-cells by other cells, either directly or indirectly, via inhibitory factors.

d) Adjuvants: An adjuvant is a substance which is able to

1) convert an apparently non-immunogenic material into an effective immunogen

2) increase the levels of circulating antibody to an antigen

3) induce delayed type hypersensitivity or its increase, and

4) lead to the production of certain disease states (White, 1967).

The most widely used adjuvant is Freund's adjuvant which is a water-in-oil emulsion either with(Freund's complete adjuvant, FCA) or without (Freund's

incomplete adjuvant) mycobacteria. Various other microbial products have adjuvant activity (Corynebacterium parvum, Bordetella pertussis, E. coli, Salmonella) and also nucleic acids, mineral substances, emulsions, organic compounds and vitamins (Jollés and Paraf, 1973).

The general mode of action of adjuvants has been reviewed by numerous workers (Munoz, 1964; Neter, 1971; Jollés and Paraf, 1973; White, 1976; Frost and Lance, 1978). The mechanisms reported (which may not apply to all adjuvants) are:-

1) Adjuvants have an effect on antigen stimula either by its slow release from a depot or its protection from destruction and rapid excretion, thus improving its distribution through the body.

2) Antigen processing may be affected by adjuvants by the mobilisation of operative cells or by the facilitation of the interaction of antigens with cell membranes.

3) Antibody producing cells may be stimulated to proliferation with an alteration in protein synthesis, e.g. a general effect on control mechanism of the body.

4) Adjuvant may affect cells not involved in the immune process so that breakdown products are released which affect immunocompetent cells.

(iii) Animals

The choice of animal for use in immunological studies is of great importance since there are differences in the abilities of various species to induce humoral and/or cell-mediated immune responses (Laky, 1978). For example, Kondo, Sakurai and Sarai (1976) have shown that staphylococcal exfoliatins A and B are immunogenic in the rabbit but not in the mouse. Similarly, it has been demonstrated that various strains of animals of the one species show constant differences in response to the same dose of antigen (Buschmann et al., 1972; Silver, McKenzie and

Winn, 1972; Nutt, Grossberg and Pressman, 1974) while Ipsen (1959) found that up to 10-fold differences occurred in the response of inbred strains of mice to tetanus toxoid.

The recognition of specific antigens as immunogens by individual animals and inbred strains is governed by the products of individual dominant immune response (Ir) genes which are linked to genes of the major histocompatibility complex (Benacerraf and Katz, 1975; Munro, 1975; Benacerraf and Germain, 1978; Fudenberg et al., 1978). These genes have been termed histocompatibility or H-linked Ir genes. Although the relevant gene products and their cellular site of action have not been fully characterised, it has been shown that subsets of T cells with helper or suppressive activities are major factors in the control and fine tuning of the immune responsiveness (Gershon, 1974; Feldman et al., 1977; Tada, 1977).

Two models have been proposed to account for the function of specific H-linked Ir genes (Benacerraf and Germain, 1978). The first model postulates that Ir genes are primarily expressed on T cells and are concerned with the production of specific helper and suppressor factors. The second model postulates that Ir genes control the immune response by coding for cell surface antigens on macrophages and B cells. Several workers have shown the importance of determinant selection by macrophages in controlling the immune response (Rosenthal, Barcinski and Blake, 1977; Niederhuber, 1978; Rosenthal, 1978; Schwartz, Yano and Paul, 1978). Recent work by Yowell et al. (1979) has indicated that the presentation by macrophages of a specific suppressor determinant on the antigen molecule, may determine the immune responsiveness of an animal by the preferential stimulation of suppressor T cells.

2. Toxoids

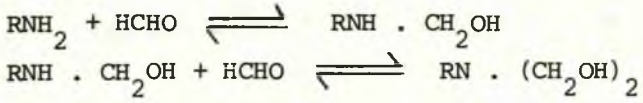
Under a variety of conditions bacterial exotoxins lose their toxicity without losing antigenicity and Ehrlich introduced the term "toxoid" to name such pharmacologically inactive toxin preparations. The use of formaldehyde to convert these bacterial toxins into non-toxic derivatives appears to have its origin in the work of Lowenstein (1909), who rendered tetanus toxin atoxic in this way. Glenny and Sudmerson (1921), Glenny and Hopkins (1923) and Ramon (1923) reported a similar use of formaldehyde to convert diptheria toxin into a non-toxic material.

The general subject of formaldehyde reactivity is reviewed by French and Edsall (1945) and subsequent studies by Fraenkel-Conrat, Branson and Olcott (1947) and Frankel-Conrat and Olcott (1948) has extended the investigation of formaldehyde as a cross-linking reagent both in model systems and with proteins. The primary reaction with many proteins is the formation of aminomethyol derivatives with the ε-amino groups of lysyl residues (Fig 1). The cross-linking reaction involves the condensation of this group with an active hydrogen on primary amides, guanidyl, phenolic, indole, and imidazolyl groups to form a methylene bridge. In addition, these and other side chain groups (e.g. aliphatic alcohols and thioalcohols) are potentially capable of initiating the crosslinking reaction (French and Edsall, 1945).

Various factors may influence methylene bridge formation and the subsequent polymerisation of toxins. The concentration of formaldehyde and toxin in the reaction mixture may not only influence the size and number of polymers obtained (Murphy, 1967) but also the rate at which the reaction occurs (Wadsworth, Quigley and Sickles, 1937). The pH of the system is also very important in the polymerisation of toxins with

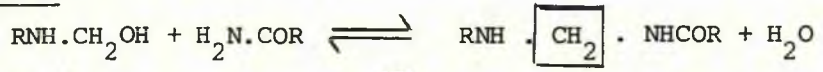
Figure 1. The Reaction of Formaldehyde with Proteins

Primary Reaction

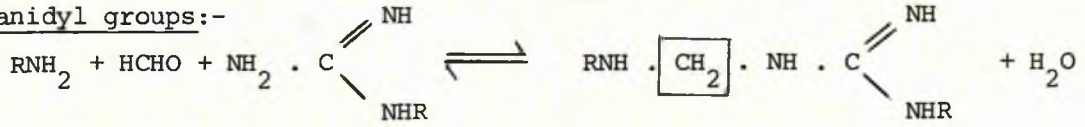


Crosslinking Reaction

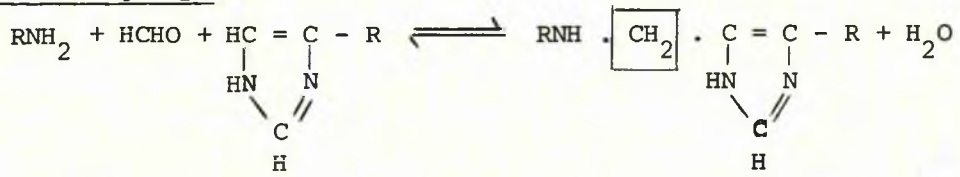
(i) Amides:-



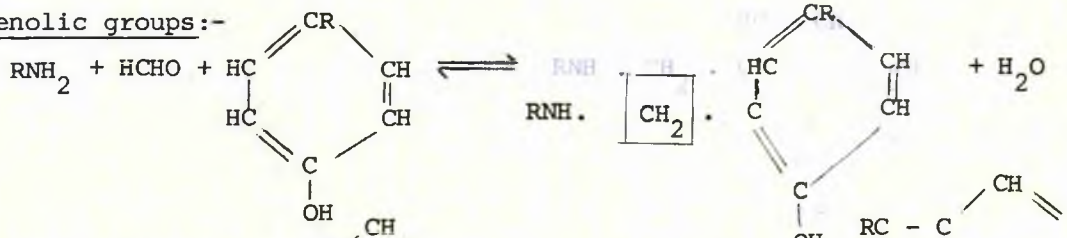
(ii) Guanidyl groups:-



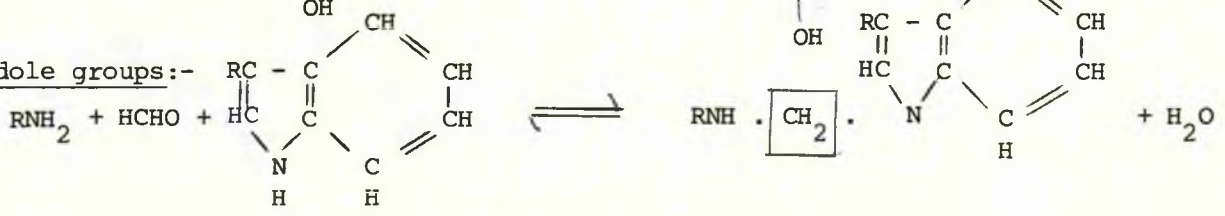
(iii) Imidazole groups:-



(iv) Phenolic groups:-



(v) Indole groups:-



formaldehyde (French and Edsall, 1945) and, in alkaline conditions, formaldehyde fails to induce any polymerisation of staphylococcal enterotoxins while, at pH 5 or pH 7.5, polymerisation is extensive (Warren et al., 1973, 1975).

Formaldehyde treatment has been reported to produce little or no change in the antigenic specificity of proteins (Habeeb, 1969; Kondo et al., 1976), however, high concentrations of formaldehyde have also been reported to lead to a marked decrease in the antigenicity of toxoids (French and Edsall, 1945). Gill et al. (1968) suggested that the formation of methylene bridges may give rise to new antigenic determinants depending on the degree of cross-linking. However, Rittenberg, Pinney and Iglewski (1976) concluded that formaldehyde treatment is more apt to destroy antigenic determinants rather than create new ones and Warren et al. (1973, 1975) found that this destruction of native determinants was influenced by the pH of the reaction. Similarly, the immunogenicity of formaldehyde-treated toxins is dependent on the pH of the system (Eaton, 1937; Moroz-Perlmutter et al., 1963; Warren et al., 1973, 1975)and also on the animal used for immunisation (Kondo et al., 1976). Loss of immunising power of some preparations of staphylococcal toxoids has been reported (Fisher, 1957).

Nevertheless, formaldehyde treatment does not involve extensive conformational alterations (Habeeb, 1969; Robinson, Picklesimer and Puett, 1975) and renders proteins relatively inert to tryptic digestion (French and Edsall, 1945), urea (Habeeb, 1969) and acid hydrolysis (Fraenkel-Conrat and Olcott, 1948). This increased stability and resistance to proteolysis of toxoids compared with toxin have been attributed to the cross-linking effects of the formaldehyde treatment (Bazaral, Goscienski and Hamburger, 1973; Pappenheimer, Uchida and Harper, 1972).

There is evidence that formalinised toxins may reactivate both *in vivo* and *in vitro*. This has been shown with diphtheria (Wadsworth *et al.*, 1937; Linggood *et al.*, 1963; Scheibel and Christensen, 1965; Stanier, 1968; Relyveld, 1969; Akama *et al.*, 1971b), tetanus (Akama *et al.*, 1971a) and cholera (Northrup and Chisari, 1972; Rappaport *et al.*, 1974) toxins. This reversion can be inhibited either by heating the toxin prior to formaldehyde treatment (Germanier *et al.*, 1976) or by the addition of certain amino acids during the detoxification process (Linggood *et al.*, 1963; Scheiber and Christensen, 1965; Stanier, 1968; Relyveld, 1969; Northrup and Chisari, 1972; Saletti and Ricci, 1974b). However, reversion to a toxic preparation was not always found with formaldehyde-treated toxins (Warren *et al.*, 1973).

Glutaraldehyde has been employed as a substitute for formaldehyde by several workers for the toxoiding of cholera (Rappaport *et al.*, 1974) and other toxins (Relyveld, Girard and Désormeau-Bedot, 1973). The efficiency of glutaraldehyde as a toxoiding agent has been attributed to the stability of the reaction products (Habeeb and Hiramoto, 1968; Richards and Knowles, 1968; Korn, Feairheller and Filachione, 1972). However, Saletti and Ricci (1974a) have shown that highly purified cholera toxin detoxified with glutaraldehyde lost its immunising capacity.

The use of other agents in detoxifying bacterial toxins has been varied both in the chemicals used and the results obtained. Carbamylation (Robinson *et al.*, 1975), maleylation (Bizzini, Turpin and Raynaud, 1973) and dinitrophenylation (Raynaud, Blass and Turpin, 1957) all produced an atoxic, non-immunogenic derivative while methylation only reduced the toxicity (Robinson *et al.*, 1975; Beugnier and Zanen, 1977). However, tetranitromethane has been successfully used for the highly selective nitration of tyrosyl residues yielding a product that was atoxic but still

immunogenic (Bizzini et al., 1973; Kondo et al., 1976; Beugnier and Zanen, 1977). The properties of the atoxic derivatives depended on the degree of nitration of the tyrosine residues, too much and both immunogenicity and toxicity were destroyed. Employment of these methods of detoxification, which are specific for certain amino acids, has indicated that particular amino acid residues are involved in toxicity and immunogenicity (Robinson et al., 1975; Kondo et al., 1976).

Object of Research

The immunogenicity of staphylococcal δ-haemolysin has been well established in the rabbit but not in the mouse. Since large numbers of animals are readily available, the mouse is a more convenient system for immunological studies. The mouse has also been employed as an animal model for several experimental staphylococcal infections and therefore induction of anti-δ-haemolysin antibodies in the mouse might be useful in determining a role for the haemolysin in the virulence of the organism.

The primary objective of this research was therefore to establish conditions necessary for the induction of an immune response to staphylococcal δ-haemolysin in the mouse. Since induction of an effective immune response might require chemical modification of δ-haemolysin, the effect of treatment with formaldehyde and glutaraldehyde on both the immune response and some in vivo and in vitro biological properties of the haemolysin was considered necessary.

Finally, it was intended to screen several strains of S. aureus from various sources for the production of δ-haemolysin which might vary in their haemolytic, antigenic and other biological properties.

MATERIALS AND METHODS

A. Production of δ-haemolysin

1. Strains of S. aureus

S. aureus strain NCTC 10345 was used throughout this investigation for large-scale production of δ-haemolysin. Other strains were obtained through the courtesy of Professor J.P. Arbuthnott, Trinity College, Dublin (JK128, JK132, JK139); Dr. S. McKay, Royal Infirmary, Glasgow (SM14); Dr. D. Taylor, Veterinary School, University of Glasgow (canine, bovine and mouse strains) and Mr. W. Irving, Department of Bacteriology, Law Hospital, Carluke (LS human isolates). These strains and their source are listed in Table 12.

2. Maintenance of cultures

On receipt, all cultures were plated on to 5% washed horse blood agar to examine the haemolysis zones surrounding the colonies, with routine gram staining to confirm culture purity. Freeze-dried samples were reconstituted in sterile saline before plating out on to blood agar plates. Cultures were maintained by lyophilization or by passage on freshly prepared nutrient agar slopes.

3. Production of crude culture supernates

All strains were subcultured from nutrient agar slopes and inoculated on to 5% washed horse blood agar. After 24 h incubation at 37°C plates were checked for culture purity and organisms from isolated colonies, which showed large haemolytic zones, were inoculated on to agar slopes and incubated overnight at 37°C. The resulting confluent growth was resuspended in 2 ml of sterile saline. One ml of this suspension was seeded into 50 ml of pre-warmed Bernheimer diffusate medium (Appendix I) contained in a 250 ml flanged Erlenmeyer flask and incubated at 37°C

Table 12. Strains of S. aureus

Strain	Source
	Human
JK128, JK132, JK139	These strains were obtained by Prof. J.P. Arbuthnott during a biochemical survey of approx. 150 clinical isolates of S. aureus
SM14	Eye infection
Wood 46 (NCTC 7121)	
LS1	Nasal swab
LS2	Pus
LS3	Eye swab
LS4	Post op infection
LS5	Nasal swab
LS6	Nasal swab
LS7	Bed sore
LS8	Mouth
LS9	Eye infection
LS10	Nasal infection
LS11	U.T.I. (female)
LS12	Varicose ulcer
LS13	Varicose ulcer
LS14	Ulcer infection
LS15	Post prostatectomy
LS16	Leg infection
LS17	Post op wound
LS18	Varicose ulcer
LS19	Varicose ulcer
LS20	Ulcer infection
LS21	Inflamed throat
LS22	Routine screen
LS23	Routine screen
LS24	Nasal screen
LS25	Nasal swab
LS26	Bunion infection
LS27	Post op infection
LS28	Septicaemia
LS29	Varicose ulcer
LS30	Umbilical cord infect.
LS31	Nasal screen
LS32	Varicose ulcer
LS33	-
	Canine
70531	nail lesion
70544	wound
70554	cellulitis
70645	Vaginal swab
70671/L	ear
	Bovine
69931	mastitis
	Mouse
Zoology Mouse (ZM)	

in an orbital shaking incubator operating at 150 rpm. This was used as a starter culture for strain NCTC 10345. In all other cases the crude culture supernates were obtained by centrifugation at 17,000 g for 20 min.

One ml of starter culture NCTC 10345 was inoculated into 500 ml of pre-warmed medium in a 2 l 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 six such 2 l flasks were processed at any one time, yielding 3 l of crude haemolysin. Crude culture supernatant fluids were obtained by centrifugation at 17,000 g for 20 min.

4. Purification

This was based on the method of Kreger et al. (1971). The crude culture supernates were stirred for 48 h with hydroxylapatite (200 g/3000 ml) at 4°C and unadsorbed material removed after centrifugation (300 g/ 10 min). The haemolysin/hydroxylapatite mixture was washed five times with 400 ml of 0.01M phosphate buffer pH 6.8 and then washed six times with 500 ml of phosphate buffer (pH 6.8, 0.4M). The mixture was stirred for 30 min each time and then centrifuged at 300 g for 10 min to separate hydroxylapatite from the supernatant fluid. Delta-haemolysin was recovered from the hydroxylapatite by washing for 30 min with 200 ml phosphate buffer (pH 7.4, 1M). Five such washes were carried out after which the supernates (containing the eluted δ-haemolysin) were bulked and centrifuged (12,000 g, 10 min) to remove traces of hydroxylapatite. Following overnight dialysis against running water, haemolysin was recovered from the dilute solution by lyophilisation. The freeze-dried preparation was redissolved in approx. 250 ml distilled water and dialysed for a further 72 h against distilled water with frequent changing of the

water. At this stage, any insoluble material was removed by centrifugation at 47,000 g for 20 min. The supernates, containing purified soluble δ-haemolysin, were lyophilized.

B. Assay for δ-haemolysin

1. Haemolytic assay

(i) Erythrocytes: Horse blood was obtained from Gibco-Bio-Cult Diagnostics Ltd., Paisley. Human blood was obtained from the Haematology Department, Western Infirmary, Glasgow. 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, University of Glasgow. Cod blood was obtained by bleeding from the dorsal aorta of freshly caught fish. These were obtained from the Firth of Clyde by the R.V. Leander, by courtesy of the University Marine Biological Station, Millport. In all cases sodium citrate (3.8% w/v) was used as an anti-coagulant.

Cod blood was stored in liquid nitrogen as described by Chao and Birkbeck (1978a) and, when required, pellets were removed, thawed and washed 4 times in 12.5% DMSO/CDS (v/v). All other blood samples were stored at 4°C and washed 4 times in PBSA.

(ii) Standardization of erythrocyte suspensions: One ml of washed packed erythrocytes was added to 100 ml CDS (for cod erythrocytes) or PBSA (for all other erythrocytes) to make an approx. 1% suspension. This suspension was standardized by haemolysing an aliquot with a few grains of saponin. The amount of haemoglobin in the lysate was measured on a Pye Unicam SP500 spectrophotometer at $E^{5\ mm}_{540nm}$. A 1% suspension of erythrocytes gave an absorbance of 1 and the approx. suspension was diluted accordingly.

(iii) Doubling dilution titration: Doubling dilutions (0.5 ml volumes) of samples were made in CDS (for cod erythrocytes), PBSA (for rabbit, horse or human erythrocytes) or PBSA containing 0.001M Mg^{2+} (for sheep erythrocytes) in 100 mm x 12 mm test tubes. Equal volumes of a 1% erythrocyte suspension were added to each dilution and tubes were incubated for 30 min at 37°C, except for tubes containing cod blood which were incubated at room temperature for 30 min. The 50% end-point (HU_{50}) was measured visually. To test for β-haemolysin when using sheep erythrocytes, tubes were incubated at 4°C for a further 1 h. Any increase in haemolytic titre after this incubation period was taken to indicate the presence of β-haemolysin.

2. Vascular Permeability enhancement

This test was based on the method of O'Brien and Kapral (1976).

(i) Animals: HAM1/CR mice were originally used but, for reasons given elsewhere, rabbits were employed in later experiments. White rabbits were obtained by cross-breeding New Zealand White and Dutch rabbits in this department.

The backs of the rabbits were shaved using Oster Professional (Model A-5) Animal Grooming Clippers. Spots were marked on the backs (to indicate injection sites) using a felt tip pen. A maximum of 80 spots per rabbit was obtained.

(ii) Procedure: The skin sites were intradermally inoculated with 0.1 ml volumes containing various doses of δ-haemolysin (1 μg - 1000 μg) or other test preparations. After various intervals (30 min - 24 h) 3-4 ml of 5% (w/v) Pontamine Sky Blue in 0.85% NaCl was given intravenously. One hour later the animals were sacrificed by intravenous injection of Nembutal at a concentration of 35 mg per Kg of body weight. The average diameter of responses was measured with Vernier Calipers.

C. Immunological Studies

1. Double Diffusion tests

The antigenic activity of test preparations was examined by the double diffusion technique of Ouchterlony (1958). Rabbit anti-δ-haemolysin antisera (Birkbeck and Whitelaw, 1979) were kindly donated by Dr. T.H. Birkbeck. Rabbit anti-α-haemolysin (Wellcome Reagents Ltd., Beckenham, England) and α-haemolysin were donated by Mr. G.M. Phimister.

A microscope slide (previously cleaned in absolute alcohol) was covered with 2 ml of 0.75% agarose (BDH Chemicals Ltd., Poole, England) in PBSA. Wells (2 mm diameter) were cut in the agar with a punch and Perspex template with well centres spaced 6 mm apart. Wells were filled with either antigen solutions, diluted to approx. 62.5 μg/ml protein, or antiserum. Plates were incubated in a moist atmosphere (sandwich box containing dampened filter paper) at room temperature for 24-48 h.

Staining of plates: The slides were washed in saline and then distilled water to remove unreacted protein and then pressed for 10 min, to remove excess water, by covering with dampened Whatman No. 1 filter paper plus several layers (2-3 cm) of soft blotting paper and finally a weight of 2-4 Kg. Two such pressings were carried out followed by two washings of the slides in saline and distilled water. Slides were dried thoroughly at 37°C after which time they were stained for 2 min with Nigrosine (saturated solution in 2% acetic acid). Destaining in 2% acetic acid was followed by drying at 37°C.

2. Immunoelectrophoresis

A glass plate (11 cm x 8 cm) was covered with 15 ml molten 0.75% agar (Oxoid L12 purified agar) in barbitone buffer (pH 8.6, I = 0.05). When the agar had set, 2 mm wells were cut 2 cm apart at a distance of

5 cm from the end of the slide. The wells were filled with samples which had been dialysed against barbitone buffer. Bromophenol Blue tracking dye was added to one well. Electrophoresis was performed at 100V until the dye was 15 mm from the anodal end of the plate. For the development of antigen-antibody precipitin arcs, troughs (8 cm x 0.3 cm) were cut parallel to the direction of migration, between the wells, and filled with antiserum.

3. Rocket Immunoelectrophoresis (RIE)

This test was based on the method of Laurell (1966). Rabbit anti-δ-haemolysin serum (0.15 ml) was mixed with 4.85 ml double strength barbitone buffer (pH 8.6). An equal volume of molten 2% agarose (BDH Chemicals Ltd., Poole, England) was added and the molten solution poured onto a 5 cm x 12.5 cm glass plate. When the gel had set, wells (2 mm diam.) were cut 3 mm apart along the cathode end of the slide. Each well was filled with 5 µl of standard δ-haemolysin solution (25 µg - 200 µg) or other test samples. Electrophoresis was carried out at 48 mA for 2 h after which time the slides were stained in Coomassie Brilliant Blue dye (Weber and Osborn, 1969) and destained in a methanol : acetic acid : water (1 : 1 : 5) solution. The height of the resulting precipitin peaks was measured and the concentration of antigen present in each sample was calculated from the standard graph.

D. Chemical modification of δ-haemolysin

1. Treatment with formaldehyde

A modification of the method of Warren et al. (1973) was employed. Delta-haemolysin was exposed for various periods (0-30 days) to aqueous formaldehyde (Analar grade, BDH Chemicals Ltd., Poole, England)

at pH 5, pH 7.5 or pH 9.5. In these experiments, lyophilized haemolysin was added to a formaldehyde-free buffer (0.15M phosphate, pH 5 or pH 7.5; 0.05M sodium carbonate/bicarbonate, pH 9.5) to a concentration of 1 or 2 mg/ml. Reagent grade 36% formaldehyde was diluted 1:6 (v/v) in the appropriate buffer and a measured volume pipetted into the haemolysin containing solutions to give a final formaldehyde concentration of 1%. Control samples contained no formaldehyde. In initial experiments the formaldehyde solutions were placed into Visking dialysis tubing, immersed in the haemolysin solutions and allowed to equilibrate for 16 h before removing the bags. However, simple mixing of haemolysin and formaldehyde solutions was found to be just as effective in toxoiding the δ-haemolysin.

The solutions were incubated at 37°C in a water bath after which time the reaction was terminated by dialysis against a large volume of formaldehyde-free 0.01M phosphate buffer (pH 6.8) containing 1:10,000 merthiolate as a preservative. Any precipitate was removed from the solutions by centrifugation (10,000 g for 20 min) and the preparations were stored at 4°C.

Such formaldehyde-treated preparations were denoted by the symbols F5, F7.5, F9.5 to indicate δ-haemolysin that had been treated with formaldehyde at pH 5, pH 7.5 and pH 9.5 respectively. Similarly, control samples were denoted by the symbols C5, C7.5, C9.5. Untreated δ-haemolysin was termed native δ-haemolysin.

2. Treatment with glutaraldehyde

Purified δ-haemolysin was treated with 1% (v/v) glutaraldehyde (25% Aqueous Solution, TAAB Laboratories, Reading, England) in the same way as with formaldehyde for up to 7 days at 37°C. The preparations obtained at pH 5, pH 7.5 or pH 9.5 were denoted by G5, G7.5 and G9.5 respectively.

3. Treatment with formaldehyde/amino acids mixtures

Delta-haemolysin was treated for 7 days at 37°C with 1% (v/v) formaldehyde and one of three amino acids, glycine, leucine, or tyrosine (BDH Chemicals Ltd., Poole, England). The first two amino acids were added to a final concentration of 0.1M. Tyrosine, which is only slightly soluble in water, was added at a concentration of 0.0026M. All reactions were done at pH 5, pH 7.5 or pH 9.5 and the reaction was terminated as for preparations treated with formaldehyde alone.

E. Properties of native and formaldehyde-treated δ-haemolysin

1. Protein estimation

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.

2. Nitrogen determination

This was done by Kjeldahl digestion and Nesslerisation. Samples of 0.5 ml were digested for 8 h with 0.2 ml acid A in boiling tubes using a Gallenkamp digestion rack. A few carborundum chips were added to prevent bumping. The cooled digested samples were diluted with 2.8 ml H_2O and 1 ml of this was mixed with 9 ml H_2O and 1 ml of Nessler's reagent. The absorbance was read at E_{450nm} using an SP500 spectrophotometer. The nitrogen content of each sample was estimated from the standard curve of a standard solution of $(NH_4)_2SO_4$ similarly treated.

3. Heat stability of δ-haemolysin

A solution of δ-haemolysin in PBSA (1 or 2 mg/ml) was immersed in a boiling water bath for up to 10 h. Aliquots of the haemolysin were removed at various times and titrated for haemolytic, enhanced vascular permeability and antigenic activities.

4. Spectral analysis

The ultraviolet adsorption spectrum was determined with a Pye Unicam SP800 spectrophotometer in 10mm path length silica cells.

5. Isoelectric focusing

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

(ii) Procedure: All procedures were done at 4°C and the total power was restricted to a maximum of 0.3W. All samples were first dialysed against 1% glycine. The apparatus was allowed to equilibrate at 4°C, 36 ml of 1% ampholines (pH 3.5 - 10; LKB Instruments, London) was added, and a pH gradient established by isoelectricfocusing for 24 h at a potential of 600V using 1% (v/v) phosphoric acid and 1% (v/v) TEMED for the anode and cathode electrode solutions respectively.

From each of the five central troughs 0.1 ml of ampholines was removed and replaced with 0.2 ml of sample (i.e. a total of 1 ml of sample was added). Focusing was continued at a potential of 800V for 18 h and finally at 1000V for a further 24 h. After focusing was completed, fractions, including any precipitated material, were removed with Pasteur pipettes 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 kept cold in an ice bucket.

The optical density of each fraction was measured at 280 nm using a Pye Unicam Model SP500 spectrophotometer with silica micro cells of 10mm light path. To detect the presence of haemolysin, doubling dilution titrations (0.05 ml volumes) were performed on all fractions with cod erythrocytes.

6. Tryptic Digestion

(i) Procedure: Solutions of native δ-haemolysin (2 mg/ml in PBSA), HCHO treated δ-haemolysin and their controls (approx. 62.5 μg/ml) were placed in bijou bottles. Trypsin (2 mg/ml solution in 0.001N HCl) was added in the ratio of 0.1 ml per ml of test solution. Buffer containing trypsin or δ-haemolysin alone served as controls. Samples were incubated at 37°C for 2 h.

(ii) Separation of tryptic peptides: A 15 mg/ml solution of δ-haemolysin was treated overnight with trypsin as described above; 10% Tween 80 was added to a final concentration of 0.1% and the sample was sonicated for a few minutes using a Polaron sonicating bath. The addition of Tween 80 was required to dissolve δ-haemolysin which is insoluble in 0.1M acetic acid (T.H. Birkbeck, unpublished observation). Tryptic peptides were separated on a Sephadex G25 (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) column (60 x 1.8 cm) and fractions of 3 ml were collected. The optical density of each fraction at 280 nm and 230 nm was measured on a Pye Unicam Model SP500 spectrophotometer with silica micro cells of 10mm light path. Haemolytic, antigenic and vascular permeability enhancing activities of each fraction were measured as described previously.

7. Inhibition by lecithin

The inhibitory effect of lecithin was tested using a 0.5 ml solution in chloroform containing 50 μg, 500 μg or 5 mg lecithin. This was evaporated to dryness under a stream of nitrogen and the lipid was dispersed in PBSA to a final concentration of 2.5 mg/ml or 250 μg/ml. The sample was then vortexed, put in the dark at room temperature for 1 h to swell after which time it was sonicated for a few minutes to form

liposomes. Purified δ-haemolysin was added to the lipid dispersion to a final concentration of 62.5 μg or 1 mg protein per ml of liposomes. Formaldehyde treated samples and their controls were similarly processed. All samples were incubated at 37°C for 45 min after which time they were tested for haemolytic, antigenic and vascular permeability enhancing activities.

8. Adsorption to cod erythrocytes

Suspensions of cod erythrocytes in CDS (50% and 10% (v/v)) were mixed with 200 μg of sample (native or formaldehyde treated δ-haemolysin in CDS) in a 3:1 volume ratio in 100 mm x 12 mm test tubes. Samples were incubated at room temperature for 30 min, centrifuged (500 g for 5 min) to remove erythrocytes, and the supernates tested for unbound antigen by double diffusion.

9. Polyacrylamide gel electrophoresis

(i) Gels: Polyacrylamide gels were prepared according to the method of Laemmli (1970).

(ii) Samples: A standard solution was prepared from four proteins (HSA, ovalbumin, myoglobin and cytochrome c) at 200 μg/ml each in 0.5M Tris HCl buffer pH 6.8. Test preparations (F5, F7.5, F9.5, C5, C7.5, C9.5) were dialysed against 1% ammonium acetate and then lyophilised. Each freeze-dried preparation (10 mg) was dissolved in 2 ml Tris HCl buffer pH 6.8.

(iii) Procedure: Solubilising buffer (0.1 ml) was mixed with 0.1 ml of sample and heated at 100°C for 5 min. Samples (25 μl) were applied to the gel and electrophoresis was performed at 20 mA per gel. Gels were stained overnight in Coomassie Brilliant Blue and then destained in acetic acid/methanol/water solution (Appendix IX).

F. Preparation and analysis of antisera

1. Injection mixtures

HAM1/CR mice were injected with 0.05 ml volumes containing equal amounts of sample and Freund's Complete Adjuvant (FCA) or saline.

2. Time Schedules

Mice were given either 1 or 3 intraperitoneal priming injections in FCA followed by 1 or 3 intraperitoneal booster injections in saline given 3 or 5 weeks later. Mice were killed in ether 7 days after the final injection and bled by cardiac puncture.

3. Rabbit immunisation

A single rabbit was immunised with F5 δ-haemolysin at a concentration of 100 μg/ml protein. Intramuscular injections of 0.5 ml of sample in FCA were given every second day for 5 days followed by a fourth priming injection 5 days later. Three booster injections in saline were given intravenously 5 weeks later. The rabbit was test bled from the ear. Rabbit antisera to native δ-haemolysin (Birkbeck and Whitelaw, 1979) was provided by Dr. T.H. Birkbeck.

4. Collection of Sera

All blood samples were allowed to clot at 4°C overnight after which time they were centrifuged to remove free erythrocytes and the sera collected.

5. Analysis of Sera

(i) Passive haemagglutination (PHA): Doubling dilutions of serum samples (0.05 ml volumes) were made in microtitre trays and 0.05 ml of tannic acid-treated sheep erythrocytes (coated with heated-δ-haemolysin) (Appendix X) added. Mixtures were shaken and the results were read

after incubation at 37°C for 1 h. The highest dilution showing complete agglutination of the tanned erythrocytes was taken as the end point.

(ii) Counter immunoelectrophoresis (CIE): Fifteen ml of 0.75% (w/v) purified agar in barbitone buffer was poured on to an 11 cm x 8 cm glass plate. Parallel rows of wells, 2 mm in diameter, were cut 3 mm apart. The anodal wells were filled with serum samples and the cathodal wells with purified δ-haemolysin (200 μg/ml in barbitone buffer). Electrophoresis was carried out for 30 min at 100V and plates examined for the presence of precipitin lines.

(iii) Haemolysin inhibition: Before this test was performed it was necessary to remove lipoproteins from the serum samples.

Each serum sample was mixed with NaBr in a 12 ml polycarbonate centrifuge tube to give a final concentration of 40% (w/v) NaBr. Two ml each of 30%, 20% and 10% NaBr in 350 μM EDTA (w/v) were carefully and sequentially layered on top of the serum sample. The tubes were finally filled with 350 μM EDTA. The samples were centrifuged in an MSE 65 superspeed centrifuge at 29,000 g for 24 h at 18°C in a 3 x 25 ml swingout rotor with 12 ml adaptors. Fractions (1 ml) were collected using an MSE tube piercer. The refractive index of each fraction was measured with an Abbé refractometer and the E^{10mm}_{280nm} was also measured. Peak fractions, containing serum proteins except lipoproteins, were pooled and tested by the haemolysin-inhibition titration.

The inhibitory titre of serum samples was determined by making serial doubling dilutions in 0.5 ml amounts in CDS in 100 mm x 12 mm test tubes and adding an equal volume of a standard haemolysin preparation containing 4 HU_{50}. After incubation at 37°C for 30 min, tubes were cooled to room temperature and 0.5 ml of a 1% suspension of cod erythrocytes was added to each tube. After a further 30 min at room temperature the 50% inhibition end-point (HI_{50}) was assayed visually.

(iv) Radial immunodiffusion: The antibody content of anti-ovalbumin sera was measured by a modification of the method of Mancini, Carbonara and Heremans (1965). Serum samples (15 µl) were applied to 3 mm diameter wells cut in 2 mm deep 0.75% agarose (BDH Ltd., Poole, England) containing 50 µg/ml ovalbumin on 75 mm x 100 mm glass plates. After 24 h incubation at 22°C in a humid chamber the diameter of zones of precipitation were measured with an eyepiece containing a millimetre graticule.

Sera giving the maximum zones of precipitation were pooled and analysed for antibody content by the quantitative precipitin test (Smith et al., 1962). The antibody content of the other sera was determined by comparison with this pooled antiserum.

(v) Absorption of sera: Absorption of antisera was done by mixing either equal volumes of serum and a 1 mg/ml solution of δ-haemolysin or by mixing serum and crude canine staphylococcal culture supernatant fluids in a volume ratio of 5:1. Mixtures were briefly allowed to react at room temperature after which time they were tested by double diffusion and counter-immunoelectrophoresis.

G. Comparison of δ-haemolysins produced by different strains of S. aureus

1. Production of crude culture supernates

Small batches (50 ml volumes) of each strain were grown in Bernheimer diffusate medium as described for strain NCTC 10345. Crude supernates thus obtained were stored at -20°C. Selected strains were grown in CCY medium under the same conditions.

2. Isoelectric focusing in polyacrylamide gels

(i) Concentration of culture supernates: Before electrofocusing was carried out in both the Talbot and Caie apparatus and in polyacrylamide

gel slabs, selected crude culture supernates were first concentrated with ammonium sulphate.

Crude supernates were mixed with ammonium sulphate to 70% (w/v) saturation and stirred at 4°C overnight. Precipitates were collected by centrifugation at 12,000 g for 15 min. Each precipitate was dissolved in 4 ml PBSA and then dialysed overnight against PBSA.

(ii) Apparatus: Electrofocusing was performed with the LKB 2117 Multiphor apparatus.

(iii) Procedure: A special template was placed on the cooling plate and the gel plate placed on top using a detergent solution to improve thermal contact. Samples were applied to the gel surface at the cathode end in 5 mm x 10 mm strips of filter paper (Whatman 3MM, Whatmans Ltd., Kent) soaked in a solution of sample (10 mg/ml purified δ-haemolysin or ammonium sulphate concentrated crude supernates. Spacing between adjacent samples was at least 5 mm. Electrofocusing strips of filter paper were soaked in the appropriate electrode solution (1M phosphoric acid; 1M sodium hydroxide), applied to the surface of the gel and the protruding ends cut off. An initial voltage of 600V was applied but, since the current dropped rapidly, the voltage was increased every 10 min until a maximum of 1000V had been reached. Focusing was continued until the current ceased to drop - about 3-4 h. After termination of focusing, the pH gradient was determined immediately with an antimony surface electrode (Beeley, Stevenson and Beeley, 1972) (Activion Ltd., Kinglassie, Fife).

(iv) Blood overlay: The haemolytic activity of bands was determined by a blood agar overlay technique. A 10% or 20% (v/v) suspension of washed cod erythrocytes in CDS was mixed with a 1% agarose solution in CDS at 56°C giving a final erythrocyte concentration of 2% or 5% (v/v).

The mixture was carefully poured on top of the gel which was surrounded by a wall of plasticine to prevent overflow. When the agar had solidified, the gel was placed in a humid chamber and incubated at 4°C overnight.

3. Formaldehyde treatment of crude culture supernates

Crude culture supernates from seven selected strains were dialysed against phosphate buffer (0.15M ; pH 5) after which time formaldehyde was added to give a final concentration of 1% (v/v). Samples were incubated at 37°C for 7 days and the reaction terminated as previously described for purified δ-haemolysin.

4. Purification with an immunosorbent

(i) Separation of γ-globulin from serum: Equal volumes (20 ml) of rabbit anti-δ-haemolysin serum and 32% (w/v) sodium sulphate solution were mixed and stirred for 20 min at room temperature. The resulting precipitate was collected by centrifugation at 10,000 g for 10 min at 20°C and re-dissolved in half the original volume (10 ml) of distilled water. An equal volume of 32% (w/v) sodium sulphate solution was added and re-precipitation was carried out as before. This second resulting precipitate was dissolved in 5 ml PBS_2 and dialysed against 1 l PBS_2.

(ii) Immunosorbent preparation: The reaction was performed such that the thiolated γ-globulin contained 6 to 10 -SH groups per molecule. The γ-globulin concentrate was mixed with N-acetyl-DL-homocysteine thiolactone (NAHTL ; 20 mg/ml in distilled water) to a ratio of 5:1. Bicarbonate buffer (2.5 ml ; pH 10.6) was added and the mixture was flushed with nitrogen. The reaction was allowed to proceed at 0°C for 1 h and the thiolated γ-globulin fraction was recovered by precipitation with an equal volume of 32% (w/v) Na_2SO_4.

The precipitate obtained above was mixed with 2 ml of cross-linking reagent and allowed to react at 0°C for 1 h. The polymer was homogenised, washed 6 times in PBS_2 and twice with glycine/HCl buffer pH 2.2. The mixture was left overnight at 4°C and the volume of the settled precipitate was noted.

(iii) Preparation of column: Sephadex G15, 5 g (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden), was allowed to swell overnight in PBS_2. Ten ml of the Sephadex slurry was mixed thoroughly with 3 ml of the polymerised immunosorbent and poured into a column 1 cm x 15 cm diam. When the mixture had settled a 2 cm layer of Sephadex was placed on top and the column was equilibrated with PBS_2 at a flow rate of 14 ml/h.

(iv) Procedure: After equilibration, 2 ml of sample (purified δ-haemolysin or ammonium sulphate concentrated crude culture supernates) was applied to the column and eluted with PBS_2 overnight. Fractions (2 ml) were collected using an LKB 2200 Ultrorac and the elution profile monitored using a Uvicord UV spectrophotometer and an LKB 2560 Chart Recorder. Adsorbed material was eluted by running glycine/HCl buffer through the column. After the second elution peak was recorded (approx 8 fractions) the column was re-equilibrated with PBS_2. The absorbance of fractions was measured at 280 nm using a SP 500 Spectrophotometer. Peak fractions were pooled and examined for protein content and haemolytic and antigenic activities.

5. Purification with hydroxylapatite

Selected strains were grown in large batch cultures (500 ml) of Bernheimer's diffusate medium and purified as previously described for strain NCTC 10345.

RESULTS

A. Preparation of purified S. aureus δ-haemolysin

1. Selection of strain

Staphylococcus aureus strain NCTC 10345 was used in this work as Chao (1976) showed that, with this strain, large quantities of δ-haemolysin could be prepared and purified by the method of Kreger et al. (1971).

2. Preparation of S. aureus δ-haemolysin

Delta-haemolysin was purified from 3 l of crude culture supernate according to the method of Kreger et al. (1971) using hydroxylapatite. The supernates from each washing step were titrated for haemolytic activity against cod erythrocytes (Table 13). The hydroxylapatite removed all haemolytic activity against cod erythrocytes. Thirty seven per cent of δ-haemolysin was recovered by washing the hydroxylapatite with 1M phosphate buffer. A final lyophilised product (34%) of 1,019 mg of soluble δ-haemolysin with a specific activity of 1024 HU_{50}/mg was obtained. Six such preparations (denoted by 76/1 ; 77/1 ; 77/2 ; 77/3 ; 78/1 ; 78/2) were made over the period of this research work and all gave similar results although the specific activity was occasionally lower.

3. Properties of purified δ-haemolysin

(i) Haemolytic spectrum: The specific activity and haemolytic spectrum of purified δ-haemolysin (Table 14) were similar to those described by Chao (1976) and Whitelaw (1978) except that the haemolytic activity against horse, human and sheep erythrocytes was somewhat lower than found by Chao (1976).

(ii) Ultra-Violet spectrum: The UV spectrum of purified δ-haemolysin, with absorption maxima at 280 nm and 291 nm and a minimum at 247 nm (Fig 2), was similar to that found by other workers (Kayser and Raynaud,

Table 13. Haemolysin activity in supernates from the purification of δ-haemolysin with hydroxylapatite

Fluid		Volume (ml)	Haemolysin titre (HU_{50}/ml cod rbc)	Total Activity (HU_{50}s cod rbc)
Culture Supt.		3,000	1024	3,072,000
Supt after addition of hydroxyl-apatite		3,000	< 2	
0.01M PB washings	1	400	2	800
	2	400	2	800
	3	400	2	800
	4	400	< 2	
	5	400	< 2	
0.4M PB washings	1	500	16	8,000
	2	500	32	16,000
	3	500	16	8,000
	4	500	16	8,000
	5	500	32	16,000
	6	500	16	8,000
1M PB washings	1	200	2048	409,600
	2	200	2048	409,600
	3	200	512	102,400
	4	200	512	102,400
	5	200	512	102,400
			Total 1M washings	1,126,400

Final lyophilised product : 1019 mg @ 1024 HU_{50}/ mg

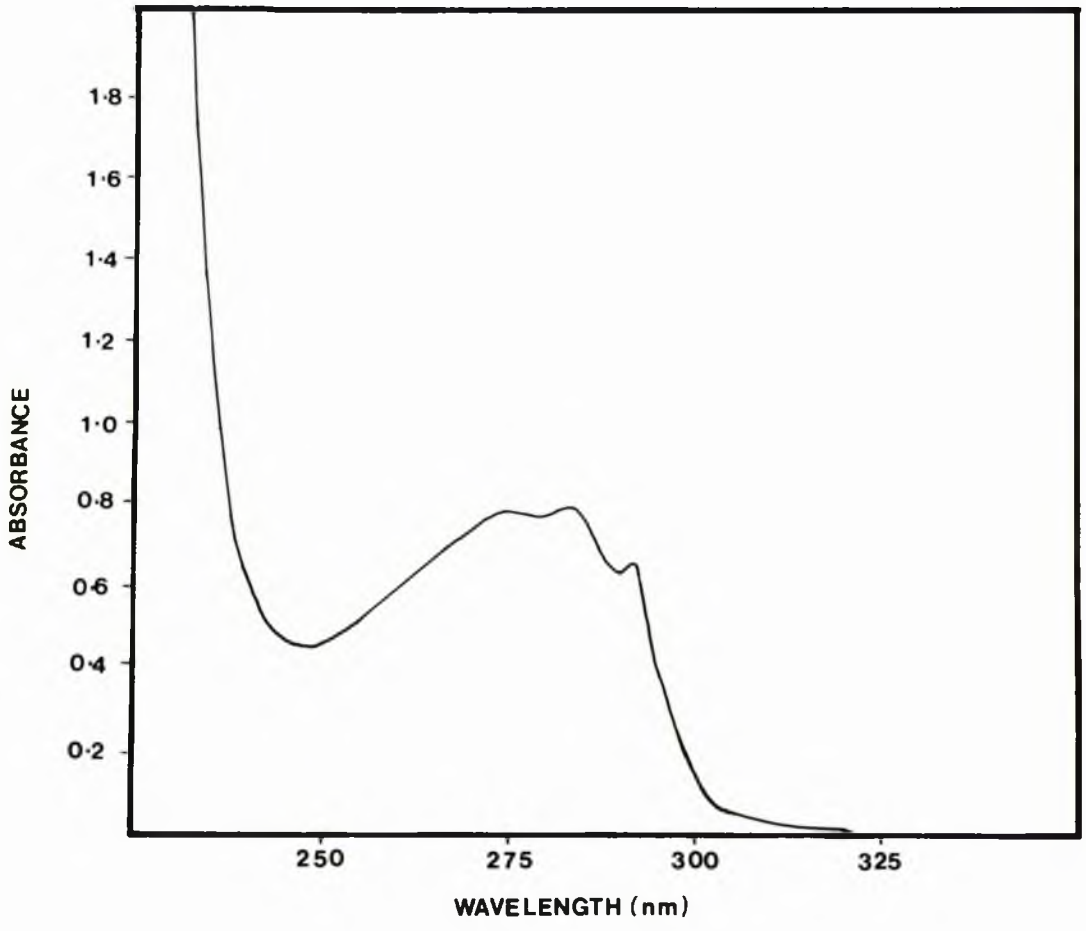
= 1,043,000 HU_{50}

Table 14 Haemolytic spectrum of purified δ-haemolysin

Erythrocytes	HU_{50}/mg
Cod	1024
Horse	64
Human	128
Rabbit	64
Sheep	< 2

Figure 2 Ultraviolet absorption spectrum of δ-haemolysin

The absorption spectrum of purified soluble δ-haemolysin (500 μg/ml) was measured in the Pye Unicam SP 800 spectrophotometer in 1 cm path length silica cells.


ABSORBANCE
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
250
275
300
325
WAVELENGTH (nm)

1965; Kreger et al., 1971; Chao, 1976; Whitelaw, 1978).

(iii) Isoelectric focusing: When δ-haemolysin was subjected to isoelectric focusing in a broad pH gradient (pH 3.5 - 10.0) all haemolytic activity was found in a single peak (pI 4.9) which corresponded to the major peak of E_{280}-absorbing material (Fig 3).

(iv) Heat stability: In preliminary experiments different preparations of δ-haemolysin varied in their sensitivity to heating at 100°C. The time taken for the haemolytic activity of δ-haemolysin (preparation 76/1) to decrease by half, after heating at 100°C, was 2.7 h. However, using a different preparation of δ-haemolysin (preparation 78/1) no loss of haemolytic activity occurred even after 6 h at 100°C. In subsequent experiments, the half-life of δ-haemolysin (preparation 78/2) was 1 h and complete loss of activity was not obtained until after 10 h incubation. Delta-haemolysin remained antigenically active against anti-δ-haemolysin antibody even after 10 h incubation at 100°C (Fig 4).

B. Immunisation of mice with purified δ-haemolysin

1. Preliminary experiment

In an initial experiment eight groups of five mice were given three intraperitoneal priming injections, at two day intervals, of 0.05 ml containing 50 μg δ-haemolysin in FCA (groups 1-4) or saline (groups 5-8). Six of the eight groups were given three intraperitoneal booster injections of δ-haemolysin (50 μg in 0.05 ml of FCA, groups 2-4, or saline, groups 6-8) 1 week (groups 2, 3, 6, 7) and/or 2 weeks (groups 3, 4, 7, 8) later. Sera, collected 6 days after the final injection, did not contain detectable levels of antibodies against δ-haemolysin when tested by CIE.

2. Variation of time schedules

Immunisation of mice with purified δ-haemolysin was further

Figure 3 Isoelectric focusing 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 against 1% cod erythrocytes

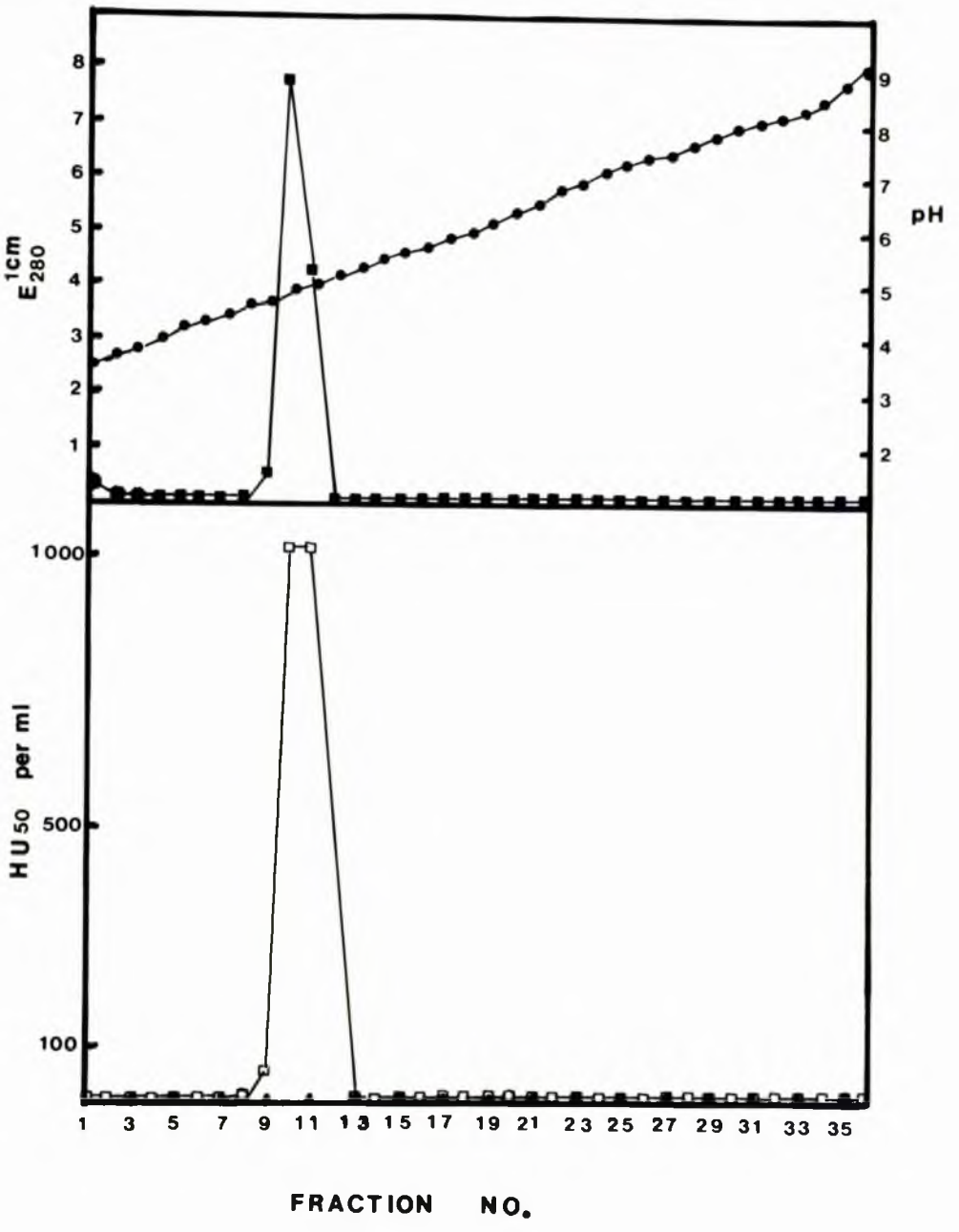

E^{1cm}_{280}
pH
HU50 per ml
1000
500
100
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
FRACTION NO.

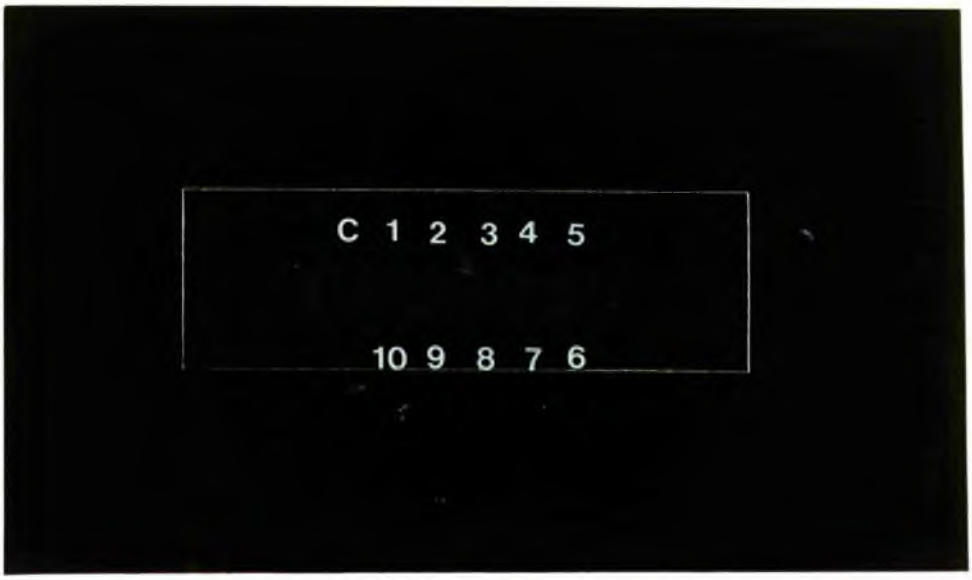
Figure 4 Antigenicity of heated δ-haemolysin

Purified δ-haemolysin was heated for 1-10 h at 100°C in a water bath and antigenic reactivity with anti-δ-haemolysin tested by double diffusion.

Nos. 1-10 - δ-haemolysin heated for 1-10 h

C - unheated δ-haemolysin

Centre wells - rabbit anti-δ-haemolysin.


C 1 2 3 4 5
10 9 8 7 6

investigated by expanding the initial experiment above to include various time schedules. Groups of 5 mice were given one or three (days 1, 3 and 5) primary injections of 50 μg δ-haemolysin in FCA followed by either one or three (at 2-day intervals) boosting injections of 50 μg of haemolysin in saline after a further 3, 5 or 6 weeks. Sera obtained 7 days after the final injection were pooled and tested by CIE and haemolysin inhibition. The presence of anti-δ-haemolysin antibodies, indicated by a strong precipitin line on CIE and an increase in haemolysin inhibition titre when compared with normal serum, was found only in the sera of mice which had received three priming and three boosting injections after an interval of 5 weeks (Table 15).

3. Variation of injection mixtures

Improvement of the response obtained previously was attempted by varying the quantity of antigen and adjuvant in the injection mixture. Mice were immunised with 3 priming injections of δ-haemolysin (6.25 μg - 100 μg/0.05 ml) in FCA containing 1.25 μg - 5 μg of Mycobacterium tuberculosis. Three booster injections of δ-haemolysin (6.25 μg - 100 μg/0.05 ml) in saline were given 5 weeks later. However, out of the 15 serum pools tested, only three gave a faint precipitin line on CIE, none of which had an increased haemolysin inhibition titre when compared with normal serum (Table 16).

4. Anti-ovalbumin sera

The reasons for the poor responses obtained on immunisation with purified δ-haemolysin were not evident. It may be that δ-haemolysin is indeed very poorly immunogenic in the mouse. However, other factors such as the mice, timing of injections, quantity or batch of adjuvant or the quantity of antigen used may be the primary cause(s). Investigations were therefore made to determine the efficacy of the immunisation schedule previously employed.

Table 15 Immunisation of mice with purified δ-haemolysin - Variation of Time Schedules

Timing of injections (days)		Analysis of sera** by:-	
in FCA	in saline	Counter- immuno- electrophoresis *	Haemolysin Inhibition (HI) Titre
1	22	-	128
1	36	-	8
1	43	-	32
1, 3, 5	22	-	32
1, 3, 5	36	-	8
1, 3, 5	43	-	32
1	22,24,26	-	128
1	36,38,40	-	64
1, 3, 5	22,24,26	-	256
1, 3, 5	36,38,40	+	256
Normal mouse serum		-	32

* + - precipitin line formed

\- - no precipitin line

** - the sera from five mice per group were pooled and analysed.

Table 16 Immunisation of mice with purified δ-haemolysin - variation of injection mixtures

Group	Injection Mixtures		Analysis of sera† by:-	
	amount of *M. tuberculosis* (µg)	amount of δ-haemolysin (µg)	counter-* immunoelectrophoresis	haemolysin inhibition (HI)
1	5	6.25	-	
2	"	12.5	+/-	2
3	"	25	+/-	2
4	"	50	-	
5	"	100	-	
6	2.5	6.25	-	
7	"	12.5	-	
8	"	25	-	
9	"	50	-	
10	"	100	-	
11	1.25	6.25	-	
12	"	12.5	-	
13	"	25	-	
14	"	50	-	
15	"	100	+/-	2

† - the sera from five mice were pooled and analysed for each group

* +/- - weak precipitin line formed
\- - - no precipitin line.

Groups of four mice were injected with 5 μg ovalbumin in 0.05 ml of FCA (containing 5 μg Mycobacterium tuberculosis) on days 1, 3 and 5 and in 0.05 ml saline on days 36, 38 and 40. Sera were collected 1-10 days after final injection and individually analysed for anti-ovalbumin antibodies by CIE and radial diffusion. All sera contained anti-ovalbumin antibodies and a maximum antibody titre of 2.5 mg per ml was obtained seven to eight days after the final injection (Fig 5).

Therefore, by employing the same mice, adjuvant and immunisation schedule as for purified δ-haemolysin, high anti-ovalbumin antibody titres could be induced, especially seven to eight days after the final injection. It was therefore concluded that purified δ-haemolysin was very poorly immunogenic in the mouse.

C. Treatment of δ-haemolysin with formaldehyde

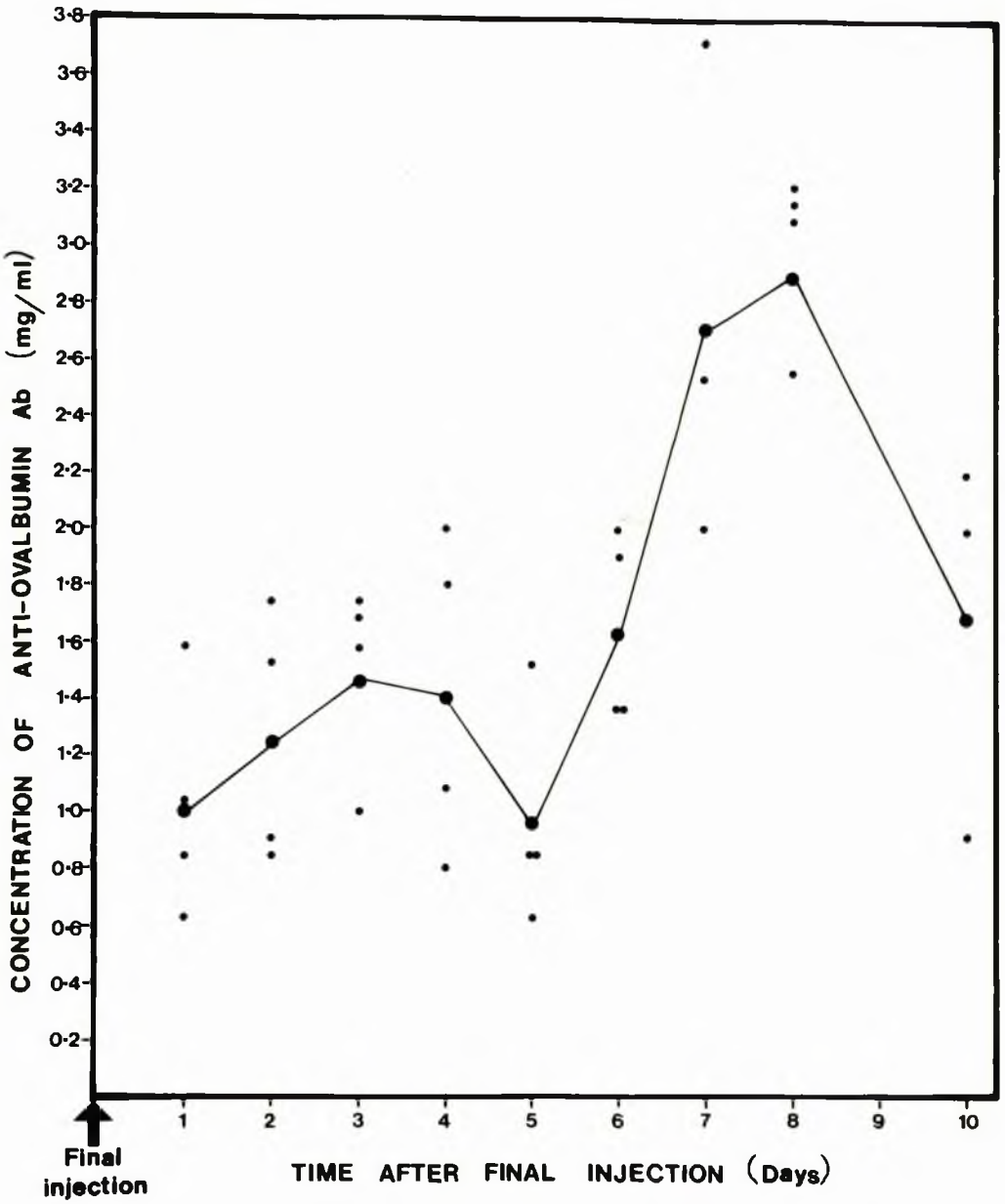
The objectives of these investigations were two-fold. First, the effects of formaldehyde on the toxicity, antigenicity and other biological properties of δ-haemolysin during the production of toxoids was monitored. Second, the immunisation of mice with formaldehyde-treated δ-haemolysin was investigated with the aim of enhancing immunogenicity by modulation of the antigenic determinants.

1. Haemolytic activity

Purified δ-haemolysin was treated with formaldehyde under acid, neutral or alkaline conditions using a modification of the method of Warren et al. (1973). In preliminary experiments, haemolytic activity was lost after incubation for as little as 1 day under acid or neutral conditions whereas, at pH 9.5, loss of activity was not complete even

Figure 5 Immunisation of mice with ovalbumin

Mice were given three primary intraperitoneal injections of 5 μg ovalbumin in FCA followed by three booster intraperitoneal injections of 5 μg ovalbumin in saline 5 weeks later. Individual sera were analysed for anti-ovalbumin antibody concentration by radial diffusion. The mean antibody concentration of four sera is shown at each time and the results of each individual serum is indicated by small dots.


CONCENTRATION OF ANTI-OVALBUMIN Ab (mg/ml)
3·8
3·6
3·4
3·2
3·0
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
1
2
3
4
5
6
7
8
9
10
Final injection
TIME AFTER FINAL INJECTION (Days)

after 6 days. Subsequent experiments indicated that, after an initial loss of 25% of activity within 1 h at 37°C, all activity was lost at pH 5 or pH 7.5 within 24 h (Fig 6). At pH 9.5, however, this initial loss of activity was not followed by any further decrease until after 24 h incubation, and some activity remained after 7 days. When δ-haemolysin was incubated for up to 7 days at 37°C in the absence of formaldehyde there was no loss of haemolytic activity.

2. Antigenic activity

Treatment of δ-haemolysin with formaldehyde did not alter its reactivity with rabbit anti-δ-haemolysin antibody; a line of identity was shown between each treated preparation and purified δ-haemolysin (Fig 7).

3. Protein and nitrogen estimations

A gradual loss in protein concentration of δ-haemolysin solutions was found after incubation with formaldehyde at pH 5 or pH 7.5; only 27% and 40% respectively of original protein being retained after 7 days (Fig 8). This loss was not so apparent after incubation of δ-haemolysin and formaldehyde under alkaline conditions, only 20% of original protein was lost after 7 days incubation.

These protein losses occurred during the incubation period since it was shown that there was no significant decrease in the protein concentration of preparations before or after the dialysis and centrifugation steps. Since formaldehyde reacts with tryptophan residues (French and Edsall, 1945) and, since this amino acid is involved in the protein assay of Lowry et al. (1951), it seems likely that reaction with formaldehyde influenced the reactivity of proteins in the Lowry assay.

Figure 6 Haemolytic activity of δ-haemolysin treated with formaldehyde

The haemolytic activity of purified δ-haemolysin was assayed with 1% cod erythrocytes after incubation with formaldehyde at 37°C at pH 5 (●——●), pH 7.5 (■——■) or pH 9.5 (▲——▲). Delta-haemolysin, incubated at pH 5 (○——○), pH 7.5 (□——□) and pH 9.5 (△——△) in the absence of formaldehyde, was also assayed.

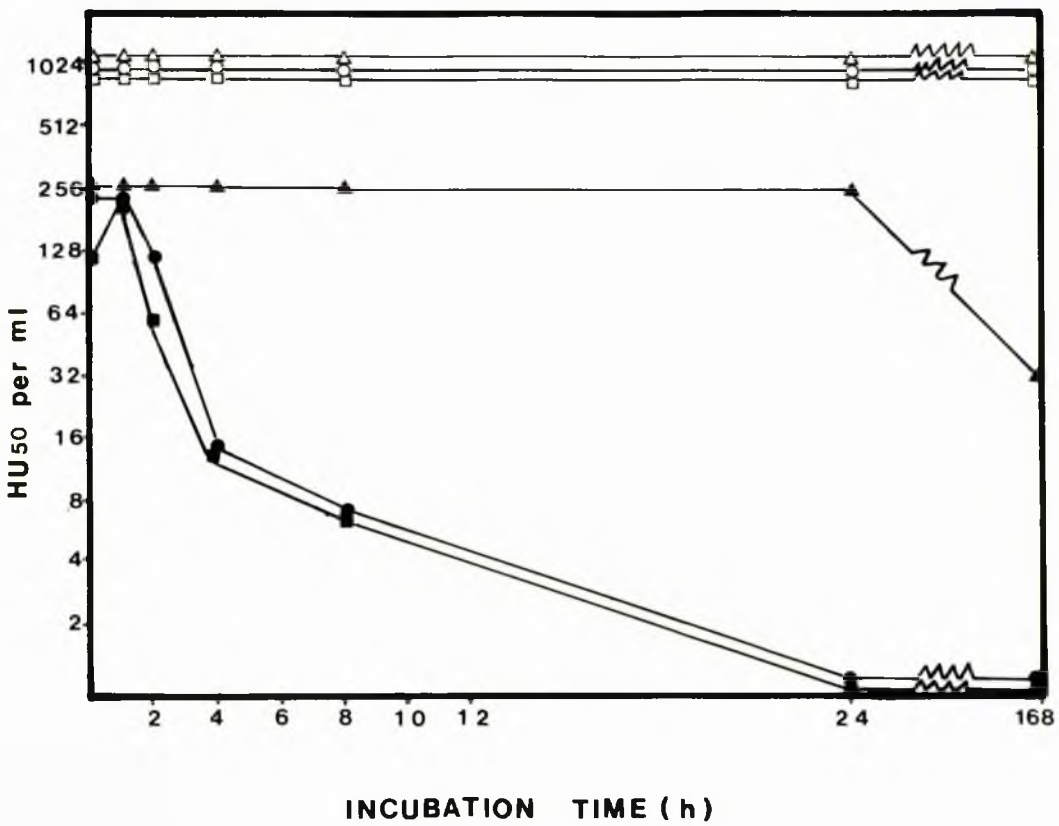

1024
512
256
128
64
32
16
8
4
2
HU50 per ml
2
4
6
8
10
12
24
168
INCUBATION TIME (h)

Figure 7 The antigenic activity of formaldehyde-treated δ-haemolysin

Delta-haemolysin, incubated at 37°C at pH 5, pH 7.5 or pH 9.5 for 7 days with formaldehyde, was tested for antigenic reactivity with rabbit anti-δ-haemolysin antibody by gel diffusion.

D - δ-haemolysin

F5 - δ-haemolysin treated at pH 5 with formaldehyde

F7.5 - δ-haemolysin treated at pH 7.5 with formaldehyde

F9.5 - δ-haemolysin treated at pH 9.5 with formaldehyde

AD - rabbit anti-δ-haemolysin antiserum.

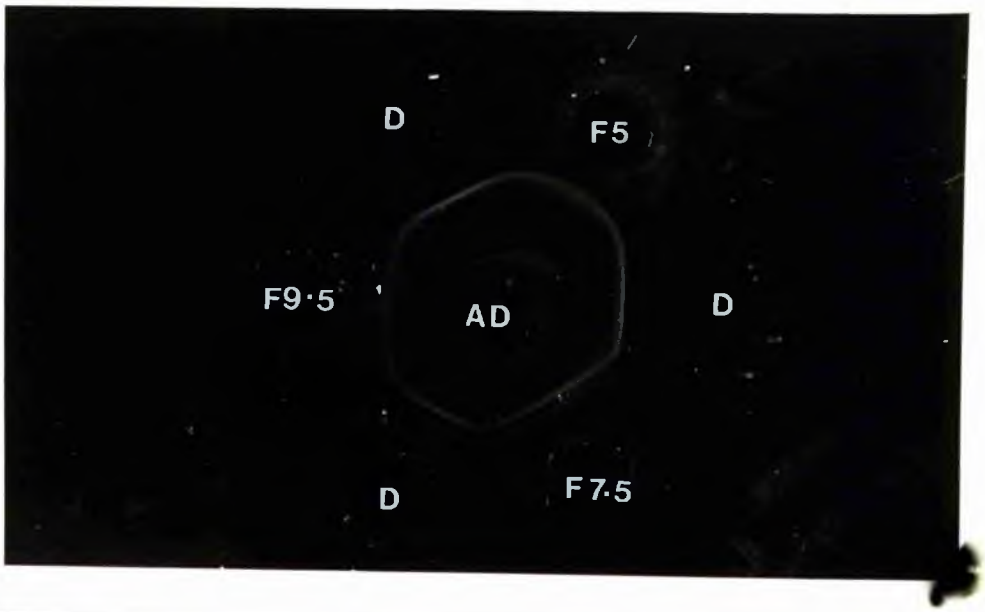

D
F5
F9·5
AD
D
D
F7·5

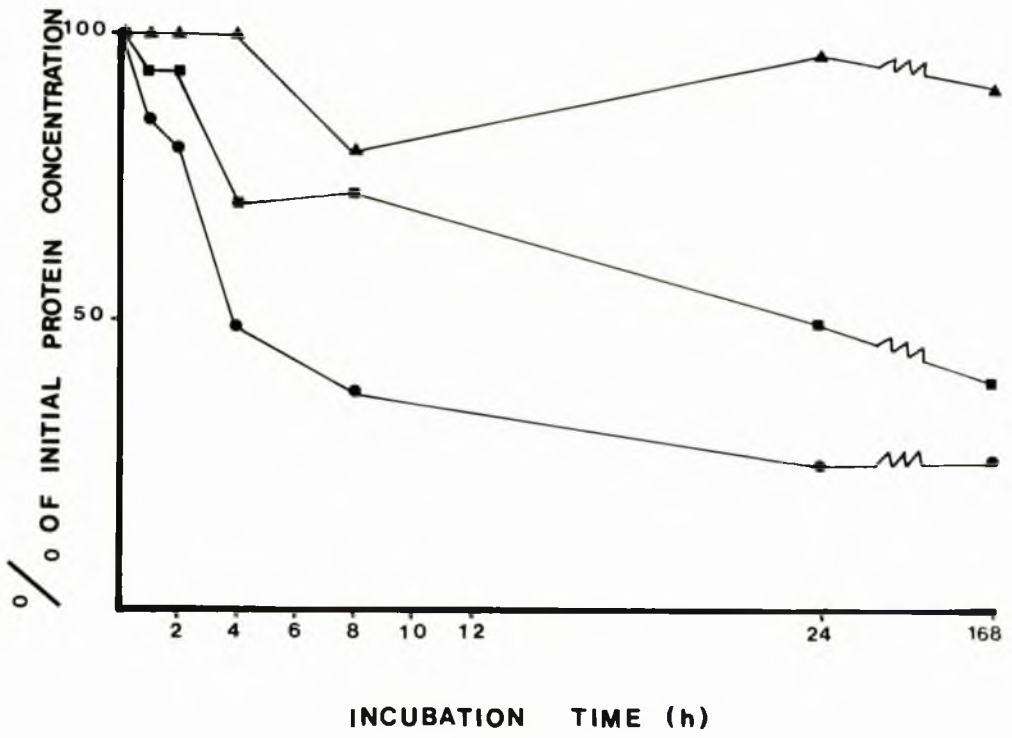
Figure 8 Lowry Reactivity of δ-haemolysin solutions treated with formaldehyde

Delta-haemolysin incubated at 37°C with formaldehyde at pH 5, pH 7.5 or pH 9.5 was assayed for protein concentration by the method of Lowry et al. (1951).

●——● - δ-haemolysin treated at pH 5 with formaldehyde

■——■ - δ-haemolysin treated at pH 7.5 with formaldehyde

▲——▲ - δ-haemolysin treated at pH 9.5 with formaldehyde


% OF INITIAL PROTEIN CONCENTRATION
100
50
2
4
6
8
10
12
24
168
INCUBATION TIME (h)

In order to investigate this, δ-haemolysin, treated for 7 days at pH 5, pH 7.5 or pH 9.5 with or without formaldehyde, was analysed for nitrogen content and the results were compared with the protein determinations of the same preparations. From Table 17 it is evident that incubation of δ-haemolysin with formaldehyde under acid or neutral conditions caused a loss of nitrogen in addition to a loss of measured protein. At pH 9.5, as well as with control samples, there was little or no loss of nitrogen. Comparison of both protein and nitrogen results indicated that the loss was greater with the former, and therefore formaldehyde might be having an influence on the assay system. However, since there was also a loss of protein in control samples there might be some other factor involved, for example, adsorption of δ-haemolysin to glass (Heatley, 1971).

4. Immunisation of mice

In preliminary experiments, twelve groups of 5 mice were immunised with δ-haemolysin treated for various times with formaldehyde at pH 5, pH 7.5 or pH 9.5 with the most successful time schedule found with native δ-haemolysin. Pooled sera were tested by CIE. The immunising ability of each antigen increased with the increased time of incubation with formaldehyde (Fig 9); samples incubated in the absence of formaldehyde failed to stimulate detectable anti-δ-haemolysin antibodies.

In subsequent experiments, mice were immunised, using varying time schedules, with δ-haemolysin treated with formaldehyde for 7 days at pH 5, pH 7.5 or pH 9.5. In 2 experiments, the percentage of pooled sera giving positive results on CIE (over the range of time schedules employed) was 100% (8/8 pooled sera), 63% (5/8 pooled sera) and 25% (2/8 pooled sera) for mice immunised with F5, F7.5 and F9.5 δ-haemolysin respectively. To determine whether this increase was due to increased antibody titres in the responding mice or merely an increase in the number of responding

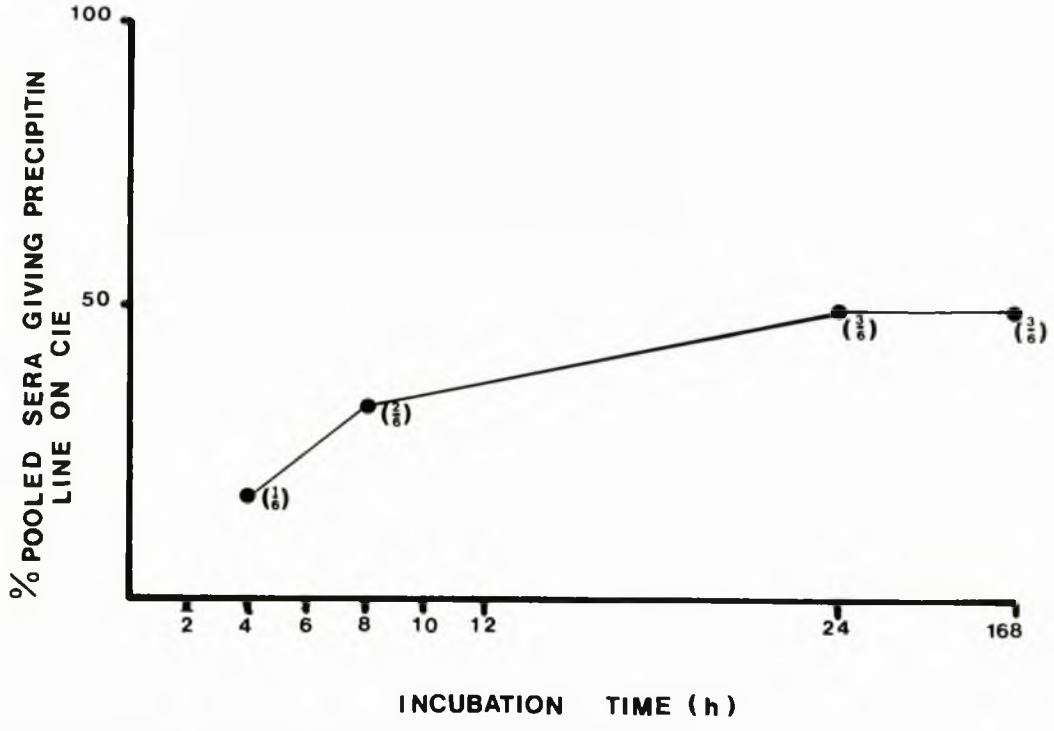
Table 17 Protein and nitrogen content of δ-haemolysin treated with formaldehyde for 7 days

pH	Protein content		Nitrogen content		% Nitrogen
	µg/ml	% of original sample*	µg/ml	% of original sample*	in protein
5	275	23	45	29	16
7.5	425	36	86	55	13
9.5	830	71	196	125	15
control 5	950	81	164	104	11
control 7.5	900	77	140	89	10
control 9.5	1100	94	210	134	12
δ-haemolysin (original sample)	1410		188		13

* - allowing for a dilution factor of 5:6 with formaldehyde

Figure 9 Immunogenicity of formaldehyde-treated δ-haemolysin in mice

Groups of five mice were immunised on days 1, 3, 5, 36, 38, 40 with δ-haemolysin which had been treated with formaldehyde for various times at pH 5, pH 7.5 or pH 9.5. The sera from mice in each group were pooled and analysed by CIE and the results of two experiments were expressed as a percentage of the total number of sera giving a positive result for each incubation time (i.e. the results of animals immunised with each immunogen which had been treated for a specific time were pooled).


100
50
% POOLED SERA GIVING PRECIPITIN LINE ON CIE
(1/6)
(2/6)
(3/6)
(3/6)
2
4
6
8
10
12
24
168
INCUBATION TIME (h)

mice, groups of 8 to 13 mice were immunised with each antigen with varying time schedules. Individual sera were analysed for anti-δ-haemolysin antibodies. The proportion of mice responding to δ-haemolysin increased with the number of injections of antigen and with the interval between primary and boosting injections (Fig 10, Table 18). Of the total mice treated with each immunogen, 23% responded to native-haemolysin and to F9.5 haemolysin; the responses to δ-haemolysin treated with formaldehyde at pH 5 or pH 7.5 were 62% and 39% respectively. Analysis of these results using the chi-squared test (χ^2) confirmed that the difference in percentages obtained between native and F7.5 haemolysin ($\chi^2 = 4.9$) and between native and F5 haemolysin ($\chi^2 = 23.2$) were significant at the 5% and 0.5% probability levels respectively (Appendix XI). In addition to an increase in the number of responding mice, immunisation of both mice and rabbits with F5 haemolysin also produced an increase in anti-δ-haemolysin antibody titre, assayed by PHA (Table 19). The discrepancies found between the PHA and CIE results may be due to the production of low avidity antibodies (Steward, Game and Petty, 1974) which were only detected by CIE because high concentrations of antigen and antibody are focused in one specific area.

All mouse antisera to formaldehyde-treated δ-haemolysin reacted with both native and formaldehyde-treated haemolysin on CIE, although on immunodiffusion only antisera with high PHA titres gave a pronounced precipitin line with both native and formaldehyde-treated haemolysin. Antisera with low PHA titres failed to precipitate with native haemolysin on immunodiffusion (Fig 11). This substantiates the above hypothesis that the antibody to native δ-haemolysin in these antisera, with low PHA titres, may be of low avidity.

The precipitinogenic activity of the mouse antisera was associated with the gamma-globulin fractions as shown by immunoelectro-

Figure 10 Immunogenicity of formaldehyde-treated δ-haemolysin in individual mice

The sera of mice immunised with δ-haemolysin treated with formaldehyde at pH 5 (A) or pH 7.5 (B) for 7 days were analysed individually by CIE for anti-δ-haemolysin antibodies.

A Anodal wells - a(i) - (v) } nine mice immunised on
b(i) - (iv) } days (1,22,24,26)

c(i) - (v) } ten mice immunised on
d(i) - (v) } days (1,3,5,22,24,26)

b(vi) - Rabbit anti-δ-haemolysin antiserum

B Anodal wells - a(iii) - (vii) } ten mice immunised on
b(iii) - (vii) } days (1,22,24,26)

c(i) - (vii) } thirteen mice immunised
d(i) - (vi) } on days (1,3,5,22,24,26)

d(vii) - Rabbit anti-δ-haemolysin antiserum

All cathodal wells contained purified δ-haemolysin (200 μg/ml).

A

B

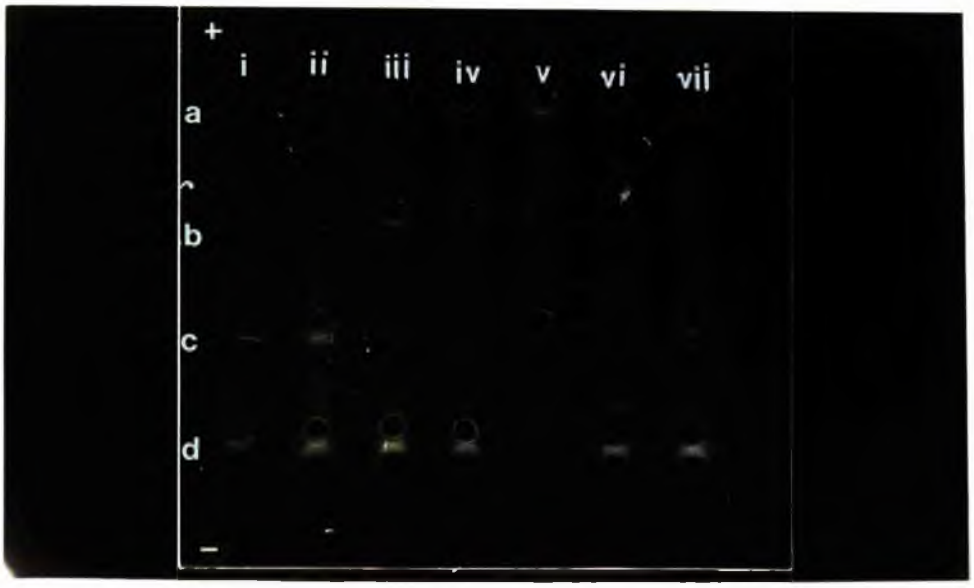


Table 18. Immunogenicity of native and formaldehyde-treated δ-haemolysin in mice

Timing of injections (days)		% animals (in groups of 8 to 13 mice) with serum antibody after immunisation with -			
in FCA*	in saline	untreated δ-haemolysin	δ-haemolysin treated with formaldehyde at pH 5	7.5	9.5
1	22	0 (0/10)	45 (5/11)	0 (0/10)	10 (1/10)
1	22,24,26	20 (2/10)	22 (2/9)	30 (3/10)	25 (2/8)
1	36	20 (2/10)	67 (6/9)	20 (2/10)	0 (0/10)
1	36,38,40	40 (4/10)	89 (8/9)	70 (7/10)	30 (3/10)
1,3,5	22	0 (0/10)	20 (2/10)	30 (3/10)	10 (1/10)
1,3,5	22,24,26	30 (3/10)	80 (8/10)	54 (7/13)	56 (5/9)
1,3,5	36	20 (2/10)	75 (6/8)	20 (2/10)	10 (1/10)
1,3,5	36,38,40	50 (5/10)	100 (10/10)	90 (9/10)	50 (5/10)
Total % Responders		23 (18/80)	62 (47/76)	39 (33/83)	23 (18/77)

* FCA = Freund's Complete Adjuvant.

Table 19. Passive haemagglutination (PHA) and counter-immunoelectrophoresis (CIE) of sera from mice and rabbits immunised with either native or formaldehyde-treated δ-haemolysin

Animal used for immunisation	Analysis of individual sera from animals immunised with native δ-haemolysin		δ-haemolysin treated with formaldehyde at pH 5		7.5		9.5		Analysis of sera from non-immunised control animals	
	PHA titre	CIE	PHA titre	CIE	PHA titre	CIE	PHA titre	CIE	PHA titre	CIE
*Mouse 1	8	+	< 2	+	< 2	+	< 2	+	< 2	-
2	32	+	< 2	+	≥ 4056	+	< 2	+		
3	8	+	≥ 4056	+	< 2	+	4	+		
4	16	+	≥ 4056	+	< 2	+	< 2	+		
5	8	+	≥ 4056	+	≥ 4056	+	< 2	+		
6	4	-	256	+	< 2	+	< 2	-		
7	< 2	-	8	+	< 2	+	< 2	-		
8	< 2	-	≥ 4056	+	≥ 4056	+	< 2	-		
9	L	-	< 2	+	< 2	+	< 2	-		
10	L	-	≥ 4056	+	< 2	-	< 2	-		
†Rabbit	Δ1280	+	5120	+					2	-

*All mice were injected intraperitoneally on days 1, 3, 5 with sample in FCA followed by three boosting injections (days 36, 38, 40) in saline.

†Rabbit was given four primary intramuscular injections of F5 haemolysin in FCA followed by 3 intravenous injections in saline five weeks later.

ΔRabbit antiserum to native δ-haemolysin supplied by Dr. T.H. Birkbeck.

\+ - precipitin line formed
\- - no precipitin line
L - sample lost

Figure 11 Immunodiffusion analysis of anti-formaldehyde-treated δ-haemolysin antisera

The activity of both mouse (A) and rabbit (B) anti-formaldehyde-treated δ-haemolysin antisera with native and formaldehyde-treated δ-haemolysin was tested by double diffusion.

Wells 1,3,5,7,8,10,12,14 - purified δ-haemolysin

Wells 2, 4, 6 - formaldehyde-treated δ-haemolysin at pH 5 (2), pH 7.5 (4) or pH 9.5 (6).

Wells 9, 11, 13 - Control samples of δ-haemolysin at pH 5 (9), pH 7.5 (11) or pH 9.5 (13).

Centre wells

a - rabbit anti-δ-haemolysin

b - mouse anti-pH 5 formaldehyde-treated δ-haemolysin antiserum with low PHA titre (A)

- rabbit anti-pH 5 formaldehyde-treated δ-haemolysin antiserum (B).


A
1 2 3 4 5 6 7
a b a b a b a b
14 13 12 11 10 9 8
B
1 2 3 4 5 6 7
a b a b a b a b
14 13 12 11 10 9 8

phoresis (Fig 12). Absorption of such sera with native δ-haemolysin did not remove all precipitinogenic activity against formaldehyde-treated haemolysin. (The figure is not illustrated as results were more clearly defined with rabbit antiserum.) Rabbit antiserum to pH 5-formaldehyde-treated δ-haemolysin reacted more strongly on immunodiffusion with native δ-haemolysin than did mouse antisera (Fig 11) and also contained antibodies to formaldehyde-treated haemolysin which were not removed by absorption with native δ-haemolysin (Fig 13).

5. Ultra-Violet spectrum

Formaldehyde treatment altered the UV-absorption spectrum of δ-haemolysin with loss of the 291 nm peak (Fig 14). This loss occurred within 24 h incubation under acid, neutral or alkaline conditions; haemolysin incubated for the same time in the absence of formaldehyde retained the 291 nm peak.

6. Immunoelectrophoresis

Reaction of δ-haemolysin with formaldehyde gave an increased electrophoretic mobility at pH 8.6 (Fig 15). Samples incubated in the absence of formaldehyde were electrophoretically similar to native δ-haemolysin.

7. Isoelectric Focusing

Delta-haemolysin treated with formaldehyde was subjected to isoelectric focusing in a broad pH gradient (pH 3.5-10.0) and all E_{280}-absorbing material was found in single peaks corresponding to pI of 4.5, 4.1 and 4.2 for F5-, F7.5- and F9.5-haemolysin respectively. Native δ-haemolysin, however, had a pI of 4.9 (Fig 16).

Figure 12 Immunoelectrophoresis of mouse anti-formaldehyde-treated δ-haemolysin sera

Wells 1, 5 - normal mouse serum

Well 2 - normal mouse serum minus lipoproteins

Well 3 - mouse anti-formaldehyde-treated δ-haemolysin

Well 4 - as for well 3 only minus lipoproteins.

Troughs a, c - purified δ-haemolysin

Troughs b, d - goat anti-mouse antiserum.

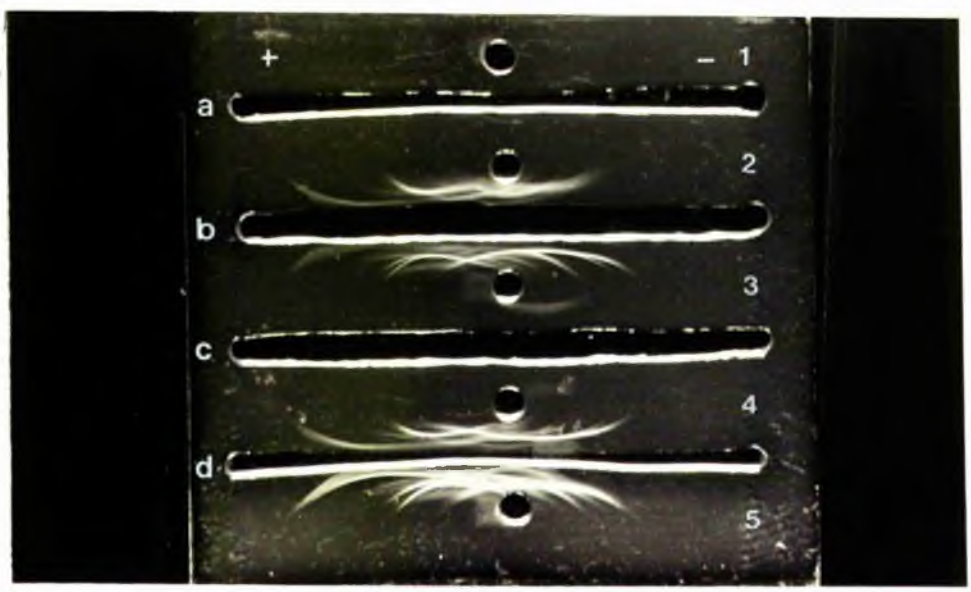

+
– 1
a
2
b
3
c
4
d
5

Figure 13 Antigenic reactivity of formaldehyde-treated δ-haemolysin with absorbed rabbit anti-F5 serum

The antigenic reactivity of formaldehyde-treated δ-haemolysins (F5, F7.5, F9.5) with rabbit anti-F5-serum absorbed with purified δ-haemolysin was tested by gel diffusion -

D - purified δ-haemolysin

F5 - δ-haemolysin treated at pH 5 with formaldehyde

F7.5 - δ-haemolysin treated at pH 7.5 with formaldehyde

F9.5 - δ-haemolysin treated at pH 9.5 with formaldehyde

C5 - δ-haemolysin treated at pH 5 without formaldehyde

C7.5 - δ-haemolysin treated at pH 7.5 without formaldehyde

C9.5 - δ-haemolysin treated at pH 9.5 without formaldehyde

Centre wells - rabbit anti-formaldehyde treated (pH 5) δ-haemolysin antiserum absorbed with purified δ-haemolysin.


D
F5
F 7·5
F 9·5
A
A
A
A
A
C 9·5
C 7·5
C 5

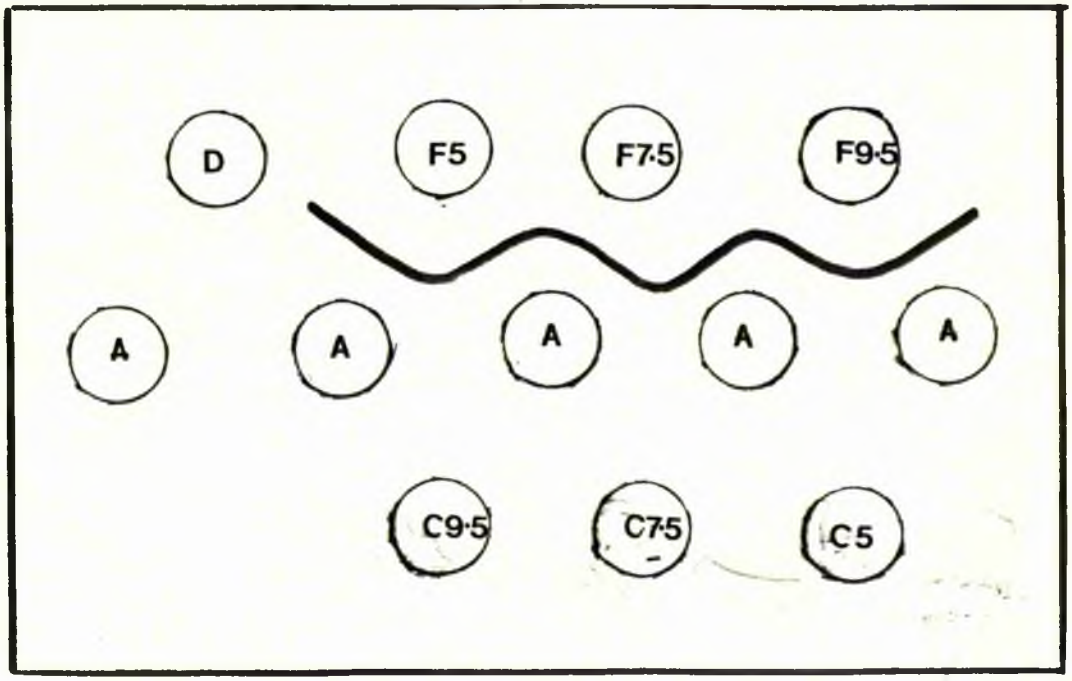

D
F5
F7·5
F9·5
A
A
A
A
A
C9·5
C7·5
C5

Figure 14 Ultraviolet absorption spectrum of formaldehyde-treated δ-haemolysin

The absorption spectrum of δ-haemolysin treated with formaldehyde at pH 5 (F5), pH 7.5 (F7.5) or pH 9.5 (F9.5) for 7 days was measured in the Pye Unicam SP800 spectrophotometer in 1 cm path length silica cells.

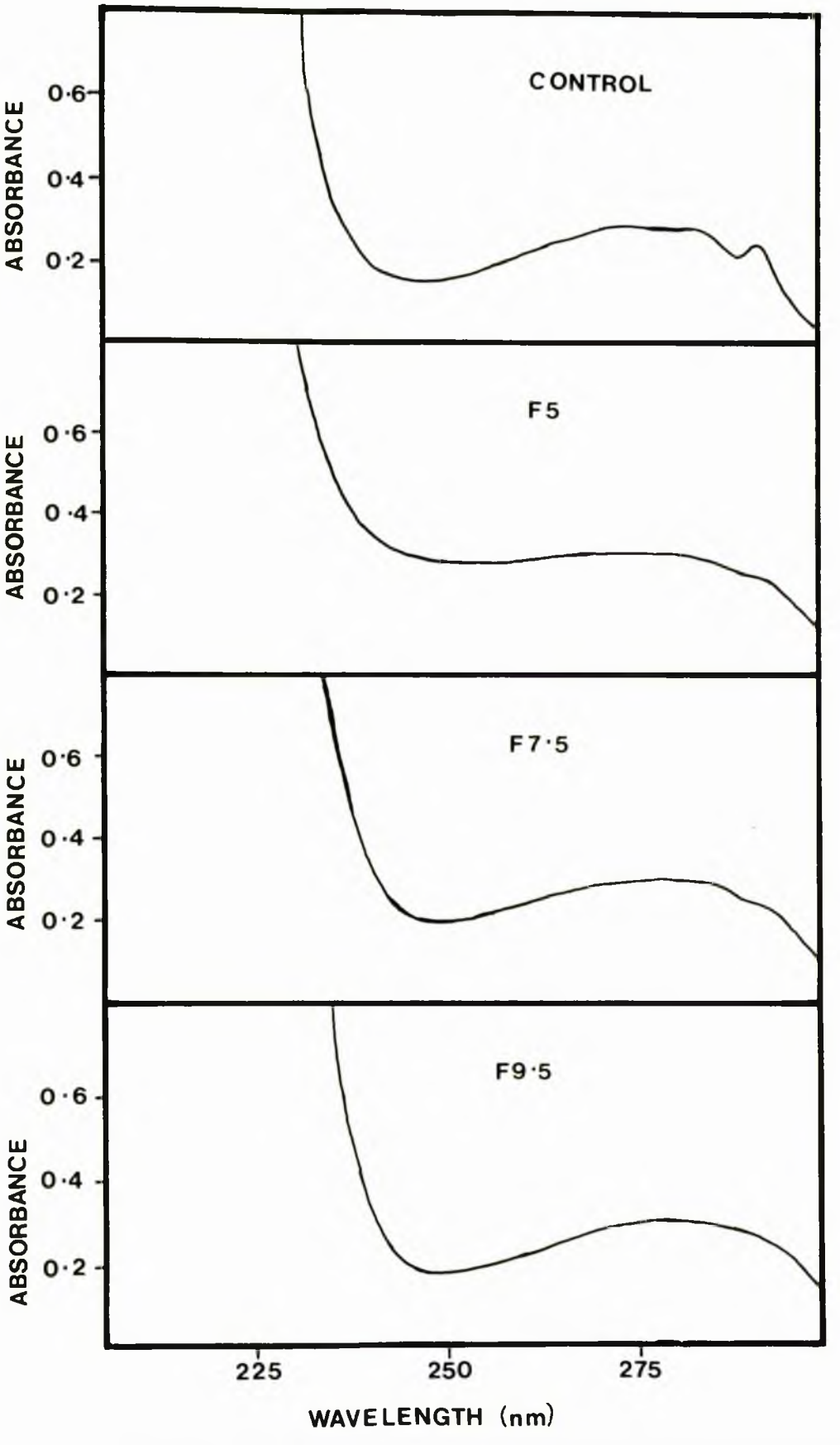

CONTROL
F5
F7·5
F9·5
ABSORBANCE
0·6
0·4
0·2
225
250
275
WAVELENGTH (nm)

Figure 15 Immunoelectrophoresis of formaldehyde-treated δ-haemolysin

F5 - δ-haemolysin treated at pH 5 with formaldehyde

F7.5 - δ-haemolysin treated at pH 7.5 with formaldehyde

F9.5 - δ-haemolysin treated at pH 9.5 with formaldehyde

C5 - δ-haemolysin treated at pH 5 without formaldehyde

C7.5 - δ-haemolysin treated at pH 7.5 without formaldehyde

C9.5 - δ-haemolysin treated at pH 9.5 without formaldehyde

D - δ-haemolysin

All troughs were filled with rabbit anti-δ-haemolysin antiserum.


+
−
F5
F7·5
F9·5
D

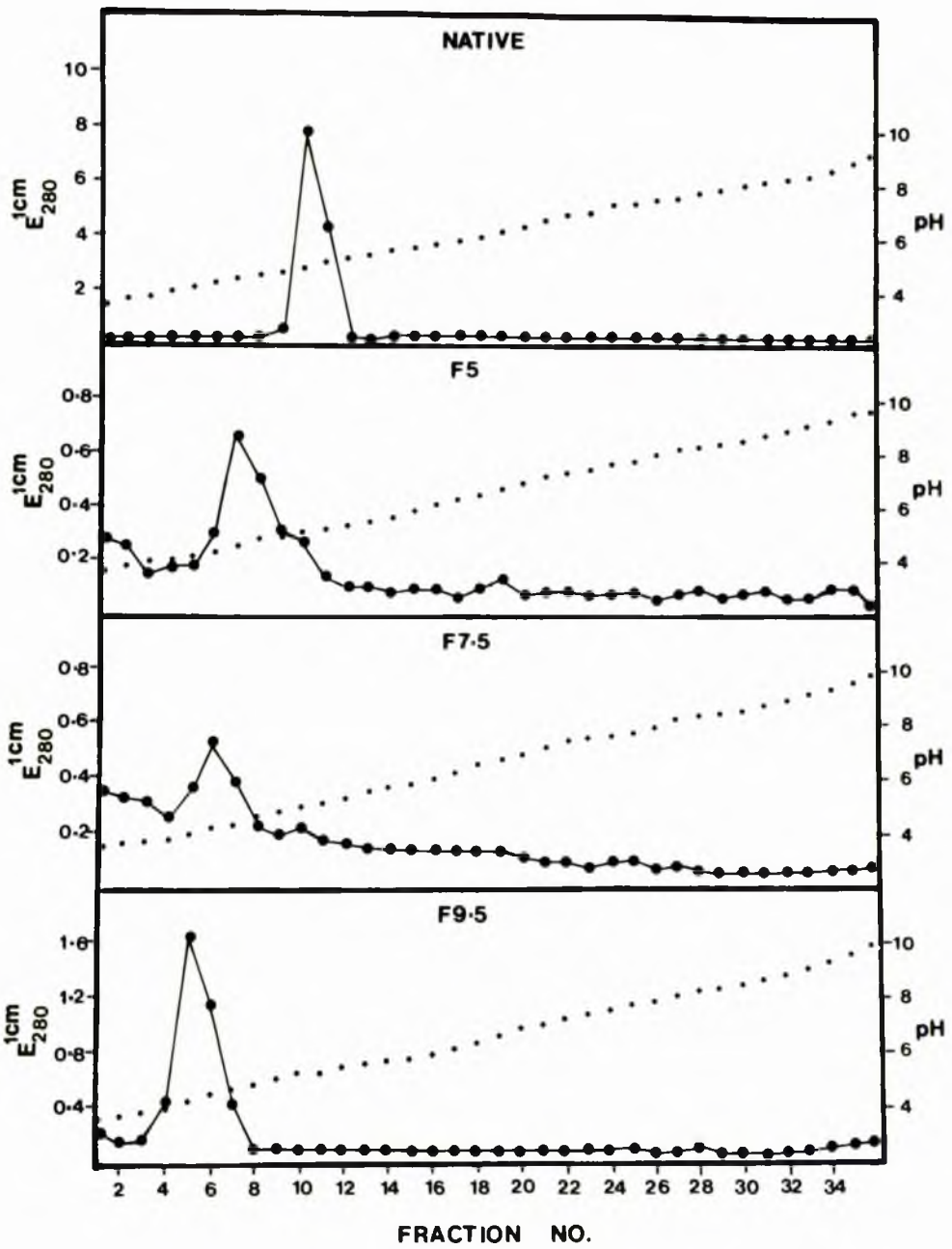

+
−
C5
C7·5
C9·5
D

Figure 16 Isoelectric focusing of formaldehyde-treated δ-haemolysin

Delta-haemolysin treated with formaldehyde at pH 5 (F5), pH 7.5 (F7.5) or pH 9.5 (F9.5) for 7 days was analysed by isoelectric focusing using the apparatus of Talbot and Caie (1975) and the E_{280} and pH of each fraction measured.

●——● - E_{280}

..... - pH


NATIVE
F5
F7·5
F9·5
E^{1cm}_{280}
pH
FRACTION NO.
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

8. Inhibition by lecithin

Since formaldehyde had altered certain physical properties of δ-haemolysin and increased its ability to stimulate the production of anti-δ-haemolysin antibodies, it was of interest to investigate the possibility that formaldehyde may also have altered the ability of δ-haemolysin to bind to phospholipids and erythrocytes. Formaldehyde-treated δ-haemolysin preparations were incubated with liposomes of lecithin at 37°C for 45 min and then tested for antigenic activity by gel diffusion. The antigenic activity of formaldehyde-treated δ-haemolysin, whether under acid, neutral or alkaline conditions was not inhibited by lecithin at a concentration of 1 mg/ml. However, native δ-haemolysin and haemolysin incubated in the absence of formaldehyde showed a much reduced gel diffusion reaction with anti-δ-haemolysin after incubation with lecithin under the same conditions (Fig 17).

9. Adsorption to erythrocytes

Incubation of formaldehyde-treated δ-haemolysin with cod erythrocytes for 30 min at room temperature did not alter its ability to react with anti-δ-haemolysin antibodies. This indicated the inability of the 'toxoids' to adsorb to the surface of the erythrocytes. Native δ-haemolysin and haemolysin incubated in the absence of formaldehyde, had a much reduced reaction with anti-δ-haemolysin antibodies after incubation with cod erythrocytes. Most of these preparations had therefore been adsorbed onto the erythrocyte surface. (Figure not shown since results were similar to those in Figure 17).

10. Tryptic digestion

Formaldehyde-treated δ-haemolysin was resistant to proteolytic digestion by trypsin, its antigenic activity being retained after

Figure 17 Immunodiffusion analysis of native- and formaldehyde-treated δ-haemolysin with anti-δ-haemolysin serum after incubation with lecithin

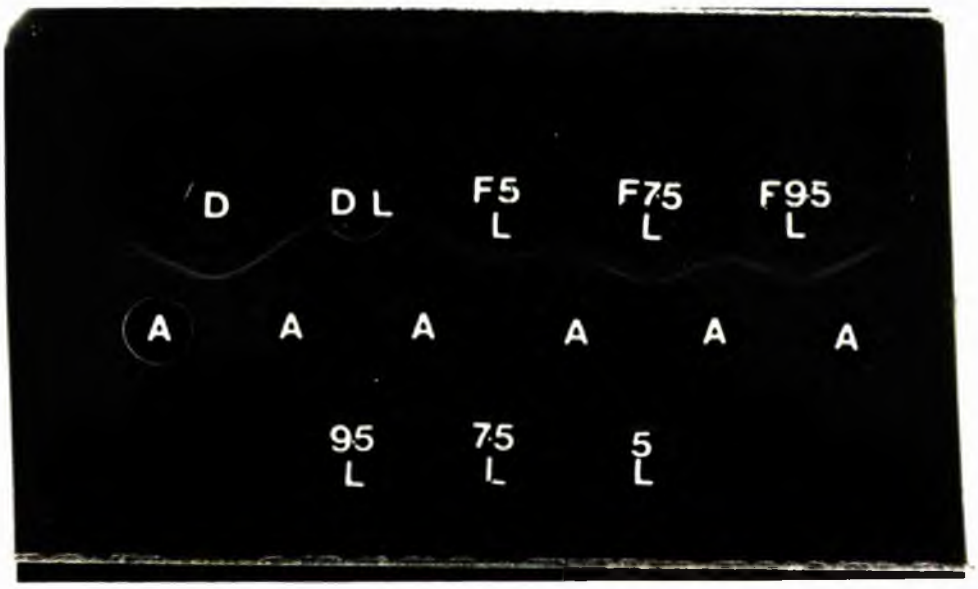
A - rabbit anti-δ-haemolysin antiserum

D - purified δ-haemolysin

DL - δ-haemolysin plus lecithin

F5L, F7.5L, F9.5L - formaldehyde-treated δ-haemolysin at pH 5, pH 7.5, or pH 9.5 plus lecithin

5L, 7.5L, 9.5L - control δ-haemolysin treated at pH 5, pH 7.5, or pH 9.5 plus lecithin.


D
D L
F5 L
F75 L
F95 L
A
A
A
A
A
A
95 L
75 L
5 L

incubation with the enzyme (Fig 18). Native δ-haemolysin and haemolysin incubated at pH 7.5 or pH 9.5 in the absence of formaldehyde lost all antigenic activity after incubation with trypsin. At pH 5, however, δ-haemolysin incubated for 7 days at 37°C remained antigenically active after treatment with trypsin even when formaldehyde was absent during the incubation period.

11. Polyacryalmide gel electrophoresis

Contrary to the work of Warren et al. (1973, 1975) there was no evidence for polymer formation of δ-haemolysin after treatment with formaldehyde at pH 5, pH 7.5 or pH 9.5 (Fig 19). However, problems in staining δ-haemolysin in polyacrylamide gels were encountered, therefore the possibility of the presence of unstained polymers cannot be excluded.

D. Treatment of δ-haemolysin with glutaraldehyde or formaldehyde/amino acid mixtures

Since toxoiding of certain bacterial toxins has been successfully carried out with glutaraldehyde (Relyveld et al., 1973; Rappaport et al., 1974) and also formaldehyde/amino acid mixtures (Linggood et al., 1963; Scheibel and Christensen, 1965; Stanier, 1968; Relyveld, 1969; Northrup and Chisari, 1972; Saletti and Ricci, 1974), δ-haemolysin was treated with these chemicals and comparisons were made between the reaction products and δ-haemolysin treated with formaldehyde alone.

1. Glutaraldehyde

Delta-haemolysin lost all haemolytic activity within 1 h incubation with glutaraldehyde at pH 5, pH 7.5 or pH 9.5. As with formaldehyde treated δ-haemolysin, there was a gradual loss in protein concentration of δ-haemolysin at pH 5 and pH 7.5 but little loss of protein at pH 9.5

Figure 18 Immunodiffusion analysis of native- and formaldehyde-treated δ-haemolysin after tryptic digestion

Wells 1,3,5,7,9,11,13 - samples before treatment with trypsin.

Wells 2,4,6,8,10,12,14 - samples after treatment with trypsin.

Wells 1, 2 - purified δ-haemolysin

3,4 - F5-δ-haemolysin

5,6 - F7.5-δ-haemolysin

7,8 - F9.5-δ-haemolysin

9,10 - C5-δ-haemolysin

11,12 - C7.5-δ-haemolysin

13,14 - C9.5-δ-haemolysin

Centre wells - rabbit anti-δ-haemolysin antiserum.


1 2 3 4 5 6 7 8
9 10 11 12 13 14

Figure 19 Polyacryalmide gel electrophoresis of formaldehyde-treated δ-haemolysin

125 μg of native- and formaldehyde-treated δ-haemolysin were electrophoresed by the method of Laemmli (1970).

Standards (5 μg)

HSA - human serum albumin

OV - ovalbumin

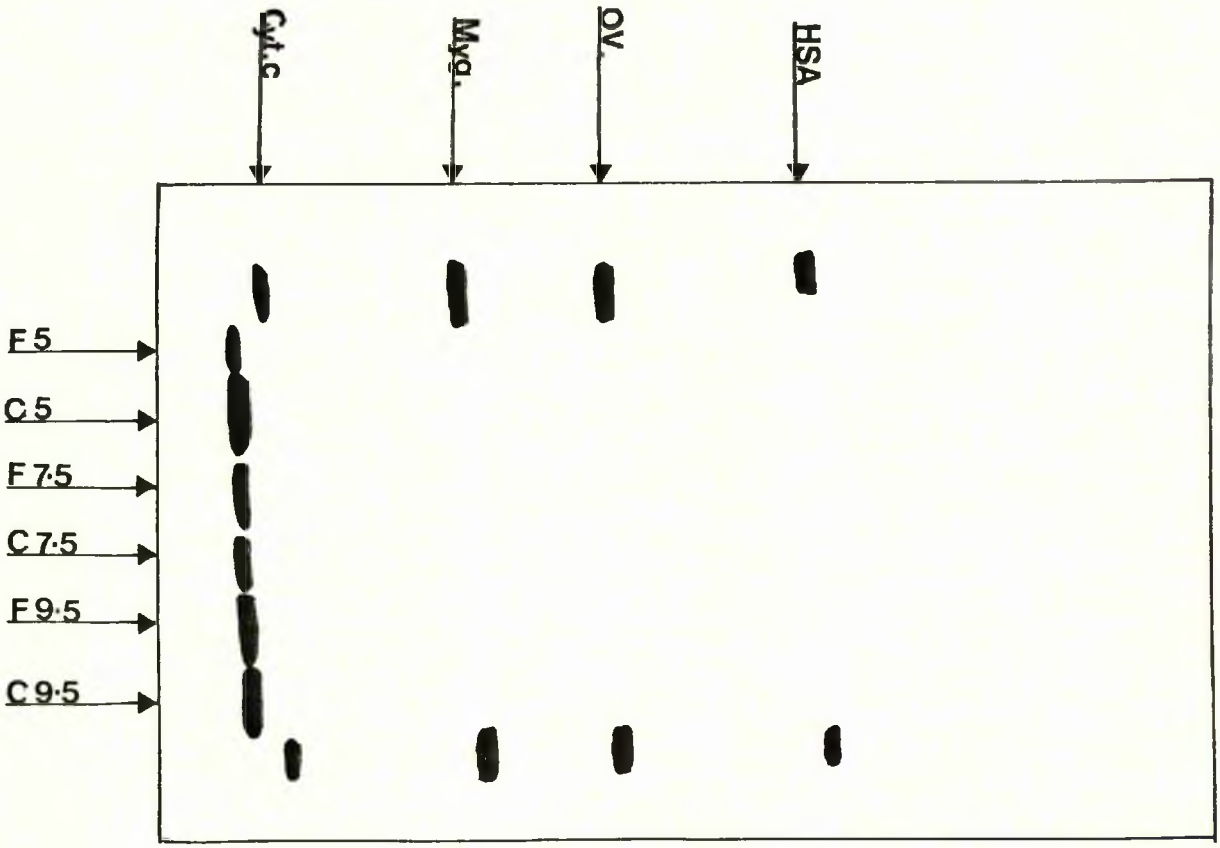
Myg - myoglobin

cytc - cytochrome c.

Test samples

F5 - F7.5 - F9.5 } δ-haemolysin treated with formaldehyde for 7 days at pH 5 (F5), pH 7.5 (F7.5) or pH 9.5 (F9.5).

C5 - C7.5 - C9.5 } δ-haemolysin treated for 7 days in the absence of formaldehyde at pH 5 (C5), pH 7.5 (C7.5) or pH 9.5 (C9.5).


Cyt.c
Myo.
OV.
HSA
F 5
C 5
F 7·5
C 7·5
F 9·5
C 9·5

(Fig 20). The reaction of these preparations with anti-δ-haemolysin antibody on gel diffusion was very weak and, after incubation for 7 days, only the preparation treated at pH 9.5 retained its antigenic activity, giving a spurring reaction with purified δ-haemolysin (Fig 21).

Mice given three primary injections of toxoid in FCA followed by three boosting injections in saline 5 weeks later, failed to stimulate the production of anti-δ-haemolysin antibodies.

2. Formaldehyde/amino acid mixtures

Incubation of δ-haemolysin for 7 days with formaldehyde plus one of three amino acids (tyrosine, glycine or leucine) produced a loss of haemolytic activity when reacted at pH 5 or pH 7.5 (Table 20). Similar results were obtained under alkaline conditions although, in the presence of tyrosine, some haemolytic activity was retained. All preparations retained their antigenic activity, and, when mice were immunised with these 'toxoids', only samples incubated at pH 5 or pH 7.5 stimulated the production of anti-δ-haemolysin antibodies.

E. Enhancement of Vascular Permeability in rabbit skin by δ-haemolysin

Enhanced vascular permeability (EVP) in the skin of guinea pigs has previously been shown (O'Brien and Kapral, 1976). The EVP activity of purified δ-haemolysin was studied in the rabbit and the effect of various treatments on this EVP activity was investigated.

Preliminary studies in mice had to be abandoned due to difficulties in measurement of zones, inadequate numbers of injection sites per animal and general inconvenience.

Figure 20 Lowry reactivity of δ-haemolysin treated with glutaraldehyde

Delta-haemolysin, incubated at 37°C with glutaraldehyde at pH 5, pH 7.5 or pH 9.5 was assayed for protein concentration by the method of Lowry *et al.* (1951).

●——● - δ-haemolysin treated at pH 5 with glutaraldehyde

■——■ - δ-haemolysin treated at pH 7.5 with glutaraldehyde

▲——▲ - δ-haemolysin treated at pH 9.5 with glutaraldehyde.

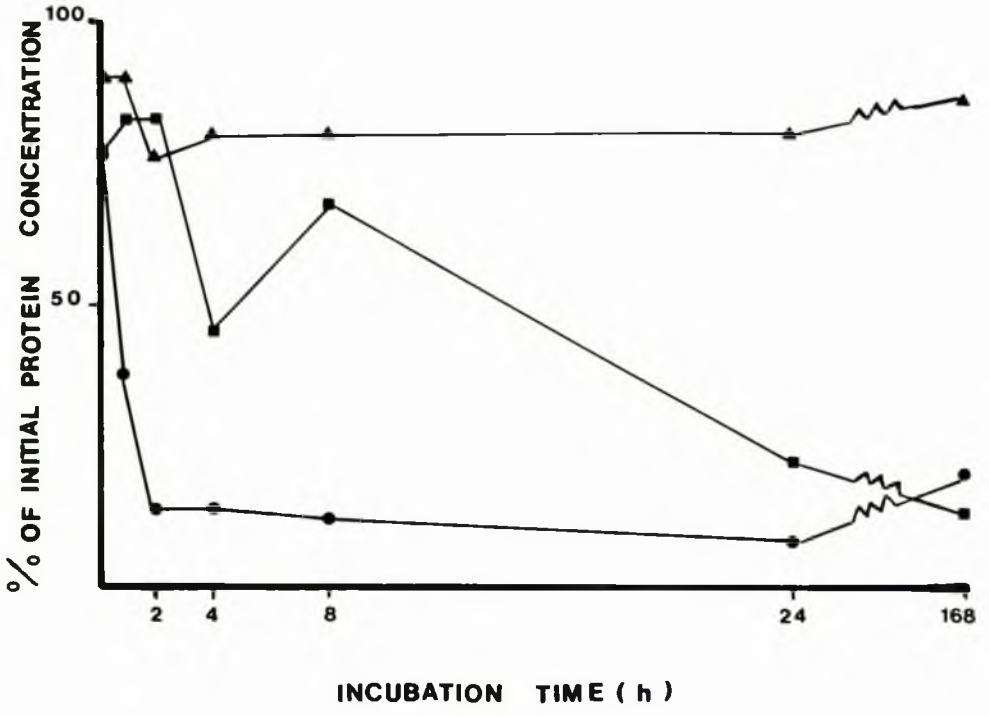

100
50
% OF INITIAL PROTEIN CONCENTRATION
2
4
8
24
168
INCUBATION TIME (h)

Figure 21 Immunodiffusion analysis of δ-haemolysin treated with glutaraldehyde for 7 days

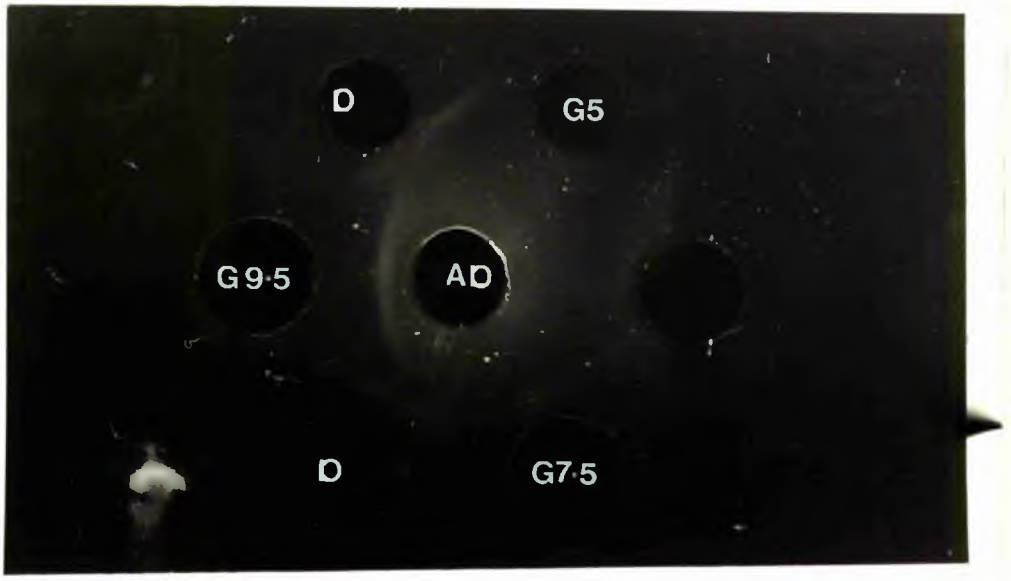
D - purified δ-haemolysin (62.5 μg/ml)

G5 - δ-haemolysin treated at pH 5 with glutaraldehyde

G7.5 - δ-haemolysin treated at pH 7.5 with glutaraldehyde

G9.5 - δ-haemolysin treated at pH 9.5 with glutaraldehyde

AD - rabbit anti-δ-haemolysin antiserum.


D
G5
G9·5
AD
D
G7·5

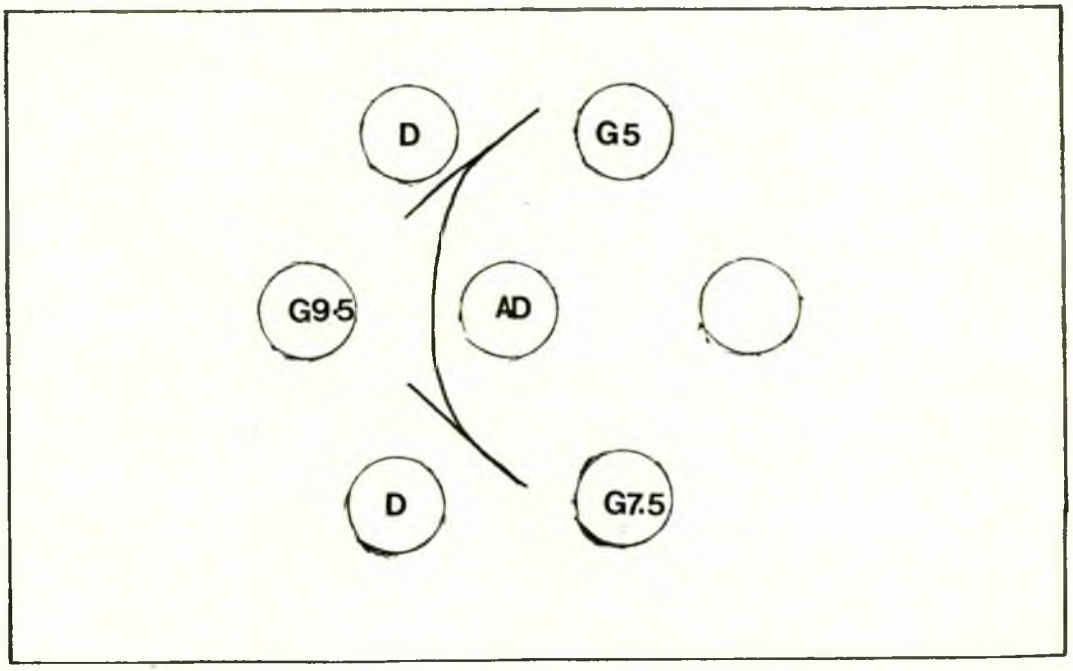

D
G5
G9·5
AD
D
G7.5

Table 20 Properties of δ-haemolysin treated with formaldehyde/amino acid mixtures for 7 days

Amino Acid	pH	Haemolytic activity (% of original preparation)	Antigenic** Activity	Immunogenic* Activity
Tyrosine	5	< 0.1	+	+
	7.5	< 0.1	+	+
	9.5	3	+	-
Glycine	5	< 0.1	+	+
	7.5	< 0.1	+	+
	9.5	< 0.1	+	-
Leucine	5	< 0.1	+	+
	7.5	< 0.1	+	-
	9.5	< 0.1	+	-

** Determined by counter immunoelectrophoresis

+ = precipitin line

- = no precipitin line

* Mice were immunised on days 1,3,5, with preparations in complete Freund's Adjuvant followed by three boosting injections (days 36, 38, 40) in saline. Sera were tested by counter immuno-electrophoresis.

1. Time of development of zones of enhanced vascular permeability

To determine the time required for maximum skin response, δ-haemolysin was injected at various times and dye was injected 30 min after the last injection of δ-haemolysin. A maximum skin response occurred within 1 h, the skin reaction fading with increased time until 6 h or later when no dye was detectable and only hard erythematous lesions were present (Fig 22). The size of these swollen areas varied with the concentration of δ-haemolysin injected. When the experiment was repeated, virtually identical results were obtained. These results differ from the findings of O'Brien and Kapral (1976) who obtained maximum skin responses after 6 h. However, their experimental animal was the guinea pig and not the rabbit.

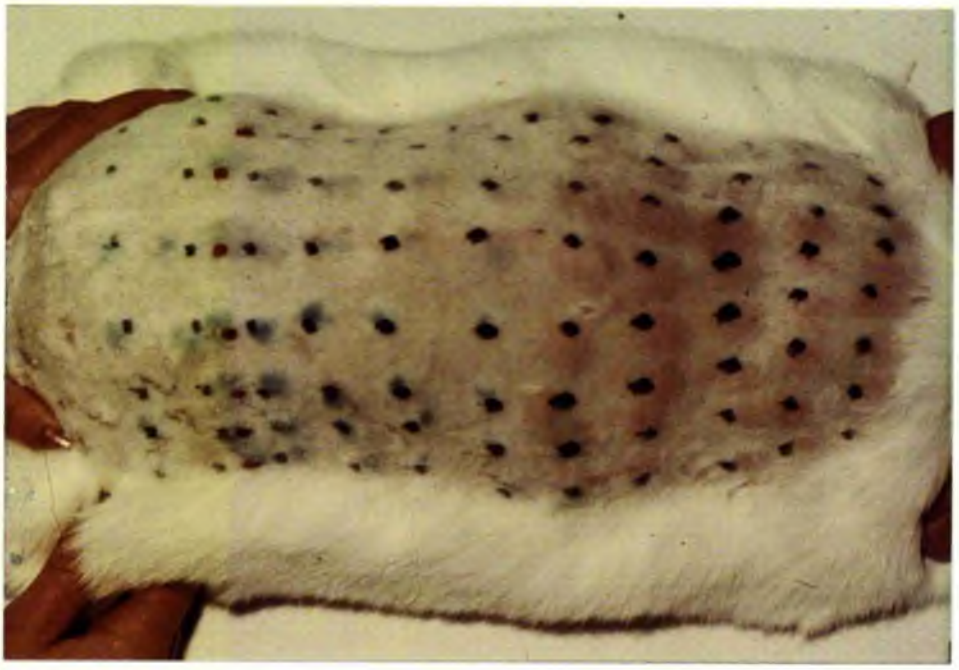
2. Dose response

Various concentrations of δ-haemolysin were injected intradermally and dye was administered 1 h later. The dose response was carried out in fifteen rabbits and, in all cases, high concentrations of δ-haemolysin (62.5 μg-1000 μg) were required to obtain a measurable result (Fig 23). In addition to animal-to-animal variation, the size of the resulting zones also varied depending upon the location of the injection site. This was investigated by injecting three rabbits with varying doses of δ-haemolysin at eight random sites per dose. The results obtained in a single rabbit are shown in Table 21 and are typical of those found in other rabbits. Figure 24 shows the range of variability found between rabbits, those giving high (R1) and those giving lower (R2) dose responses. Although different preparations of δ-haemolysin were used in R1 (78/2) and R2 (78/1), the variabilities obtained cannot be attributed to the preparations themselves since the dose responses of nine rabbits all

Figure 22 Enhanced Vascular Permeability in the Rabbit - timing of injection of dye

The rabbit was injected intradermally with varying doses of δ-haemolysin at various times (30 min to 24 h) and Pontamine Sky Blue dye was injected intravenously 30 min after the final injection. The resulting zones measured 1 h later.

Injection sites are indicated by black or red dots.



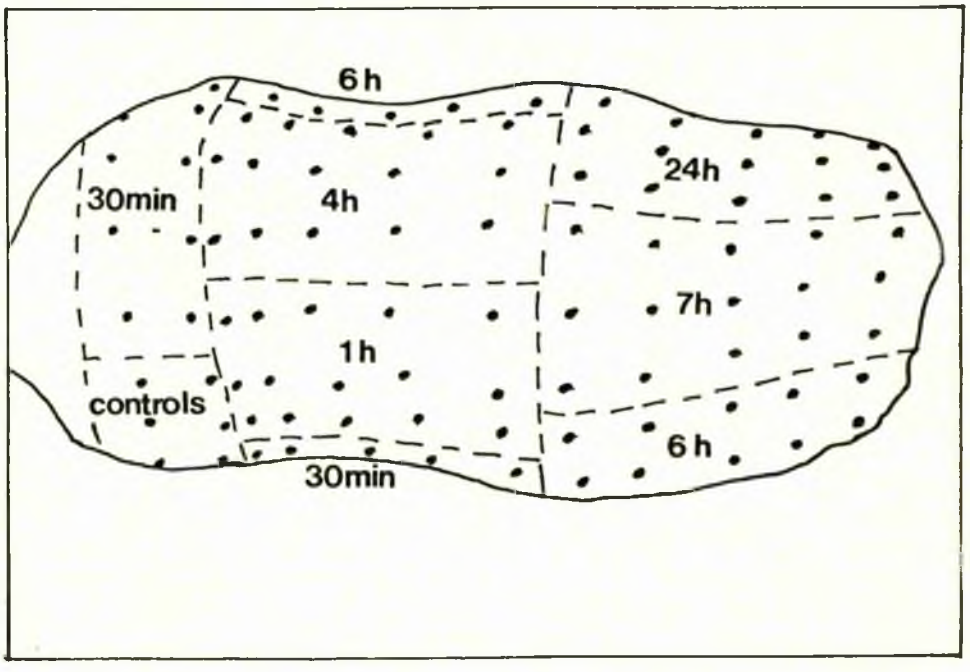

6 h
30min
4h
24h
7h
1h
controls
6 h
30min

Figure 23 Enhanced Vascular Permeability in the Rabbit - dose response

The rabbit was injected intradermally with 1000 (1), 500 (2), 250 (3), 125 (4) or 62.5 (5) μg δ-haemolysin. After 1 h, Pontamine Sky Blue dye was injected intravenously and the resulting blue zones measured 1 h later.

Injection sites are indicated by black dots.

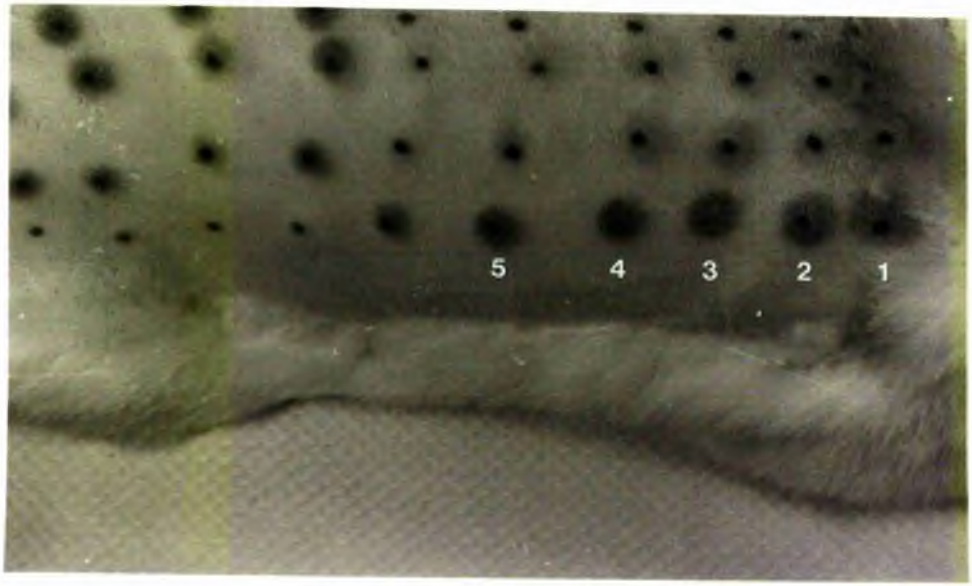
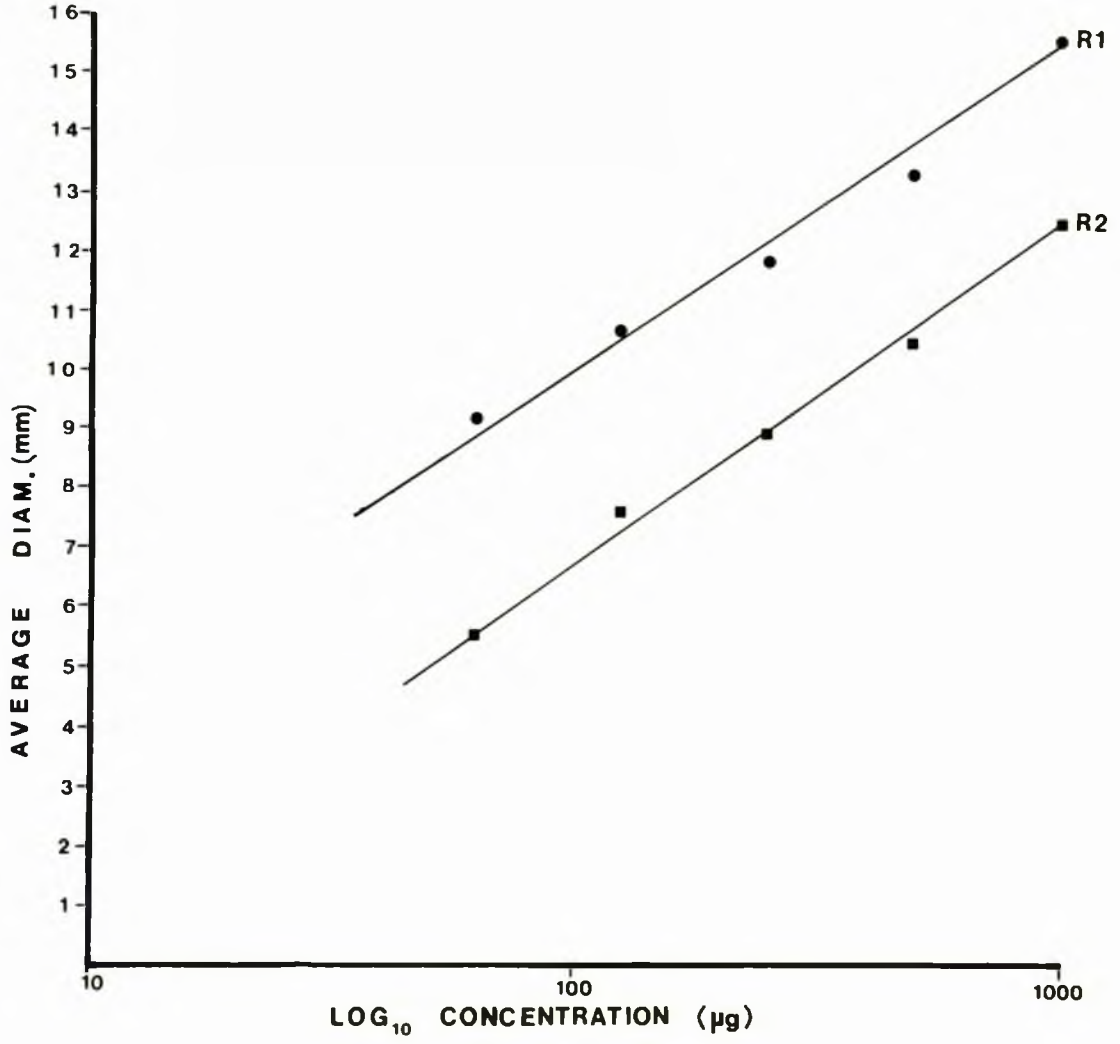

5
4
3
2
1

Table 21. Site-to-site variation in the enhanced vascular permeability activity of δ-haemolysin in rabbit skin

Concentration of δ-haemolysin (μg)	Individual Zone diameters (mm)	Average zone diameter (mm)
1000	17.5 ; 14.1 ; 14.4 ; 17.4 ; 16.2 ; 15.8 ; 13.7 ; 14.2	15.4
500	14.5 ; 11.9 ; 12.9 ; 15.0 ; 11.6 ; 13.4 ; 12.7 ; 12.8	13.1
250	10.9 ; 8.3 ; 11.9 ; 11.9 ; 11.3 ; 10.5 ; 11.5 ; 11.6	10.9
125	7.3 ; 7.5 ; 8.9 ; 9.9 ; 8.3 ; 7.3 ; 8.9 ; 9.2	8.4
62.5	5.5 ; 5.3 ; 6.9 ; 7.1 ; 6.1 ; 6.7 ; 7.7 ; 5.8	6.3

Figure 24 Animal-to-animal variation in the enhanced vascular permeability activity of δ-haemolysin in rabbit skin

For clarity only the highest (•——•) and lowest (■——■) responses obtained in the nine rabbits tested are shown. In all other cases a straight line dose response was obtained which fell between the limits shown.


16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
AVERAGE DIAM. (mm)
R1
R2
10
100
1000
LOG10 CONCENTRATION (µg)

fell within this range and were independent of the preparation used.

Despite these variations, a linear response was obtained in all cases. Therefore, to investigate the effect of various treatments on the EVP activity of δ-haemolysin, each test sample was administered in triplicate at random sites and the results expressed in μg equivalents of the dose response on each individual rabbit. In this way, the results obtained in different rabbits could be compared.

3. Effect of various treatments on the ability of δ-haemolysin to enhance vascular permeability

(i) Heat: Delta-haemolysin retained a substantial amount of EVP activity after incubation at 100°C (Fig 25). After 5 h at 100°C, 30% of EVP activity was retained compared with 6% of haemolytic activity. While longer incubation periods resulted in the complete loss of haemolytic activity, the EVP activity of δ-haemolysin was greatly increased. It must be noted that the antigenic activity of δ-haemolysin was retained after 10 h incubation at 100°C (Table 22).

(ii) Lecithin: The inhibitory effect of lecithin on the haemolytic activity of δ-haemolysin has been well documented (Kreger et al., 1971; Kapral, 1972; Whitelaw and Birkbeck, 1978). The effect of lecithin on the EVP activity of δ-haemolysin was therefore investigated. Incubation of δ-haemolysin with lecithin, at a concentration of 1.25 mg lecithin per mg of haemolysin, abolished haemolytic and antigenic activities but 61% of EVP activity was retained (Table 22). At lower lecithin concentrations (125 μg/mg haemolysin), haemolytic activity was reduced, antigenic activity was retained and 80% of EVP activity remained.

(iii) Trypsin: The EVP activity of δ-haemolysin was greatly increased after incubation with trypsin (100 μg/mg haemolysin) while the haemolytic

Figure 25 Effect of heat on the haemolytic and enhanced vascular permeability activities of δ-haemolysin

Purified δ-haemolysin was heated at 100°C for 1-10 h and assayed for haemolytic activity (1% cod erythrocytes) and EVP activity in rabbit skin.

●——● - EVP activity

■——■ - Haemolytic activity

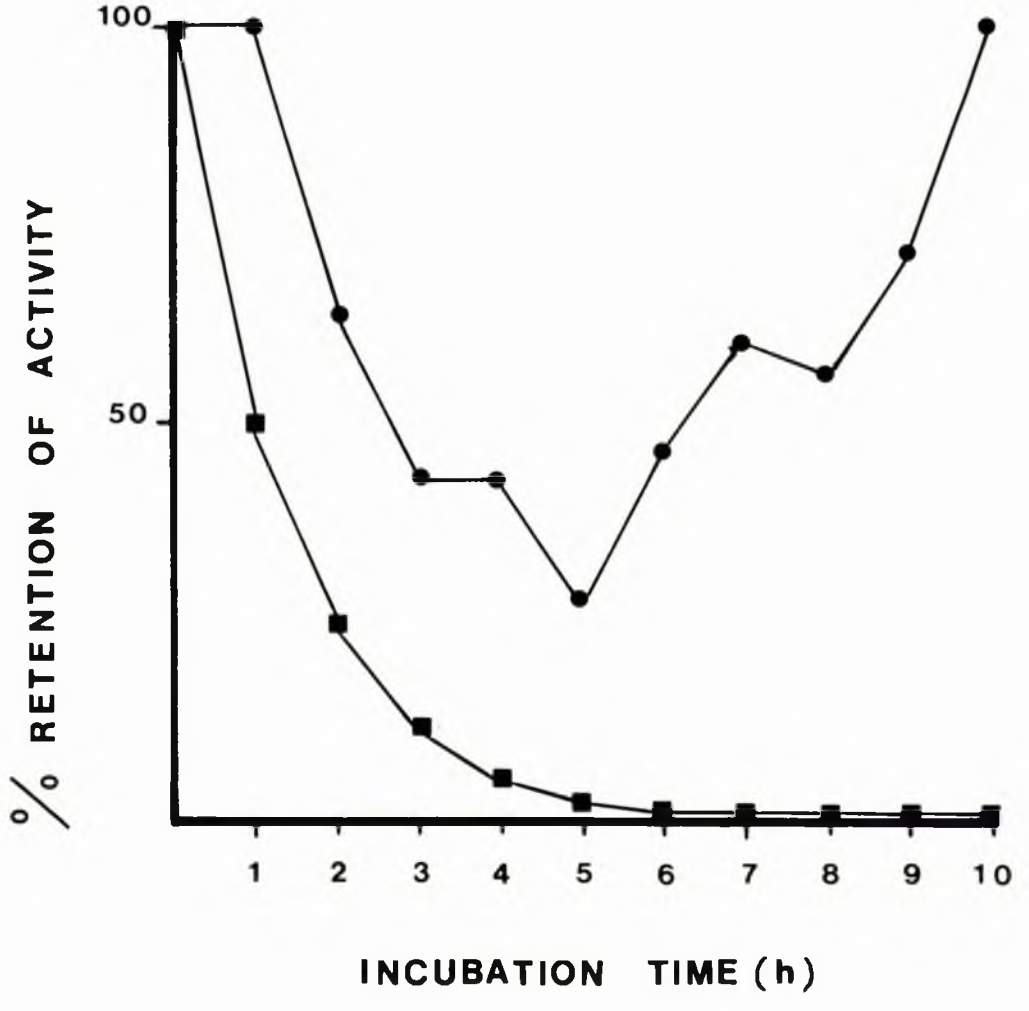

100
50
% RETENTION OF ACTIVITY
1
2
3
4
5
6
7
8
9
10
INCUBATION TIME (h)

Table 22 Effect of various treatments on the haemolytic, antigenic and enhanced vascular permeability activities of δ-haemolysin

Treatment	% activity retained		Antigenic*
	Haemolytic	EVP	activity
Heating at 100°C for 10 h	< 0.1	100	+
Lecithin			
1.25 mg/mg haemolysin	< 0.1	61	-
125 µg/mg haemolysin	50	80	+
Trypsin			
100 µg/mg haemolysin	< 0.1	244	-
Anti-δ-haemolysin antibody			
Neat/200 µg δ-haemolysin	100	65	+
Neat/100 µg δ-haemolysin	100	85	+
Neat/50 µg δ-haemolysin	100	126	+
Formaldehyde/7 days at 37°C			
pH 5	< 0.1	52	+
pH 7.5	< 0.1	58	+
pH 9.5	3	69	+

* + = reacts with rabbit anti-δ-haemolysin antibodies

\- = no reaction with rabbit anti-δ-haemolysin antibodies.

and antigenic activities of the haemolysin were destroyed (Table 22). These results suggested that tryptic fragments of δ-haemolysin were sufficient to induce EVP activity in the rabbit. Further investigations into the tryptic digestion of δ-haemolysin were therefore examined with a 15 mg/ml solution of the haemolysin. The tryptic peptides were separated by molecular exclusion chromatography and each fraction was analysed for haemolytic activity, antigenic activity by RIE and EVP activity in the rabbit.

Six tryptic peptides (D_1 to D_6), absorbing at $E_{230\ nm}$, were consistently isolated, only four of which (D_1, D_2, D_5, D_6) absorbed at $E_{280\ nm}$ (Fig 26). Peptides of high molecular weight (D_1) contained all haemolytic and antigenic activities and corresponded to either free or partially digested δ-haemolysin since untreated δ-haemolysin was eluted in a single peak at the same point as fraction D_1. Similarly, free tryptophan could be eluted in fractions corresponding to D_6 peptides. The EVP activity of these tryptic fractions was shown in two experiments to be associated with a number of peptides which lacked both haemolytic and antigenic activities and which correlated well with the fragments absorbing at $E_{230\ nm}$ and $E_{280\ nm}$ (D_2, D_3, D_4, D_5). Buffer containing 10% Tween failed to induce an EVP response in rabbits.

(iv) Formaldehyde: Treatment of δ-haemolysin with formaldehyde reduced, but did not eliminate, all EVP activity of the haemolysin (Table 23). Even after incubation for 7 days, 52%, 58% and 69% of EVP activity was retained when δ-haemolysin was treated with formaldehyde at pH 5, pH 7.5 or pH 9.5 respectively (Table 22).

(v) Neutralisation by anti-δ-haemolysin antibody: Complete neutralisation of EVP activity was not found after incubation of various

Figure 26 Tryptic Digestion of purified δ-haemolysin

Delta-haemolysin (15 mg/ml) was incubated overnight at 37°C with trypsin and peptides separated on Sephadex G25 column.

●——● - E_{230}

○——○ - E_{280}

□——□ - Haemolytic activity (1% cod erythrocytes)

■——■ - Antigenic activity (by RIE)

▲——▲ - EVP activity in rabbit skin

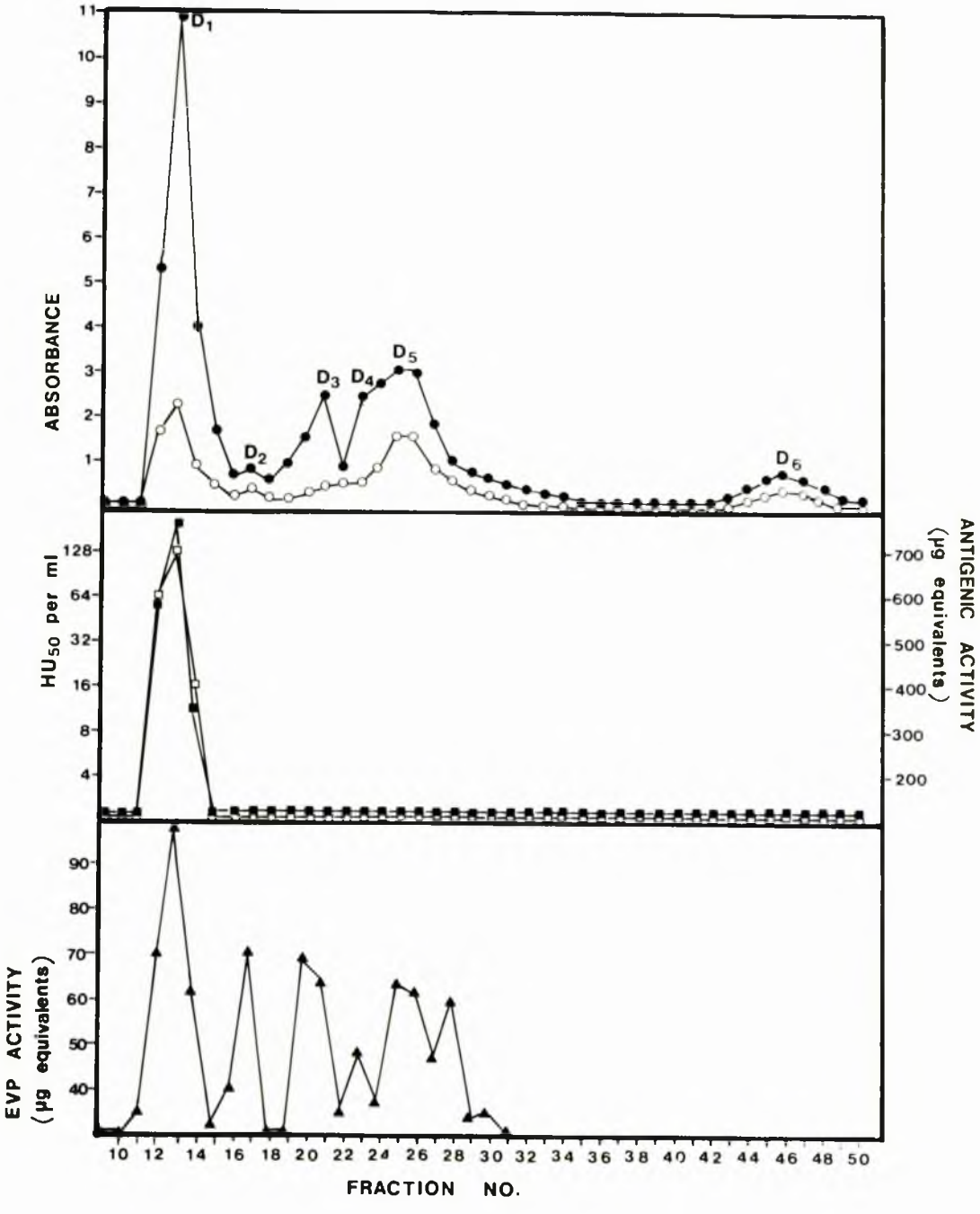

ABSORBANCE
D1
D2
D3
D4
D5
D6
HU50 per ml
ANTIGENIC ACTIVITY (µg equivalents)
EVP ACTIVITY (µg equivalents)
FRACTION NO.

Table 23 Enhanced Vascular Permeability in three different rabbits after Injection of Formaldehyde-treated δ-haemolysin

pH*	Incubation* time	Protein injected (μg)	EVP activity (μg equivalents) **R1	R2	R3	Average	% Retention of Activity†
5	0 h	150	92	74	90	85	57
	1 h	122	80	56	86	74	61
	2 h	116	66	42	94	67	58
	4 h	92	37	39	72	49	53
	8 h	58	-·	14	48	31	53
	24 h	58	-·	14	50	32	55
	7 days	58	-·	30	-	30	52
7.5	0 h	150	32	48	62	47	31
	1 h	138	54	19	50	41	30
	2 h	132	68	17	58	48	36
	4 h	128	72	48	60	60	47
	8 h	116	68	39	48	52	45
	24 h	104	72	20	56	49	47
	7 days	980	74	40	58	57	58
9.5	0 h	150	66	37	50	51	34
	1 h	150	84	49	50	61	41
	2 h	144	88	29	48	68	47
	4 h	138	96	19	50	73	53
	8 h	128	76	58	54	63	49
	24 h	144	96	90	100	95	66
	7 days	132	100	98	74	91	69

* Time and pH of incubation of δ-haemolysin with HCHO.

** Average results from each rabbit.

† Percentages calculated from concentration of protein injected.

· Zones of blue present but difficult to read.

concentrations (200, 100 or 50 μg) of δ-haemolysin with a gamma globulin concentrate of rabbit anti-δ-haemolysin (Table 22). There was, however, a decrease in EVP activity but this was associated with increasing concentrations of δ-haemolysin. Haemolytic and antigenic activities of δ-haemolysin were both retained. It is therefore probable that, due to the high concentrations of δ-haemolysin required to cause EVP, there was not sufficient antibody present to completely neutralise the haemolytic, antigenic and EVP activities of the haemolysin.

(vi) Serum proteins: To investigate the effect of other proteins on increasing the vascular permeability of rabbits, BSA and HSA (100 μg) were injected into rabbit skin. There was no visible blue zoning at injection sites indicating that neither of these proteins had the ability to cause EVP activity in the rabbit.

F. Comparison of δ-haemolysin from different strains of S. aureus

The aim of this section of the present study was the selection of different strains of S. aureus which produced a δ-haemolysin which differed in haemolytic spectrum and/or antigenic reactivities. Having selected various strains on this basis, various other biological properties were studied and compared with that of strain 10345.

1. Growth conditions

Several strains of S. aureus were grown in Bernheimer's and/or CCY medium in approx. 50 ml volumes. Each culture supernate was collected after centrigugation and tested for haemolytic activity against cod, sheep, horse, human and rabbit erythrocytes (Tables 24, 25).

With the exception of five strains of S. aureus, all crude culture supernates showed haemolytic activity against at least three

Table 24 Haemolytic spectrum of crude culture supernates from various strains of S. aureus grown in Bernheimer's yeast extract and CCY media

Strain	Haemolysin titre (HU_{50}/ml) with the following erythrocytes									
	cod		sheep*		rabbit		horse		human	
	B	C	B	C	B	C	B	C	B	C
10345	512	256	8	< 2	64	32	128	128	256	16
SM14	1024	-	8	-	512	-	64	-	512	-
JK128	256	64	< 2	16	32	32	16	16	64	4
JK132	64	32	< 2	4	4	4	4	< 2	16	< 2
JK139	16	64	< 2	< 2	< 2	4	< 2	2	2	< 2
Wood 46	512	-	2	-	≥4096	-	64	-	512	-
70531	512	512	< 2	16	256	256	64	128	256	128
70554	256	64	4	8	128	16	64	8	128	16
70544	512	64	8	4	512	32	128	16	256	16
70645	32	32	< 2	< 2	16	16	16	16	16	4
70671/L	64	256	< 2	16	8	256	8	32	8	64
ZM	≥ 1024	512	4	16	≥1024	128	128	16	512	16
69931	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2

- - not done
B - grown in Bernheimer's yeast extract medium
C - grown in CCY medium
* - the recorded titre with sheep erythrocytes is the difference in titre obtained after incubation at 37°C followed by incubation at 4°C.

Table 25 Haemolytic spectrum of crude culture supernates from various strains of *S. aureus* of human origin grown in Bernheimer's yeast extract medium

Strain	Haemolysin titre (HU_{50}/ml) with the following erythrocytes: cod	sheep*	rabbit	horse	human
LS1	512	2	≥ 1024	64	256
2	2048	2	≥ 1024	128	256
3	512	< 2	32	16	64
4	2048	< 2	≥ 1024	32	32
5	256	< 2	64	16	16
6	2048	2	≥ 1024	128	256
7	512	< 2	16	8	8
8	2048	2	≥ 1024	64	128
9	2048	4	≥ 1024	128	256
10	< 2	< 2	< 2	< 2	< 2
11	128	2	256	32	128
12	2048	< 2	64	16	32
13	< 2	< 2	< 2	< 2	< 2
14	2048	2	512	64	128
15	2048	2	≥ 1024	64	64
16	2048	16	≥ 1024	64	128
17	512	8	256	32	64
18	512	16	≥ 1024	64	128
19	64	< 2	128	16	16
20	512	< 2	64	16	16
21	2048	4	≥ 1024	64	128
22	256	< 2	32	16	32
23	< 2	< 2	< 2	< 2	< 2
24	2048	8	≥ 1024	32	64
25	2048	2	≥ 1024	256	512
26	64	< 2	512	< 2	< 2
27	256	< 2	32	16	16
28	2048	< 2	≥ 1024	16	16
29	512	< 2	≥ 1024	128	128
30	32	< 2	16	4	< 2
31	2048	4	512	128	256
32	2048	2	512	256	512
33	< 2	< 2	< 2	< 2	< 2

* The recorded titre with sheep erythrocytes is the difference in titre obtained after incubation at 37°C followed by incubation at 4°C.

erythrocyte types. In most cases, incubation with sheep erythrocytes was not associated with any significant difference in titre obtained after incubation at 37°C followed by incubation at 4°C. These results indicate that very little (if any) β-haemolysin was produced under these growth conditions. High titres against rabbit erythrocytes showed high concentrations of α-haemolysin. Activity against cod, human and horse erythrocytes was variable but, in most strains of human origin, a high titre against cod erythrocytes was found, indicating high concentrations of δ-haemolysin were produced.

Only the canine strain 70671/L showed any increase in haemolysin production when grown in CCY medium as opposed to Bernheimer's medium (Table 24).

2. Antigenic activity

The crude culture supernates were tested for antigenic reactivity with rabbit anti-δ-haemolysin antiserum. Except for supernates with no haemolytic activity and that of strain LS26, a line of complete identity was shown between each crude supernate and purified (10345) δ-haemolysin with anti-δ-haemolysin antiserum (Fig 27). However a "split" precipitin line was found when δ-haemolysin (10345) was compared with the crude supernates of certain canine strains (70554, 70544 - grown in Bernheimer's yeast extract medium - and 70671/L - grown in CCY medium) (Fig 28). When the anti-δ-haemolysin serum was absorbed with either purified δ-haemolysin or crude 70544 culture supernate, neither purified δ-haemolysin nor crude supernates from these canine strains gave a precipitin line with the antiserum (Fig 28). These results therefore indicated that, despite the "split" line on immunodiffusion, these canine strains were antigenically identical to purified (10345) δ-haemolysin.

Only strain LS26 gave an atypical reaction with anti-δ-haemolysin antibody (Fig 27, Slide C, well 12). Purified δ-haemolysin gave a line

Figure 27 The Antigenicity of crude culture supernates

Centre Wells - Rabbit anti-δ-haemolysin antiserum

Wells 1,3,5,7,9,11,13,15,17,19 - purified δ-haemolysin

Wells 2,4,6,8,10,12,14,16,18,20 - A, LS1-10 respectively

B, LS11-20 respectively

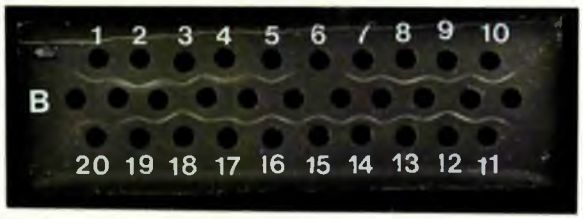
C, LS21-30 respectively

D, 10345(4), SM14 (6)

JK128 (8), JK132(10)

JK139(12), Wood 46(14).


1 2 3 4 5 6 7 8 9 10
A
20 19 18 17 16 15 14 13 12 11


1 2 3 4 5 6 7 8 9 10
B
20 19 18 17 16 15 14 13 12 11


1 2 3 4 5 6 7 8 9 10
C
20 19 18 17 16 15 14 13 12 11


1 2 3 4 5 6 7 8 9 10
D
20 19 18 17 16 15 14 13 12 11

Figure 28 The antigenic reactivity of crude culture supernates from S. aureus of canine origin

Wells 1,3,5 - purified δ-haemolysin

Wells 2 - 70544

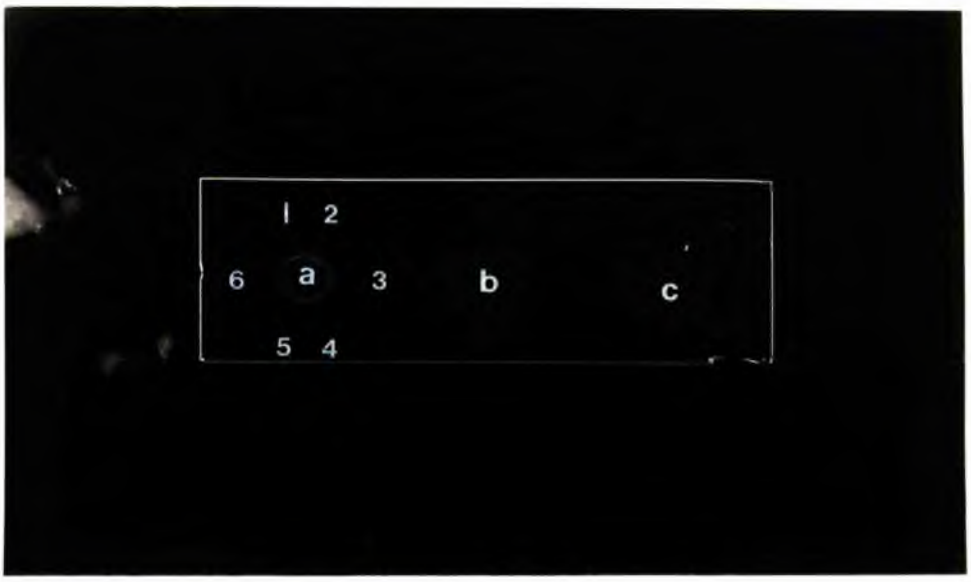
4 - 70554

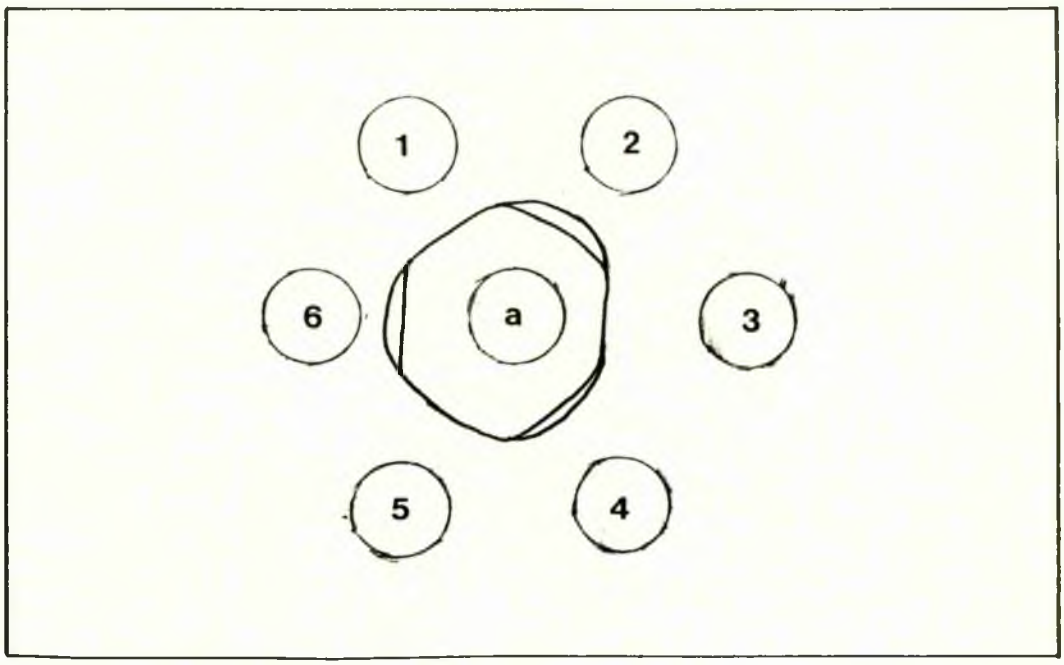
6 - 70671/L

Centre wells a - Rabbit anti-δ-haemolysin antiserum

b - as for (a) and absorbed with purified δ-haemolysin

c - as for (a) and absorbed with 70544 crude culture supernate.


1 2
6 a 3 b c
5 4


1
2
6
a
3
5
4

of partial identity with the crude culture supernate of this strain. When anti-δ-haemolysin antiserum was absorbed with purified δ-haemolysin, the homologous antibody still reacted with the crude supernate (Fig 29). Anti-δ-haemolysin antisera produced in four different rabbits also precipitated with LS26 crude culture supernate on gel diffusion. The γ-globulin fraction of one of these rabbit anti-δ-haemolysin antisera also reacted with crude LS26, whereas other serum fractions failed to react with the crude supernate. Sera from ten mice and 1 rabbit not previously immunised with δ-haemolysin did not precipitate with the crude culture supernate of LS26 on gel diffusion. Only one electrophoretic form was found by immunoelectrophoresis (Fig 30).

R_e-cultivation of this organism in Bernheimer's and CCY media yielded high concentrations of δ-haemolysin. Unfortunately, the δ-haemolysin produced did not react in a similar way with anti-δ-haemolysin antisera - a line of complete identity with purified δ-haemolysin was obtained.

3. Immunoelectrophoresis

Several crude culture supernates were selected for further study based on their cod:human haemolytic titre ratios:- high, low or intermediate. Seven of the fifteen strains tested had a greater electrophoretic mobility than purified δ-haemolysin (Fig 31).

4. Isoelectric focusing

Isoelectric focusing in the Talbot and Caie apparatus, as well as in polyacrylamide gels, was carried out on four selected crude culture supernates. The choice of these supernates was based on their haemolytic spectrum, electrophoretic mobilities and the animal from which each

Figure 29 Reactivity of crude culture supernates on gel diffusion with anti-δ-haemolysin antiserum absorbed with purified δ-haemolysin

Wells 1,3,5 - purified δ-haemolysin

2 - 70554

4 - LS26

6 - 10345

Centre Wells - A - rabbit anti-δ-haemolysin antiserum

B - as for (a) and absorbed with purified δ-haemolysin.

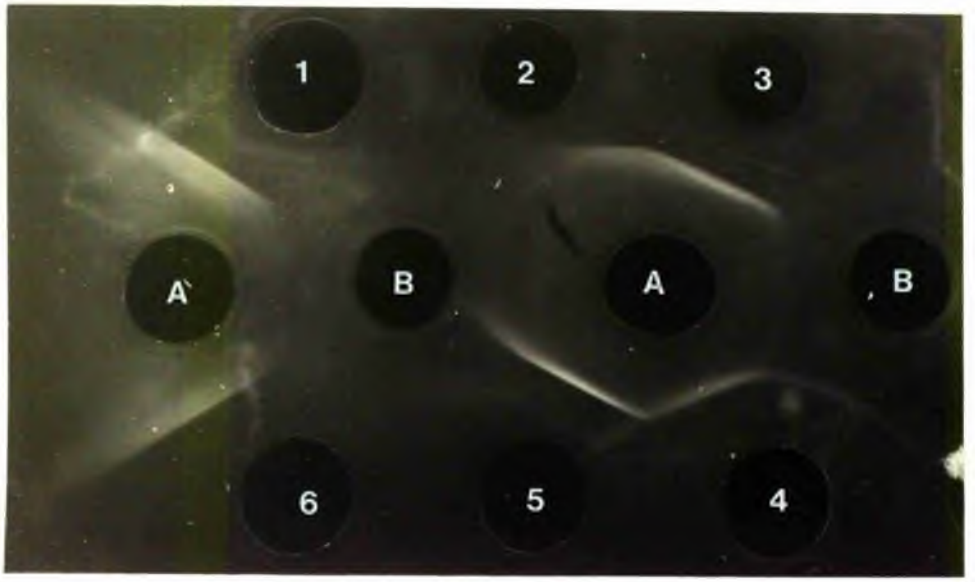

1
2
3
A
B
A
B
6
5
4

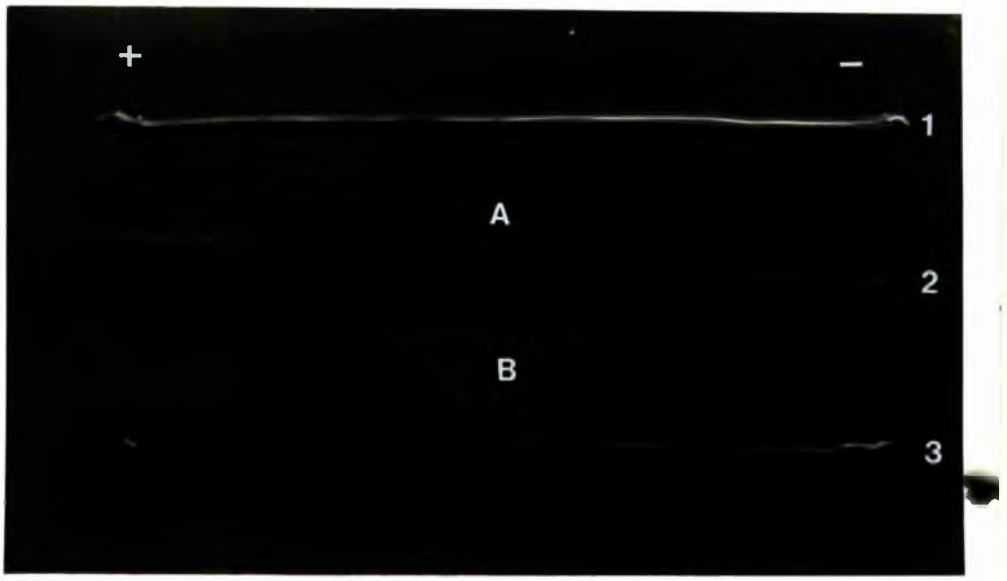
Figure 30 Immunoelectrophoresis of the crude culture supernate of strain LS26

Trough 2 - Rabbit anti-δ-haemolysin antiserum

Trough 1,3 - as for (2) and absorbed with purified δ-haemolysin

Well A - LS26

B - purified δ-haemolysin.


+
−
1
A
2
B
3

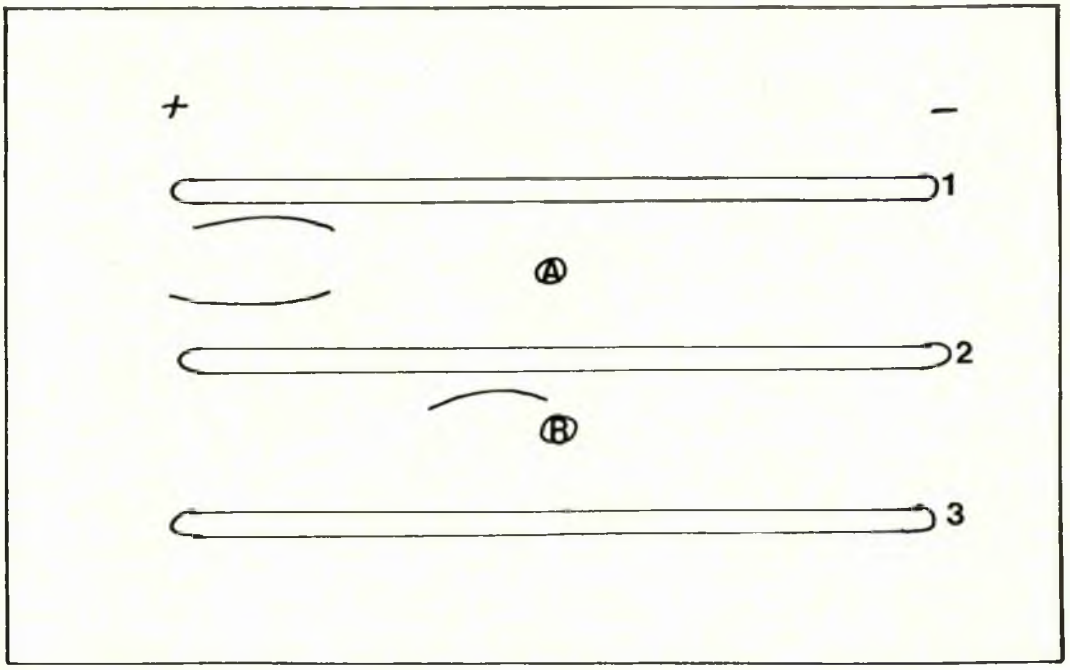
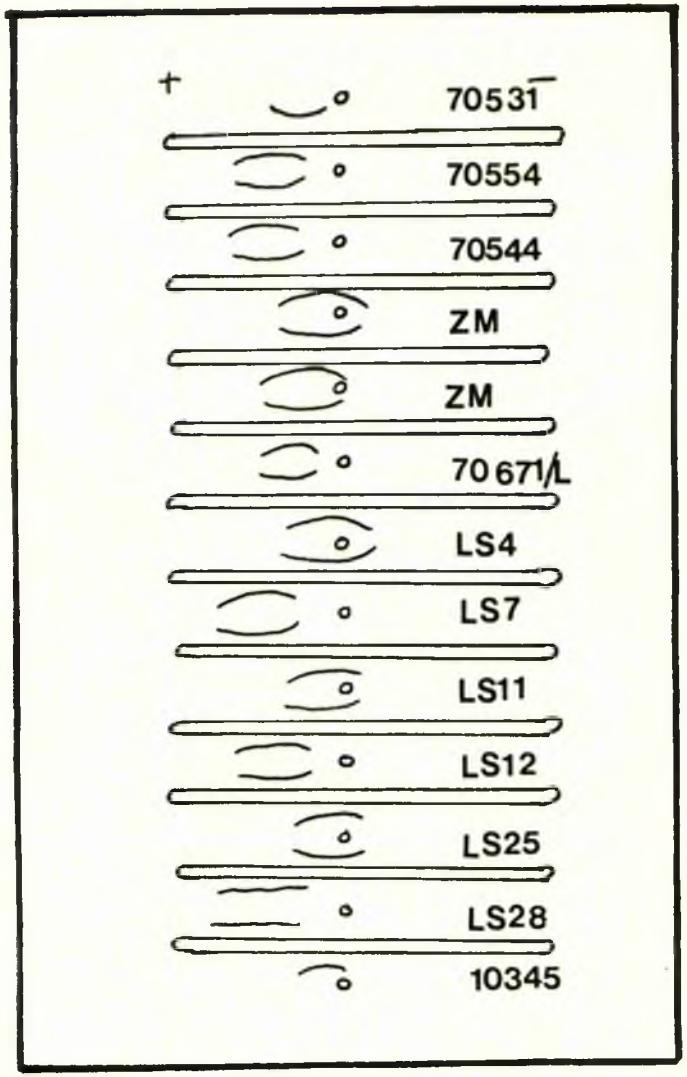

+
−
1
A
2
B
3

Figure 31 Immunoelectrophoresis of crude culture supernates from different strains of S. aureus

All troughs were filled with rabbit anti-δ-haemolysin antiserum.


+
−
70531
70554
70544
ZM
ZM
70671/L
LS4
LS7
LS11
LS12
LS25
LS28
10345


+
−
70531
70554
70544
ZM
ZM
70671/L
LS4
LS7
LS11
LS12
LS25
LS28
10345

respective strain had been isolated. The canine (70544) and the mouse (ZM) strains had similar haemolytic spectra although they differed in their electrophoretic mobilities. The human strains, LS4 and LS28, were similarly chosen and they also differed from the two animal strains in their activity against horse and human erythrocytes.

(i) Zone convection isoelectric focusing: A major peak of E_{280} absorbing material was found at pI 4.75 (LS28), pI 5.1 (70544 and LS4) and pI 5.2 (ZM) (Figs 32, 33, 34, 35). Apart from strain ZM, all haemolytic activity against cod, human and horse erythrocytes was found in this major peak. Haemolytic activity against rabbit erythrocytes was also found in fractions of pH 7 to 8. Since the principal form of α-haemolysin has a pI of 8 (McNiven et al., 1972), it is probable that these fractions contain this haemolysin. The antigenic activity of the fractions, determined by RIE, was also found in the major E_{280}-absorbing peak although, with strain 70544, this was only detected by CIE in fractions 3 to 11, and not by RIE. The mouse strain (ZM) differed from the other strains in that fractions of pI 7.6 to 8.1 also contained haemolytic activity (against cod erythrocytes) and antigenic activity.

(ii) Polyacrylamide gels: In preliminary experiments, with purified δ-haemolysin, it was found that a 10 mg/ml solution of haemolysin was required to give a distinct band on the gel; fixing in 1% glutaraldehyde did not improve the resolving power of the stain. Crude culture supernates were concentrated by precipitation with 70% saturated ammonium sulphate and applied to the gels. Multiple bands were found with all supernates although the number detected varied. However, bands were found to be more distinct when electrofocusing was carried out in gels containing 9M urea (Fig 36). The multiple bands could be arranged into groups of varying pI, ie 1) pI 4.6 to 4.9, 2) pI 5 to 5.9 and 3)

Figure 32 Isoelectric focusing of the crude culture supernate of strain LS28

Crude culture supernate of strain LS28 were analysed by isoelectric focusing in the apparatus of Talbot and Caie (1975) and the E_{280}, HU_{50} (cod, horse, human, rabbit erythrocytes) and antigenic activity by RIE measured.

○——○ - E_{280}

..... - pH

●——● - Haemolytic activity, cod erythrocytes

■——■ - Haemolytic activity, rabbit erythrocytes

▲——▲ - Haemolytic activity, human erythrocytes

✖——✖ - Haemolytic activity, horse erythrocytes

□——□ - Antigenic activity (by RIE).

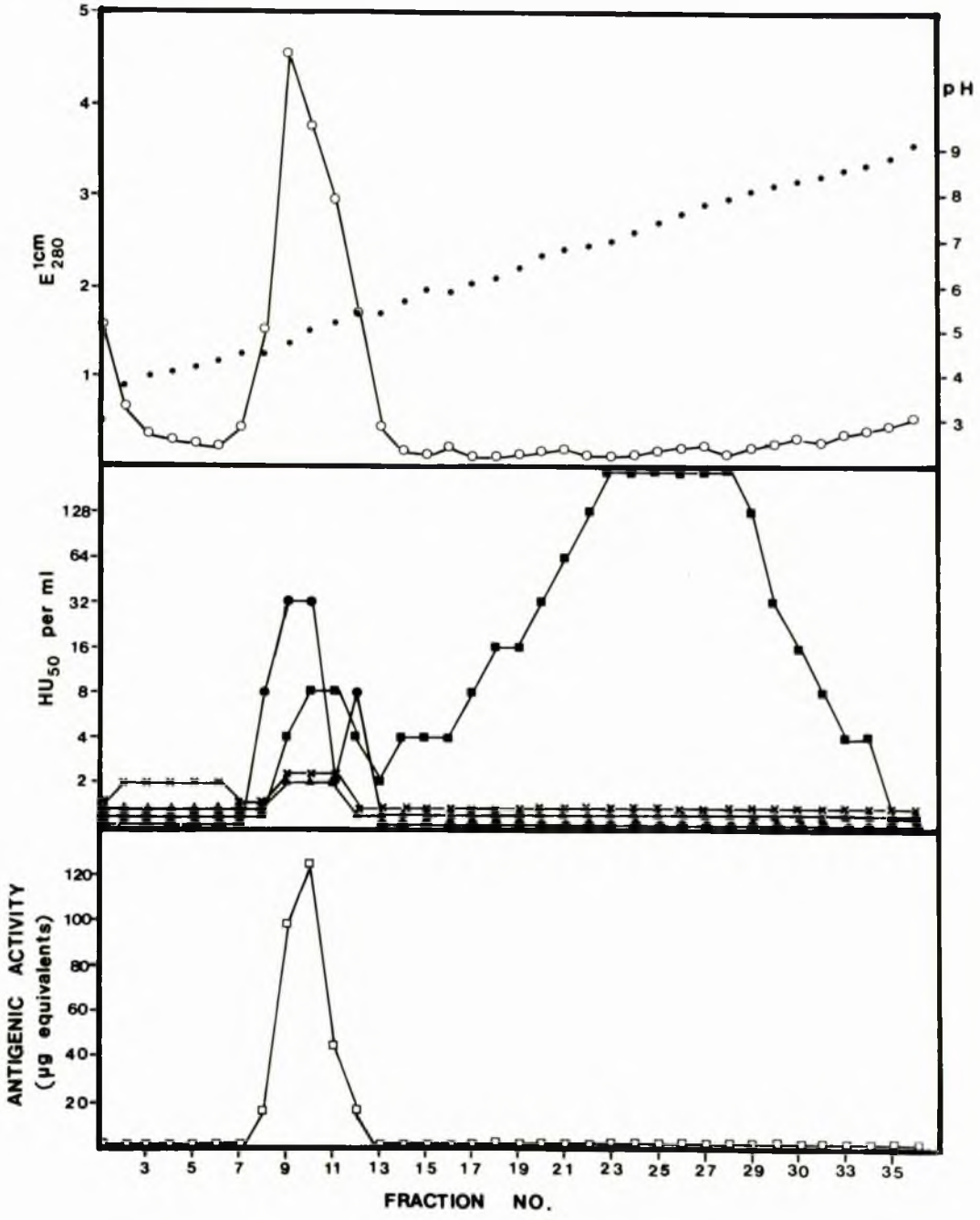

E^{1cm}_{280}
pH
HU_{50} per ml
ANTIGENIC ACTIVITY
(µg equivalents)
FRACTION NO.

Figure 33 Isoelectric focusing of the crude culture supernate of strain 70544

Crude culture supernate of strain 70544 were analysed by isoelectric focusing in the apparatus of Talbot and Caie (1975) and E_{280}, HU_{50} (cod, horse, human, rabbit erythrocytes) and antigenic activity by RIE measured.

○——○ - E_{280}

..... pH

●——● - Haemolytic activity, cod erythrocytes

✖——✖ - haemolytic activity, horse erythrocytes

▲——▲ - Haemolytic activity, human erythrocytes

■——■ - Haemolytic activity, rabbit erythrocytes

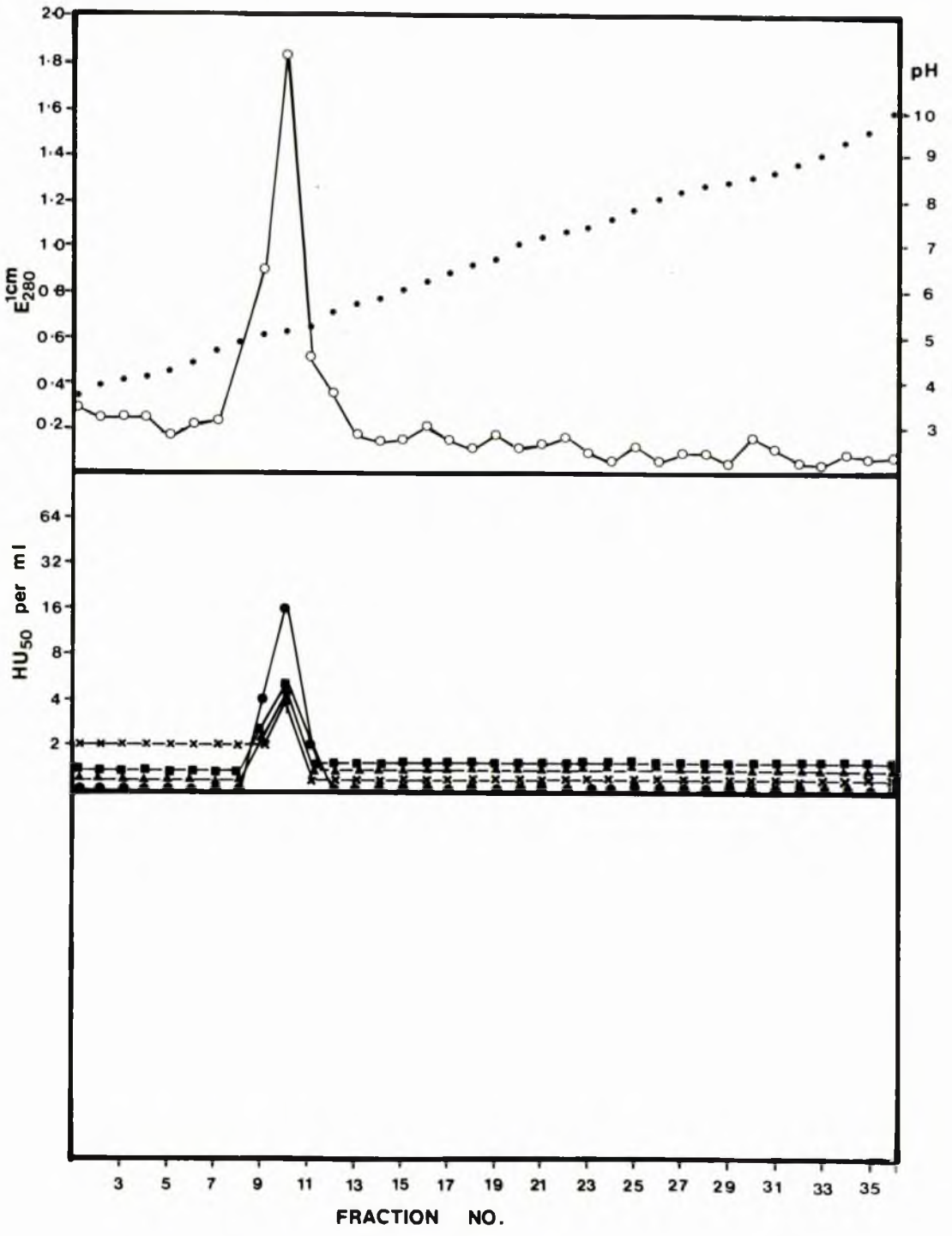

2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
E^{1cm}_{280}
pH
10
9
8
7
6
5
4
3
64
32
16
8
4
2
HU_{50} per ml
3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
FRACTION NO.

Figure 34 Isoelectric focusing of the crude culture supernate of strain LS4

Crude culture supernate of strain LS4 were analysed by isoelectric focusing in the apparatus of Talbot and Caie (1975) and E_{280}, HU_{50} (cod, horse, human, rabbit erythrocytes) and antigenic activity by RIE measured.

○——○ - E_{280}

●——● - Haemolytic activity, cod erythrocytes

✖——✖ - Haemolytic activity, horse erythrocytes

▲——▲ - Haemolytic activity, human erythrocytes

■——■ - Haemolytic activity, rabbit erythrocytes

□——□ - Antigenic Activity (by RIE).

..... - pH

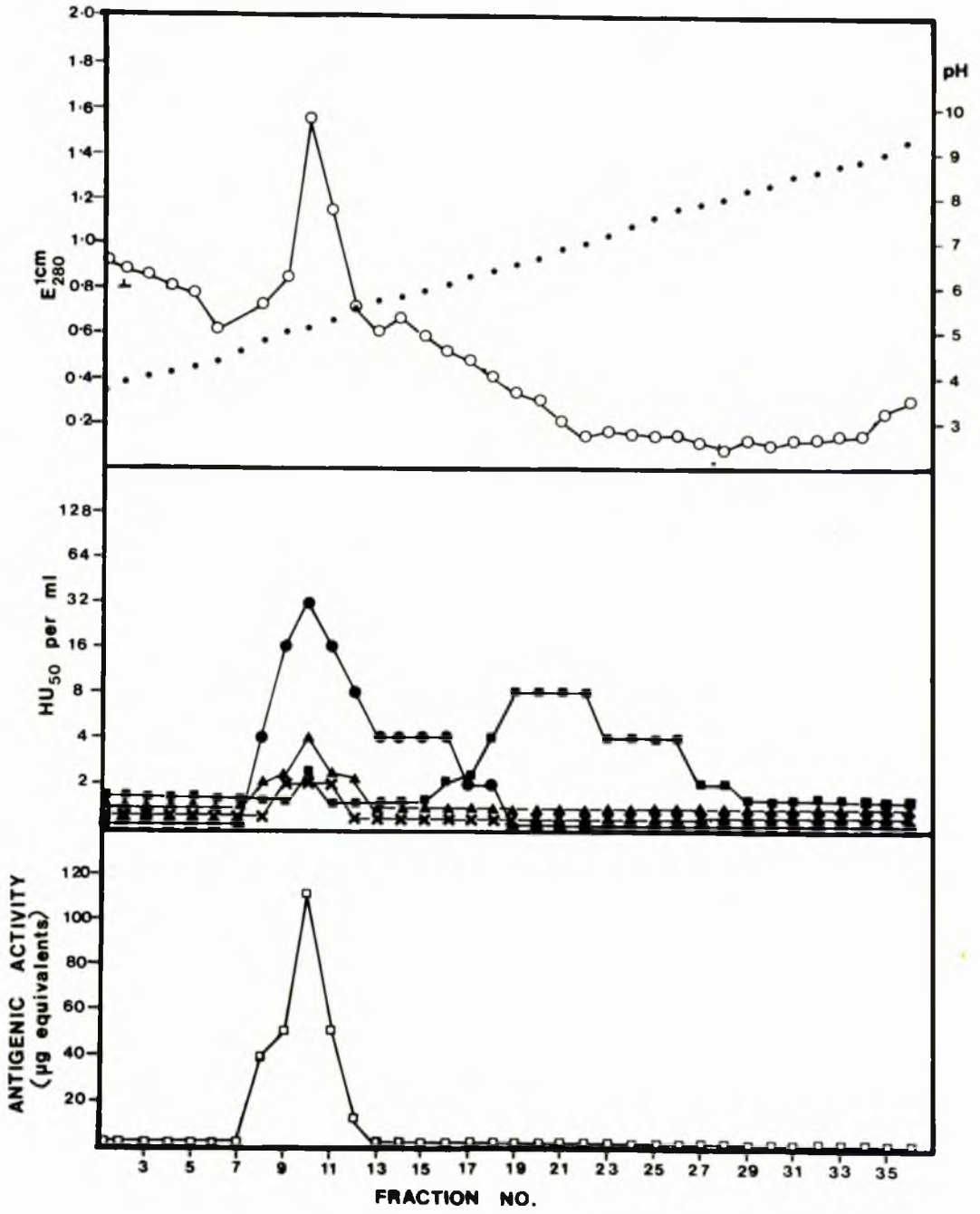

2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
E^{1cm}_{280}
pH
10
9
8
7
6
5
4
3
128
64
32
16
8
4
2
HU_{50} per ml
120
100
80
60
40
20
ANTIGENIC ACTIVITY (µg equivalents)
3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
FRACTION NO.

Figure 35 Isoelectric focusing of the crude culture supernate of strain ZM

Crude culture supernate of strain ZM were analysed by isoelectric focusing in the apparatus of Talbot and Caie (1975) and E_{280}, HU_{50} (cod, horse, human, rabbit erythrocytes) and antigenic activity by RIE measured.

○——○ - E_{280}

····· - pH

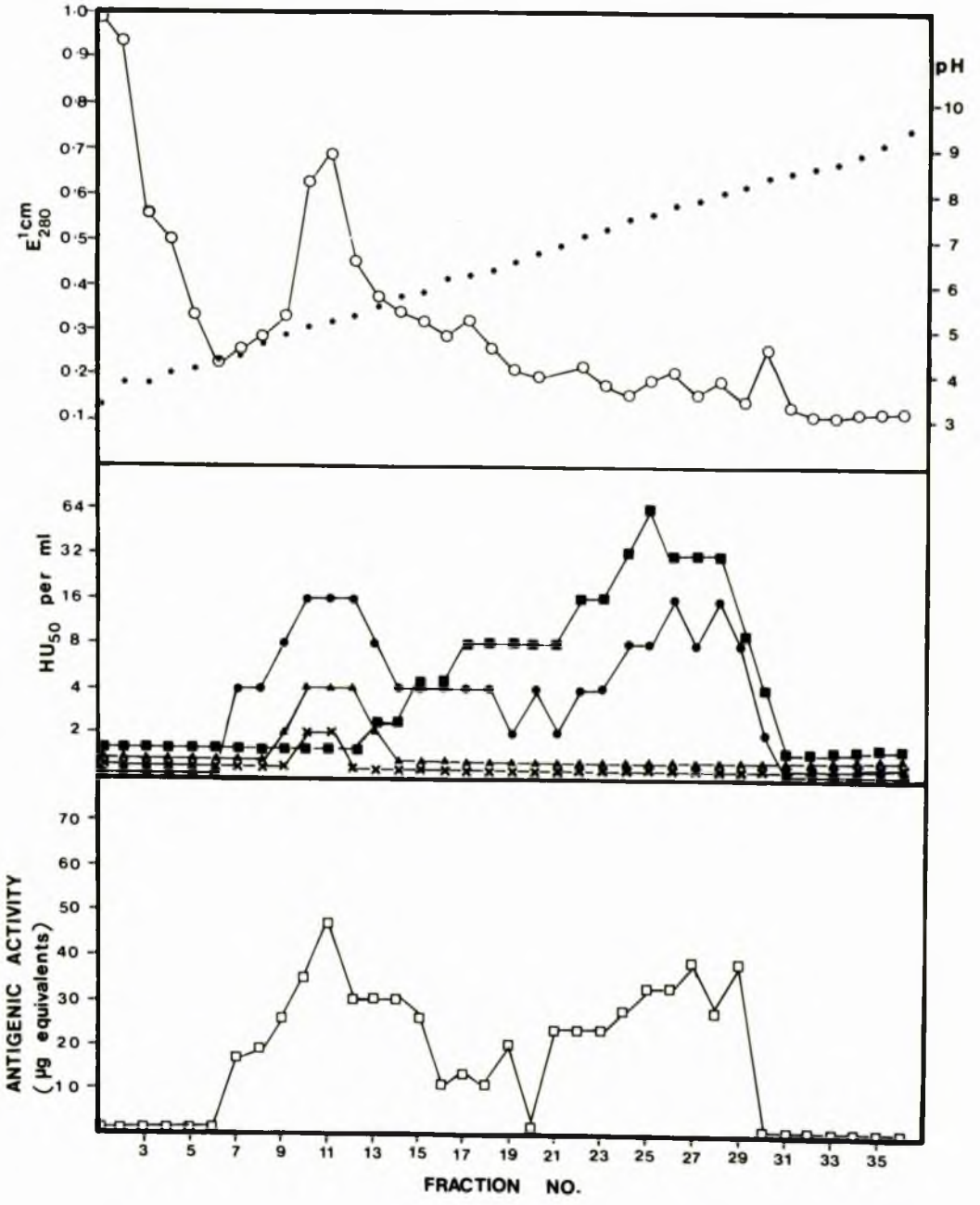
●——● - Haemolytic activity, cod erythrocytes

✖——✖ - Haemolytic activity, horse erythrocytes

▲——▲ - Haemolytic activity, human erythrocytes

■——■ - Haemolytic activity, rabbit erythrocytes

□——□ - Antigenic Activity (by RIE).


E^{1cm}_{280}
pH
HU_{50} per ml
ANTIGENIC ACTIVITY
(µg equivalents)
FRACTION NO.

pI 7 to 8. Purified δ-haemolysin had bands appearing in the last two of these categories.

Experiments with cod erythrocyte overlays were less clear and areas of lysis were found from the sample application point to the component with the lowest pI (Fig 37).

5. Purification

(i) Immunoadsorbent: Purification of the crude culture supernates was attempted.by adsorption chromatography. The adsorbing capacity of the column was determined by applying a 2 mg/ml solution of purified δ-haemolysin and elution with PBS2. After the unadsorbed protein had been removed, the adsorbed material was eluted using 0.2M glycine HCl pH 2.2 buffer. Peak fractions were pooled and the pH adjusted to 7 with NaOH. Each sample was analysed for protein content and haemolytic activity.

Thirty-eight per cent of original protein sample was recovered from the column by elution at pH 2.2 out of a total of 70% protein recovered (Table 26). A loss of haemolytic activity was found after adsorption to the column. This loss was not due to acid inactivation by the buffer since a solution of δ-haemolysin, equilibrated against the buffer, did not lose any haemolytic activity. Similarly, equilibration against carbonate/bicarbonate buffer pH 11 did not decrease the haemolytic activity of δ-haemolysin.

Crude culture supernates of LS26, grown in Bernheimer's yeast extract or CCY medium were respectively eluted in glycine HCl pH 2.2 and carbonate/bicarbonate buffer pH 11 (Stephen, Gallop and Smith, 1966) (Figs 38, 39).

Figure 36 Isoelectric focusing of crude culture supernates in polyacrylamide gels containing 9M urea

Crude culture supernatant fluids were analysed by isoelectric focusing in the LKB 2117 Multiphor apparatus and the gel was stained in Coomassie Brilliant Blue.

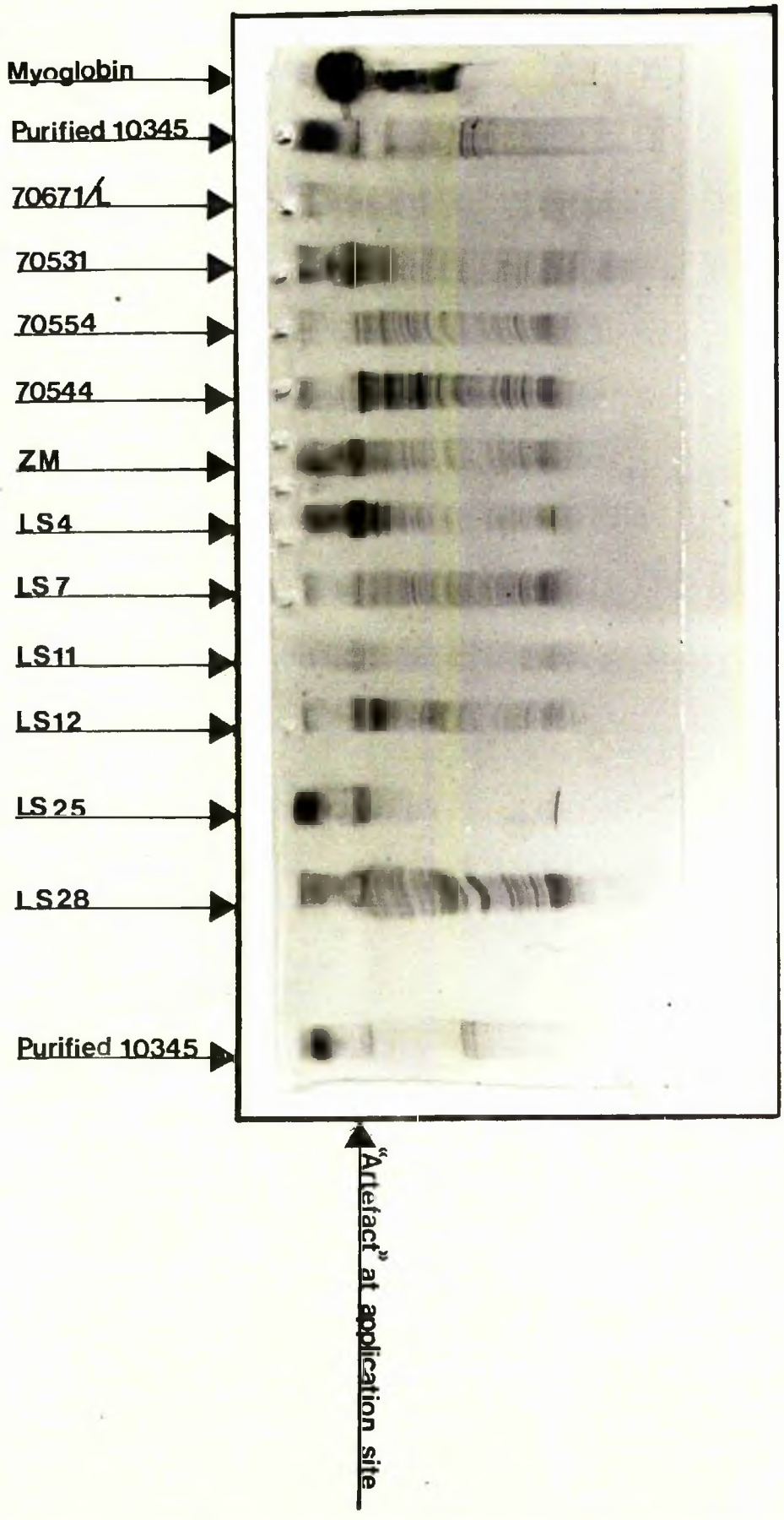

Myoglobin
Purified 10345
70671
70531
70554
70544
ZM
LS4
LS7
LS11
LS12
LS25
LS28
Purified 10345
"Artefact" at application site

Figure 37 Isoelectric focusing of crude culture supernates in polyacrylamide gels with a cod erythrocyte overlay

Crude culture supernatant fluids were analysed by isoelectric focusing in the LKB 2117 Multiphor apparatus and 5% (v/v) cod erythrocytes/agarose overlay layered on top. Areas of lysis were observed after overnight incubation at $4^{o}C$.

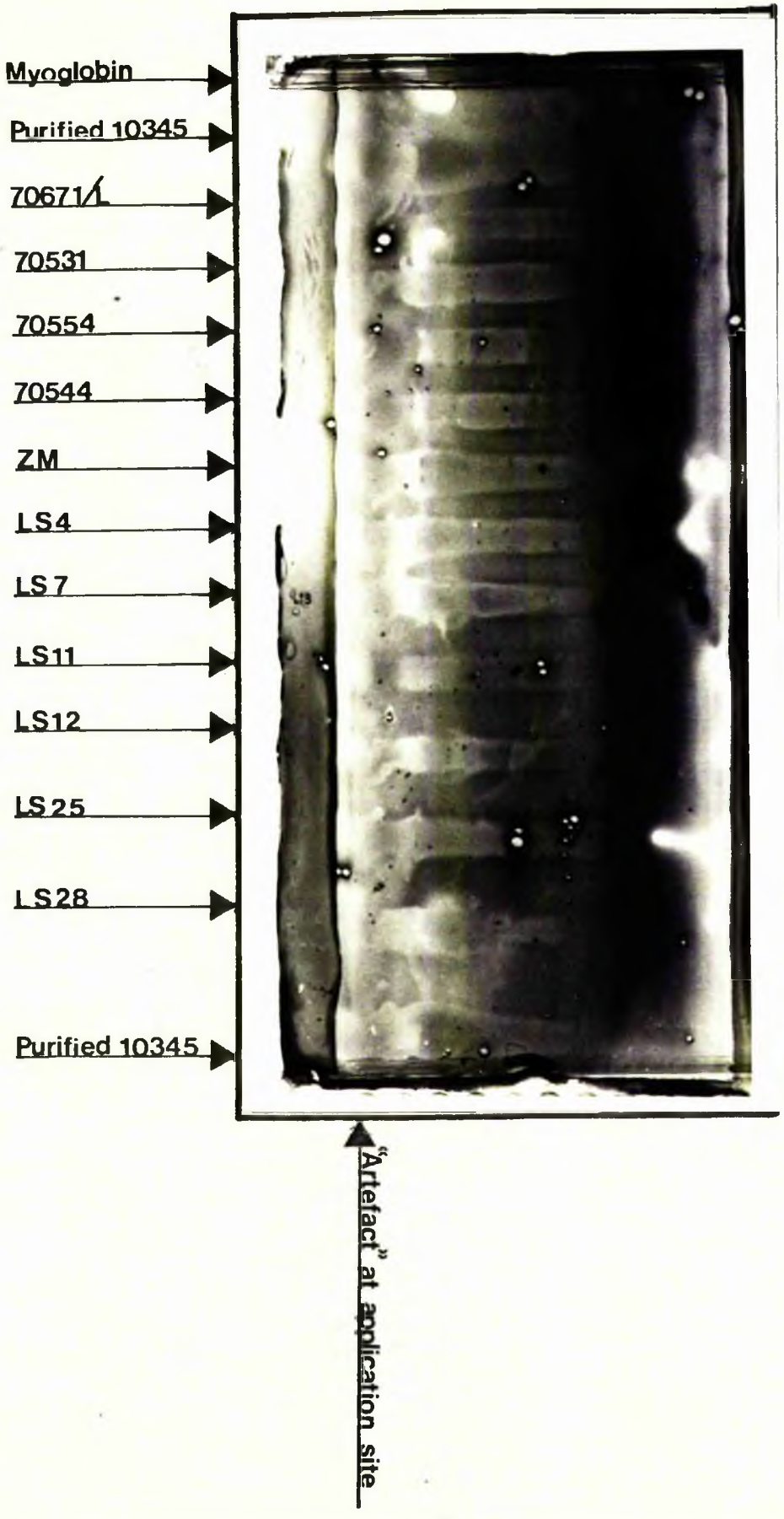

Myoglobin
Purified 10345
70671
70531
70554
70544
ZM
LS4
LS7
LS11
LS12
LS25
LS28
Purified 10345
"Artefact" at application site

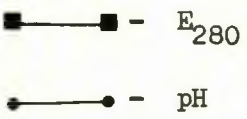
Table 26 Immunosorbent purification of δ-haemolysin - adsorbing capacity of the column

Fraction	Volume (ml)	Protein concentration (μg/ml)	Haemolysin titre (1% horse erythrocytes)**	% recovery*	
				protein	haemolysin
Sample Applied	2	1730	128	100	100
Unadsorbed	4.2	256	4	32	6.6
Desorbed	4.6	290	4	38	7.2

* percentage of sample applied

**horse erythrocytes were used because cod erythrocytes were unavailable at the time of the experiment.

Figure 38 Adsorption chromatography of the crude culture supernate of LS26 - elution with glycine HCl buffer



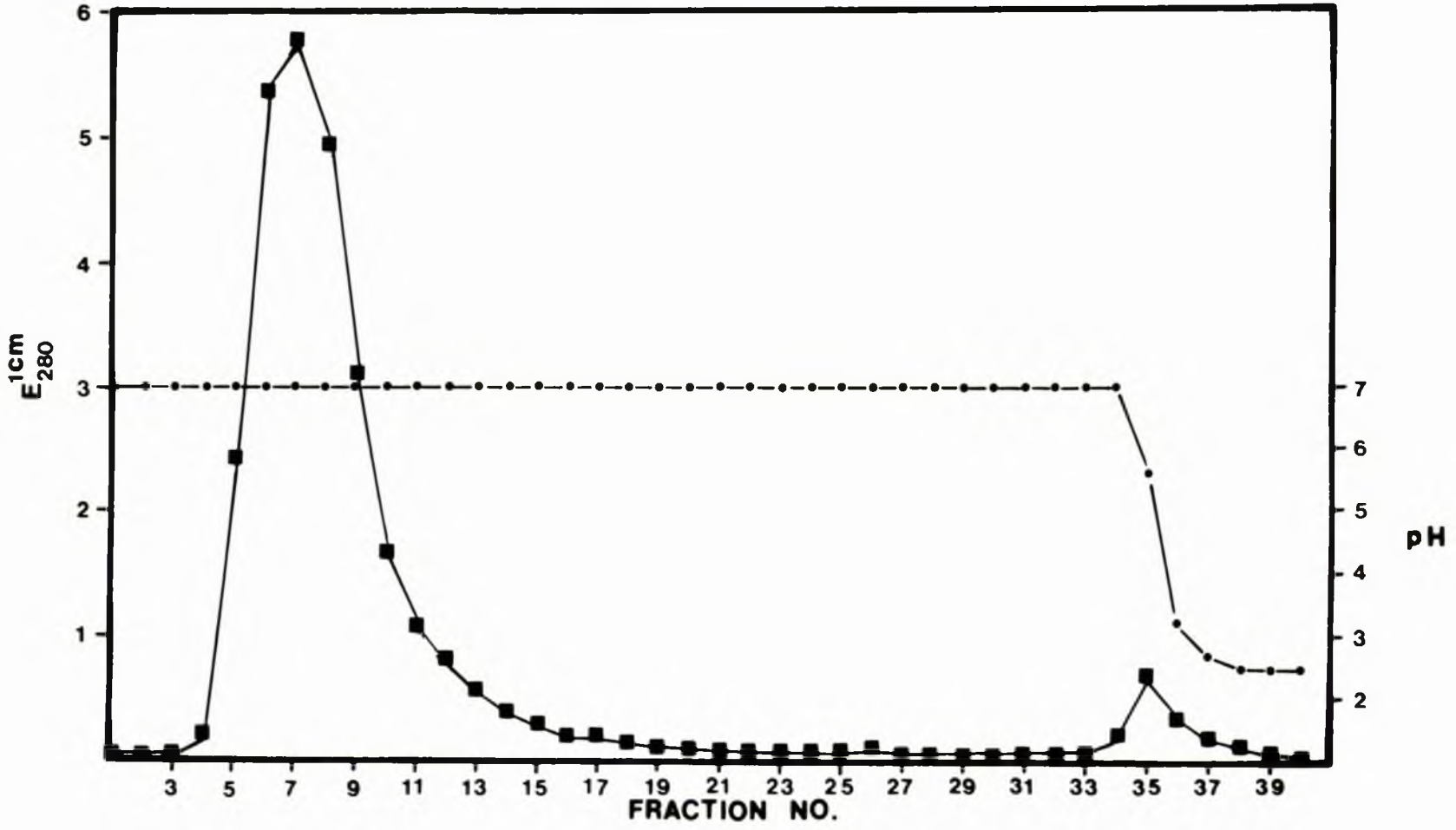
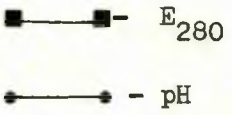

E^{1cm}_{280}
6
5
4
3
2
1
7
6
5
4
3
2
pH
3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
FRACTION NO.

Figure 39 Adsorption chromatography of the crude culture supernate of LS26 - elution with carbonate/bicarbonate buffer



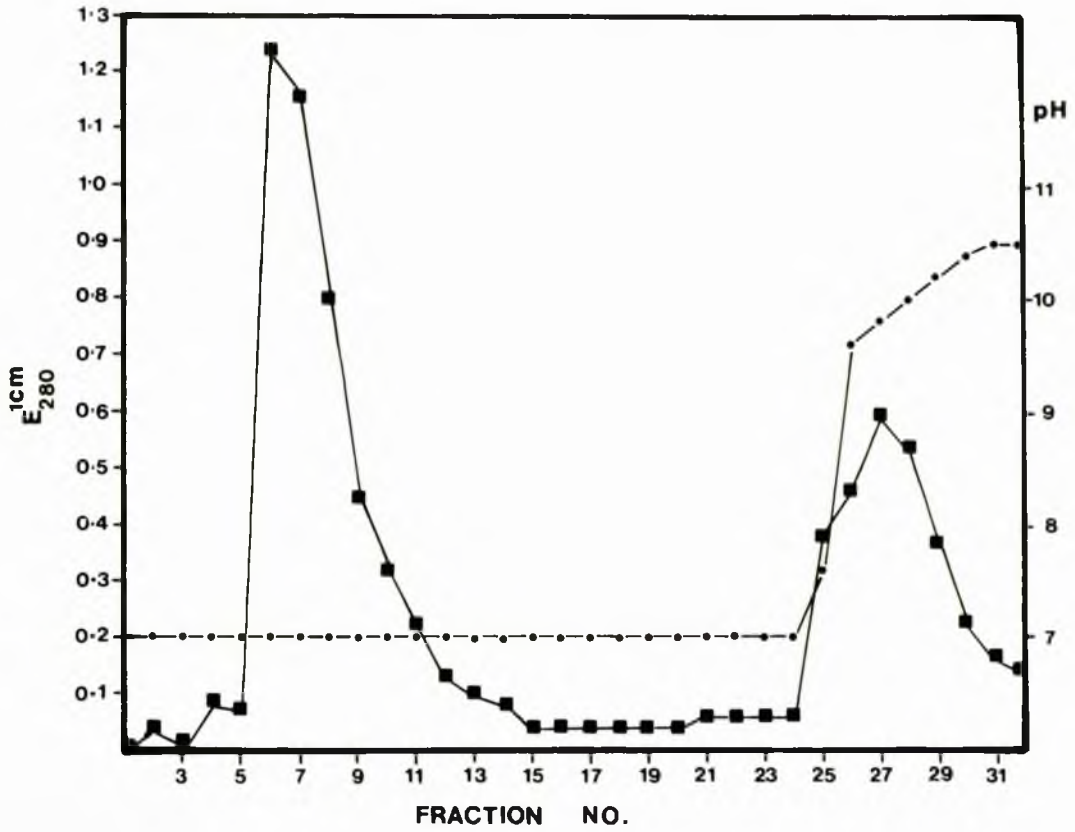

1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
E^{1cm}_{280}
pH
11
10
9
8
7
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
FRACTION NO.

Table 27 Purification of δ-haemolysin from strain LS26 with an immunosorbent-comparison of elution with different buffers

Fraction	Volume (ml)		Protein concentration (μg/ml)		Haemolysin titre (HU_{50}/ml) (1% cod erythrocytes)		% recovery protein		% recovery haemolysin	
	pH 2.2	pH 11	pH 2.2	pH 11	pH 2.2	pH 11	pH 2.2	pH 11	pH 2.2	pH 11
Sample Applied	3	3	1300	920	1024	1024	100	100	100	100
Unadsorbed	6.6	7.5	520	276	64	64	88	75	14	16
Desorbed	5.2	5.5	40	230	16	64	5.4	46	3	11

Comparison of the two methods indicated that a greater recovery of desorbed protein was obtained after elution at pH 11 (Table 27). However, loss of haemolytic activity was still found. Immunoelectrophoresis (Fig 40) and gel diffusion of the desorbed material indicated the presence of δ-haemolysin. The adsorbing capacity of the column, however, decreased after continual use and therefore this method of purification had to be abandoned.

(ii) Hydroxylapatite: Several strains of S. aureus were grown in 500 ml volumes of Bernheimer's yeast extract medium. However, except for strain LS28, none produced high haemolysin titres. Strain LS28 gave a titre of 1024 (1% cod erythrocytes) and therefore purification of δ-haemolysin by hydroxylapatite, as employed for strain 10345, was attempted. Delta-haemolysin was not recovered after washing the hydroxylapatite in 1M phosphate buffer pH 7.2 or with 1M NaH_2PO_4 pH 4.5.

This purification method was therefore also abandoned.

6. Immunogenicity

Since purification of the crude culture supernates proved to be unsuccessful using the above methods, the immunogenicity of the δ-haemolysins was investigated using the crude preparations.

Seven selected crude culture supernates were treated with formaldehyde at pH 5 for 7 days at 37°C. In addition to loss of haemolytic activity (Table 28), three of the seven formaldehyde-treated supernates also lost antigenic activity with rabbit anti-δ-haemolysin antiserum (Fig 41). Groups of 9-10 mice were immunised and sera were tested individually by CIE. Antibodies to native δ-haemolysin (strain NCTC 10345) were present only in the sera from mice immunised with LS28 or ZM antigens (Table 28). All sera gave multiple lines when tested

Figure 40 Immunoelectrophoresis of peak fractions from adsorption chromatography of strain LS26

All troughs contained rabbit anti-δ-haemolysin antiserum.

S - Sample applied to column

P1 - unadsorbed fraction

P2 - desorbed fraction

D - purified δ-haemolysin

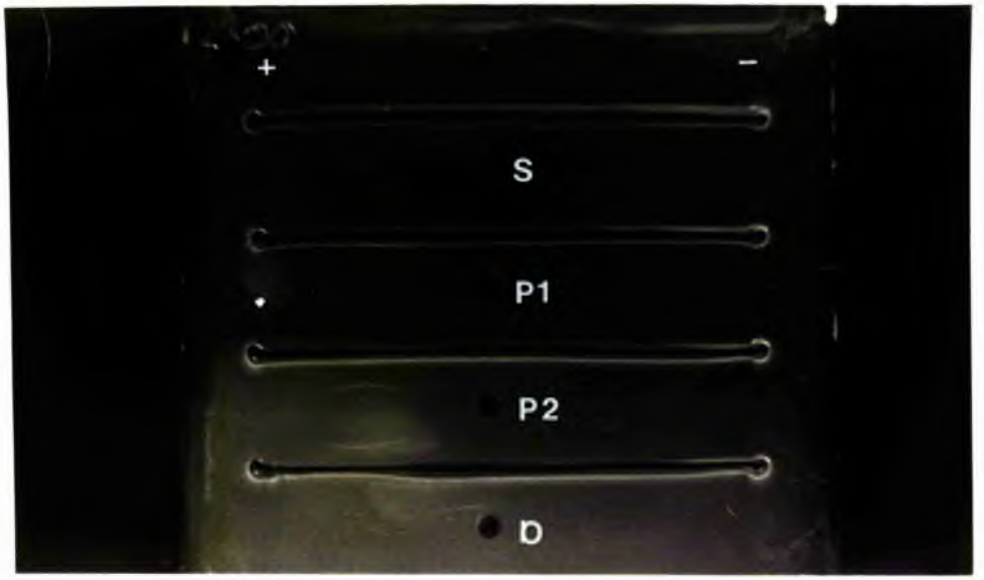

+
−
S
P1
P2
D

Table 28 Haemolytic, antigenic and immunogenic properties of crude culture supernates treated with formaldehyde at pH 5 for 7 days

Crude Culture Supernate	Haemolytic Activity (HU_{50})	Antigenic activity*	Immunogenic activity - % animals with serum Ab after immunisation with δ-haemolysin or crude culture supernates treated with HCHO at pH 5**
LS7	< 2	+	0
LS26	< 2	+	0
LS28	< 2	+	40 (4/10)
70671/L	< 2	-	0
70531	< 2	-	0
70554	< 2	-	0
ZM	< 2	+	10 (1/10)
Purified 10345 δ-haemolysin	1024	+	44 (4/9)

* + - reacts with anti-δ-haemolysin antiserum

\- - does not react with anti-δ-haemolysin antiserum

** - groups of 9-10 mice were used.

Figure 41 Antigenic reactivity of crude culture supernates with anti-δ-haemolysin antiserum after treatment with formaldehyde for 7 days at pH 5

Centre wells - rabbit anti-δ-haemolysin antiserum

Wells 2 - LS7

4 - LS26

6 - LS28

8 - 70671/L

10 - 70531

12 - 70554

14 - ZM

Wells 1,3,5,7,9,11,13 - purified δ-haemolysin.


1 2 3 4 5 6 7
14 13 12 11 10 9 8

against homologous toxoids by CIE. Antisera containing anti-δ-haemolysin antibodies gave multiple lines on gel diffusion with crude toxin and toxoid whereas only one line of identity was shown against purified δ-haemolysin (Fig 42: Line of identity was clearly shown on original slide although is difficult to see on figure shown). None of the mouse antisera reacted with purified α-haemolysin.

Figure 42 Reaction of anti-ZM antiserum with crude toxin, toxoid and purified δ-haemolysin and α-haemolysin

Well 1, 3, 5 - purified δ-haemolysin

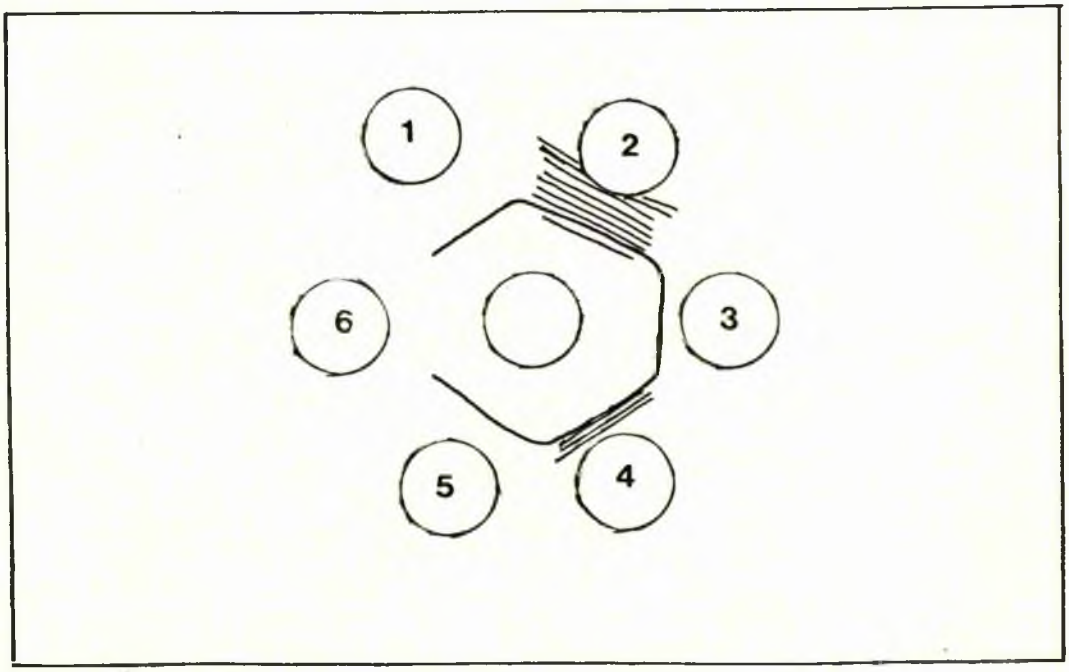
Well 2 - crude culture supernate of strain ZM

Well 4 - crude formaldehyde-treated culture supernate of strain ZM

Well 6 - purified α-haemolysin.

Centre well - anti-ZM antiserum.


1
2
6
3
5
4


1
2
6
3
5
4

DISCUSSION

The immunogenicity of staphylococcal δ-haemolysin in rabbits is now well established but the immunogenicity of this haemolysin in mice has not previously been reported. There were two reasons for wanting to use mice: first, when investigating the effect of various factors (time schedules, chemical modification etc.) on the relative immunogenicity of proteins, mice are useful since they are easy to handle and large numbers are readily available. Secondly, since the mouse has been used as an animal model to study various staphylococcal infections (Table 11), the production of anti-δ-haemolysin antibody in the mouse may prove advantageous in determining a role for δ-haemolysin in these infections.

The principal objective of this investigation was therefore to establish conditions for inducing an immune response to δ-haemolysin in the mouse. In parallel, the chemical modification of δ-haemolysin was investigated and a large part of the study involved comparison, both *in vivo* and *in vitro*, of treated and untreated δ-haemolysin. Also, as part of a programme to select variants of δ-haemolysin which might be of use in determining the mode of expression of the biological properties of δ-haemolysin, a number of different strains of *S. aureus* were screened for antigenic reactivity and also other properties.

Preparation and properties of δ-haemolysin

The preparation of δ-haemolysin by the procedure of Kreger *et al.* (1971) has been used routinely in this laboratory for a number of years and the preparation and properties of the haemolysin have been discussed by Chao (1976) and Whitelaw (1978). The six preparations used in this study, with a specific activity of 1024 HU_{50}/mg with 1% cod erythrocytes, were essentially similar to those of Chao (1976) and Whitelaw (1978) in specific activity, haemolytic spectrum, ultraviolet spectrum, isoelectric

focusing, SDS-polyacrylamide gels and lack of detectable α-haemolysin (as judged by the low activity against rabbit erythrocytes and the lack of detectable anti-α-haemolysin antibody on immunisation). The only difference found between the six preparations in this study and that of Whitelaw (1978) was in their sensitivity to heating at 100°C. Preparation 78/1 retained all haemolytic activity after 6 h at 100°C while preparation 78/2 had lost 50% of its activity within 1 h at 100°C. The results of the latter preparation are in agreement with those of Kreger et al. (1971), however, Whitelaw (1978) reported the half-life of δ-haemolysin to be 4½ h at 100°C. The reason for the high thermostability of preparation 78/1 is unknown. Whether the different preparations of δ-haemolysin exist in various aggregated forms (Kantor et al., 1972) which may render them thermostable to varying degrees depending on their state of aggregation, remains to be investigated.

Although contamination by other staphylococcal products was not investigated in this study, it has been shown previously that purification by hydroxylapatite yields a product that is free of most staphylococcal extracellular enzymes and toxins (Kreger et al., 1971; Whitelaw, 1978). The one known contaminant in the δ-haemolysin prepared by Whitelaw (1978) was a phosphatidyl inositol specific phospholipase C (Low and Finean, 1976, 1977), present at a level of 0.0014% (w/w) (M.G. Low, personal communication). However, unlike δ-haemolysin, this enzyme has little haemolytic activity (< 2%) (Low and Finean, 1977) and it hydrolyses very little lecithin in erythrocyte ghosts (Low & Finean, 1976). Therefore, if this enzyme was present in the preparationsused in this study it would be unlikely to interfere with the results obtained.

Immunogenicity of native δ-haemolysin

Native δ-haemolysin was poorly immunogenic in the mouse inducing

a response in, at most, 50% of immunised animals (Table 18) whereas, in the rabbit, an immune response was obtained in all animals tested by Birkbeck and Whitelaw (1979). Similarly, Kondo et al. (1976) found staphylococcal exfoliatin A and B to be immunogenic in the rabbit but non-immunogenic in the mouse and Laky (1978) reported that the response in mice to chicken erythrocytes was lower than that obtained in rabbits.

These results therefore suggest that genetic factors are responsible for the unresponsiveness of mice. The structure of δ-haemolysin may render the protein more suitable for induction of an immune response in the rabbit than in the mouse. For example, the amino acid sequences of the polypeptide chains may be more "foreign" in the rabbit than in the mouse (Burnet and Fenner, 1949) and therefore more immunogenic in the former species.

Genetic unresponsiveness may also be explained by 1) differences in the ease and speed with which cells are induced into specific antibody production (Ipsen, 1959), 2) the number of immunocompetent B cells present (Shearer, Mozes and Sela, 1971) or 3) greater susceptibility to tolerance of high affinity cells as a direct result of defective macrophage function at the level of antigen processing and presentation to immunocompetent cells (Steward et al., 1974). A further possibility may be a suppressor determinant on the haemolysin molecule which specifically activates T-suppressor cells in the non-responding mice (Adorini, Miller and Sercarz, 1979). A suppressor determinant, which specifically induces suppressor T cells in non-responding mice, has recently been identified in hen egg-white lysozyme (Yowell et al., 1979). When this determinant was removed, the mice generated a vigorous T-cell response directed against a large fragment of the antigen and the authors suggested that genetic responsiveness is controlled at the level of the macrophage in the presentation of different determinants to the competing subpopulations of regulatory T cells.

In this study, therefore, it is possible that "expression" of a suppressor determinant may occur in the non-responding mice but not in rabbits in which the macrophages may present different antigenic determinants to the immuno-competent cells.

Chemical modification of δ-haemolysin

Since native δ-haemolysin failed to induce an acceptable immune response in the mouse, chemical treatment of the haemolysin with formaldehyde, glutaraldehyde or formaldehyde/amino acid mixtures was examined in an attempt to increase its immunogenicity.

Treatment of δ-haemolysin with formaldehyde at 37°C and at pH 5 or pH 7.5 caused complete loss of haemolytic activity within 24 h of incubation. However, under alkaline conditions, some activity was retained even after 7 days incubation. The initial loss of haemolytic activity at "zero time" compared with control samples can be explained by the reaction which occurred whilst setting up the dialysis and during the initial period of dialysis (Myers and Hardman, 1971).

Eaton (1937) reported that greater quantities of formaldehyde were needed to convert diphtheria toxin into toxoid at acid pH than at alkaline pH which is contrary to what was found here. However, in excess formaldehyde, the reaction may be reversible (Linggood et al., 1963) and may account for the slower rate of detoxification under alkaline conditions.

Marks and Vaughan (1950) reported a loss of haemolytic activity within 48-72 h when δ-haemolysin was treated with 0.5% formaldehyde but, unfortunately, the pH of the reaction was not recorded.

The importance of pH in the reaction of formaldehyde with toxins and the resulting properties has been reported for the toxin of the palestine viper, Vipera palestinae. Neurotoxic and antigenic activities

of the toxin were retained at acidic pH but lost at alkaline pH while the haemorrhagic property of the toxin was destroyed over a pH range of 5.5 to 9.2 (Moroz-Perlmutter et al., 1963).

The antigenic activity of all formaldehyde-treated δ-haemolysin preparations was retained and all produced a line of identity with native δ-haemolysin against anti-δ-haemolysin antibody (Fig 7). Other workers also failed to detect any differences in antigenic specificity of BSA (Habeeb, 1969) or staphylococcal exfoliatin A and B (Kondo et al., 1976) after treatment with formaldehyde. Destruction of antigenic determinants by formaldehyde has been reported with diphtheria toxin (Rittenberg et al., 1976) but not found by other workers (Bazaral et al., 1973). However, destruction of native determinants is pH-dependent since there is a greater loss with increasing pH (Warren et al., 1973, 1975). These results were obtained from quantitative precipitin tests of toxoids with antibody to native enterotoxins. Since this test is more sensitive than gel diffusion it is possible that some native determinants may have been destroyed in this study. Indeed, the precipitin reaction with samples treated at pH 9.5 was frequently weaker than that obtained with the other toxoids. This is therefore in agreement with the work of Eaton (1937) that, in alkaline conditions and in excess formaldehyde, there is a loss in flocculating ability of toxin.

Antisera raised against the toxoids in both the rabbit and the mouse contained antibodies directed against new antigenic determinants not found in native δ-haemolysin (Fig 13). This is probably due to the intramolecular cross-linking caused by formaldehyde (Gill et al., 1968). Also, if the destruction of native determinants and the creation of new antigenic determinants occurred at different rates, it is possible that the antibody response against the native determinants would be weaker (Schirmacher and

Wigzell, 1974; Naor and Galili, 1977; Onica, Lenkei and Ghetie, 1978) or of lower avidity (Steward et al., 1974) than that directed against the new determinants. This would explain the inability of native δ-haemolysin to precipitate with certain mouse anti-toxoid antisera on immunodiffusion (Fig 11). However, since differences in antibody avidity can occur between individual mice (Steward et al., 1974) and δ-haemolysin is more immunogenic in the rabbit (Birkbeck and Whitelaw, 1979), the response against the native determinants would be greater in the rabbit and in certain mice and therefore detectable by immunodiffusion (Fig 11).

Treatment of δ-haemolysin with glutaraldehyde caused a rapid loss of haemolytic activity. Since glutaraldehyde is a bifunctional aldehyde its mechanism of reaction is probably analogous to that of formaldehyde at each functional group, but the reaction occurs more rapidly (Habeeb and Hiramoto, 1968; Habeeb, 1969) and the binding to proteins and introduction of cross-linkages is greater than with formaldehyde (Bowes and Cater, 1966). A decrease in antigenic activity was found (Rappaport et al., 1974) and, after incubation for 7 days, only samples treated at pH 9.5 retained antigenic activity (Fig 21). This may be due to the polymerisation of glutaraldehyde in alkaline conditions which affects its efficiency as a cross-linking reagent (Bowes and Cater, 1966). The destruction of native determinants, therefore, may not occur appreciably under alkaline conditions. Similarly, under acid or neutral conditions, formaldehyde exists as a polymer but is depolymerised by changing to alkaline conditions (Tomiyama, 1935). This would similarly account for the activities of the formaldehyde-treated preparations.

The addition of tyrosine, glycine or leucine during "toxoiding" did not significantly alter the reaction of formaldehyde with δ-haemolysin. The antigenic activity of δ-haemolysin was retained in all preparations and, at pH 5 or pH 7.5, haemolytic activity was lost. The main difference between δ-haemolysin treated with formaldehyde alone or with formaldehyde/amino acid mixtures was the complete loss of haemolytic activity after incubation for 7 days at pH 9.5 with glycine or leucine. Formaldehyde may preferentially react with these free amino acids (French and Edsall, 1945) and, therefore, the reversible reaction occurring under alkaline conditions due to excess formaldehyde (as postulated above) may not occur due to this "mopping up" effect of the amino acids. In this way, detoxification could occur more rapidly.

The primary reaction of formaldehyde with proteins occurs at lysyl residues (Fraenkel-Conrat and Olcott, 1948) but methylene bridges may also be formed between lysine and tyrosine, histidine or tryptophan residues (Fraenkel-Conrat and Olcott, 1948; Blass, Bizzini and Raynaud, 1967) and other amino groups (Dunlop, Marini and Martin, 1971). However, since δ-haemolysin lacks tyrosine and histidine (Kreger et al., 1971; Heatley, 1971; Kantor et al., 1972; Whitelaw, 1978), the reaction of δ-haemolysin with formaldehyde can be regarded as occurring between lysine and tryptophan, and possibly also asparagine and glutamine residues. The reaction with tryptophan residues occurs mainly in alkaline conditions (Fraenkel-Conrat et al., 1947).

Treatment of δ-haemolysin with formaldehyde or glutaraldehyde caused a gradual loss in protein concentration with increasing incubation times. This loss was extensive under acid conditions, while,

at pH 9.5, there was little decrease in the protein concentration of δ-haemolysin. It was initially considered that the apparent loss of protein by the Folin assay was due to an altered reactivity caused by the modification of the tryptophan residues. However, there was also a loss in the total nitrogen content of these preparations which paralleled the loss of measurable protein. Since the decrease in protein concentration was greater than the decrease in nitrogen content, formaldehyde may have influenced the assay system, but the results nevertheless indicate that there was a genuine loss of protein.

The loss of protein occurred during the incubation period of δ-haemolysin with formaldehyde since there was no significant decrease in the protein concentrations of preparations before or after the dialysis and centrifugation steps. The reason for this loss of protein was not investigated, however, one explanation may be that the haemolysin had adsorbed to the glass surface of the test tubes during incubation. Heatley (1971) reported that dilute solutions of δ-haemolysin (about 2 HU/ml) adsorbed quickly and strongly to glass. If this occurred in these experiments, then the pH of the reaction of formaldehyde with δ-haemolysin is an important factor in the ability of the haemolysin to adsorb to glass. The alteration in the overall charge of δ-haemolysin due to modification of lysyl residues probably does not influence the binding of δ-haemolysin to glass since the pI of all 'toxoids' was very similar. However, varying degrees of cross-linking at different pH's may influence the adsorption of δ-haemolysin to glass. For example, if there is a greater degree of crosslinking in alkaline conditions this may prevent the binding of the haemolysin to glass. The

crosslinking reaction of formaldehyde with tryptophan residues may cause this (Fraenkel-Conrat et al., 1947). However, as mentioned earlier, the crosslinking efficiency of glutaraldehyde is decreased under alkaline conditions (Bowes and Cater, 1966) and therefore some other factors must be involved either in the adsorption of δ-haemolysin to glass or causing the loss of protein.

The ultraviolet absorption spectrum of formaldehyde-treated preparations indicated a loss of the small peak at 291 nm (Fig 14). This may be due to reaction of formaldehyde with tryptophan residues. However, the slight increase in absorption at 265 nm with pH 5 samples may be caused by the reaction of the formaldehyde with lysine residues (Korn et al., 1972; Onica, 1978).

Immunogenicity of chemically modified δ-haemolysin

Treatment of δ-haemolysin with formaldehyde at pH 5 gave a product with enhanced immunogenicity. An increase in immunogenicity has been reported after formaldehyde treatment of staphylococcal enterotoxins (Warren et al., 1973, 1975). However, unlike the results of these workers, the preparation treated at pH 7.5 was less immunogenic than the preparation treated at pH 5. The failure of formaldehyde to increase the

immunogenicity of δ-haemolysin under alkaline conditions confirms the findings of other workers using diphtheria toxin (Eaton, 1937), snake toxins (Moroz-Perlmutter et al., 1963), staphylococcal enterotoxins (Warren et al., 1973, 1975) and staphylococcal exfoliatin A and B (Kondo et al., 1976). A greater destruction of native determinants may account for the decreased immunogenicity of these preparations (Warren et al., 1973) and therefore there may not be the correct number of antigenic determinants present to induce an immune response (Desaymard and Feldmann, 1975; Desaymard and Howard, 1975; Dintz et al., 1976).

Various experiments were performed to try and explain the increased immunogenicity of the formaldehyde-treated preparations. Polymerisation caused by formaldehyde treatment has been considered as the contributing factor in the enhanced immunogenicity of staphylococcal enterotoxins (Warren et al., 1973, 1975). However, extensive crosslinking giving large polymers was not evident in the soluble fractions used for the immunisation of mice. Other workers also failed to detect polymerisation of staphylococcal α-haemolysin (Bernheimer et al., 1968) or BSA (Habeeb, 1969) after treatment with formaldehyde. This may be due to not only the pH of the reaction (Warren et al., 1973, 1975) but also to the concentration of formaldehyde and toxin used (Murphy, 1967).

The reaction with formaldehyde resulted in an increased electrophoretic mobility (Fig 15) and reduced isoelectric point (Fig 16) of δ-haemolysin. This is probably due to the reaction with free amino groups (Murphy, 1967; Habeeb, 1969). However, this increase in negative charge is unlikely to affect the immunogenicity of the protein (Maurer, 1964; Reichlin et al., 1970).

The enhanced immunogenicity of δ-haemolysin treated with formaldehyde may be explained by its reduced affinity for lecithin (Fig 17).

During immunisation, this would leave δ-haemolysin free to stimulate an immune response rather than being bound to lipoproteins or cell membrane phospholipids. Also, formaldehyde rendered δ-haemolysin resistant to tryptic digestion, probably due to the crosslinking effect of formaldehyde (Pappenheimer et al., 1972; Bazaral et al., 1973). This stability may render the protein resistant to proteolysis by other enzymes (Fraenkel-Conrat and Olcott, 1948; Eaton, 1937) which may cause metabolic inertness or diminished renal excretion (Crumpton, 1974). However, resistance to both lecithin inhibition and tryptic digestion was found in all toxoid preparations and therefore some other factors must be involved to explain the increased immunogenicity of the pH 5 treated preparations. An important point is, however, the resistance to tryptic digestion of δ-haemolysin treated at pH 5 in the absence of formaldehyde. If acidity influences the structure of δ-haemolysin in such a way as to render the susceptible sites inaccessible to the enzyme, then further treatment with formaldehyde may result in δ-haemolysin being extremely resistant to metabolic digestion.

An increased rigidity of δ-haemolysin due to formation of cross-linkages (Eaton, 1937) may also explain the increase in immunogenicity (Haurowitz, 1952; de Weck, 1974). If little cross-linking occurred at pH 9.5 this would leave the protein in a flexible conformation (Colacicco et al., 1977) which may not be suitable for immunising purposes. However, as stated earlier, the formation of cross-linkages may occur more readily under alkaline conditions, therefore rigidity of the molecule may not be an important factor here.

In addition to an increase in the titre of anti-δ-haemolysin antibodies produced, the enhanced response to δ-haemolysin also resulted from an increase in the number of responding mice. There are therefore

strong implications that genetic factors may be important in the enhanced immunogenicity. The results of the present study are in agreement with those of Nutt et al. (1974) in that the mice appeared to have the potential to make an immune response against δ-haemolysin but may not be able to express that potential. However, by treating the haemolysin with formaldehyde at pH 5 the antibody production was affected in such a way that the phenotypic expression of specific antibody was carried out. Whether the increased response is due to a selective increase in immunocompetent B cell precursors (Shearer et al., 1971), T-helper cells (Dennert and Tucker, 1972; Kahan et al., 1976) or merely an increased affinity for B cells (Parish and Ada, 1972) remains to be investigated. The crosslinking caused by formaldehyde however, may mask or destroy a suppressor determinant on the native haemolysin (Adorini et al., 1979) and therefore other determinants would be free to induce the proliferation of T-helper cells (Yowell et al., 1979).

Immunisation of mice with δ-haemolysin treated with formaldehyde/amino acid mixtures produced a similar response as found in mice immunised with δ-haemolysin treated with formaldehyde alone. Only preparations treated at pH 5 or pH 7.5 induced the production of detectable anti-δ-haemolysin antibody.

Delta-haemolysin treated with glutaraldehyde failed to stimulate a detectable immune response to the haemolysin. Loss or decrease in immunogenic activity of cholera toxin (Saletti and Ricci, 1974a), sheep erythrocytes and spleen cells (Ramos, Zavala and Hoecker, 1979) has been found after treatment with glutaraldehyde. Ramos et al. (1979) have suggested that the reason for this decrease in immunogenicity may be due to either 1) a delay in the digestion of the toxoid by macrophages which may disrupt the proper time-sequence of the processes required for a

complete induction of an immune response or 2) a decrease in the number of native determinants remaining on the surface of the molecule.

Erythrocytes treated with glutaraldehyde or formaldehyde induced a very poor primary response in mice but induced a high secondary response, implying that the treated cells were potent memory inducers (Dennert and Tucker, 1972; Kahan et al., 1976; Ramos et al., 1979). However, production of a secondary response depended upon the timing of the second challenge with antigen and, if given too late, inhibitory humoral factor(s) decreased the capacity of the primed mice to mount a secondary response (Kahan et al., 1976). The production of some "inhibitor" may therefore account for the necessity of three priming and three boosting injections to give a maximum antibody response to formaldehyde-treated δ-haemolysin: if only one primary and one booster injection was given the inhibitor may have prevented immunocompetent cells from being stimulated by antigen whereas, when three injections were administered, the stimulus on the immunocompetent cells would be too high for the inhibitor to have any effect on antibody production. Also, if the inhibitor is regarded as having a specific half-life, then the timing of the booster injections may also be critical: after three weeks inhibitor would still be present but absent after five weeks. If the above proposal is correct then this would explain the differences in the immune response to δ-haemolysin with different time schedules.

Enhanced Vascular Permeability

Since formaldehyde had altered certain physical and biological properties of δ-haemolysin, the in vivo activity of δ-haemolysin was studied and the effect of formaldehyde, and other treatments, on this activity was investigated.

The vascular permeability enhancing effect of δ-haemolysin was observed optimally by intravenous injection of Pontamine Sky Blue dye 1 h after injection of haemolysin into the skin of rabbits. If dye was injected after 2 h or later the blue zones faded until, after 24 h, only hard erythematous lesions were present which were devoid of any bluing. These results differ from those of O'Brien and Kapral (1976) who obtained maximal results after 6 h. These differences may have been due to the different animal models employed. However, although Craig (1971) noted differences in the size of the resulting zones after administration of equal quantities of cholera toxin into guinea pigs and rabbits, there was no evidence that the response in guinea pigs took longer to occur than in rabbits (i.e. 18-24 h). Nevertheless, since the mechanism of induction of EVP by δ-haemolysin may differ from that of cholera toxin (O'Brien and Kapral, 1977), animal variation may be more significant in this case. Another possibility is that the preparation used here differed in activity or physical state from that of O'Brien and Kapral (1976) due to different purification techniques (Kapral and Miller, 1971).

Two skin permeability factors have been found in culture filtrates of Salmonella typhimurium (Sandefur and Peterson, 1976). A rapid acting factor had a critical bluing time of 1 h after completion of skin sites, had no associated induration and was suggested to be or associated with endotoxin (Peterson and Sandefur, 1979). A delayed factor was also isolated, after chromatography of the crude culture supernatant fluid on Sephadex G100, which caused a marked induration of rabbit skin within 18 h and was accompanied by bluing. This factor was very similar to cholera toxin in both physical and antigenic properties (Sandefur and Peterson, 1977; Peterson and Sandefur, 1979). It was suggested that the effects of the delayed factor were blocked or masked by an inhibitor-like substance

present in the crude culture filtrates (Sandefur and Peterson, 1976). The data presented by O'Brien and Kapral (1976) show that, although the maximum EVP activity developed by 6 h post inoculation, there was an early response at 30 min - 2 h before the development of this maximum activity. In this study, the response to δ-haemolysin was of a similar degree and time-course to this early response found by O'Brien and Kapral (1976). Whether δ-haemolysin exists in two or more different physical states (possibly depending on the method of purification) which may have different biological activities, remains to be investigated.

Another interesting observation by these workers which is analogous to the results in the present work, was the thermostability (4 h at 100°C) (Sandefur and Peterson, 1976) and the resistance to enzymic digestion (Peterson and Sandefur, 1979) of the rapid factor. The enhanced vascular permeability activity of δ-haemolysin was retained after heating for 10 h at 100°C and after tryptic digestion, while lecithin only reduced, but did not abolish, the EVP activity of the haemolysin. O'Brien and Kapral (1976) obtained a greater reduction in EVP activity after treatment with lecithin than was found in this investigation, although the lower doses of δ-haemolysin required for activity may account for this difference. However, if the hypothesis of two separate factors holds true, the delayed factor of O'Brien and Kapral may be more susceptible to inhibition by lecithin.

Animal-to-animal variation was found here, and also by O'Brien and Kapral (1976). Craig (1971) found rabbit pairs varied 16-fold in susceptibility to cholera toxin. However, in this study, comparisons were made on each individual rabbit to overcome this problem.

Delta-haemolysin (62.5 μg - 1 mg) was found to give a linear

log dose/response curve. From the results of O'Brien and Kapral (1976) it appeared that their preparation was more active than the one used here, but animal variation may be a complicating factor as found by Craig (1971) with cholera toxin.

Unlike cholera toxin (Craig, 1966), formaldehyde treatment of δ-haemolysin did not eliminate all EVP activity (Tables 22, 23). It is possible that a longer incubation period is required to lose EVP activity than haemolytic activity. Silverman, Espeseth and Schantz (1969) found formaldehyde treatment of staphylococcal enterotoxin B altered the emetic, lethal and pyrogenic activities of the toxin at different rates. The possible reason for these differences is that the amino acids affected by formaldehyde (i.e. lysine and tryptophan) may not be so important in EVP and antigenic activities as in the haemolytic activity of the haemolysin.

Complete neutralisation of EVP activity was not obtained with anti-δ-haemolysin antibody. This may be explained by the high concentrations of δ-haemolysin required to produce a measurable response and insufficient antibody for neutralisation. This is endorsed by the fact that the haemolytic activity was also present after addition of antibody. However, the reason why increasing concentrations of δ-haemolysin was associated with decreasing EVP activity in the presence of a constant amount of antibody, is at present unknown.

The EVP activity of δ-haemolysin was not a non-specific response as shown by the inability of serum proteins to produce a response. However, the possibility that a factor other than δ-haemolysin is responsible for EVP activity cannot be disregarded. The most positive evidence that δ-haemolysin produced this in vivo response comes from the results showing the EVP activity to be associated with tryptic peptides

which lacked both haemolytic and antigenic activities. It is, however, recognised that the study of these peptide fragments of δ-haemolysin is of a preliminary nature and several limitations must be taken into account such as the sensitivity of the separation technique and the possible presence of artefacts, etc. Also, it remains to be investigated whether the action of the peptide fragments of δ-haemolysin is specific or may be mediated by a wide variety of small peptides. Nevertheless, the results were reproducible and therefore provide a basis for further experiments.

The increased vascular permeability caused by V. cholera enterotoxin and E. coli heat-labile enterotoxin is assumed to be a cAMP mediated response in that these toxins stimulate mucosal adenyl cyclase (Craig, 1965; Evans et al., 1972). However, unlike these toxins, δ-haemolysin failed to cause other cAMP-mediated responses such as morphogenesis and steroidogenesis in cultured tissue cells, (O'Brien and Kapral, 1977) although it did increase cAMP levels in guinea pig ileum (O'Brien and Kapral, 1976). Therefore, δ-haemolysin may elevate cAMP levels by a mechanism different from that of cholera or E. coli toxins.

Stimulation of adenyl cyclase is an unlikely mechanism of increasing the levels of intracellular cAMP by δ-haemolysin since both melittin (Cook and Wolff, 1977) and non-ionic detergents (Sutherland, Rall and Menon, 1962; Levey, 1970), the membrane-disrupting properties of which are similar to that of δ-haemolysin, inhibit or decrease the activity of adenyl cyclase and disrupt the membrane integrity required for the enzyme activity (Engelhard et al., 1976). However, O'Brien and Kapral (1977) have suggested that the elevation in cellular cAMP is a secondary response to δ-haemolysin, which primarily alters the movement of ions across the mucosa, and the EVP activity is a reflection of an increase in intercellular leakage (O'Brien et al., 1978).

Structural-functional relationship of δ-haemolysin

Table 22 lists the effects of various treatments on the haemolytic, EVP and antigenic activities of δ-haemolysin. Since haemolytic and antigenic activities may be lost independently of EVP activity (as with lecithin and trypsin treatments) while haemolytic activity can also be lost independently of antigenic activity (as in heating and formaldehyde treatments), then these findings strongly suggest that different mechanisms and areas of the molecule may be involved in different functions.

The EVP activity was associated with tryptic peptides which lacked haemolytic and antigenic activities. A fairly rigorous treatment was carried out, i.e. 10:1 ratio of toxin:enzyme for 2 h, and yet an increased EVP activity was recorded, which suggests that the fragments containing EVP activity were resistant to tryptic digestion. Six peptides were identified in this study with absorbance at E_{230}, while Kantor *et al.* (1972) separated eight peptides by electrophoresis and chromatography. Since the amino acid composition of the preparation used was similar to that of Kantor *et al.* (1972) (Whitelaw, 1978), it is possible, as suggested earlier, that the differences observed may be due to the differences in the sensitivity of the separation and detection techniques employed.

Since δ-haemolysin lacks arginine residues (Kreger *et al.*, 1971; Heatley, 1971; Kantor *et al.*, 1972; Whitelaw, 1978) cleavage would be restricted to lysine residues. However, low level tryptic cleavage at asparagine residues may also occur (Casey and Lang, 1976; Rice, Watts and Brown, 1979). Delta-haemolysin may therefore contain a unique sequence of amino acid residues which lacks lysine or asparagine residues (i.e. resistant to tryptic digestion) and which is involved in the EVP activity of the haemolysin. The reason for so many fragments having

EVP activity may be due to either various cleavage products containing this unique sequence of amino acids or the inability of the separation technique to resolve individual peptides. Müller-Eberhard (1975) has reported that a small segment of both C_{3a} and C_{5a} anaphylatoxins is involved in the expression of the biological activity of these proteins. Removal of the COOH-terminal arginine residue, in either protein, by carboxypeptidase B caused a total loss of biological activity.

Peptide digestion has been employed by several workers to determine the structure-functional relationship of various toxins. Watanabe and Kato (1978) isolated a lethal fragment from staphylococcal α-haemolysin by tryptic digestion. This was devoid of haemolytic activity and had very little dermonecrotic activity. Similarly, papain digestion of tetanus toxin gave two fragments which were antigenically distinct and yet both were atoxic (Helting and Zwisler, 1977a). However, the major peptide (Fragment C) contained the binding site for gangliosides and therefore anti-fragment C antibody was sufficient to neutralise the lethal effect of tetanus toxin *in vivo* since binding to brain receptors is the necessary primary step required for expression of toxicity (Helting and Zwisler, 1977b).

Binding of δ-haemolysin to erythrocytes and lecithin not only caused lysis of erythrocytes but also a loss of antigenic activity. However, on treatment with formaldehyde, the haemolytic activity was lost but the antigenic activity was retained. The amphipathic polypeptide, bee melittin, shares many properties with δ-haemolysin (Freer and Arbuthnott, 1976) and the initial binding to membranes is thought to be mediated by ionic interaction followed by post-binding conformational changes resulting in membrane penetration (Dawson *et al.*, 1978). It is possible, therefore, that the binding of δ-haemolysin to erythrocyte

membranes may involve an initial ionic interaction with the membrane surface which causes some conformational change in the haemolysin resulting in penetration of the bilayer (Colacicco et al., 1977). In this way, entry of δ-haemolysin into the membrane would not only cause lysis of the cell but may also render the antigenic determinants unavailable for reaction with specific antibody. If lysine or tryptophan residues are critical to the initial binding of haemolysin to erythrocyte membranes, then formaldehyde treatment of the haemolysin may prevent this binding due to alteration of these residues. The antigenic determinants would therefore be free to precipitate with anti-δ-haemolysin antiserum.

The results therefore suggest that lysine and perhaps tryptophan residues are important in the binding of δ-haemolysin to erythrocytes. Several treatments have been employed to determine the amino acid residues involved in the biological activities of proteins. Tetranitromethane is highly specific for tyrosine residues (Kondo et al., 1976; Beugnier and Zanen, 1977) and reductive methylation and carbamylation are specific for lysine residues (Means and Feeny, 1968; Robinson et al., 1975; Beugnier and Zanen, 1977). The less specific treatments of diazotization and formaldehyde have also been employed to determine the amino acids or the different sites involved in different functions (Habermann, 1973; Kondo et al., 1976). By using either one or several of the above techniques it has been discovered that

1) tyrosine residues, at least, are essential for the exfoliatin of staphylococci to manifest or maintain its exfoliative activity (Kondo et al., 1976)

2) the enzymic site of diphtheria toxin contains one essential tyrosine residue implicated in the binding of nicotinamide adenine dinucleotide and at least one lysine residue is not implicated in the dinucleotide binding

(Beugnier and Zanen, 1977) and

3) different sites of the tetanus toxin molecule are implicated in the binding process to brain matter, toxicity, and immunogenicity (Kryzhanousky, 1973; Habermann, 1973; Robinson et al., 1975). However, the excellent research programme carried out by Atassi (1975, 1978), which included immunochemical analysis of both modified chemical derivatives and peptide fragments of proteins, has resulted in the production of a precise and complete map of the antigenic structure of sperm-whale myoglobin and hen egg-white lysozyme.

Comparison of δ-haemolysins from different strains of S. aureus

Since the previous studies had indicated that different sites of the δ-haemolysin molecule from strain 10345 may be involved in haemolytic, antigenic and EVP activities, preliminary investigations into possible differences between δ-haemolysins produced by different strains of S. aureus were carried out. High titres of α- and δ-haemolysins were produced by the human strains, confirming previous findings that α- and δ-haemolysins are frequently associated with strains from human origin (Elek and Levy, 1950; Fraser, 1964). However, unlike the reports of other workers (Elek and Levy, 1950; Fraser, 1964) the animal strains produced little or no β-haemolysin and several of the canine strains produced fairly high α-haemolysin titres. The reason for these differences may be the cultural conditions employed. Sharma and Haque (1973) found that aeration by shaking was inhibitory to β-haemolysin production in synthetic medium while the production of α-haemolysin depends on the growth rate of the organisms (Arbuthnott, 1970). A high growth rate is accompanied by a lower α-haemolysin production while the reverse is true for a lower growth rate (Coleman and Abbas-Ali, 1977).

In most cases, growth in CCY medium was accompanied by a lower haemolysin production. However, with the canine strain 70671/L, there was an increase in α-, β- and δ-haemolysin production when the organism was grown in CCY medium. The finding that different cultural conditions may be required by different strains of S. aureus for haemolysin production is supported by Turner (1978b) who found that δ-haemolysin production depended on the composition and volume of medium and also on the strain of S. aureus employed.

The strain isolated from a case of bovine mastitis (69931) did not produce any haemolysins although the cultural conditions employed may not have been suitable for haemolysin production by this strain. Nevertheless, production of haemolysins in vivo may not be a prerequisite factor for the virulence of strains causing bovine mastitis (Anderson, 1976).

Contrary to the work of Turner (1978a), antigenic differences between crude culture supernates of canine strains and purified δ-haemolysin were not detected on gel diffusion against anti-purified δ-haemolysin. Similarly, all human strains (except strain LS26) produced a δ-haemolysin which was antigenically identical to purified (10345) δ-haemolysin. However, several of the canine strains gave a split line of identity with purified δ-haemolysin. Sometimes this was mistaken for a spurring reaction (usually in unstained preparations) but, by absorption of the antisera with either the crude canine culture supernate or with purified δ-haemolysin, it was shown that these canine δ-haemolysins were antigenically indistinguishable from that of strain 10345. Turner (1978a) also reported two precipitin lines with crude culture supernates of bovine strains but, unlike the findings in this study, only one of these lines gave identity with purified δ-haemolysin. Turner suggested that this second precipitin line may have been caused by the presence of protein A in the crude culture supernates. Since both precipitin lines found in this study

gave a reaction of identity with purified δ-haemolysin it is possible that they were caused by different aggregated forms of the haemolysin (Kantor et al., 1972). Turner (1978a) did not report split precipitin lines with any of his canine crude culture supernates. However, he failed to test the crude supernates against antisera absorbed with either crude or purified δ-haemolysin which would have proved that these strains did produce an immunologically distinct haemolysin.

The human strain, LS26, produced δ-haemolysin which precipitated with antibodies in anti-purified δ-haemolysin antiserum which were directed against antigenic sites not present on the homologous haemolysin. This reaction occurred with several anti-δ-haemolysin antisera. These results suggest that hidden antigenic determinants are "exposed" during immunisation with δ-haemolysin which induce specific antibody formation. Detection of antibodies directed against buried determinants has been shown using proteolytic peptides of HSA (La Presle and Durieux, 1958) and BSA (Ishizaka, Campbell and Ishizaka, 1960). Exposure of these buried determinants in vivo may be due to aggregation, conformational changes or local enzymic reactions (Goetzl and Peters, 1972).

Therefore, during production of δ-haemolysin, in strain LS26, the protein may be assembled and secreted in such a way as to uncharacteristically expose these hidden determinants which would therefore be free to precipitate with specific antibody.

The general mechanism of protein secretion has been reported by Blobel and Dobberstein (1975) while Gerda and Kreil (1977) have described the mode of secretion of melittin. As mentioned earlier, δ-haemolysin shares many properties with melittin and therefore it may also be secreted by cells in a similar way. Therefore, secretion of δ-haemolysin may involve synthesis of a signal sequence of a few amino acids which interacts

with the membrane and in some way allows the translocation of the rest of the peptide across the membrane as it is synthesised. The pro-haemolysin secreted may be in an inactive form which requires some enzymic cleavage to produce an active δ-haemolysin. If this pro-sequence of amino acids is cleaved at different points, then it may result in an altered conformation which could "expose" the normally hidden antigenic determinants. The results of strain LS26 could, however, also be explained if strain 10345 contained a certain percentage of organisms which produced this atypical form of δ-haemolysin and therefore the antisera raised against the haemolysin would contain a small proportion of antibodies directed against the "hidden" determinants. Normally these may not be identified due to the lack of sufficient antigen but if, as in the case of strain LS26, a greater proportion of cells produced this atypical haemolysin, then there is a better chance for these different antigenic determinants to be identified. This may also explain why, after recultivation of the organism, the haemolysin produced was antigenically identical to purified δ-haemolysin since production of the antigenically distinct form may be extremely sensitive to cultural conditions (Turner, 1978b). For example, cultural conditions may affect the activity of the enzyme involved in cleavage of the pro-sequence.

The immunoelectrophoresis of crude supernates revealed that several strains had an increased electrophoretic mobility compared with strain 10345. This indicated that those strains produced a haemolysin with an increased surface charge. However, by isoelectric focusing in the Talbot and Caie apparatus, the pI of the different haemolysins were very similar to that of strain 10345. The different electrophoretic mobilities may therefore be due to differences in molecular size. A basic component of pI 8 was also identified in strain ZM. However, since two

components were not found on immunoelectrophoresis, adsorption of the haemolysin to ampholines (Cann and Stimpson, 1977; Jonsson et al., 1978) or some pH-dependent conformational transition (Stimpson and Cann, 1977; Porcelli, Small and Brewer, 1978) may account for the basic component.

Isoelectric focusing in polyacrylamide gels confirmed previous findings that different strains of S. aureus differ in the number of extracellular products released (Wadstrom et al., 1974). For example, strain LS25 produced 4-5 bands while strain 70544 produced 20-26 bands. The two closely associated bands of purified δ-haemolysin are probably not due to different conformations of the molecule since focusing was carried out in 9M urea which should reduce the influence of conformational alterations (Zechel, 1977). Adsorption to ampholines may therefore explain this double banding (Cann and Stimpson, 1977; Jonsson et al., 1978).

Attempts to purify some of those crude preparations proved unsuccessful. Apart from strain LS28, none of the strains grown in 500 ml volumes of Bernheimer's yeast diffusate medium produced high titres of δ-haemolysin. These results are in agreement with those of Turner (1978b) who found that the volume of the medium can, to a great extent, influence the amount of δ-haemolysin produced. Turner (1978a) also reported the inability to purify δ-haemolysin from a canine source by hydroxylapatite, which was found with strain LS28. Desorption of the haemolysin from hydroxylapatite did not occur after washing in 1M phosphate buffer pH 7.2 or with 1M NaH_2PO_4 pH 4.5. The pI of δ-haemolysin from strain LS28 was very similar to that of strain 10345 and so a stronger binding of the haemolysin to hydroxylapatite cannot be explained by an increased charge. However, it is possible that conformational alterations may influence the binding of the haemolysin.

Purification of δ-haemolysin by affinity chromatography indicated

that the haemolysin could be successfully removed from crude culture supernates using this technique. Preliminary experiments showed that only 38% of total haemolysin applied to the column could be de-sorbed and the eluted sample contained little haemolytic activity. These results are similar to those reported by Heatley and Gladstone (1977). Desorption of δ-haemolysin from strain LS26 was successful with carbonate/bicarbonate buffer at pH 11. The eluted material retained its antigenic properties (Fig 40) although its haemolytic activity was decreased. Further experiments with this technique were abandoned due to the loss of adsorbing capacity of the column and the inability to recover haemolytically active material.

The inability to obtain purified δ-haemolysin from different strains of S. aureus was disappointing since it was envisaged that those preparations would have been useful to determine difference in immunogenic properties of the haemolysins. However, mice were immunised with crude preparations which had been treated with formaldehyde at pH 5 for 7 days to investigate the problem. Only two out of seven crude supernates induced the production of anti-δ-haemolysin antibodies. However, all antisera contained antibodies against each homologous toxoid. Three of the crude preparations had lost their antigenic activity against anti-δ-haemolysin antisera which could explain their inability to stimulate anti-δ-haemolysin antibody production. However, the problem of antigenic competition (Liacopoulos and Ben-Efrain, 1975), due to the presence of many antigenic species in the crude preparations, may explain both the inability of strains LS7 and LS26 to induce anti-δ-haemolysin antibody production and also the weak responses obtained with strains LS28 and ZM. Since more formaldehyde is required to toxoid a crude preparation than a purified preparation, due to the influence of other proteins in the medium (Wadsworth et al., 1937), a longer incubation period may be required to

produce a better immunogen. The importance of the length of incubation time and the resulting immunogenicity was shown with purified δ-haemolysin (Fig 9). This also explains the low results obtained with strains LS28 and ZM.

Possible areas for future research

The present work has outlined conditions suitable for the successful immunisation of mice against staphylococcal δ-haemolysin which may prove useful in determining a role for δ-haemolysin in the virulence of S. aureus. Adlam et al. (1977) have shown that immunisation with alpha-toxoid can modify the outcome of experimentally induced staphylococcal mastitis in rabbits. In a similar manner, the effect of immunisation with 'delta-toxoid' on experimentally induced infections in mice may be investigated. The study of skin necrosis in mice may be important in this respect since Van der Vijver et al. (1975b) have shown mutant strains, lacking δ-haemolysin, had a reduced dermonecrotic activity. Purified δ-haemolysin from different strains of S. aureus would also be useful in such experiments, since the effect of immunisation on the course of infection may depend on the strain employed. However, the results of the present study were disappointing due to the inability to purify these different haemolysins. A more extensive programme is therefore required to investigate this problem of purification by possibly involving the techniques of other workers (Heatley, 1971; Kantor et al., 1972) to purify δ-haemolysin. The recently described single-step purification of δ-haemolysin by hydrophobic interaction chromatography (Nolte and Kapral, 1979) also may be of use.

A detailed study of the growth requirements of strain LS26 for the production of the "unique" form of δ-haemolysin is another area of

interesting research. A purified preparation of this haemolysin could be exploited in several ways. For example, amino acid analysis may provide some information into the method of release of δ-haemolysin (cf. Gerda and Kreil, 1977), as would in vivo studies for enhanced vascular permeability activity. If the 'unique' antigenic determinants found in this haemolysin are indeed due to some conformational alteration during assembly, as suggested above, then the site of the molecule involved in EVP activity may be masked by such a conformational change.

The present work has suggested that different mechanisms and areas of the molecule are involved in different biological activities of δ-haemolysin. For example, a requirement for lysine residues in the binding of δ-haemolysin to lecithin or erythrocytes has been implicated while the EVP activity of the haemolysin may not rely to such a great extent on these residues. These results could be confirmed by employing more specific chemical modification techniques, such as reductive methylation and carbamylation, which are highly specific for lysine residues.

Similarly, the separation of tryptic fragments by molecular exclusion chromatography was only a preliminary investigation and therefore more sensitive separation techniques, such as ion exchange or paper chromatography, may provide a more efficient isolation of the fragments. Purification and amino acid analysis of such peptides would be important in determining the position and composition of the different sites of δ-haemolysin involved in haemolytic, antigenic and EVP activities.

A further area of research regarding the EVP activity of δ-haemolysin would be to determine the effect of various chemical treatments on the ability of δ-haemolysin to form indurated lesions at 24 h. Preliminary studies (not shown) indicated that formaldehyde-treated δ-haemolysin failed to cause this response at 24 h, while control samples did

produce such lesions. Therefore, it is possible that the bluing and lesion-forming responses of δ-haemolysin may involve two separate mechanisms.

Shier (1979) has recently suggested that δ-haemolysin may interact with the plasma membrane and stimulate intrinsic phospholipase A2 activity leading to formation of the lytic agent, lysolecithin, in the membrane. The effect of formaldehyde and other treatments on this stimulation by the haemolysin may also provide information on the mechanism of action of δ-haemolysin.

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APPENDICES

APPENDIX I

MEDIA

1. Yeast diffusate medium (Bernheimer and Schwartz, 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 h at 4^{o}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.

2. CCY medium (Gladstone and van Heyningen, 1957)

Bacto Casamino acids (Difco)	160 g
Yeast diffusate	1600 ml
Sodium lactate (70% syrup)	113.6 ml

Sodium glycerophosphate	160 g
$MgSO_4.7H_2O$	0.16 g
$MnSO_4.4H_2O$	0.084 g
0.32% (w/v) $FeSO_4.7H_2O$	16 ml
0.32% (w/v) citric acid	
KH_2PO_4	3.28 g
$Na_2HPO_4.12H_2O$	50 g
Distilled water to 8 l.	

Medium was autoclaved at 15 lb/in^2 for 15 min.

Yeast diffusate

Yeast extract (Difco)	200 g
Distilled water	200 ml

Suspension was dialysed (2.3/4" Visking tubing) against 1800 ml distilled water at 4°C for 24 h with constant stirring.

APPENDIX II

Buffers and diluents

1. Citrate/Dextrose/Sodium chloride solution, CDS. (Hodgins and Ridgway, 1964).

Glucose	2.05 g
Trisodium citrate	0.80 g
Sodium chloride	0.40 g
Distilled water to	100 ml

2. Dulbecco's A phosphate buffered saline, PBSA.

Phosphate buffered saline (Oxoid)	1 tablet
Distilled water	100 ml

The buffer was autoclaved at 15 lb/in^2 for 15 min.

3. Dulbecco's A phosphate buffered saline $_2$, PBS_2.

Sodium chloride	11.7 g
PBSA	1 l

Sodium azide (0.05%, w/v) was added to prevent contamination.

4. Potassium phosphate buffers (PB).

i) 1.0M phosphate (pH 7.4) buffer.

Stock solutions:

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.

5. Phosphate buffer, pH 5. 0.15M

A: 0.15M solution of potassium dihydrogen orthophosphate

(20.4 g KH_2PO_4 in 1 l of distilled water)

B: 0.15M solution of disodium hydrogen orthophosphate

(26.7 g $Na_2HPO_4.2H_2O$ in 1 l of distilled water.

Nine hundred and eighty-eight ml of solution A and 12 ml of solution B were mixed and the pH adjusted to 5.0.

6. Phosphate buffer, pH 7.5. 0.15M

A: 0.15M solution of sodium dihydrogen orthophosphate

(23.4 g $NaH_2PO_4.2H_2O$ in 1 l of distilled water)

B: 0.15M solution of disodium hydrogen orthophosphate

(53.7 g $Na_2HPO_4.12H_2O$ in 1 l of distilled water)

One hundred and sixty ml of solution A and 840 ml of solution B were mixed and the pH adjusted to 7.5.

7. Sodium-carbonate/sodium bicarbonate buffer, pH 9.5, 0.05M

(Delory and King, 1945)

A: 0.1M solution of sodium carbonate

(28.62 g $Na_2CO_3.10H_2O$ in 1 l of distilled water)

B: 0.1M solution of sodium bicarbonate

(8.4 g $NaHCO_3$ in 1 l of distilled water)

Forty ml of solution A and 60 ml of solution B were mixed and the pH adjusted to 9.5. This solution was diluted 1:2 to give 0.05M buffer.

8. Bicarbonate buffer pH 10.6

Na_2HCO_3	15 g
K_2CO_3	170 g

Distilled water to 1 l, and the pH adjusted to 10.6 and 0.05% (w/v) sodium azide added.

9. Carbonate-bicarbonate buffer pH 11, 0.2M (Stephen et al., 1966)

A: 0.2M solution of anhydrous sodium carbonate

(21.2 g Na_2CO_3 in 1 l of distilled water)

B: 0.2M solution of sodium bicarbonate

(16.8 g $NaHCO_3$ in 1 l of distilled water)

Four hundred and fifty ml of solution A and 15 ml of solution B were mixed and pH adjusted to 11 and 0.05% (w/v) sodium azide added.

10. Glycine HCl buffer pH 2.2

A: 0.2M glycine solution (15.01 g/l) was adjusted to pH 2.2 with concentrated HCl and 0.05% (w/v) sodium azide added,

11. Barbitone buffer, pH 8.6

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

12. Double strength barbitone buffer, pH 8.6

Barbitone sodium	24.74 g
Calcium lactate	1.54 g
Sodium azide	0.2 g
Distilled water	900 ml

The pH was adjusted to 8.6 and buffer was made up to 1 l with distilled water.

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 $4^{\circ}C$.

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 : 1% copper sulphate ($CuSO_4.5H_2O$) in distilled water.

Reagent C : 2% aqueous solution of potassium sodium tartrate.

Reagent D : Equal volumes of reagent 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 phenophthalein indicator and diluted to give a 1N solution.

APPENDIX V

Immunosorbent

SH titration

Solutions:

MMD	0.078 g/l
Sodium nitroprusside	10% (w/v)
$Na_2[Fe(CN)_5NO], 2H_2O$	in distilled water

The protein solution (0.5 ml) was mixed with 0.5 ml of bicarbonate buffer pH 10.6 (Appendix II) and 2 drops of sodium nitroprusside indicator and was titrated with 0.00025N MMD. The titration was complete when the colour of the solution had changed from pink to yellow.

One ml of 0.00025N MMD is equivalent to 2.5×10^{-7} -SH groups and 1 mg/ml γ-globulin is equal to 6.35×10^{-9} moles. Therefore the number of -SH groups/mole in the immunosorbent preparation were calculated from the following equation:

$$\text{-SH/mole} = \frac{\text{ml MMD} \times 2.5 \times 10^{-7}}{\text{mg/ml } \gamma G \times 6.35 \times 10^{-9}}$$

Crosslinking agent

$K_3Fe(CN)_6$	1.82 g
NH_4Cl	4.86 g
Aqueous NH_3	7.1 ml

Distilled water to 100 ml.

APPENDIX VI

Polyacrylamide gel electrophoresis (Laemmli, 1970)

I. Stock solutions

A. Acrylamide/Bis

Acrylamide	30 g
N,N'-bis-methylene acrylamide	0.8 g

distilled water to 100 ml.

B. Tris-HCl 1.5M, pH 8.8

Tris (hydroxymethyl)aminomethane	18.15 g

distilled water to 50 ml, pH adjusted to 8.8 with 1N HCl and distilled water to 100 ml.

C. 10% SDS

Sodium dodecyl sulphate	10 g

distilled water to 100 ml

D. Tris/HCl, 0.5M, pH 6.8

Tris	6 g

Distilled water to 50 ml, pH adjusted with 1N HCl and distilled water to 100 ml.

E. Running buffer (5X conc) pH 8.3

Glycine	144 g
Tris	30 g
SDS	5 g

Distilled water to 800 ml, pH adjusted and distilled water to 1 l.

F. Ammonium persulphate (10%)

Ammonium persulphate	0.1 g

Distilled water to 1 ml

G. Bromophenol blue (0.1%)

Bromophenol Blue 0.1 g

Dissolved in 100 ml 10^{-4}M sodium azide (6.5 mg/l).

H. TEMED

N,N,N',N'-tetramethylethylenediamine

I. 2-Mercaptoethanol

J. Glycerol

II. Solubilising buffer

	Stock solution	Volume	Final concentration (in sample)
D	Tris/HCl pH 6.8, 0.5M	2.5 ml	0.0625M
C	SDS (10%)	4.0	2%
J	Glycerol	2.0	10%
I	β-mercaptoethanol	1.0	5%
G	Bromophenol blue (0.1%)	0.2	0.001%
	H_2O	0.3	
		10 ml	

III. Separating gel - 10%

	Stock solution	Volume	Final concentration
A	Acrylamide/Bis	13.3 ml	10%
B	Tris/HCl, pH 8.5	10.0	0.375M
C	SDS (10%)	0.4	0.1%
H	TEMED	0.04	0.1%
F	Ammonium persulphate	0.4	0.1%
	H_2O	15.8	
		40 ml	

IV. Stacking gel

	Stock solution	Volume	Final concentration
A	Acrylamide/bis	2 ml	6%
D	Tris HCl, pH 6.8	2.5	0.125M
C	SDS (10%)	0.1	0.1%
H	TEMED	0.01	0.1%
F	Ammonium persulphate	0.1	0.1%
	H_2O	5.3	
		10 ml	

V. Staining solution

Coomassie Brilliant Blue - see Appendix IX

VI. Destaining solution

Acetic acid	75 ml
Methanol	50 ml
Water	875 ml

APPENDIX VII

Polyacryalmide gels for isoelectric focusing

Solutions

29.1% (w/v) Acrylamide	5 ml
0.9% (w/v) Bis	5 ml
Ampholines (pH 3.5-10)	0.5 ml
Ampholines (pH 3-5)	0.2 ml
Distilled water	18.3 ml
Sucrose	3.5 g
TEMED	20 µl
0.002% (w/v) Riboflavin	0.2 ml
Urea	16.2 g
Nonidet-P40	0.6 ml

The solutions were mixed in the order above and, after thorough degassing under reduced pressure, were polymerised between glass plates, separated by gaskets, using a fluorescent light source. After polymerisation, the top plate was gently removed and the bottom plate, containing the gel, was placed on the LKB 2117 Multiphor apparatus.

Staining of gels

Stain. Coomassie Brilliant Blue G250, Seva, (0.4 g) was dissolved in 200 ml distilled water and mixed with an equal volume of 2N H_2SO_4. The solution was left at room temperature for 3 h after which time it was filtered through Whatman No. 1 filter paper and the volume of the filtrate measured. Potassium hydroxide (10N) was added in a volume 1/9 of the filtrate volume and 100% (w/v) TCA was added to give a final concentration of 12%.

The gels were stained overnight and destained in tap water with frequent changing of the water.

APPENDIX VIII

Freund's Complete Adjuvant

Mycobacterium tuberculosis (Stewart-Tull and White, 1967)	4 mg
Bayol F	17 ml
Arlacel A	3 ml

The M. tuberculosis was mixed with the Bayol F using an MSE 100 watt Ultrasonic disintegrator. The arlacel A was then added and the suspension re-sonicated.

APPENDIX IX

Stains

Bromophenol blue

Bromophenol blue	10 mg
Distilled water	100 ml

Coomassie Brilliant Blue (Weber and Osborn, 1969)

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

APPENDIX X

Binding of δ-haemolysin to sheep erythrocytes

Solutions

Tannic acid

PBS

Borate succinate buffer

36% aqueous formaldehyde

Delta-haemolysin (2 mg/ml) heated at 100°C for 10 h

Sheep erythrocytes (fresh)

Borate-succinate buffer, pH 7.5, 0.15M

Solution A: 0.05M solution of sodium tetraborate

(19.0 g $Na_2B_4O_7.H_2O$ dissolved in 1 l distilled water)

Solution B: succinic acid, 0.05M

(5.9 g dissolved in 1 l distilled water)

Sodium chloride

Horse serum (heat inactivated at 56°C for 45 min)

One l of A was mixed with solution B until pH 7.5 was reached. Sodium chloride was added to 0.14M and solution was mixed with 1% horse serum (final concentration).

Sheep blood was washed three times in PBS and the final suspension was adjusted to 4%. Fifty ml of PBS, containing 2.5 mg of tannic acid, was mixed with 50 ml of the 4% erythrocyte suspension and incubated at 37°C for 15 min. The erythrocytes were collected by gentle centrifugation (to prevent agglutination) at 100 g for 20 min and divided into two aliquots. Each aliquot was washed in PBS and one was used for antigen coating and the other was used as control cells. The test cells were resuspended in 10 ml PBS, mixed with 10 ml of δ-haemolysin (2 mg/ml)

and incubated at 37°C for 30 min. Cells were collected by centrifugation (100 g, 20 min) and resuspended in 20 ml borate-succinate buffer. Control cells were also suspended in 20 ml borate-succinate buffer. Two ml of 36% formaldehyde was added to both cell suspensions, while stirring, over a period of 30 min and the cells were allowed to settle overnight at 4°C. A further 2 ml of formaldehyde was added and the cells allowed to re-settle at 4°C overnight. The supernatant was decanted off and the cells were resuspended in borate-succinate buffer with vigorous shaking. The cells were allowed to settle overnight and, after a final washing in borate-succinate buffer, the suspensions were adjusted to 1% (v/v) and 0.2% formaldehyde (final concentration) was added as a preservative. The cells were stored at 4°C.

APPENDIX XI

Statistical Analysis of Table 18

The results of Table 18 were analysed by the chi-square test using the Yates correction for continuity.

	Non-Responders	Responders	Total
Untreated haemolysin	a	b	a + b
Treated haemolysin	c	d	c + d
Total	a + c	b + d	N

$$\chi^2 = \frac{N\,(|ad - bc| - n/2)^2}{(a+b)\,(c+d)(a+c)(b+d)}$$

There is one degree of freedom and tabulated chi-square values at 1 degree of freedom are:-

3.84 ; $P = 0.05$

6.63 : $P = 0.01$

7.88 = $P = 0.005$

(i) Analysis of native / F7.5 results

	Non-Responders	Responders	Total
Native	62	18	80
F7.5	50	33	83
Total	112	51	163

$$\therefore \quad \chi^2 = \frac{163(2046 - 900 - 81.5)^2}{80 \times 83 \times 112 \times 51}$$

$$= \underline{4.9}$$

Calculated χ^2 value = 4.9 which is greater than 3.84 and therefore the variance between the number of responding mice to native and to F7.5

haemolysin is significant at the 5% probability level.

(ii) Analysis of native/F5 results

	Non-Responders	Responders	Total
Native	62	18	80
F5	29	47	76
Total	91	65	156

$$\therefore \quad x^2 = \frac{156(2914 - 522 - 78)^2}{80 \times 76 \times 91 \times 65}$$

$$= 23.2$$

This value is much greater than 7.88 and therefore the variance between the number of responding mice to native or F5-haemolysin is highly significant at the 0.5% probability level.

The Reaction of Formaldehyde with Staphylococcal Delta-Haemolysin

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Staphylococcal δ-haemolysin is cytolytic towards a wide range of different cell types (1), elevates cyclic AMP levels and inhibits water adsorption in the guinea pig ileum and causes increased vascular permeability in the skin (2,3). Delta-haemolysin also binds to phospholipids (4) and lipoproteins (5). We have investigated the reaction of formaldehyde with δ-haemolysin to form 'toxoids' for the immunisation of mice and to determine whether some of the above properties of δ-haemolysin are mediated by common mechanisms.

Delta-haemolysin was treated with formaldehyde at pH 5, pH 7.5 or pH 9.5 for up to 7 days at 37°C; haemolytic activity was lost rapidly at pH 5 or pH 7.5 but more slowly at pH 9.5. All HCHO-treated preparations had an increased electrophoretic mobility at pH 8.6 with a concommitant decrease in isoelectric point, but soluble polymers of the basic subunit of δ-haemolysin were not detected by SDS-polyacrylamide gel electrophoresis. Delta-haemolysin treated with formaldehyde retained its antigenicity assessed by counter-immunoelectrophoresis and gel diffusion. This precipitinogenic activity of HCHO-treated δ-haemolysin was not inhibited by lecithin or destroyed by trypsin. Formaldehyde treatment also reduced (or abolished) the ability of δ-haemolysin to bind to erythrocytes. Haemolysin treated with HCHO for 7 days at pH 5 or pH 7.5 lost all detectable haemolytic activity but still induced increased vascular permeability in rabbit skin although to a lesser degree than native δ-haemolysin.

Native δ-haemolysin was poorly immunogenic in the mouse. However, on reaction with HCHO at pH 5, the immunogenicity of δ-haemolysin was enhanced. Antisera to HCHO-treated haemolysin reacted with both native and HCHO-treated haemolysin on counter-immunoelectrophoresis. The enhanced immunogenicity of δ-haemolysin following reaction with HCHO may result from the reduced affinity for phospholipids rather than by polymerisation (6).

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