THE ASSAY OF STAPHYLOCOCCAL DELTA-HAEMOLYSIN WITH FISH ERYTHROCYTES

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SUMMARY

The purpose of this thesis was to investigate the suitability of cod and other fish erythrocytes for the assay of staphylococcal δ-haemolysin. The investigation adopted two broad approaches: (1) determining conditions for storage of fish erythrocytes, (2) the development of an assay system using fish erythrocytes as an indicator of haemolysis.

The preservation of erythrocytes from cod (Gadus morhua), saithe (Pollachius virens) and mackerel (Scomber scombrus) was studied using diemthyl sulphoxide (DMSO) as a protective additive. Equal volumes of DMSO solution (15-30% in citrate dextrose saline) and citrated blood were mixed, rapidly frozen and stored in ampoules in liquid nitrogen. The optimum concentration of DMSO was 12.5% and erythrocyte recoveries of greater than 90% were obtained from all species after storage for up to five months. Larger quantities of blood were stored by removal of plasma from citrated blood prior to addition of DMSO solution, and by storage of pelleted frozen blood in aluminium canisters in liquid nitrogen. Maximum recoveries of washed intact erythrocytes required thawing of pellets in 12.5% DMSO solution and washing with citrate/dextrose/saline solutions containing decreasing concentrations of DMSO. Washed erythrocytes could be kept at 4^{o} for at least two days without appreciable haemolysis and were morphologically similar to fresh erythrocytes and equally susceptible to staphylococcal δ-haemolysin.

The δ-haemolysin of Staphylococcus aureus strain NCTC 10345 was purified by absorption to and selective elution from hydroxylapatite and, after dialysis against phosphate buffered saline, concentrated by ultra-filtration.

On polyacrylamide disc gel electrophoresis purified δ-haemolysin gave a single broad band in acid gels (separation at pH 4.3) and two bands in

alkaline gels (separation at pH 9.3). After isoelectric focusing, all haemolytic activity was associated with a single component of isoelectric point 4.5. No α- or β-haemolysin was detectable in the preparation.

Haemolysis of fish erythrocyte by δ-haemolysin occurred rapidly and haemolysin titre was independent of incubation temperature over the range 0^{o} to 37^{o}. Activity was not affected by 2-mercaptoethanol, and with blood from twelve individual cod and five saithe no significant individual variation was found in erythrocyte sensitivity. Incubation of constant amounts of δ-haemolysin with varying concentrations of human, cod or mackerel cells showed that erythrocytes of different species did not respond to δ-haemolysin in the same way. With fish erythrocytes, the percentage cell lysis was a linear function of log erythrocyte concentration whereas for human erythrocytes a curve was obtained.

Fish erythrocytes were incorporated into agar for assay of δ-haemolysin by eight strains of staphylococci. As the fish erythrocytes tested were thermolabile at 37^{o}, staphylococci were inoculated onto nutrient agar plates, incubated in air for 24 hr at 37^{o} and the plates overlaid with blood agar. In all cases zones of haemolysis were observed after 24 hr at 4^{o} which were larger than those obtained on human or horse blood agar. Incubation of the nutrient agar plates in an atmosphere of 25% CO_2 rather than air yielded smaller zones of haemolysis with all strains in cod or human blood agar but larger zones on sheep blood agar.

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ABBREVIATIONS

BSS	basal salt solution
CDS	citrate/dextrose/salt
cm	centimetre
CM-	carboxymethyl-
DEAE-	diethylaminoethyl-
DMSO	dimethylsulphoxide
E	extinction coefficient
EDTA	ethylenediaminetetra-acetate
g	gram
hr	hour
HU	haemolytic unit
kg	kilogram
M	molar
ma	milliamp
mg	milligram
mm	millimetre
µg	microgram
µl	microlitre
NCTC	National Collection of Type Cultures
PB	phosphate buffer
PBS	phosphate-buffered saline
p.s.i.	pounds per square inch
pI	isoelectric point
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TEAE-	triethylaminoethyl-
UV	ultraviolet
V	volt
v/v	volume for volume

INTRODUCTION

Since Sir Alexander Ogston (1882) introduced the name "staphylococcus" for a cluster-forming coccus which he considered responsible for certain abscesses in man, much progress has been made in isolating and purifying the extracellular products of staphylococci as an aid to understanding the mechanisms of staphylococcal pathogenicity.

For reviews on haemolysins (toxins), see Bernheimer (1965, 1968, 1970), Gladstone (1966), Arbuthnott (1970), Wiseman (1970), Jeljaszewicz (1967, 1972) and Kwarecki et al. (1972); for reviews on some of the diffusible enzymes including staphylocoagulase, hyaluronidase, phosphatase and nuclease, see Abramson (1972). Other factors such as enterotoxins are discussed by Bergdoll (1967, 1970, 1972) and leucocidin by Woodin (1970, 1972). Farrer & McLeod (1960) and Shulman & Nahmias (1972), have dealt with clinical aspects of staphylococcal infections.

The Pathogenicity of Staphylococcus aureus

S. aureus, which is present in the nose and on the skin of a high proportion of healthy people, is capable of initiating a wide variety of acute infections. It is most liable to infect new-born infants, surgical patients, old and malnourished persons and patients with diabetes or other chronic and debilitating diseases.

It is generally agreed that staphylococcal infections can be facilitated by damage to skin (e.g. burns, wounds, primary skin diseases), defects in leucocyte function (e.g. in phagocytosis, chemotaxis or intracellular bacterial killing), prior viral infections (e.g. influenza, measles), presence of foreign bodies (e.g. intravenous catheters, sutures) and the alteration of host defence mechanisms of bacterial interference by antibiotics to which the infecting S. aureus is not susceptible.

Shulman & Nahmias (1972) divided staphylococcal infections into two major groups:

(1) infections occurring locally or involving contiguous sites, e.g. skin, eye, nose and throat, gastrointestinal tract, urethra and vagina:

(2) infections which are spread primarily via the bloodstream, giving metastatic sites in bones and joints, lungs, skin, muscle, heart, kidney or central nervous system.

Fortunately, S. aureus usually only causes local skin infections; few individuals get serious local or contiguous infections and even fewer develop bacteraemia with subsequent death.

Staphylococcal infection of the skin is perhaps the most common of all bacterial infections. It ranges from folliculitis to more severe forms such as boils, hidradenitis suppurativa and carbuncles which involve the subcutaneous tissues in addition to the follicle.

In new-born infants, impitigenous lesions or T.E.N. (Toxic Epidermal Necrolysis) of Ritter's type (also known as Lyell's disease or "scalded skin" syndrome) are among the other skin manifestations of S. aureus infection. Impitigenous lesions, which occur frequently, may be caused by either S. aureus or group A streptococci alone or by both, and such infections are difficult to differentiate clinically. The Ritter's type "T.E.N." has recently been shown to be caused by phage group II staphylococci and is particularly associated with phage type 71 (Jefferson, 1967; Lyell, Dick & Alexander, 1969; Arbuthnott et al., 1969, 1971, 1972; Melish & Glasgow, 1970). A soluble extracellular epidermolytic toxin containing no detectable haemolytic activity has been isolated and demonstrated in vivo to cause extensive splitting of the epidermis in newborn mice (Arbuthnott et al., 1971). Apart from the enterotoxic strains

of staphylococci this is at present the only instance in which a soluble product of staphylococci has been unequivocally shown to play a role in staphylococcal pathogenicity.

In addition to this recently observed epidermolytic toxin, S. aureus strains from human and animal origin also produce, in vitro, a large number of extracellular, enzymic or toxic factors including staphylocoagulase, leucocidin, enterotoxins, hyaluronidase, phosphatase, nuclease, penicillinase, proteases, lipase, catalase, lactate dehydrogenase and lysozyme. Also there are various haemolysins (cytolytic toxins) which are believed to play a role in the infected host. Many attempts have been made to correlate staphylococcal virulence with these factors but in spite of the several toxins and enzymes which have been identified there is little clear understanding of virulence in chemical terms. Indeed the existence of an unidentified "virulence factor" produced only in vivo has been postulated by Abramson (1972). However, the potential importance of these enzymes and toxins in staphylococcal pathogenicity should not be disregarded - certainly not the haemolysins, some of which have a broad spectrum of cytolytic activities. In this thesis only the haemolysins will be considered in any detail.

Staphylococcal haemolysins

It is now established that at least four different haemolysins, (α-, β-, γ- and δ-haemolysins) are produced by staphylococci. The α- and δ-haemolysins are more frequently produced by coagulase-positive strains of human origin whereas β-haemolysin is produced principally by strains from animals (Elek & Levy, 1950). Alpha-, β- and δ-haemolysins are the only toxic factors of S. aureus which have been purified and characterized. The physical properties of γ-lysin were described by Möllby & Wadström (1971) who reported

it to be a protein with a pI of 9.5. A further haemolysin (ε-) has been postulated (Elek & 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, ε-haemolysin was later demonstrated to be identical with δ-lysin by Kleck & Donahue (1968).

Many early reports of haemolysins, particularly with respect to biological properties, are difficult to interpret because the preparations were of unknown purity. The following sections on the physical form and haemolytic activity of α-, β- and δ-haemolysin therefore deal only with reports of the last dozen years.

Alpha-haemolysin

Physical properties. It appears that α-haemolysin can exist in three physical states:

(1) biologically active 3S soluble lysin - α3S (Bernheimer & Schwartz, 1963; Lominski, Arbuthnott & Spence, 1963; Cooper, Madoff & Weinstein, 1966; Coulter, 1966)

(2) biologically inactive 12S soluble lysin - α12S (Bernheimer & Schwartz, 1963; Lominski *et al.*, 1963)

(3) biologically inactive insoluble lysin (Coulter, 1966; Arbuthnott, Freer & Bernheimer, 1967).

The 12S and insoluble form of α-lysin were found to possess little or no haemolytic or toxic properties but were capable of inducing specific antibody and hence can be considered as toxoids. They are assumed to be aggregates of the active α3S, as Arbuthnott *et al.* (1967) disaggregated these two inactive forms with 8 M urea to give a product which possessed similar physical and biological properties to native 3S α-lysin.

Further evidence of the ability of α-haemolysin to aggregate was provided by electron microscopic examination of negatively-stained purified α12S component which showed a uniform appearance of 10.0 nm diameter rings containing a hexagonal arrangement of six subunits with a diameter of 2.0 to 2.5 nm (Bernheimer & Schwartz, 1963). Freer, Arbuthnott & Bernheimer (1968) also observed ring-like structures on the surface of artificial lipid spherules and on human or rabbit erythrocyte ghosts after treatment with 3S α-lysin. Arbuthnott et al. (1967) suggested that the formation of aggregates probably depends on several factors including purity, concentration, pH, ionic strength and temperature. Heat inactivated (60^{o}, 1 min) α-haemolysin preparations have been subsequently reactivated by treatment with 8 M urea (Arbuthnott et al., 1967).

The characteristic biologically active α3S form is heterogeneous in molecular weight and electrophoretic mobility.

The molecular weight of this haemolysin is controversial (Table 1). Bernheimer (1968) proposed several forms with molecular weights ranging from 21,200 to 50,000 probably due to differing states of aggregation. Previously, Madoff (1965) and Coulter (1966) postulated that the lysin associates to give several polymeric forms in equilibrium. Hence it is possible to understand the reported molecular weights by assuming that the lysin exists both as a monomer of molecular weight 22,000 and a dimer of 44,000 and that estimates around 30,000 might represent mixtures of the two (McNiven, 1973).

Bernheimer & Schwartz (1963) demonstrated four distinct peaks (α_a - α_d) in sucrose density-gradient electrophoretic analysis of α-haemolysin. Similar reports of components having different electrophoretic mobilities were also made by Madoff & Weinstein (1962) and Kitamura, Shelton & Thal (1964). Isoelectrofocusing has provided further confirmation of the multiple forms of

Table 1. Molecular weights of Staphylococcal α-haemolysin

Method	Molecular weight	Reference
Sedimentation equilibrium	44,000	Bernheimer & Schwartz, (1963)
Sedimentation equilibrium	21,000	Coulter (1966)
Sedimentation velocity	29,600	Coulter (1966)
Gel filtration	41,000	Bernheimer & Grushoff (1967)
SDS gel electrophoresis	34,000	Bernheimer & Shapiro
Gel filtration	33,000	Bernheimer & Lamb (1971)
SDS gel electrophoresis	36,000	McNiven (1973)

α-haemolysin; Wadström (1968) and Bernheimer (1970) separated several components with haemolytic activity against rabbit erythrocytes (Table 2) but the reported isoelectric points disagree. The most conclusive evidence of molecular heterogeneity was provided by McNiven (1973) who observed four α-haemolytic components in purified preparations (Table 2). The α-haemolytic properties of each component were confirmed by neutralisation studies, haemolytic spectrum, disc gel electrophoresis and refocussing in the presence or absence of urea.

Haemolytic spectrum. The haemolytic properties of α-haemolysin are well defined despite the difficulty of direct comparisons of data from different laboratories due to the different titration techniques and to the different susceptibility of erythrocytes from different individuals of the same species - this latter may vary by up to fivefold with the same batch of haemolysin (Bernheimer & Schwartz, 1963; Bernheimer, 1965; Cooper et al., 1966). It is generally agreed that rabbit erythrocytes are highly susceptible while those from other species are less so. Human erythrocytes, which are susceptible to δ-haemolysin, are particularly resistant to α-haemolysin (Table 3).

The susceptibility of fish erythrocyte to α-haemolysin has not been systematically studied although it was reported that α-haemolysin was active against dogfish erythrocytes (Hunter, Bullock & Rawley, 1949). However, this work is in doubt since crude preparations of haemolysin, possibly contaminated with δ-haemolysin, were used. Birkbeck & Arbuthnott (unpublished, 1972) observed that cod erythrocytes were resistant to α-haemolysin.

Mode of action. The "split titration" experiments of Lominski & Arbuthnott (1963) showed that the rate of haemolysis was directly proportional to the concentration of haemolysin at low concentration (2.5 MHD/ml to 160 MHD/ml).

Table 2. Isoelectric points of α-haemolysin components separated by isoelectric focusing

Strain (and investigator)	Component	Isoelectric point (pI)
Wood 46 (Wadström, 1968)	αII	7.1
	αI	8.1
	αI	8.3
	αI	8.7
Wood 46 (Bernheimer, 1970)	αIII	4.5 - 5.5
	αII	6.5 - 7.5
	αIa	8.0 - 8.7
	αIb	9.1 - 9.2
Wood 46 (McNiven, 1973)	αD	6.26 ± 0.11
	αC	7.36 ± 0.03
	αa	8.55 ± 0.12
	αb	9.15 ± 0.07
V8 (Wadström, 1968)	αIII	5.0
	αII	7.0
	αI	8.6

Table 3. Relative sensitivity of erythrocytes from different species to staphylococcal α-haemolysin*

Species	Haemolytic activity compared to that of rabbit (%)
Rabbit	100
Wallaby	20
Hamster	11 - 27
Dog	10 - 25
Rat	10
Mouse	9
Cat	9
Deer	5
Wood duck	4
Bear	1
Sheep	0.6 - 1.0
Human	0 - 0.8
Chicken	0 - 0.5
Guinea pig	0 - 0.1
Horse	0 - 0.06
Monkey	0

* From Bernheimer (1968)

Kinetic studies by Bernheimer (1970) on the effect of constant amounts of α-haemolysin on varying concentrations of erythrocytes also favoured an enzymatic action by α-haemolysin.

According to fluorescence observations (Klainer et al., 1964) and kinetic studies (Marucci, 1963; Madoff, Cooper & Weinstein, 1964) of the haemolysis of rabbit erythrocytes three steps are involved in lysis. The first two, reversible, steps occurred in the pre-lytic phase: (1) rapid adsorption of α-lysin on to the erythrocyte surface; (2) reaction of α-lysin with the erythrocyte membrane resulting in loss of selective permeability as indicated by leakage of potassium ions; (3) lysis of the damaged erythrocytes. The addition of antitoxin in the early, pre-lytic phase prevented lysis, and osmotic stabilizers (sucrose and polyethylene glycol) protected the irreversibly-damaged erythrocyte membrane from osmotic shock and thus prevented lysis (Cooper, Madoff & Weinstein, 1964).

Recently, Wiseman, Caird & Fackrell (1975) proposed that α-haemolysin was produced as a zymogen which could be converted to an active toxin by trypsin or activating enzymes at the erythrocyte surface. Protoxin activated by trypsin coupled to carboxymethyl cellulose acquired proteolytic activity as indicated by hydrolysis of tosyl-arginine methyl ester, and the N-terminal amino acid of active toxin.

Although the above work and previous kinetic studies of α-lysin suggested an enzymatic action, the substrate in the erythrocyte membrane has not been identified and the nature of the complicated interaction between α-lysin and cell membrane is not fully understood.

Beta-haemolysin

Physical properties. Beta-haemolysin was recognized as being serologically distinct from α-haemolysin by Glenny & Stevens (1935). Like α-haemolysin, multiple forms have been reported by various investigators using different strains.

Thaysen (1958) described a β_2-lysin in the culture filtrates of S. aureus isolated from furunculosis in dogs and showed that it differed from normal β-lysin in antigenic properties and kinetics of haemolysis. More recently, Maheswaran, Smith & Lindorfer (1967) obtained a major "cationic" and a minor "anionic" β-lysin on both DEAE-cellulose and carboxymethylcellulose chromatography, confirming the previous reports of Hague & Baldwin (1963). Chesbro et al. (1965), Doery et al. (1965) and Wiseman & Caird (1967) also detected two antigenic components in their purified preparations of β-haemolysin.

Wadström (1968) purified β-haemolysin from S. aureus strain R-1 by isoelectrofocusing and chromatography with CM-Sephadex C-25 and Biogel-P-10 and also reported a major cationic (pI 9.4 ± 0.1) and a minor anionic form (pI 3 ± 0.4) in isoelectrofocusing. However, in the presence of 6 M urea little of the anionic component was found. Wadström suggested that the cationic β-lysin might form aggregates spontaneously with acidic components as previously reported for another basic protein, egg-white lysozyme.

An approximate molecular weight of 59,000 was calculated by sucrose-gradient centrifugation (Chesbro et al., 1965). Gow & Robinson (1969) showed that α- and β-haemolysins were eluted together on Sephadex G-100 chromatography indicating that they had similar molecular weights. Wadström & Möllby (1971), also using Sephadex G-100, found a molecular weight of only 38,000. Moreover, neither of the above molecular weight estimates agreed with the reported

sedimentation coefficient of 1.7 (Gow & Robinson, 1969). A summary of the reported physical properties of β-haemolysin is presented in Table 4.

Haemolytic activity. The characteristic "hot-cold" haemolytic reaction - the enhanced haemolytic activity of this haemolysin if incubation at 37^{o} is followed by incubation at 4^{o} or room temperature - was first described by Bigger, Bolland & O'Meara (1927). This "hot-cold" reaction of β-haemolysin was also observed in blood agar plates in which the zone of darkened erythrocytes surrounding the β-toxigenic S. aureus colony progressed to haemolysis if the plates were subsequently cooled. The mechanism of "hot-cold" reaction is not fully understood.

Wiseman (1965) demonstrated the high susceptibility of sheep, ox and human cells to β-haemolysin from the R-1 and 252F strains of S. aureus (Table 5) although Haque & Baldwin (1964) were unable to detect lysis of human and rabbit erythrocytes by β-haemolysin from their Paris strain. Haemolytic activity is greatly enhanced by Mg^{+2}, Mn^{2+} and Co^{2+} ions at concentrations as low as 0.001 M and is inhibited by EDTA and citrate (Jackson & Mayman, 1958; Wiseman, 1965).

Mode of action. Doery et al. (1963) in a study of 10 strains of S. aureus producing α- and β-haemolysins, found that all hydrolysed sphingomyelin, a common constituent of cell membranes, to yield N-acylsphingosine and phosphorylcholine. Wiseman & Caird (1966, 1967), Maheswaran & Lindorfer (1966, 1967) and Wadström & Möllby (1972) confirmed the phospholipase activity of β-haemolysin. Thus the high level of sphingomyelin in the sheep and ox erythrocyte cell membrane may well explain their high susceptibility to the action of β-haemolysin (Wiseman & Caird, 1967; Maheswaran & Lindorfer, 1967; Wadström & Möllby, 1972). The sphingomyelinase activity of β-haemolysin has led to its use as an agent for the study of membrane structure (Low, Freer & Arbuthnott, 1974).

Table 4. Physical properties of staphylococcal β-haemolysin

Molecular weight	Sedimentation coefficient (S_{20w})	Isoelectric point (pI)	References
59,000 (Ultracentrifugation)		8.6 - 8.9	Chesbro et al. (1965)
		10.1	Wadström (1968)
	1.7		Gow & Robinson (1969)
		9.5	Maheswaran & Lindorfer (1971)
38,000 (gel filtration)		9.4	Wadström & Mollby (1971)

Table 5. Susceptibility of erythrocytes from different animal species to staphylococcal β-haemolysin*

Erythrocyte species	Haemolytic activity compared to that of sheep (%)
Sheep	100
Ox	25
Man	12.5
Cat	3
Rabbit	3
Pig	1.5
Fowl	0.19
Rat	0.19
Mouse	0.19
Frog	0.19
Dog	0.19
Guinea Pig	0.19
Horse	0.19

Delta-haemolysin

Purification and physicochemical properties. Williams & Harper (1947) demonstrated the existence of δ-haemolysin as a separate staphylococcal haemolysin characterised by a wide spectrum of haemolytic activity and having a synergistic action with β-haemolysin on sheep erythrocytes. These findings were confirmed by Marks & Vaughan (1950) who partially purified the haemolysin by extraction of crude culture supernatants with ethanol. The haemolysin was soluble in ethanol, insoluble in acetone, non-dialysable, thermostable (100^{o}/2 hours), inactivated by formaldehyde and absorbed by alumina below pH 8.

A crystalline preparation of δ-haemolysin was obtained by Yoshida (1963) from S. aureus strain Foggie by chromatography of concentrated, heated culture supernatants on calcium phosphate gel and TEAE-cellulose. The haemolytic fraction was lyophilised and recrystallised 2 - 3 times by dissolution in Tris buffer and dialysis against water. Ultracentrifugation showed the preparation to be highly homogeneous (sedimentation coefficient 6.1S) and from amino acid analysis a molecular weight of 68,230 was calculated. Lysine, aspartic acid, phenylalanine and isoleucine were the amino acids present in greatest amount; cystine was absent and tyrosine, proline, histidine and arginine were detected as minor components (Table 6). Purified δ-haemolysin was trypsin-sensitive, and, in contrast to crude haemolysin preparations, was quite heat sensitive and insoluble in chloroform/methanol (2:1). However, further study by Gladstone & Yoshida (1967) showed this preparation to be contaminated with ribonuclease.

Caird & Wiseman (1970) purified the δ-haemolysin of strain E-delta by isoelectric precipitation (pH 4.0), ammonium sulphate precipitation and DEAE-cellulose chromatography. Purified haemolysin obtained in 16% yield with

Table 6. 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, Temple & Shaw (1972)
Lysine	16.55	16.66	13.6	16.2
Histidine	0.41	-	tr	-
Arginine	0.94	-	-	-
Aspartic acid	12.53	13.46	10.9	13.6
Threonine	6.98	8.02	7.5	9.4
Serine	5.12	2.46	3.2	4.3
Glutamic acid	7.99	4.90	5.2	4.6
Proline	0.45	-	-	-
Glycine	3.73	3.60	8.1	6.2
Alanine	4.06	3.09	5.9	4.8
Half cystine	-	0.09	-	-
Valine	4.55	6.64	5.5	6.7
Methionine	4.88	4.56	4.3	4.0
Isoleucine	9.71	18.17	10.5	17.1
Leucine	7.41	6.04	6.3	4.9
Tyrosine	1.68	-	-	-
Phenylalanine	10.4	7.93	7.6	5.5
Trytophan	2.61	3.30	4.0	2.5
Ammonia	1.63	1.08	7.4	

*Percentages calculated from the data presented, assuming 4% tryptophan

a 32-fold increase in specific activity, yielded a single precipitation line on immunodiffusion or immunoelectrophoresis against antiserum to δ-haemolysin (vide infra) and the sole N-terminal amino acid was proline. A single protein band was noted on polyacrylamide gel electrophoresis (pH 9.5) but ultra-centrifugation revealed two components (2.8S and 9.8S).

A mutant of S. aureus strain Wood 46, which was deficient in α-haemolysin production (W46M), was used by Kreger et al. (1971) who purified δ-haemolysin by adsorption to hydroxylapatite and elution with phosphate buffers of increasing molarity. On dialysis against water, both soluble and insoluble forms of δ-haemolysin were recovered. 'Soluble' haemolysin was soluble in water, various buffer solutions, chloroform-methanol (2:1) but insoluble in chloroform, methanol, acetone or ether. The haemolysin was shown to be molecularly heterogeneous by gel filtration, sucrose density gradient centrifugation, ion exchange chromatography, polyacrylamide gel electrophoresis and isoelectric focussing. Two bands were obtained on polyacrylamide gel electrophoresis at pH 4.3 and at pH 9.5 and on isoelectric focusing two protein peaks (pI 9.5 and 5.0) corresponding to the peaks of haemolytic activity were found. Refocussing of the basic haemolysin yielded additional acidic haemolysin but this did not yield basic haemolysin on refocussing. Electron microscopy of fractions of purified haemolysin showed that two forms were present, first a fibrous, high molecular weight, 11.9S fraction of pI 5.0 and second a granular lower molecular weight fraction, 4.9S of pI 9.5. The amino acid composition was similar to that reported by Yoshida (1963), although histidine, arginine, proline and tyrosine were not detected (Table 6).

The solubility of δ-haemolysin in 2:1 chloroform-methanol was exploited by Heatley (1971) who obtained pure preparations from strain 186X by ammonium sulphate precipitation, chloroform-methanol extraction and cyclic

transfer between organic and aqueous phases of a two-phase system (chloroform-methanol-water) by adjustment of the pH. Purified haemolysin was soluble in water, saline or chloroform-methanol (2:1), sparingly soluble in methanol and insoluble in chloroform, acetone or hexane. A single, polydisperse peak (4.9S) was observed on ultracentrifugation, while polyacrylamide gel electrophoreses at pH 4.6 or pH 8.4 showed a main diffuse band with a faint diffuse secondary band. The amino acid composition was similar to that given by Kreger et al. (1971).

Using the S. aureus strain W46M, Kantor et al. (1972) achieved a 20-fold purification of δ-haemolysin by adsorption to aluminium hydroxide gel and elution with 0.5 M phosphate buffer pH 7.2. When examined by polyacrylamide gel electrophoresis, isoelectric focusing in the presence of 0.1% Tween 80 and gel filtration in 6 M guanidine hydrochloride, the preparation was homogeneous. By gel filtration and sedimentation velocity experiments in the presence of increasing amounts of Tween 80, Kantor et al. (1972) concluded that δ-haemolysin consisted of a basic subunit of molecular weight 21,000 daltons composed of individual polypeptide chains of molecular weight 5100-5200 daltons. Pentameric and decameric association of the basic subunit was assumed to yield polymers of molecular weights consistent with those determined in the absence of Tween 80 by sucrose gradient centrifugation, analytical ultracentrifugation and gel filtration. The amino acid analysis (Table 6) was consistent with a molecular weight of 5200 daltons and tryptic digestion, yielded 7 - 8 peptides, also in agreement with the determined seven lysine residues.

The model proposed by Kantor et al. (1972) provides a reasonable explanation for the conflicting published data on molecular weight, sedimentation coefficient, isoelectric point and heterogeneity of purified δ-haemolysin (Table 7).

Table 7. Molecular weight estimates and sedimentation coefficients of staphylococcal δ-haemolysin

Method	$S_{20,w}$	Molecular weight	Reference
Sedimentation velocity	6.1	74,000	Yoshida (1963)
Amino acid analysis		68,230	
Sedimentation equilibrium		72,000 - 150,000	
Sedimentation velocity	5.5		Kayser & Raynaud (1965)
Sedimentation velocity	1.4	12,000	Kayser & Raynaud (1965)
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 centrifugation (PBS)	6.19	191,000 61,850 (crude) 102,500 (purified)	"
Sucrose gradient centrifugation (0.1% Tween 80)		21,000	"
Sucrose gradient centrifugation (1% Tween 80)		<10,000	"
SDS gel electrophoresis		<10,000	"
Gel filtration (PBS)		195,000	"
(6M guanidine HCl)		5,200	
Amino acid analysis		5,100	"

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

Haemolytic spectrum. Delta-haemolysin, in contrast to α- and β-haemolysins, has a wide haemolytic spectrum, and Gladstone (1966) stated that all erythrocyte species tested were susceptible. Human erythrocytes have generally been found most sensitive (Table 8). Synergistic action between β- and δ-haemolysins has been shown with sheep erythrocytes (Williams & Harper, 1947; Marks & Vaughan, 1950; Kreger et al., 1971) and human erythrocytes (Heatley, 1971). Kapral (1972) demonstrated synergistic action between α- and δ-haemolysins on rabbit erythrocytes. Electron micrographs have also shown interaction of the two haemolysins to yield rectilinear arrays on the erythrocyte membrane (Bernheimer et al., 1972).

Lysis of other cells. Tissue culture cells (Gladstone & Yoshida, 1967; Hallander & Bengtsson, 1967; Thelestam, Möllby & Wadström, 1973) leucocytes (Jackson & Little, 1957; Gladstone & Yoshida, 1967; Hallander & Bengtsson, 1967) and bacterial protoplasts and spheroplasts (Bernheimer, Avigad & Grushoff, 1968; Kayser, 1968; Kreger et al., 1971) were lysed by δ-haemolysin. Cellular organelles (lysosomes and mitochondria) (Bernheimer & Schwarz, 1964; Gladstone & Yoshida, 1967; Evans & Leck, 1969; Kantor et al., 1972; Kreger et al., 1971; Rahal, 1972) and lipid spherules (Freer et al., 1968; Kreger et al., 1971) were also disrupted by this haemolysin.

Table 8. Relative sensitivity to δ-haemolysin of erythrocytes of different animal species

Species	Haemolytic activity compared to that of human (%)			
	Wiseman (1970) strain Newman	Wiseman (1970) strain E-delta	Kreger et al. (1971) strain W46M	Kantor et al. (1972) strain W46M
Human	100	100	100	100
Rabbit	25	25	200	50
Sheep	25	12.5	40	50
Horse	3	6		
Guinea pig	6	12.5	50	
Pig			40	
Calf/Bovine	12.5	12.5	40	
Goat			20	
Cat			20	
Chicken			20	
Monkey		50		

Toxicity. Marks & Vaughan (1950) injected δ-haemolysin intradermally into man and observed slight erythematous lesions which disappeared within 48 hours. Larger amounts injected into rabbit or guinea pig skin induced indurated lesions which in the guinea pig became necrotic. Similar findings were reported by Kreger et al. (1971); intradermal injection of 1 mg purified haemolysin resulted in large erythematous indurated lesions which became necrotic within three days. Incorporation of serum (Marks & Vaughan, 1950) or lecithin (Kreger et al., 1971) into the injection mixture prevented dermonecrosis. The minimum lethal doses of δ-haemolysin for mice and guinea pigs were 110 mg/kg and 30 mg/kg respectively (Kreger et al., 1971).

Mode of action. Wiseman & Caird (1968) suggested that δ-haemolysin possessed phospholipase-C activity from the observation that, as with β-haemolysin, water-soluble organic phosphorus was released from phospholipid extracts of various species of mammalian erythrocytes by purified δ-haemolysin from two staphylococcal strains. The amount of organic phosphorus released was in direct proportion to haemolytic sensitivity. Water-soluble phosphorus was also released from phosphatidylinositol, a constituent of the erythrocyte membranes (Neville, 1967; Wiseman & Caird, 1968) which suggested that phosphatidylinositol was the substrate in the erythrocyte.

On the contrary, Kreger et al. (1971), Heatley (1971) and Rahal (1972) failed to detect phospholipase-C activity in their purified haemolysin preparations. Furthermore, incubation of constant amounts of δ-haemolysin with varying concentrations of erythrocytes did not show a typical enzymic response but was similar to the action of detergents (Bernheimer, 1970). The δ-haemolysin preparations of Kreger et al. (1971) and Heatley (1971) were both surface-active and had effects on mitochondria similar to that of the detergents Triton X100 and sodium deoxycholate (Rahal, 1972). The liberation

of ^{3}H-nucleosides from tissue culture cells by δ-haemolysin was independent of temperature over the range 4^{o} to 37^{o} and also resembled the action of Triton X-100 (Thelestam et al., 1974). This again suggested that δ-haemolysin is a surface-active polypeptide with a wide spectrum of activities.

Immunogenicity. Several workers have attempted to raise antiserum to δ-haemolysin and almost all have concluded that it is non-antigenic. However, as almost all normal sera have been shown to produce a precipitin line on gel diffusion against δ-haemolysin and to neutralise haemolytic activity (vide infra), results are difficult to interpret. Gladstone & Yoshida (1967) and Kantor et al. (1972) were unable to demonstrate an immune response to δ-haemolysin. On the other hand McLeod (1963), Kayser & Raynaud (1965) and Caird & Wiseman (1970) reported the production of specific antibodies to δ-haemolysin although no evidence was presented to show that the non-specific inhibitors of δ-haemolysin had been removed.

More recently Fackrell & Wiseman (1974) obtained γ-globulin fractions of serum from rabbits immunised intensively with 16 injections of δ-haemolysin. Such fractions yielded specific quantitative precipitin curves with δ-haemolysin and a single precipitin line on diffusion against purified δ-haemolysin which in blood agar coincided with the edge of the lysis zone. Immunogenicity of δ-haemolysin prepared by Kreger et al. (1971) was also demonstrated by Fackrell & Wiseman (1974) who also found Kreger's haemolysin to be contaminated with α- and γ-haemolysins.

Neutralisation of δ-haemolysin by normal sera and by phospholipids. Marks & Vaughan (1950) found that δ-haemolysin was neutralised by normal human, sheep, bovine, rabbit and guinea-pig serum. Also, normal sera yielded a precipitin line with δ-haemolysin (Gladstone & Yoshida, 1967) which, when serum was subjected to immunoelectrophoresis, occurred in the α-globulin region (Kantor

et al., 1972). Fractionation of serum to identify the inhibitory components showed that crude globulin fractions of human serum were inhibitory (Jackson & Little, 1958) and all Cohn fractions of serum were inhibitory, particularly fractions I, III and IV (fibrinogen, β- and α-globulins respectively) (Gladstone & Yoshida, 1967). The inhibitory components have not been identified although Donahue (1969), who found a relatively constant inhibitory titre in normal human sera suggested that the inhibitors are lipoproteins which have been shown to neutralise streptolysin S (Stollerman et al., 1950).

In addition to neutralisation by normal serum, δ-haemolysin is neutralised by many phospholipids, although conflicting reports are found in the literature. Whereas Gladstone & van Heyningen (1957) and Caird & Wiseman (1970) found cholesterol to be inhibitory, the opposite was reported by Gladstone & Yoshida (1967), Kreger et al. (1971) and Kapral (1972). Similarly lecithin was reported as inhibitory by Gladstone & Yoshida (1967) but not by Kreger et al. (1971) or by Kapral (1972). The inhibitory activity of several phospholipids is summarised in Table 9. Kapral (1972) concluded that the minimum inhibitory unit was phosphatidic acid and that components attached to this unit did not interfere with neutralisation. He also stated that the phospholipid content of serum could readily account for the neutralisation of δ-haemolysin by serum as discussed above.

Assay of δ-haemolysin

Investigations of some of the above topics have been limited by the available assay techniques since δ-haemolysin has a much lower specific haemolytic activity than α- or β-haemolysins. Using horse or human erythrocytes as indicators of haemolysis, titres of up to 300 HU/mg purified haemolysin have been reported (Kreger et al., 1971; Heatley, 1971). Lysis

Table 9 Inhibition of δ-haemolysin by phospholipids

Inhibitory phospholipids	Reference
Cholesterol	Gladstone & van Heyningen (1957); Caird & Wiseman (1970)
Phosphatidyl choline (lecithin)	Kreger et al. (1972); Kapral (1972)
Phosphatidic acid (natural)	Kreger et al. (1971) "
Phosphatidyl serine	" "
Phosphatidyl inositol	" "
Cardiolipin	" "
Sphingomyelin	" "
Phosphatidylethanolamine	" "

Non-inhibitory agents	
Cholesterol	Gladstone & Yoshida (1967); Kreger et al. (1971); Kapral (1972)
Phosphatidyl choline	Kapral (1972)
Myristic acid	"
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)	"

of protoplasts (Bernheimer et al., 1972) is more sensitive (610 units/mg) but inconvenient when screening large numbers of fractions. The ability of δ-haemolysin to lyse mammalian cells was exploited by Thelestam et al. (1973). HeLa cells or human diploid embryonic lung fibroblasts (HEL) were labelled with ^{3}H-uridine and treated with purified α-, β-, γ- and δ-haemolysins. Purified α-, β- and γ-haemolysins had no effect on HEL cells but δ-haemolysin (1 HU/ml, human erythrocytes) liberated 93% of releaseable soluble radioactive substances from the cells. Release of label was not temperature-dependent over the range 4^{o} to 37^{o} but was abolished by serum. This technique was 3 - 4 times more sensitive than haemolytic assays with human erythrocytes and had the further advantage that purified α- and β-haemolysins did not affect the assay.

The haemolytic assay is however more convenient and Birkbeck & Arbuthnott (1972) (unpublished observations) showed that cod erythrocytes were more sensitive to δ-haemolysin than human or rabbit erythrocytes. Concentrated culture supernatants from broth cultures of S. aureus strain Wood 46 (McNiven, Owen & Arbuthnott, 1972) showed no loss in titre against cod erythrocytes after heating at 100^{o} for 10 minutes whereas the titre against rabbit erythrocytes was reduced from 10,000 HU/ml to 80 HU/ml by this treatment (Table 10). Purified α- and β-haemolysins were inactive against cod erythrocytes at the highest concentrations tested, whereas purified δ-haemolysin was four times as active against cod erythrocytes as against rabbit. No synergistic action was detected in mixtures of purified δ- and β-haemolysins and the haemolytic activity against cod or mackerel erythrocytes was temperature-independent over the range 0 to 37^{o}. Serum, however, abolished the haemolytic activity of purified δ-haemolysin.

Table 10. Sensitivity of cod erythrocytes to staphylococcal haemolysins

after Birkbeck & Arbuthnott (1972, unpublished observations)

Haemolysin preparation	Titre (HU_{50}/ml) on assay with erythrocytes of		
	rabbit	cod	sheep
Crude filtrate (Wood 46)	10,000	320	
Heated crude filtrate	80	320	
Purified α-haemolysin[1].	3,200	< 10	
Purified β-haemolysin[2].		<200	12,800
Purified δ-haemolysin[3].	320	1,280	

1. Prepared by Dr. J.P. Arbuthnott
2. Prepared by Dr. T. Wadström
3. Prepared by Dr. A. Kreger.

Birkbeck & Arbuthnott concluded that because of the greater sensitivity of fish erythrocytes to δ-haemolysin and the lack of interference by α- and β-haemolysins, fish erythrocytes would provide an improved assay system for δ-haemolysin.

The aim of this thesis is to explore the utility of fish erythrocytes for the assay of δ-haemolysin and in this context the properties of fish erythrocytes and suitable methods for collection and long term storage of erythrocytes will be reviewed.

Nature of fish erythrocytes and serum

The blood of fish like that of the other vertebrates consists of a suspension of erythrocytes and leucocytes in a solution of proteins and electrolytes which constitutes the plasma. There are several reviews on the nature of fish blood (Holmes & Donaldson, 1969; Satchell, 1971; Love, 1970) although few authors have considered the species of fish used in this thesis.

The total blood volume of many fish species has been measured by dilution techniques in which a fixed quantity of tracer (Evans blue dye, fluorescein-labelled or ^{131}I-labelled serum proteins or ^{51}Cr-labelled erythrocytes) was injected into fish and, after a period of equilibration, the concentration of tracer in the blood was measured to determine the total blood volume (Holmes & Donaldson, 1969). Ronald *et al.* (1964) showed by injection of fluorescent-labelled serum proteins into the Atlantic cod (*Gadus morhua*) that the total blood volume was directly proportional to the body weight (24 ml/kg). For teleostei the total blood volumes are generally within the range 2.2 - 3.1 ml/kg body weight, whereas blood volumes of the more primitive chondrostei are higher (3.0 - 8.0 ml/kg) but with a corresponding reduction in erythrocyte concentration in the blood to 16.8 - 30%, compared to 28 - 39% for teleostei (Holmes & Donaldson, 1969).

The erythrocytes. Klontz et al. (1971) stated that the fish erythrocyte was derived from the smaller haemocytoblast and, as in all vertebrates other than mammals, had a central nucleus.

The erythrocytes have been found to vary in shape and size from one species to another. Hartman & Lessler (1964) found that the erythrocytes of Dasyatis centrura (Northern stingray) were oval (19.7 $\pm$ 0.38 μm x 13.8 ± 0.24 μm) whereas the erythrocytes of Lepomis macrochirus (Bluegill) were reported to be smaller and circular (10.9 μm diameter) (Smith, Lewis & Kaplan, 1952).

The erythrocyte count in teleostei has been found to be approximately 0.2 - 3.1 x 10^6 cells/mm^3, although two extreme examples of erythrocyte count have been reported. Saunders (1966) reported that Acanthurus bahianus had a count of 6.48 x 10^6 cells/mm^3 which exceeded the normal range for human blood (4.6 - 6.2 x 10^6 cells/mm^3). In contrast, the blood of Chaenocephalus aceratus (ice fish), showed no absorption band for haemoglobin and was presumed to totally lack erythrocytes (Ruud, 1954).

As in mammalian erythrocytes, different blood group antigens have been demonstrated on fish erythrocytes of several species and this topic has been reviewed by de Ligny (1969). However, to the author's knowledge no published data exists on the chemical nature of the fish erythrocyte plasma membrane, in contrast to the extensive studies on mammalian erythrocytes (Zwaal, Roelofsen & Colley, 1973).

Biochemical properties of fish serum

Electrolytes. Most fishes, because of their limited salinity-tolerance, are restricted to either salt or fresh water and are able to maintain distinct concentration differences of one or more of their main inorganic constituents

(Na^+, Cl^-, K^+, Ca^{+2}, Mg^{+2} and SO_4^{-2}) between their body fluid and the external medium. Sodium and chloride are known to be most important inorganic constituents of the body fluid. Smith (1929, 1931b) demonstrated that in the marine teleosts which have a lower salt content in the blood and body tissues than the surrounding sea, water losses due to outward diffusion under the action of the osmotic gradient were made good by drinking sea-water. Monovalent ions and water are absorbed from the intestine, but salt balance is maintained by the secretion of the absorbed ions against a concentration gradient through the gill membranes. The urine is very concentrated and produced in small amount. On the other hand, freshwater fish maintain a large osmotic gradient by the production of a dilute urine (Smith, 1931a).

The osmolality of fish serum has been determined by freezing point depression (Green & Hoffman, 1953) but it is clear that serum osmolality may vary according to the environmental salinity and temperature. The freezing point of Gadus ogac (Greenland cod) plasma was -1.47^{o} in winter, but in the summer rose to -0.8^{o}. At the onset of winter, when the plasma freezing point dropped, there was little change in the concentration of chloride, and Scholander et al. (1957) suggested that non-protein nitrogen might help to prevent freezing in ice-cold sea water.

Woodhead & Woodhead (1959, 1964, 1965) have studied the response of Gadus morhua to low temperatures and showed that the plasma sodium, potassium and chloride may increase by up to 25% during winter. Fish serum osmolality varies widely in different species (Green & Hoffman, 1953) and even for one species may vary within wide limits.

A more extensive study of the response of Gadus morhua to low temperature was performed by Harden-Jones & Scholes (1974). In over 200 fish maintained at various temperatures (16^{o} to -1.5^{o}) for up to 40 days, the plasma

osmolality and chloride ion concentrations were increased by low temperatures. Also it was found that the plasma osmolality was influenced by the method of killing the fish prior to withdrawal of blood.

Preservation of fish erythrocytes

Isotonic solutions for fish erythrocytes. Many balanced salt solutions (BSS) which provide suitable conditions of pH and osmolality for maintenance of cells from warm-blooded animals have been described (Tyrode, 1910; Gey, 1936, 1945; Simms, 1941; Earle, 1943; Hanks, 1946; Dulbecco, 1954). For fish cells a number of balanced salt solutions have been formulated and some are compared to Dulbecco's BSS in Table 11. Sodium and chloride ions are the most important inorganic constituents of the body fluid and some 'physiological' solutions for fish tissues have contained only NaCl (Mackie et al., 1975).

Since the osmolality of fish plasma has been found to vary widely in different species (Green & Hoffman, 1953) suitable salt solutions for maintenance of erythrocytes will vary accordingly.

The osmolality of body fluids is commonly determined by freezing-point depression and this technique has been applied to fish blood by Botazzi (1897), Garrey (1905) and Krogh (1939). In these studies only a few fish species were used in comparisons with other aquatic animals.

Other techniques for preparation of isotonic solutions for fish erythrocytes have been used to measure the erythrocyte volumes both in NaCl solution and in serum (haematocrit, haemoglobin concentration and densitometric methods). The concentration of NaCl in which the cells maintained the same volume as in serum was taken as isotonic (Green & Hoffman, 1953). However, it was found that the isotonic concentration of NaCl determined by depression of

Table 11. Constitution of various physiological salt solutions for tissues from different sources

(Grams per litre)

Constituent (g/litre)	Dulbecco (1954) Dulbecco & Vogt (1954)	Holfreter (1931)	Cortland	Holmes & Stott (1960)	Keynes & M-Ferreira (1953)
	(Mammals)	(Amphibia and fish)	(Brown trout)	(Cutthroat trout)	(Electric eel)
NaCl	8.00	3.50	7.25	7.41	9.88
KCl	0.20	0.05	0.38	0.37	0.37
$CaCl_2.2H_2O$	0.13	0.10	0.23		0.44
$MgCl_2.6H_2O$	0.10				0.30
$MgSO_4.7H_2O$			0.23	0.31	
$NaH_2PO_4.H_2O$			0.41	0.40	0.04
$Na_2HPO_4.2H_2O$	1.15			0.20	0.21
KH_2PO_4	0.20			0.17	
$NaHCO_3$		0.20	1.00	0.31	
Glucose			1.00		

the freezing point was higher than that obtained by the haematocrit or haemoglobin concentration methods for some marine teleosts and elasmobranchs. It was not established which method gave the correct results but it was suggested that water exchange and urea diffusion from the cells occurred during the relatively long period required for haematocrit and haemoglobin measurements thereby giving lower values.

Protection of erythrocytes during freezing. Human erythrocytes for blood transfusion are routinely stored in liquid nitrogen.

Glycerol, used to protect human erythrocytes from freezing damage (Gibbs et al., 1962; Huntsman et al., 1962), was reported to protect erythrocytes from the shiner, seaperch, albacore, goosefish and three species of salmon by Cushing et al. (1957). Hodgins & Ridgway (1964) successfully stored erythrocytes from salmon and trout in liquid nitrogen or at -70^{o} in glycerol but found that removal of glycerol was difficult without causing extensive haemolysis.

Using glucose as a protective additive, as much as 89% of salmonid erythrocytes were recovered from liquid nitrogen, but the half-life of washed cells was less than 1 - 2 hours (Hodgins & Ridgway, 1964).

Polyvinylpyrrolidone successfully protected the erythrocytes from immature red salmon, but not adult red salmon or chinook salmon against lysis.

Dimethyl sulphoxide has been used by Lovelock & Bishop (1959) to protect human and bovine erythrocytes and bull sperm from freezing damage and by Ashwood-Smith (1961), Dougherty (1962), Porterfield & Ashwood-Smith (1962), Perry (1963), Greaves, Nagington & Kellaway (1963) and Huggins (1963) to freeze and recover mammalian bone marrow, tissue and peripheral blood cells. Using dimethyl sulphoxide, Hodgins & Ridgway (1964) recovered 70% of erythrocytes

from salmon and trout after freezing and thawing with the advantage that dimethyl sulphoxide was readily washed from cells without causing appreciable haemolysis or detectable antigenic changes in washed cells. Preservation in liquid nitrogen also gave better recovery and stability of erythrocytes than freezing at -20^{o} or -70^{o}.

Object of Research

The above work demonstrates that long-term storage of fish erythrocytes by freezing in liquid nitrogen should be feasible. In this thesis the objectives were firstly to determine suitable conditions for storage of blood so that sufficient quantities of blood were available in the laboratory for routine use in titrations. Secondly, conditions should be found which permit recovery of blood which is stable for a period of one day to allow several comparative haemolysin titrations to be carried out using one batch of washed cells. Thirdly, the optimum conditions for assay of delta haemolysin (time, temperature, ion requirements, etc.) need to be established to determine whether the assay sensitivity may be improved. Finally the possible use of cod erythrocytes in blood agar plates is to be investigated as a potential means of screening staphylococci for the production of delta haemolysin.

MATERIAL AND METHODS

A. Bacterial cultures

1. Strains of S. aureus

The strains of S. aureus used in this investigation were obtained through the courtesy of Dr. J.P. Arbuthnott, Department of Bacteriology, Royal Infirmary, Glasgow, and of Miss S. McKay, Department of Microbiology, University of Glasgow (strains SM 6 - 15). Isolates were received freeze-dried in ampoules or on nutrient agar slopes. These strains and their more important known characteristics were as shown below:

Strain	Characteristics
JK 128, JK 132, JK 138, JK 139	These four strains were obtained by Dr. J.P. Arbuthnott during a biochemical survey of approximately 150 clinical isolates of S. aureus. All produce δ-haemolysin and small amounts of α-haemolysin.
NCTC 10345	A mutant of the Wood 46 strain of S. aureus which produces mainly δ-haemolysin.
NCTC 7121 (Wood 46)	Produces α-haemolysin in high titre and also δ-haemolysin.
SM 6	Coagulase negative (S. epidermidis); non-toxinogenic clinical isolate.
SM 9	Weakly α- and δ-toxinogenic clinical isolate.
SM 10	An α-toxinogenic clinical isolate.
SM 14	Clinical isolate producing α- and β-haemolysins and small amount of δ-haemolysin.
SM 15	A β-toxinogenic bovine mastitis isolate (strain BB).
JK 21	A clinical isolate producing epidermolysin.

2. Maintenance of cultures and checks for purity

On receipt, all cultures were plated on to tryptone soya agar (Oxoid) and isolated colonies subcultured twice on the same medium, with routine gram staining to confirm culture purity. Selected colonies were subcultured onto 10% washed horse blood agar to examine the haemolysis zones surrounding the colonies.

Cultures were maintained by lyophilization or by passage on freshly prepared nutrient agar slopes. The latter method was employed with those strains which were used regularly.

All strains, whether lyophilized or maintained on agar slopes stored at 4^{o}, were plated on to tryptone soya agar to check for contaminants and for uniformity of colonial morphology before experimental use.

B. Production and purification of staphylococcal δ-haemolysin

1. Production of crude culture supernatants

The contents of a freeze-dried ampoule of S. aureus (strain NCTC 10345) were dissolved in sterile 0.85% saline and inoculated on to a 10% horse blood agar plate. After 24 hr incubation at 37^{o}, organisms from isolated colonies which showed large haemolytic zones were inoculated onto 4 agar slopes and incubated 24 hr at 37^{o}. The confluent growth on each slope was resuspended in 2 ml Dulbecco 'A' saline. Seven 2 litre flanged Erlenmeyer flasks containing 750 ml Bernheimer diffusate medium (Appendix 1) were inoculated with 1 ml of suspension ($E^{1\ cm}_{660nm}$ = 0.49) and incubated for 20 hr at 37^{o} on a rotary shaker operating at 135 r.p.m. 1 ml of octan-2-ol was added to reduce foaming. The cultures were pooled and centrifuged at 17,000 x g for 10 min at 4^{o} to remove the cocci. The crude culture supernatant (approx. 5 litres) was used for preparation of purified δ-haemolysin.

2. Purification of δ-haemolysin

All procedures were done at 4°. Crude culture supernatant was stirred for 3 hr with hydroxylapatite (Appendix III) (20 g/l) and allowed to stand overnight. The supernatant fluid was discarded by decantation and the hydroxylapatite washed 5 times with 2 l phosphate buffer (pH 6.8, 0.01 M) to remove culture supernatant. The hydroxylapatite was stirred with 500 ml phosphate buffer (pH 6.8, 0.4 M) for 30 min, centrifuged (300 g, 10 min) and the supernatant discarded. After six such washes the hydroxylapatite was stirred for 30 min with 400 ml phosphate buffer (pH 7.4, 1 M), centrifuged (300 g, 10 min), washed a further five times and the supernatants of the 1 M phosphate buffer washes (containing the eluted δ-haemolysin) were bulked and centrifuged (12,000 g, 10 min, 4°) to remove traces of hydroxylapatite. In an initial experiment, δ-haemolysin was recovered from the dilute solution as described by Kreger et al. (1971) by exhaustive dialysis against water, centrifugation (1,000 g, 20 min) to remove 'insoluble δ-lysin' and lyophilisation. In later experiments, the dilute δ-haemolysin solution was dialysed against Dulbecco 'A' PBS and concentrated to approximately 5% of its original volume by ultrafiltration (Amicon ultrafiltration cell with PM10 membrane, 20 p.s.i. pressure). The concentrate was clarified by centrifugation (50,000 g, 30 min) and stored at -70° in 2 ml or 5 ml quantities in screw cap bottles.

Dialysis against phosphate buffered saline and concentration by ultrafiltration gave a higher yield of soluble δ-haemolysin with little 'insoluble' δ-haemolysin which was discarded. Concentrated, purified δ-haemolysin solutions used in the experiments described in this thesis had haemolytic activities of 750 and 150 HU/mg protein against 0.8% cod and human erythrocytes respectively.

C. Physico-chemical properties of purified δ-haemolysin

1. Protein estimation

The method of Lowry et al. (1951) was used with crystalline bovine serum albumin as a standard.

2. Spectral analysis

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

3. Polyacrylamide disc gel electrophoresis

a) Acid gels. This was performed at pH 4.3 in 7.5% polyacrylamide gels as described by Reisfeld et al. (1962). Electrophoresis was done at 6 ma per gel until the tracking dye (Pyronin Y, Difco) reached the final few millimetres, at which time the current was increased to 8 ma per gel. Migration was towards the cathode.

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

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

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

4. Isoelectricfocusing

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

b) Procedure. All procedures were carried out in a 4^{o} cold room and the total power was restricted to a maximum of 0.25 watts. The apparatus was allowed to equilibrate at 4^{o}, 30 ml of 1% ampholines (pH 3.5 - 10; LKB Instruments, London) was added and a pH gradient established by isoelectric focussing for 24 hr at a potential of 600 volts. The sample (up to 1 ml dialysed against 1% glycine) was introduced into the three centre troughs and focussing was continued at a potential of 800 V for 20 hr and finally at 1,000 V for a further 24 hr. After focussing, fractions, including any precipitated material, were removed by Pasteur pipette and retained for analysis.

c) Analysis of fractions. The pH of each fraction was measured with a Pye Model 46A Vibret Lab pH Meter with the samples maintained at 4^{o} in a cooled water bath.

The optical density of each fraction was measured at 280 nm using a Pye Unicam Model SP 500 spectrophotometer with silica micro-cells of 1 cm light path. To detect the presence of haemolysins a preliminary test was done by adding 0.5 ml of a 10-fold dilution of each fraction to 0.5 ml 1% washed red blood cells (cod, rabbit, human or sheep erythrocytes).

Doubling dilution titrations were performed on those fractions which showed haemolytic activity in preliminary tests. In titrations with sheep erythrocytes, 0.001 M Mg^{+2} was added to detect β-haemolysin.

D. Assay of δ-haemolysin

1. Doubling dilution titration

The haemolytic titre of δ-haemolysin preparations was determined by making serial doubling dilutions in 0.5 ml volumes of Dulbecco 'A' PBS (mammalian erythrocytes) or citrate/dextrose/sodium chloride solution (CDS solution) (fish erythrocytes) in 4" x ½" test tubes, and adding 0.5 ml of 0.8% erythrocyte suspension to each tube. Erythrocytes incubated in buffer served as controls. Test mixtures were incubated for 30 min at 37° (mammalian erythrocytes) or at 15° or 20° (fish erythrocytes) and centrifuged (700 x g for 2 min). The optical density of each supernatant was measured at 541 nm in a Pye Unicam SP 500 series 2 spectrophotometer with 1 cm path length silica micro-cells; supernatant from the control tube served as the blank. The haemolytic titre (HU_{50}) was taken as the reciprocal of the highest dilution of haemolysin causing 50% haemolysis of a suspension under the above conditions. When making serial two-fold dilutions, pipettes were changed at every second transfer and when comparing titrations, a one-tube difference in the end-point was not considered significant.

2. Titration of δ-haemolysin at different temperatures

Doubling dilutions of δ-haemolysin were made in 5 ml volumes of CDS solution and 0.5 ml amounts of each dilution transferred into a series of nine 4" x ½" tubes to give nine replicate series of dilutions. To each tube was added 0.5 ml of a 0.8% suspension of washed mackerel erythrocytes in CDS solution. The mixtures were incubated at 0° (ice bath), 5°, 10° (Grant Instruments Ltd., cooled glycol baths), 15°, 20°, 25°, 30°, 37° or 45° (Grant SB2 water baths) for 30 min. All tubes were centrifuged and the titration end-points determined spectrophotometrically as described above.

3. Effect of 2-mercaptoethanol on haemolysin titre

A mixture of δ-haemolysin (0.9 ml) and 2-mercaptoethanol (10 mM, 0.1 ml) was incubated for 5 min at 37° and serial 1 ml dilutions of mercaptoethanol treated and control, untreated, δ-haemolysin were made in CDS solution. After addition of 1 ml of saithe erythrocyte suspension (1%) to each tube the mixtures were incubated at 15° and the titration end-points determined spectrophotometrically as described previously.

4. Effect of erythrocyte concentration on the degree of haemolysis by a fixed amount of δ-haemolysin

Doubling dilution titrations of δ-haemolysin were performed using human, cod and mackerel erythrocytes to establish the concentration of haemolysin which lysed approx. 50% of a 1% cell suspension (HU_{50}). Human, cod and mackerel erythrocytes were suspended in Dulbecco 'A' (human) or CDS solution (cod and mackerel) in 0.5 ml volumes at concentrations from 8% to 0.1% v/v and one HU_{50} (0.5 ml) δ-haemolysin added to each suspension (1/256 dilution of stock δ-haemolysin for human cells; 1/1600 for cod and 1/3200 for mackerel cells) and after incubation for 30 min at 37° (human) or 15° (cod and mackerel) the proportion of cells lysed was measured by spectrophotometry and comparison to cell suspensions lysed by addition of saponin.

E. Mammalian erythrocytes

Rabbit blood was collected by bleeding from the marginal ear vein and sheep blood by jugular venepuncture. Sterile sodium citrate 3.8% (w/v) was used as an anticoagulant for both. Defibrinated horse blood (Oxoid, London) containing no preservative was used as a source of horse erythrocytes and citrated human blood was obtained from the Haematology Department, Western Infirmary, Glasgow.

F. Fish blood

1. Collection of fish

The fish used in this work were obtained by courtesy of Professor N. Millott, University Marine Biological Station, Millport, Isle of Cumbrae, and were caught by trawling in the Firth of Clyde using the research vessel m.v. 'Mizpah'. The fish obtained by trawling for periods of about one hour were sorted on deck and selected individuals kept alive in open 40-gallon plastic baths prior to bleeding.

A variety of species of fish were obtained (Table 12) but daily and seasonal variations in the abundance of individual species permitted the use of several different species in both storage and haemolysin-sensitivity experiments. Saithe and cod predominated although, occasionally, both were totally absent.

2. Collection of blood

Fish were killed by stunning and blood withdrawn from the dorsal aorta using a syringe and needle; with fish less than 500 g in weight, a 2.5 ml syringe and 23 g needle were used, but with larger fish a 10 ml or 20 ml syringe with 21 g or 18 g needle was taken. After withdrawal, blood was immediately mixed with 3.8% sodium citrate to prevent coagulation and blood from several fish was normally bulked and maintained at 0^{o} in an ice-filled Dewar flask for transport to the laboratory. Serum samples were obtained by allowing blood to clot in Universal containers. Serum was withdrawn after transport to the laboratory, centrifuged to remove traces of erythrocytes and stored frozen at -20^{o}.

3. Storage of blood in liquid nitrogen

Equal volumes of citrated fish blood and dimethyl sulphoxide

Table 12. Species of fish obtained by trawling

Family	Species	Common name
GADIDAE	Gadus morhua	Cod
	Melanogrammus aeglifinus	Haddock
	Pollachius virens	Saithe
	Pollachius pollachius	Pollack
	Merlangius merlangus	Whiting
	Trisoplerus minutus	Poor Cod
	Merluccius merluccius	Hake
GLUPIDEAE	Clupea harengus	Herring
PLEURONECTIDAE	Pleuronectes platessa	Plaice
	Eutriglia gurnadus	Grey Gurnard
SCOMBRIDAE	Scomber scombrus	Mackerel

(7.5 to 15% v/v in CDS solution), cooled to 0^{o}, were mixed and allowed to stand at 0^{o} for 5 - 10 min. Blood samples containing DMSO were distributed into 2 ml polypropylene screw cap ampoules (Sterilin Ltd., Richmond, Surrey) (0.8 ml per ampoule). Ampoules were mounted in aluminium canes and the blood frozen quickly by immersion in liquid nitrogen (British Oxygen Company 10 litre Vivostat liquid nitrogen storage container).

For storage as frozen pellets, the blood/CDS/DMSO mixture was frozen by dropwise addition into liquid nitrogen in a Dewar flask; the liquid nitrogen was removed by decantation and pellets stored in screw cap aluminium canisters (18.5 cm x 3.5 cm diameter with 1 mm holes drilled at intervals along the length to allow free exchange of liquid nitrogen).

4. Measurement of erythrocyte concentration in blood

In experiments designed to test the effect of blood concentration on storage properties, the packed cell volume of citrated blood samples was measured using the micro-haematocrit technique. A heparinised haematocrit capillary-tube (Sherwood Medical Ind. Inc., St. Louis, Missouri) was filled with blood, one end was sealed with 'Cristaseal' (Gelmer-Hawksley, Lancing, Sussex), the capillaries centrifuged (2,000 rpm, 10 min, MSE bench centrifuge, micro-haematocrit head assembly) and the percentage packed cell volume measured from the relative lengths of the erythrocyte and plasma columns (MSE Microhaematocrit reader).

Erythrocyte concentrations in citrated blood were adjusted by removal of the calculated volume of citrated plasma after centrifugation (500 g, 5 min).

5. Recovery of frozen blood

Ampoules of frozen blood were removed from their storage canes and

rapidly thawed at 35^{o} in a water bath.

Pelleted blood was recovered by pouring pellets into a Universal bottle containing CDS solution which was immersed in a 35^{o} water bath to accelerate thawing.

6. Measurement of the degree of lysis on storage

The amount of free haemoglobin in the supernatants of erythrocyte suspensions was used as a measure of lysis.

Blood stored in ampoules was thawed at 35^{o} and 0.3 ml added to 20 ml CDS solution containing the same concentration of DMSO as the stored blood. After centrifugation (700 g, 5 min) the supernatant optical density at 541 nm was measured in a Pye Unicam SP 600 spectrophotometer. A 100% lysis standard was prepared by addition of saponin (approx. 1 - 2 mg) to the blood suspension and centrifugation to remove erythrocyte debris. The percentage lysis =

$$\frac{E_{541}\ \text{sample}}{E_{541}\ \text{saponin lysis}} \times 100$$

Pelleted blood was weighed into a tared Universal bottle and CDS/DMSO solution added to give a 1% w/v suspension; erythrocyte lysis on storage was then determined spectrophotometrically as described above.

Erythrocyte washing procedure

Erythrocytesfrom rabbit, human, horse and sheep were washed three times by centrifugation and resuspension in Dulbecco 'A' phosphate buffered saline (10 - 20 volumes each time) before use. Dimethyl sulphoxide was washed from fish erythrocytes by centrifugation and resuspension in 10 volumes of CDS/DMSO solution containing decreasing proportions of DMSO (12.5%, 10%, 7.5%, 5.0%, 2.5% and 1.0%) before washing twice in CDS solution.

G. Properties of fish sera

1. Osmolality

The osmolalities of fish sera and buffer were determined with a Fiske clinical osmometer (Advanced Instruments, Mass., U.S.A.) by courtesy of the M.R.C. Blood Pressure Unit, Western Infirmary, Glasgow. The instrument was calibrated with a standard solution of sodium chloride (500 milliosmoles/kg H_2O) and osmolalities were measured on duplicate 0.3 ml samples of serum. Variation between duplicate samples was not greater than 1% and readings were taken directly in milliosmoles/kg. Serum samples, which had been stored at -20^{o}, were thawed and allowed to reach room temperature before use.

2. pH

The pH of sera and buffer was measured with a Pye Model 292 pH meter (PyeUnicam) with a special long-reach combination electrode CMAT (Russell pH Ltd., Auchtermuchty, Fife).

H. Assay of δ-haemolysin on blood agar plates

1. Radial diffusion haemolytic assay for δ-haemolysin

Blood agar plates were prepared by pouring 5 ml molten 1% to 5% (v/v) blood agar (Oxoid blood agar base No. 2) into a 5 cm diameter plastic petri dish (Sterilin Ltd., Richmond, Surrey). Sodium azide was incorporated into blood agar(to inhibit bacterial growth)at a concentration (0.01%) shown to have no effect on the haemolytic activity of δ-haemolysin. Sodium chloride (0.1 M) was incorporated into fish blood agar to increase the osmolality and to give clearer lysis zones. Six 3.5 mm wells were cut in the agar by means of a cork borer and perspex template with holes equidistant from the centre of the plate. The holes were filled with 10 µl amounts of δ-haemolysin at

concentrations of 0.5%, 1.0%, 2.0%, 4.0%, 6.0%, 8.0% of a stock solution. The plates were then incubated at 37^{o} (human) and at 4^{o} (cod and mackerel) for 24 and 48 hr respectively. The radius of the haemolytic zone (colony edge to zone edge) around each well was then measured with an eyepiece containing a millimetre graticule. The optimum time of incubation was found to be 48 hr.

2. Growth of S. aureus on blood agar plates

Cod, human, horse, rabbit and sheep erythrocytes were used in this study. The preparation of 5% blood agar plates was as described above but with the omission of sodium azide.

a) Inoculation of cultures. A stab-inoculation method was used which was essentially as described by Williams & Harper (1947) and Marks & Vaughan (1950). It was done with a straight wire charged from a colony on a horse blood agar plate by stabbing perpendicularly into the blood agar in a 5 x 1 cm petri dish. Each strain was inoculated onto the five species of blood agar. Though there was no detectable difference in the size of the haemolytic zones produced by 10 colonies arising from 10 consecutive stabs made without recharging the wire, a duplicate experiment was always performed. In one row, the stab-inoculation was carried out in the order: cod, human, horse, sheep, rabbit, while the other row was done in the reverse order.

b) Incubation condition and measurement of zones. The mammalian blood agar plates were incubated at 37^{o} and the cod at 25^{o} and the radii of zones measured after 24 hr and then 48 hr incubation. The radii of colonies and their lytic zones were measured from the reverse side of the plate.

3. Blood-agar overlay assay

Because of the instability of fish erythrocytes at temperatures above

25^{o} a blood-agar overlay technique was used in several tests. Cultures were inoculated on to a nutrient agar plate instead of blood agar. After 24 hr growth at 37^{o} an overlay of 5 ml molten 5% blood agar (45^{o}) containing NaN_3 was then poured onto the top of the growth culture. The plates were then incubated at 4^{o} and the sizes of colonies and their lytic zones were measured as described above after 24 hr and then 48 hr.

RESULTS

Preparation of purified S. aureus δ-haemolysin

Selection of strain

The haemolytic activity of five staphylococcal strains, JK128, JK132, JK138, JK139, NCTC 10345, which had been observed to produce high levels of haemolysin not neutralisable by anti α-haemolysin antiserum were examined to select a suitable δ-haemolysin producer.

Crude culture supernatants of each strain were prepared as described for S. aureus strain NCTC 10345 but on a smaller scale (60 ml). Doubling-dilution titrations against human and rabbit erythrocytes were then performed on the crude culture supernatant of each strain. The ratio of the haemolysin titres (human/rabbit) was assumed to reflect the ratio of δ-haemolysin to α-haemolysin in the culture supernatant.

The results (Table 13) showed that of the five strains tested NCTC 10345 yielded both the highest titre against human erythrocytes and the highest titre ratio. This strain was therefore selected for production of δ-haemolysin.

Preparation and characterisation of S. aureus δ-haemolysin

Delta-haemolysin was purified from 1 litre of crude culture supernatant according to the method of Kreger et al. (1971) using hydroxylapatite. The supernatants from each washing step were titrated for haemolytic activity against rabbit and cod erythrocytes (Table 14). Addition of hydroxylapatite to the culture supernatant removed all haemolytic activity against cod erythrocytes, although haemolysin active against rabbit erythrocytes (presumably α-haemolysin) remained in the supernatant. Forty five per cent of δ-haemolysin

Table 13. Titration of crude culture supernatants of five staphylococcal strains against human and rabbit erythrocytes

Staphylococcal strain	Haemolysin titre (HU/ml)		Ratio of haemolysin titres (Human/Rabbit)
	Human	Rabbit	
NCTC 10345	160	80	2
JK 128	40	160	0.25
JK 132	80	320	0.25
JK 138	40	80	0.5
JK 139	80	320	0.25

Table 14. Haemolysin activity in supernatant liquids from the purification of δ-haemolysin with hydroxylapatite

Fluid	Volume (ml)	Haemolysin titre (HU/ml)	
		Rabbit erythrocytes	Cod erythrocytes
Culture supernatant	1000	640	640
Supernatant after addition of hydroxylapatite	1000	40	0
0.01 M P.B.* washings (bulked)	2000	0	0
0.4 M P.B.* washings			
(supernatant 1)	600	40	160
(supernatant 2)	500	20	80
(supernatant 3)	200	20	80
(supernatant 4)	200	20	40
1.0 M P.B.* washings			
(supernatant 1)	100	320	1280
(supernatant 2)	100	160	640
(supernatant 3)	100	80	640
(supernatant 4)	100	20	160
(supernatant 5)	100	20	160

* P.B.; phosphate buffer

was recovered by washing the hydroxylapatite with 1.0 M phosphate buffer and the specific activity of the concentrated preparation was 750 HU/mg protein on titration with cod erythrocytes. Similar results were obtained in subsequent preparations of δ-haemolysin.

Ultraviolet spectrum of purified δ-haemolysin

The UV spectrum of δ-haemolysin (Fig. 1) showed absorption maxima at 273 nm and 291 nm with a minimum at 250 nm and was similar to that found by Kreger et al. (1971).

Disc-gel electrophoresis of purified δ-haemolysin

Electrophoresis of purified δ-haemolysin in polyacrylamide gels revealed a single broad band in acidic gels (pH 4.3) and two bands in alkaline (pH 9.5) gels. Densitometer traces of disc gels of both systems are shown in Fig. 2.

Isoelectricfocusing of purified δ-haemolysin

When δ-haemolysin was subjected to isoelectricfocusing in a broad pH gradient (pH 3.5 - 10), all haemolytic activity was found in a single peak (pI 4.45) corresponding to the major peak of E_{280}-absorbing material (Fig. 3). The haemolytic activity of the peak fraction against cod erythrocytes was 128 X greater than against rabbit erythrocytes and 8 X greater than against human erythrocytes. Heating (100^{o}, 10') caused no loss in haemolytic activity but titration of the peak fraction in 5% human serum resulted in an eightfold reduction in titre. No α- or β-haemolysin was detectable as judged by the absence of haemolysins with a pI greater than 7.

This δ-haemolysin preparation was considered satisfactory for the further examination of the haemolysin assay using cod erythrocytes.

Figure 1. Ultraviolet absorption spectrum of δ-haemolysin

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

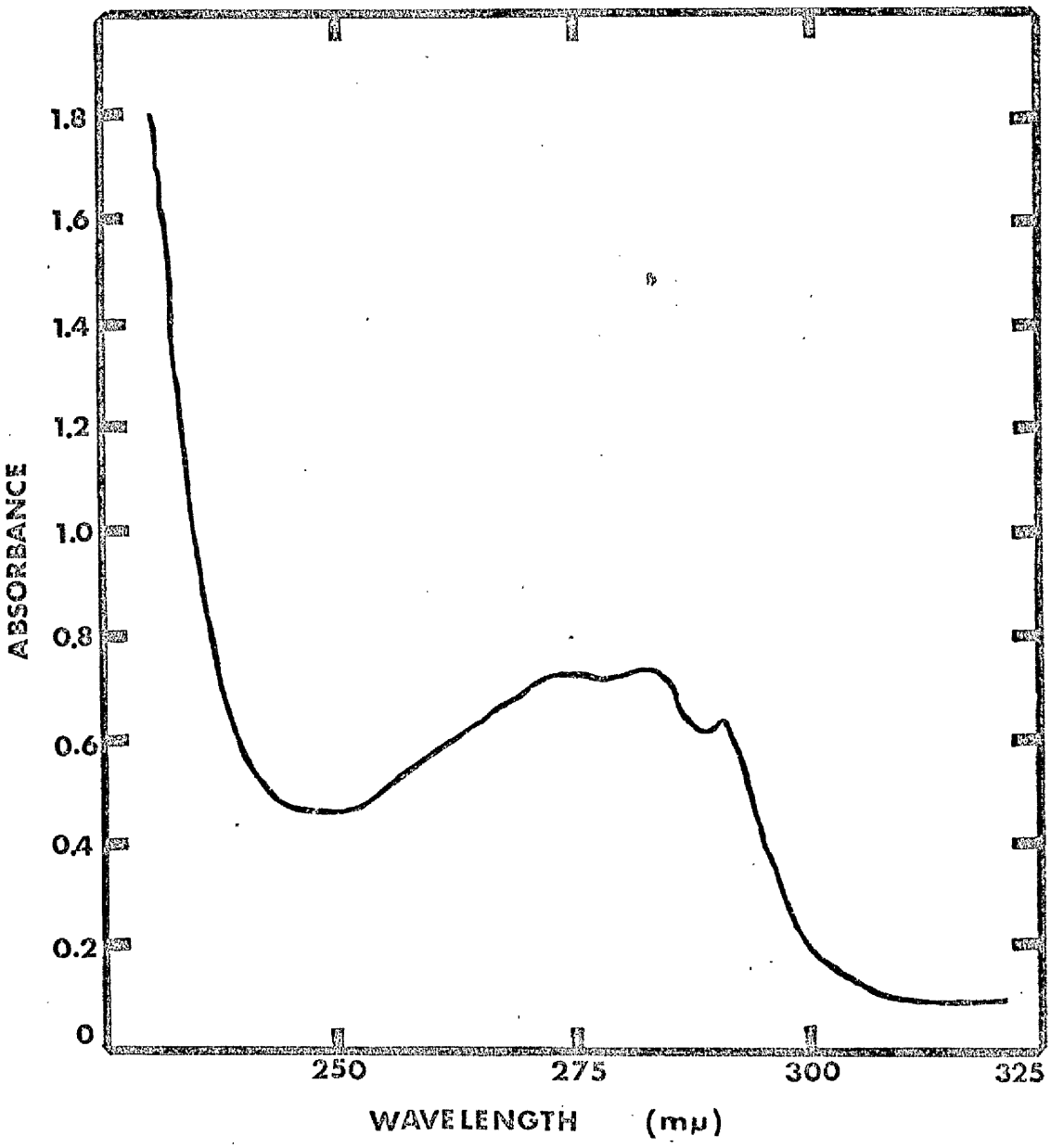

ABSORBANCE
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
250
275
300
325
WAVELENGTH (mμ)

Figure 2. Disc gel electrophoresis of δ-haemolysin

a) Densitometer trace of 27 μg δ-haemolysin analysed in an acidic (pH 4.3) 7.5% polyacrylamide gel.

b) Densitometer trace of 13 μg δ-haemolysin analysed in an alkaline (pH 9.5) 7.0% polyacrylamide gel.

S.G. stacking gel.

T.G. tracking dye. An absorption band corresponding to the tracking dye was found in blank gels in both acidic and alkaline gels.

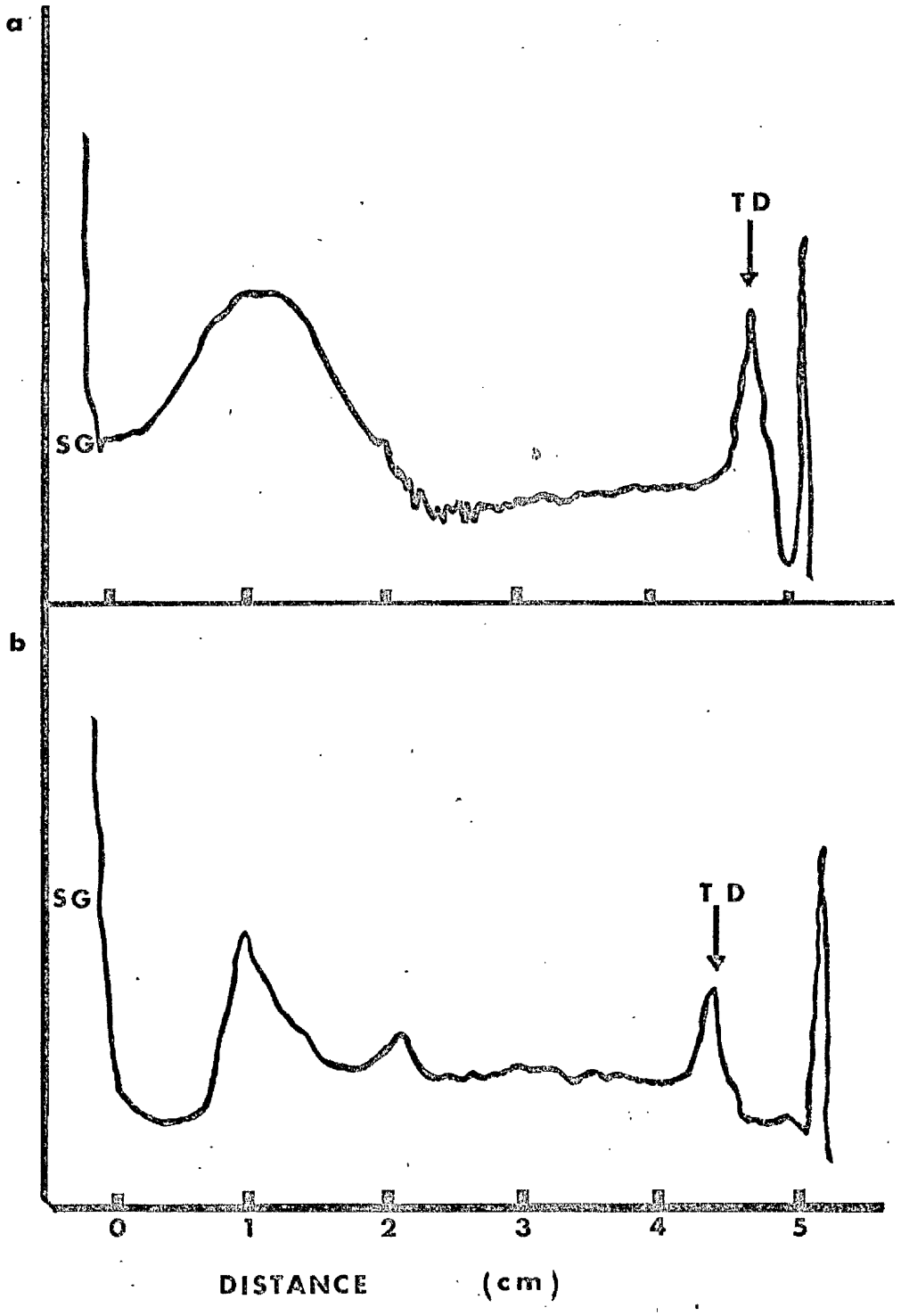
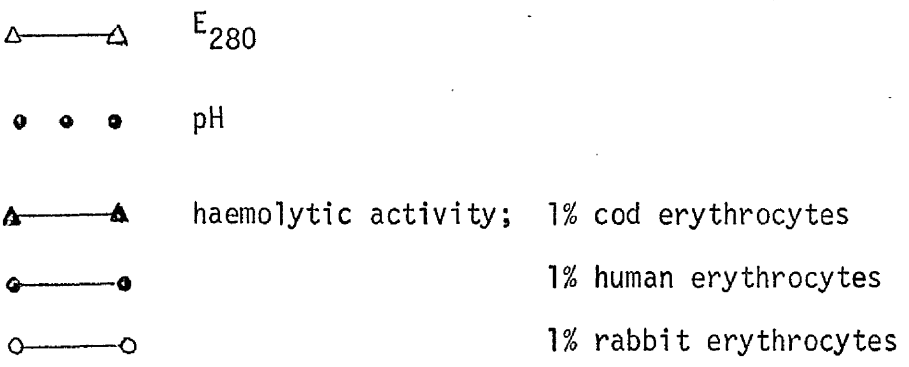
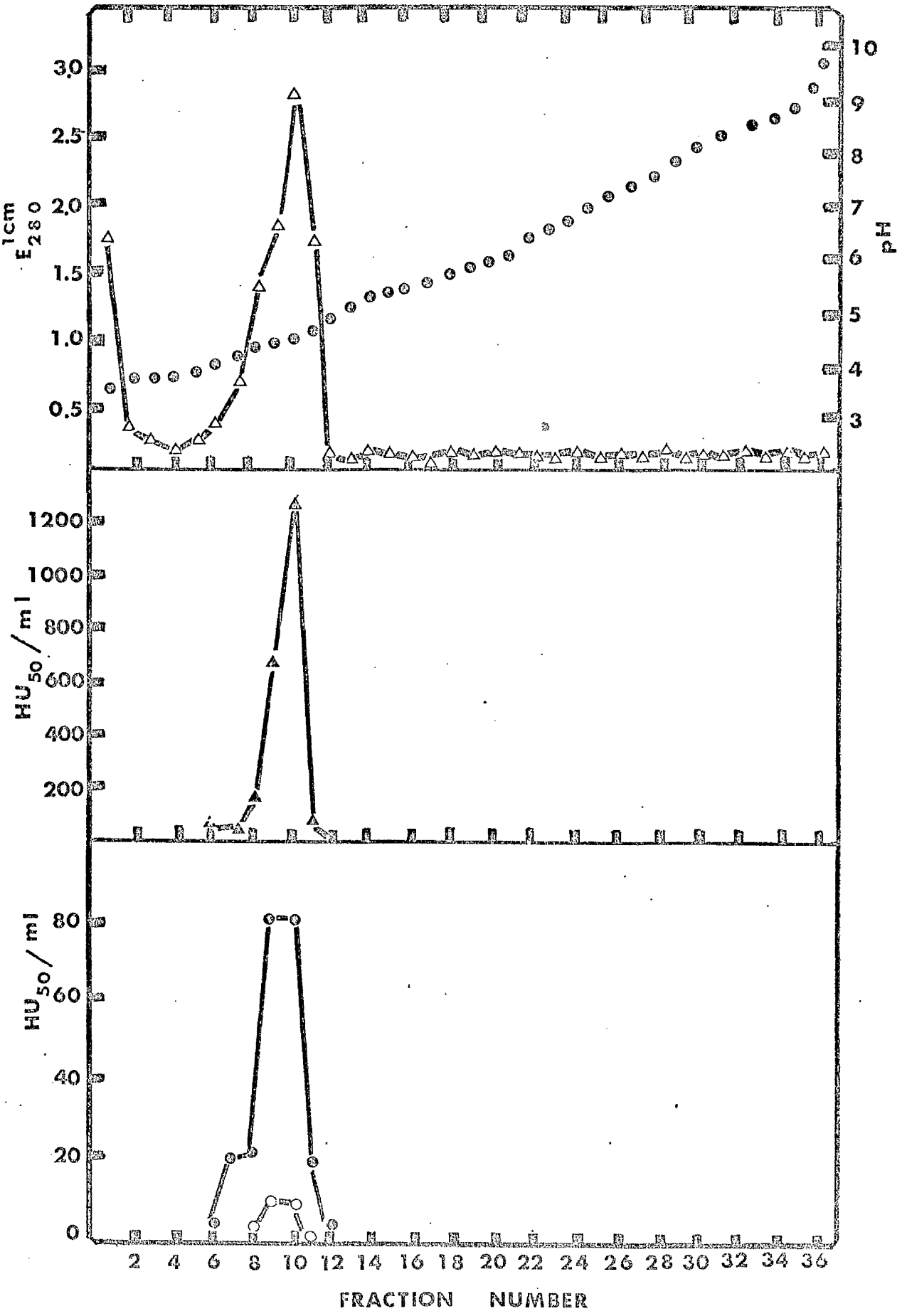

a
TD
SG
b
SG
TD
0
1
2
3
4
5
DISTANCE (cm)

Figure 3. Isoelectric focusing of δ-haemolysin

Purified δ-haemolysin (2 mg) was analysed by isoelectric focusing using the apparatus of Talbot & Caie (1975) and the E_{280}, pH and haemolytic activity of each fraction assayed.




E^{1cm}_{280}
3.0
2.5
2.0
1.5
1.0
0.5
pH
10
9
8
7
6
5
4
3
HU_{50} / ml
1200
1000
800
600
400
200
HU_{50} / ml
80
60
40
20
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
FRACTION NUMBER

Properties of Fish Serum

Selection of a suitable suspending solution for fish erythrocytes is complicated by the wide variation in serum osmolality between different fish species.

In preliminary experiments, frozen fish blood (vide infra) was thawed and diluted in several buffer systems (CDS, Hodgins & Ridgway, 1964; PBS buffers of Holmes & Stott, 1960; Cortland, ; and Dulbecco & Vogt, 1954). Although little lysis occurred in CDS solution, the pH and osmolality of several fish sera and buffers was measured to assess the suitability of this buffer system (Table 15). The osmolalities of saline and of rabbit serum were measured as controls.

Cod serum had a mean osmolality of 401 milliosmoles/kg and a pH of 7.15; the serum osmolality of other fish species varied widely but the serum pH was, as with cod, slightly alkaline. The CDS solution although hypotonic for all fish sera tested appeared to be satisfactory for use in the handling of fish erythrocytes.

Storage of Fish Blood

Because of the difficulty in obtaining regular supplies of fresh fish blood, the conditions required for preservation were examined. In preliminary experiments, washed erythrocytes suspended in a variety of buffers were stable for approx. 3 days at 0^{o} to 4^{o}. Citrated whole blood was more stable; less than 1% lysis of cod blood occurred after 7 days storage at 0^{o}, but lysis increased rapidly during the second week of storage. Citrated haddock, saithe or herring blood were not visibly lysed after one week at 4^{o}. Experiments were therefore made to determine the conditions for storage of fish blood in liquid nitrogen using dimethyl sulphoxide (DMSO) as a cryopreservative.

Table 15. Osmolality and pH of fish sera and buffers

Fluid	Osmolality milliosmoles/kg H_2O	pH
SERA*		
Cod	392	7.1
	407	6.7
	402	7.35
	402	7.2
	n.t.	7.35
Saithe	398	7.3
Pollack	359	7.1
Hake	413	7.2
Whiting	428	7.3
Gurnard	430	7.45
Herring	501	n.t.
Mackerel	414	7.2
Plaice	390	n.t.
Rabbit	304	n.t.
BUFFERS AND SOLUTIONS		
3.8% sodium citrate	371	9.85
0.9% NaCl	290	n.t.
Dulbecco A	256	7.25
CDS	320	7.35

* Five individual cod sera & a pool of 3 saithe sera were tested. The remainder were individual samples.

n.t. = not tested.

Several species of fish were used for these experiments since the availability of particular fish varied during the year.

Storage of cod blood

Aliquots of pooled citrated cod blood were mixed with equal volumes of DMSO (15 to 30% in CDS solution) to give final DMSO concentrations of 7.5 to 15% v/v. Blood was frozen in ampoules in liquid nitrogen which, after various storage times, were thawed and the degree of lysis measured (Table 16). The optimum concentration of DMSO in the freezing mixture was found to be 10 to 12.5%. Initially 10% DMSO was considered the optimum storage concentration and was used in several experiments until it became apparent that 12.5% DMSO gave marginally better recovery of erythrocytes. Cod blood was also stored in 7.5 to 15% DMSO for up to 5 months with little increase in lysis on thawing during this period (Table 17).

Storage of blood of other fish species

Citrated blood of saithe, haddock, mackerel and herring was stored in liquid nitrogen for one month using 7.5 to 15% DMSO as a cryoprotective agent (Table 18). Insignificant lysis of saithe, haddock and mackerel erythrocytes occurred with DMSO concentrations of 10 and 12.5%, but extensive lysis of herring erythrocytes occurred especially at the higher concentrations of DMSO.

Effect of erythrocyte concentration on storage of fish blood

To maximise the quantity of fish blood which could be stored in liquid nitrogen three approaches were considered.

Firstly, instead of adding an equal volume of 20% DMSO in CDS to citrated blood, neat DMSO or 30-50% DMSO in CDS was added to citrated saithe blood to a final concentration of 10% DMSO. Significantly greater lysis of

Table 16. Lysis of cod erythrocytes stored one month in various concentrations of dimethyl sulphoxide in liquid nitrogen

DMSO concentration (% v/v)	Lysis (%) after storage for one month	
	mean*	standard error
7.5	6.8	1.5
10.0	2.1	1.0
12.5	1.5	1.0
15.0	3.4	1.2

* Ten determinations in two separate experiments

Table 17. Lysis of cod erythrocytes during storage in various concentrations of dimethyl sulphoxide in liquid nitrogen

DMSO concentration (% v/v)	Lysis (%)* after storage for stated period					
	1 week	2 wks	1 month	2 months	3 months	5 months
7.5	6.2	9.1	6.6	8.9	5.5	8.2
10.0	0.8	4.9	2.7	7.1	4.7	6.8
12.5	0.0	0.2	0.0	11.6	3.3	5.5
15.0	0.0	0.0	0.0	10.9	7.2	7.0

* Mean of two determinations

Table 18. Lysis of erythrocytes of various fish species stored one month in various concentrations of dimethyl sulphoxide in liquid nitrogen

DMSO concentration (% v/v)	Fish species			
	Saithe	Haddock	Mackerel	Herring
7.5	4.2	7.6	6.6	11.5
10.0	1.8	5.8	5.3	21.9
12.5	2.3	3.3	5.2	48.7
15.0	9.1	8.5	9.6	68.9

erythrocytes occurred as the DMSO concentration in the added fluid was increased (Table 19); least lysis occurred when equal volumes of 20% DMSO in CDS and citrated blood were mixed.

Secondly, the erythrocyte concentration was increased by removal of citrated plasma from the blood before addition of an equal volume of 20% DMSO in CDS. With both saithe (Table 20) and haddock blood (Table 21) insignificant lysis occurred when the erythrocyte concentration was raised to 34% and 20% respectively.

Thirdly, the blood/CDS/DMSO mixture was added dropwise into liquid nitrogen to form pellets which could be stored in canisters rather than ampoules. Cod, saithe and mackerel blood could be satisfactorily stored in this way (Table 22) and mackerel blood was less than 8% lysed over a period of six months.

Blood was routinely stored by removal of plasma to increase the erythrocyte concentration, adding an equal volume of CDS/DMSO and dropping into liquid nitrogen to form pellets. In this way citrated blood from one collection (300 ml) was stored and used over a period of three months without deterioration.

Recovery of blood from the frozen state

Extensive haemolysis usually occurred if the thawed blood was suspended directly in CDS solution instead of in the CDS-DMSO buffer used for storage. Saithe and cod blood stored in 7.5 to 15% of DMSO in CDS for 1 week and 21 weeks respectively was thawed and two samples of each were diluted in CDS or CDS + DMSO. In Table 23 the haemolysis after suspension in CDS or in DMSO-CDS solution was compared and this showed that the degree of haemolysis in CDS was high and also highly variable but was more reproducible in DMSO-CDS. Similar results were obtained for pelleted mackerel erythrocytes (Table 24).

Table 19. Lysis of saithe erythrocytes after storage in 10% DMSO at various erythrocyte concentrations in liquid nitrogen

Composition of freezing mixture			erythrocyte concentration (p.c.v., %)	lysis (%) after two week storage
citrated blood volumes	CDS/DMSO volumes	% DMSO		
1	1	20	11	8.2
2	1	30	14.7	15.0
4	1	50	17.6	26.8
9	1	100	19.8	28.2

Table 20. Lysis of saithe erythrocytes stored in 10% DMSO at various erythrocyte concentrations in liquid nitrogen

erythrocyte concentration in freezing mixture (% p.c.v.)*	Lysis (%) after storage	
	2 days	37 days
9	6.0	5.5
15	6.6	3.0
18	6.8	5.9
27	1.7	0.9
34	2.6	5.2

* Plasma was removed from citrated blood to increase the erythrocyte concentration to 18 - 68% and an equal volume of 20% DMSO in CDS was added

Table 21. Lysis of haddock erythrocytes stored in 10% DMSO at various erythrocyte concentrations in liquid nitrogen

Erythrocyte concentration in freezing mixture (% p.c.v.)	Lysis (%) after storage	
	3 days	37 days
9	2.4	0.0
10	2.8	0.0
13	9.8	5.5
15	3.4	1.0
20	3.0	5.0

Table 22. Lysis of erythrocytes stored as pellets

Erythrocytes	Time stored (weeks)	% lysis
cod (10% DMSO)	2	2.6
	4	6.2
	6	7.3
mackerel (12.5% DMSO)	2	7.7
	3	0.5
	9	7.0
	26	3.5

Table 23. Lysis of cod and saithe erythrocytes after thawing and dilution in CDS solution or DMSO/CDS

Saithe or cod blood, stored in ampoules for 1 week and 21 weeks respectively in 7.5 - 15% DMSO/CDS was thawed and 0.3 ml diluted with 20 ml CDS or 20 ml CDS + DMSO (at the same concentration used for storage). Cod erythrocytes were also diluted in CDS + NaCl (total concentration 0.25 M) or CDS + DMSO + NaCl (total concentration 0.25 M).

Fish species and % DMSO in storage buffer	% lysis on dilution in			
	CDS	CDS + DMSO	CDS + NaCl	CDS + DMSO + NaCl
Saithe				
7.5	52.2, 54.2	9.6, 10.5	-	-
10.0	76.8, 82.0	6.1, 10.1	-	-
12.5	84.0, 90.0	6.7, 10.8	-	-
15.0	91.3, 92.6	7.6, 6.9	-	-
Cod				
7.5	54.1	4.2, 7.9 6.6, 5.2	7.6, 8.7 -	 4.0, 3.0
10.0	82.4	3.4, 9.2 10.0, 6.3	24.4, 23.6	 9.2, 3.3
12.5	81.2	9.1, 12.2 14.7, 13.5	70.0, 79.3	 10.3, 10.9
15.0	-	13.2, 18.4 12.5, 10.6	93.0, 96.2	 8.0, 9.4

Table 24. Lysis of mackerel erythrocytes after thawing and dilution in CDS solution or CDS + DMSO

Pelleted mackerel blood, stored two weeks in 12.5% DMSO/CDS was weighed in tared Universal bottles and CDS solution or CDS solution + 12.5% DMSO was added to give a 5% w/v suspension.

Suspending solution	% lysis
CDS	25.5, 57.4
CDS + 12.5% DMSO	7.0, 8.4

DMSO was washed from erythrocytes with graded dilutions of DMSO in CDS solution through five or six steps to remove both DMSO and serum proteins which inhibit the action of δ-haemolysin. Duplicate samples of cod or saithe blood which had been stored in 7.5 to 15% DMSO in CDS respectively for 2 weeks were thawed in a 35° water bath. Erythrocytes were washed successively in solutions containing decreasing concentrations of DMSO in CDS, the erythrocytes being collected by centrifugation at 600 x g/10 min between each washing. Slight haemolysis occurred during the first three or four washing steps (Table 25). The washed erythrocytes remained fairly stable and the erythrocyte-CDS suspension could be kept at 4° for at least two days without appreciable haemolysis. In a number of tests, the titration results using fish erythrocytes frozen with dimethyl sulphoxide were similar to those obtained using fresh blood.

Finally, no morphological differences were visible between fresh erythrocytes and stored erythrocytes either before or after washing to remove serum proteins and DMSO (Plate 1).

Assay of δ-haemolysin

Preliminary work by Birkbeck & Arbuthnott established that fish erythrocytes had advantages over mammalian erythrocytes for the assay of δ-haemolysin. The conditions required to give a reproducible and sensitive assay for δ-haemolysin were investigated in this section of the work.

Incubation temperature

Doubling dilutions of δ-haemolysin were incubated for 30 minutes with 1% mackerel erythrocytes over the temperature range 0 - 37° and the degree of lysis in each tube measured spectrophotometrically. The haemolysin titre (1000 HU/mg protein) was essentially constant over the temperature range 0 - 37° confirming the results obtained by Birkbeck & Arbuthnott for cod erythrocytes.

Plate 1. Morphology of cod erythrocytes

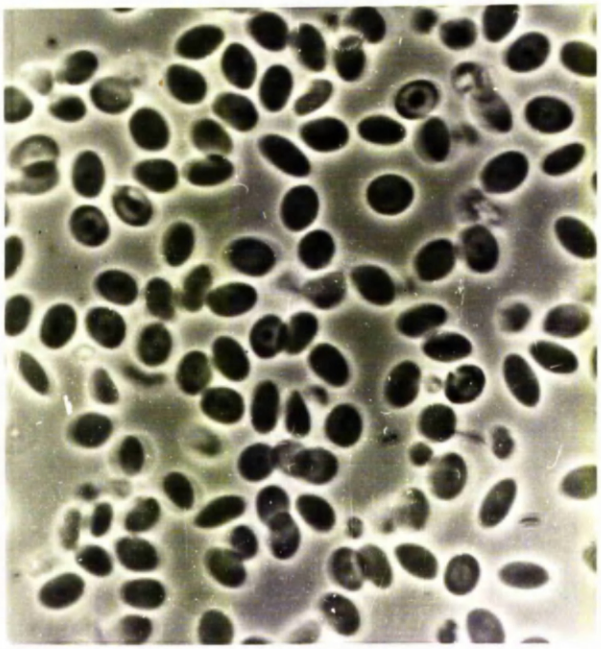
Cod erythrocytes were examined with a Leitz Orthoplan microscope and photographed using Ilford Pan F film.

a) fresh citrated cod blood

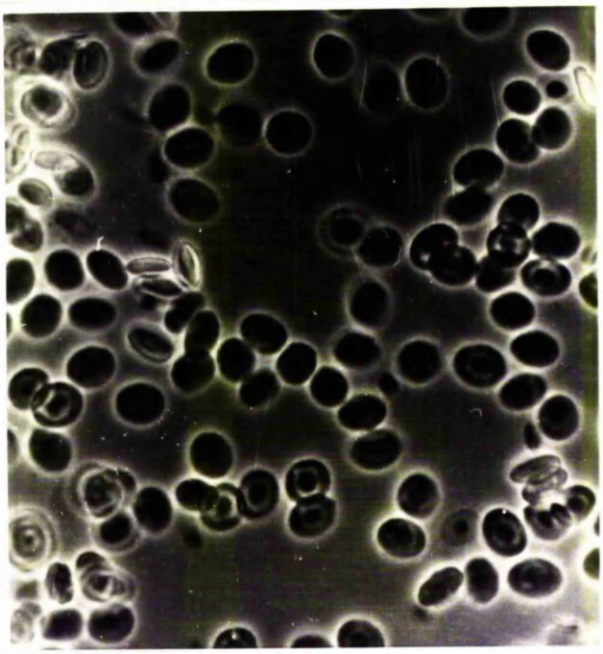
b) fresh citrated cod blood washed once in CDS solution

c) cod erythrocytes after storage in liquid nitrogen (50% citrated blood, 40% CDS, 10% DMSO)

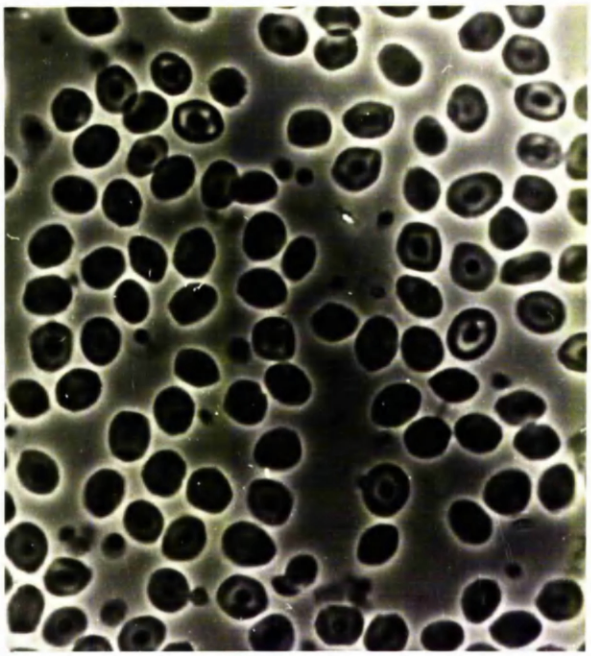
d) stored cod erythrocytes after successive washes in 10, 7.5, 5.0, 2.5, 1.0 and 0% DMSO in CDS solution.



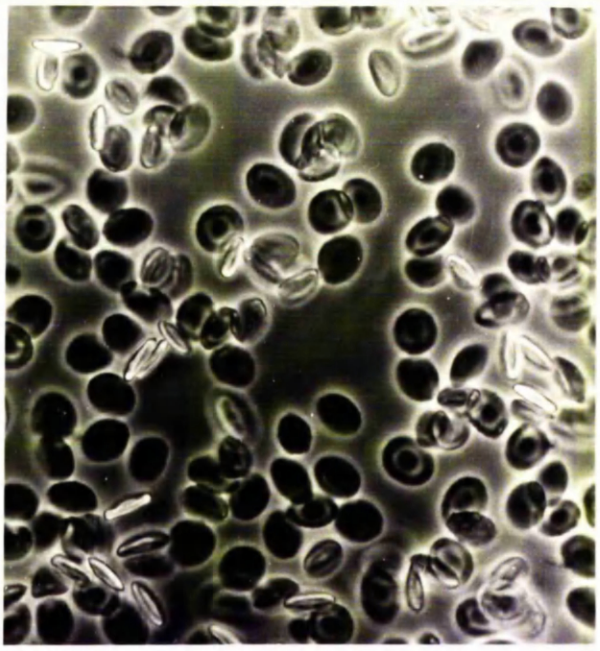
(a)



(b)



(c)



(d)

Table 25. Lysis of cod erythrocytes on washing in CDS solutions containing decreasing amounts of DMSO

Eight ampoules of cod blood containing 7.5 - 15% DMSO in CDS and stored in liquid nitrogen for two weeks were thawed, diluted in 20 ml CDS containing 7.5 - 15% DMSO, centrifuged and successively resuspended in 20 ml CDS containing decreasing amounts of DMSO.

DMSO concentration in storage buffer (%)	% lysis on successive washings in CDS solutions containing the stated concentration of DMSO								recovery (%)	% lysis after 18 hr in CDS solution (+ 4°)
	15	12.5	10.0	7.5	5.0	2.5	1.0	0.0		
15.0	10.5	2.7	0.9	0.2	0	0.9	3.8	2.7	78.3	8.5
	6.8	2.9	0	0	0	1.3	4.7	3.2	81.1	8.4
12.5	-	4.4	0	0.8	0	0	0.3	0	94.5	2.8
	-	6.8	2.0	0.2	0.5	0	0.2	0	90.3	2.0
10.0	-	-	5.1	2.2	0	1.7	0.7	0.5	89.8	4.1
	-	-	7.4	0.8	0	1.6	0.5	1.1	88.6	2.9
7.5	-	-	-	9.2	2.0	1.0	1.2	1.2	85.4	6.7
	-	-	-	10.7	1.4	1.9	1.9	0.6	83.5	4.5

All further work was carried out at an incubation temperature of 15^{o}, as cod erythrocytes were slightly more sensitive to δ-haemolysin at this temperature.

Time of incubation

In the doubling dilution assay of δ-haemolysin, fish erythrocytes lysed rapidly. The kinetics of lysis in the test were determined by performing four parallel doubling dilution assays which were terminated by centrifugation (2000 g/5 min) after 10, 20, 30 and 60 min respectively, and the degree of lysis in each tube measured spectrophotometrically. Erythrocyte lysis was rapid and at the titration end-point the degree of lysis increased only slightly after 10 min incubation (Fig. 4).

All doubling dilution assays were incubated for 30 min although, in practice, acceptable results would be obtained after only 10 min incubation.

Composition and ionic strength of diluent

For assay of complement or of S. aureus β-haemolysin, the composition of the assay buffer has a marked influence on the titres obtained. Delta-haemolysin was therefore assayed using mackerel erythrocytes in buffer containing varying amounts of sodium chloride to increase the ionic strength.

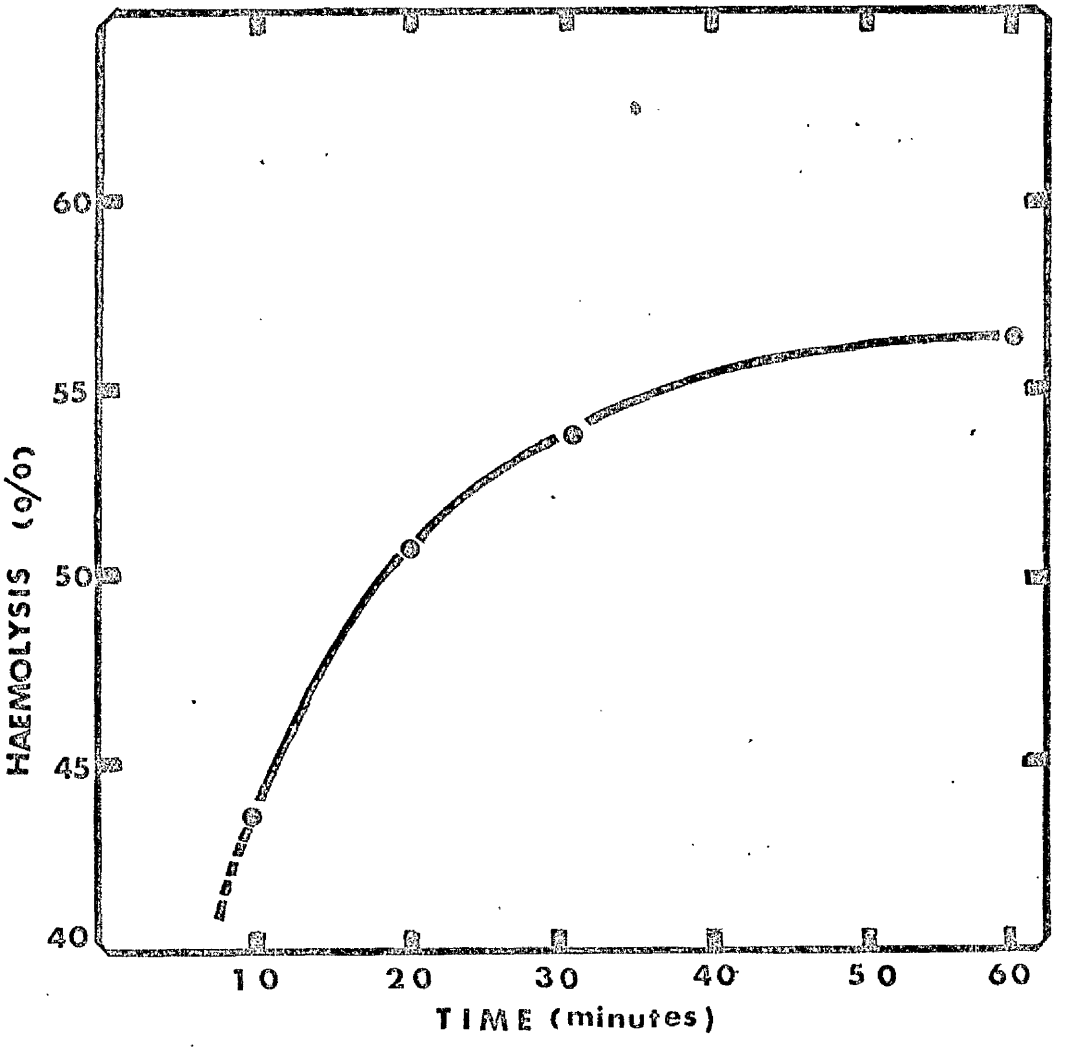
No significant change in titre was found on assay in Dulbecco 'A' PBS, CDS solution, CDS solution with added NaCl (2, 4, 6, 8, 10, 12 and 14 g/litre), Dulbecco 'A' PBS with added NaCl (2, 4 or 6 g/litre). Therefore although CDS solution was slightly hypotonic for fish erythrocytes no increase in sensitivity to δ-haemolysin was observed with solutions closer to ideal tonicity and CDS solution was used in all titrations.

Activation by 2-mercaptoethanol

When δ-haemolysin was incubated with 1 mM 2-mercaptoethanol no increase in activity was found when assayed using cod erythrocytes.

Figure 4 Kinetics of haemolysis of cod erythrocytes by δ-haemolysin

Delta-haemolysin (approximately 1 HU_{50} in 0.5 ml) was added to 0.5 ml aliquots of 0.8% cod erythrocytes and after 10, 20, 30 or 60 min the suspension was centrifuged and the percentage lysis estimated by spectrophotometry.


60
55
50
45
40
HAEMOLYSIS (%)
10
20
30
40
50
60
TIME (minutes)

The effect of erythrocyte concentration on haemolysin titre

The variation in haemolysin titre with erythrocyte concentration was investigated to determine the optimum concentration for assay and also to investigate the mode of action of δ-haemolysin on fish erythrocytes. Mackerel erythrocytes at concentrations of 2 to 0.1% v/v were used and the haemolysin titre (HU_{50}) at each concentration determined from a graph of percentage lysis against haemolysin concentration for each cell concentration used. The variation of haemolysin titre with erythrocyte concentration (Fig. 5) showed that halving the erythrocyte concentration increased the haemolysin titre by approx. 60%. Using 0.3% erythrocyte suspensions, end-points could be measured satisfactorily by spectrophotometry at 540 nm but 0.5 to 1% cell suspensions were more suitable for visual estimation.

To obtain information on the mode of action of δ-haemolysin on fish erythrocytes, haemolysin (1 HU_{50}; determined separately for each erythrocyte species using a 1% cell suspension) was added to human, cod or mackerel erythrocytes of varying concentrations and the percentage cell lysis after 30 min incubation was determined spectrophotometrically.

With fish erythrocytes, the percentage cell lysis was a linear function of log erythrocyte concentration whereas for human erythrocytes a curve was obtained (Fig. 6) indicating that although δ-haemolysin acts on a wide range of erythrocytes, erythrocytes of different species do not respond to δ-haemolysin in the same way.

Haemolytic spectrum of δ-haemolysin

The sensitivity of various erythrocyte species to δ-haemolysin was investigated and the titration end-points determined by spectrophotometry. From Table 26, it can be seen that all fish erythrocyte species tested were

Figure 5 The effect of cod erythrocyte concentration on δ-haemolysin titre

Delta-haemolysin was assayed using 0.1 - 2% cod erythrocytes. The results of three separate experiments are shown.

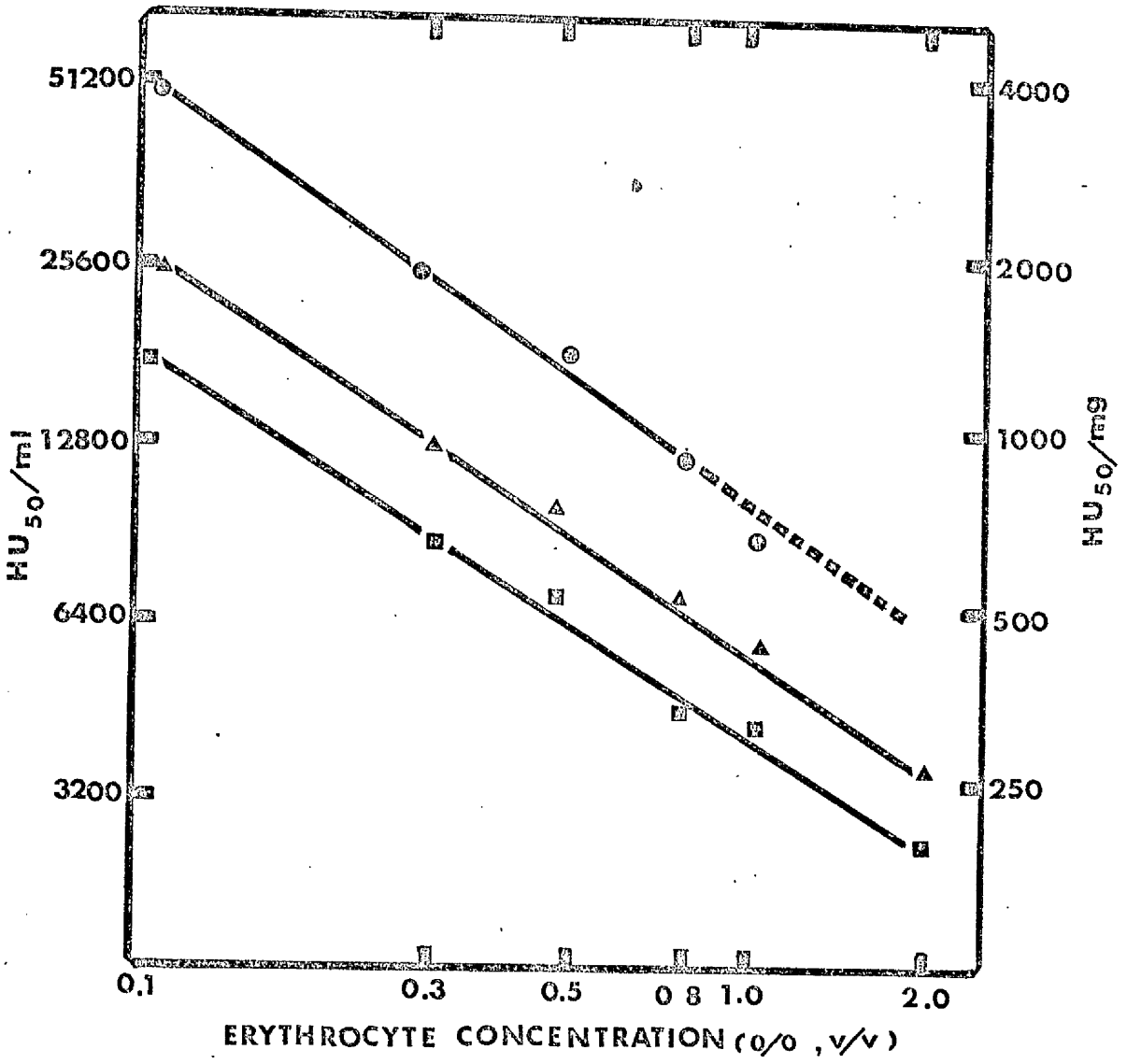
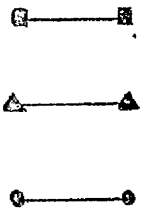

51200
25600
12800
6400
3200
HU_{50}/ml
4000
2000
1000
500
250
HU_{50}/mg
0.1
0.3
0.5
0 8
1.0
2.0
ERYTHROCYTE CONCENTRATION (o/o , v/v)

Figure 6 Degree of haemolysis on incubation of a fixed quantity of δ-haemolysin with various concentrations of erythrocytes

Delta-haemolysin (approx 1 HU_{50}/0.5 ml for a 1% suspension of each erythrocyte species) was added to an equal volume of human, cod or mackerel erythrocytes (0.25 - 8.0% v/v). After 30 min incubation the degree of haemolysis was measured by spectrophotometry.



human

cod

mackerel

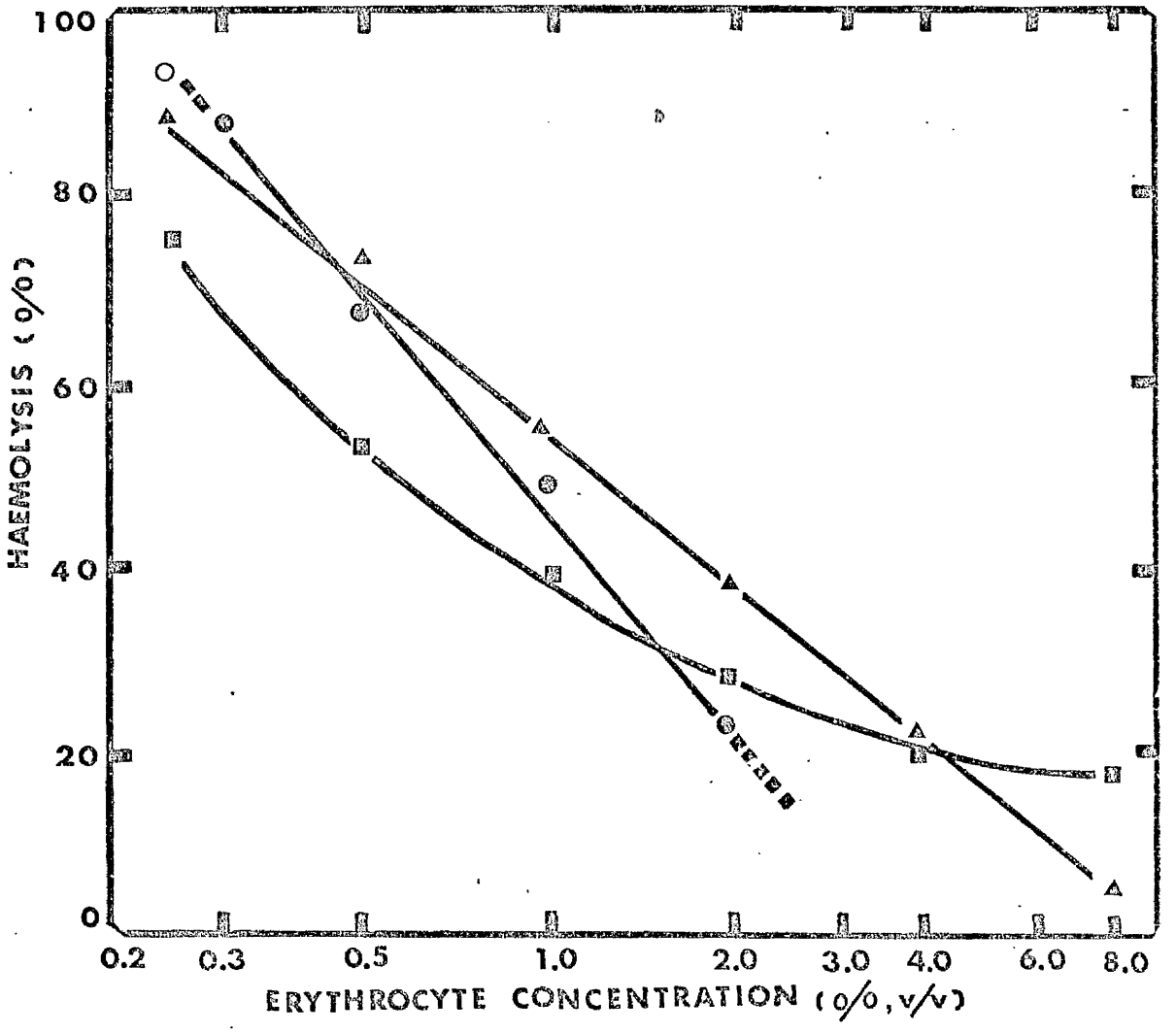

100
80
60
40
20
0
HAEMOLYSIS (%)
0.2
0.3
0.5
1.0
2.0
3.0
4.0
6.0
8.0
ERYTHROCYTE CONCENTRATION (%, v/v)

Table 26. Haemolytic spectrum of δ-haemolysin

Erythrocyte species[1]	sensitivity relative to human (%)
Human	100
Horse	100
Rabbit	60
Sheep	20
Cod	400
Poor Cod	400
Saithe	400
Haddock	400
Hake	400
Whiting	400
Plaice	200
Gurnard	200
Mackerel	1600

1. 1% suspension of mammalian erythrocytes were titrated at 37^{o}, fish erythrocytes at 15^{o}.

more sensitive to δ-haemolysin than mammalian species. Mackerel erythrocytes were particularly sensitive, being up to sixteen times more sensitive than human or horse erythrocytes.

To test for variations in the haemolysin sensitivity of blood from individual specimens of cod and saithe, blood samples from twelve cod and four saithe were collected separately and tested for haemolysin sensitivity in a single experiment. Two-fold dilutions of δ-haemolysin were made in CDS solution and 0.5 ml volumes of each dilution were added to 0.5 ml aliquots of 1% suspensions of erythrocytes from the individual fish. The degree of lysis in each mixture was measured by spectrometry and 50% end-points determined by plotting percentage lysis against haemolysin dilution. The maximum variation in titre between individual cod was two-fold (mean titre 6000 HU/ml; standard error = 350 HU/ml) which was within the experimental error of the test. Similar results were obtained using saithe erythrocytes, from which it was concluded that there was no significant variation in individual sensitivity within these species.

Assay of δ-haemolysin by Radial Diffusion in Blood Agar Plates

Kantor *et al*. (1972) assayed δ-haemolysin by radial diffusion in human blood agar plates and found that the diameter of the zone of haemolysis was proportional to the logarithm of the haemolysin concentration. Similar experiments were therefore performed here to compare the effectiveness of fish and human erythrocytes for δ-haemolysin assay.

Blood agar plates were prepared using cod or mackerel erythrocytes but these were thermolabile and deteriorated rapidly at 37^{o} and plates were therefore incubated at room temperature. Although fish blood could be collected aseptically by swabbing the skin with ethanol this was not done routinely and

addition of sodium azide (0.01%) to blood agar plates prevented growth of contaminating microorganisms on the agar.

Effect of sodium chloride concentration on the diameter of haemolysis zones

The osmolality of fish blood is greater than that from mammals and, in order to determine the optimum NaCl concentration for assay, cod blood agar plates were prepared with NaCl levels from 0.085M to 0.75M. Wells (4 mm diameter) were punched in the agar and 10 µl δ-haemolysin applied to each well; after incubation at room temperature for 24 and 48 hr the diameter of each lysis zone was measured with an eyepiece and graticule. Fig. 7 shows that at both observation times the zones of maximum diameter with clearly defined edges were obtained at sodium chloride concentrations of 0.2 to 0.3M. In subsequent experiments 5.8 g/l NaCl was added to Oxoid Blood Agar Base No. 2 to achieve a final NaCl concentration of 0.25M.

The experiment was repeated with mackerel erythrocytes and, again, maximum zone diameters occurred with 0.25M NaCl although the difference in zone diameters was not so great as with cod erythrocytes.

Rate of development of zones of lysis

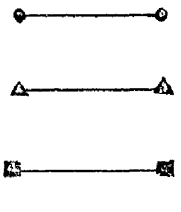
In cod, mackerel and human blood agar plates at room temperature, lysis zones with δ-haemolysin were visible within 4 hr but required 48 hr to grow to maximum size (Fig. 8). At this time the zone diameter was proportional to log haemolysin concentration. The size of the haemolytic zone was markedly dependent on the blood concentration in the agar plate (Fig. 9).

Comparison of human and fish blood for radial diffusion assay of δ-haemolysin

Human, cod and mackerel blood agar plates (2% v/v) were compared for the assay of δ-haemolysin (128 HU/ml) by inoculation of 10 µl serial dilutions into 4 mm diameter wells in each plate. The lysis zone diameters were measured

Figure 7 The effect of increasing sodium chloride concentration on the diameter of lysis zones produced by δ-haemolysin in fish blood agar plates

Delta-haemolysin (10 µl) was applied to 4 mm diameter wells in 1% cod blood agar plates containing NaCl to a total concentration of 0.085M to 0.75M and the zones of lysis measured after 24 and 48 hr at room temperature.



16 HU_{50}/ml δ-haemolysin

32 HU_{50}/ml δ-haemolysin

80 HU_{50}/ml δ-haemolysin

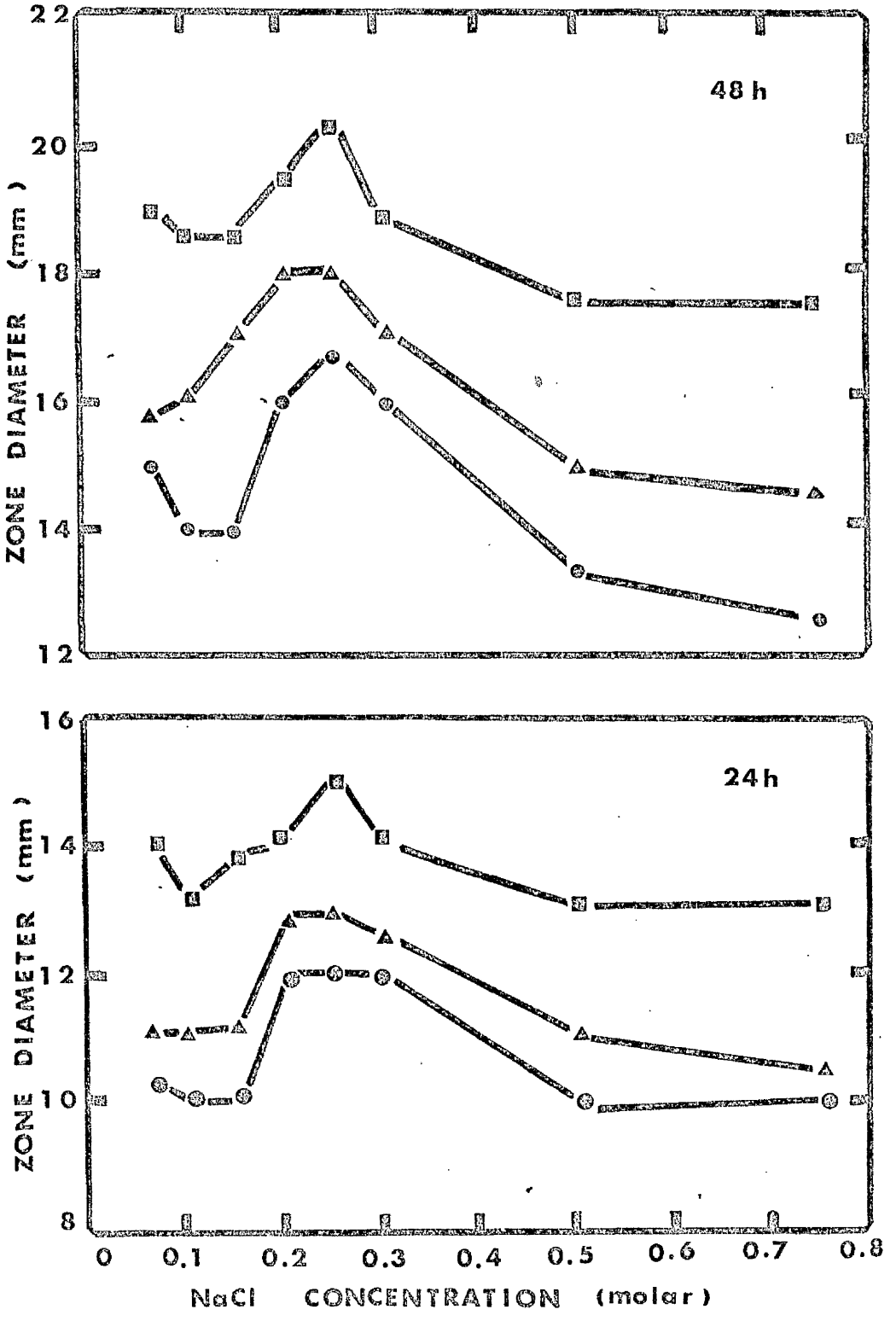

48 h
24 h
ZONE DIAMETER (mm)
22
20
18
16
14
12
10
8
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
NaCl CONCENTRATION (molar)

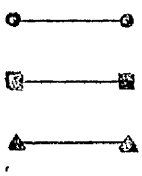
Figure 8. Rate of development of lysis zones in blood agar plates

Delta-haemolysin (10 µl of solutions containing 16-128 HU_{50}/ml assayed with cod erythrocytes) was applied to 4 mm diameter wells in blood agar plates and lysis zones measured after 4, 24 and 48 hr incubation at room temperature.

A 2% human blood agar

B 2% cod blood agar

C 2% mackerel blood agar



4 hr

24 hr

48 hr

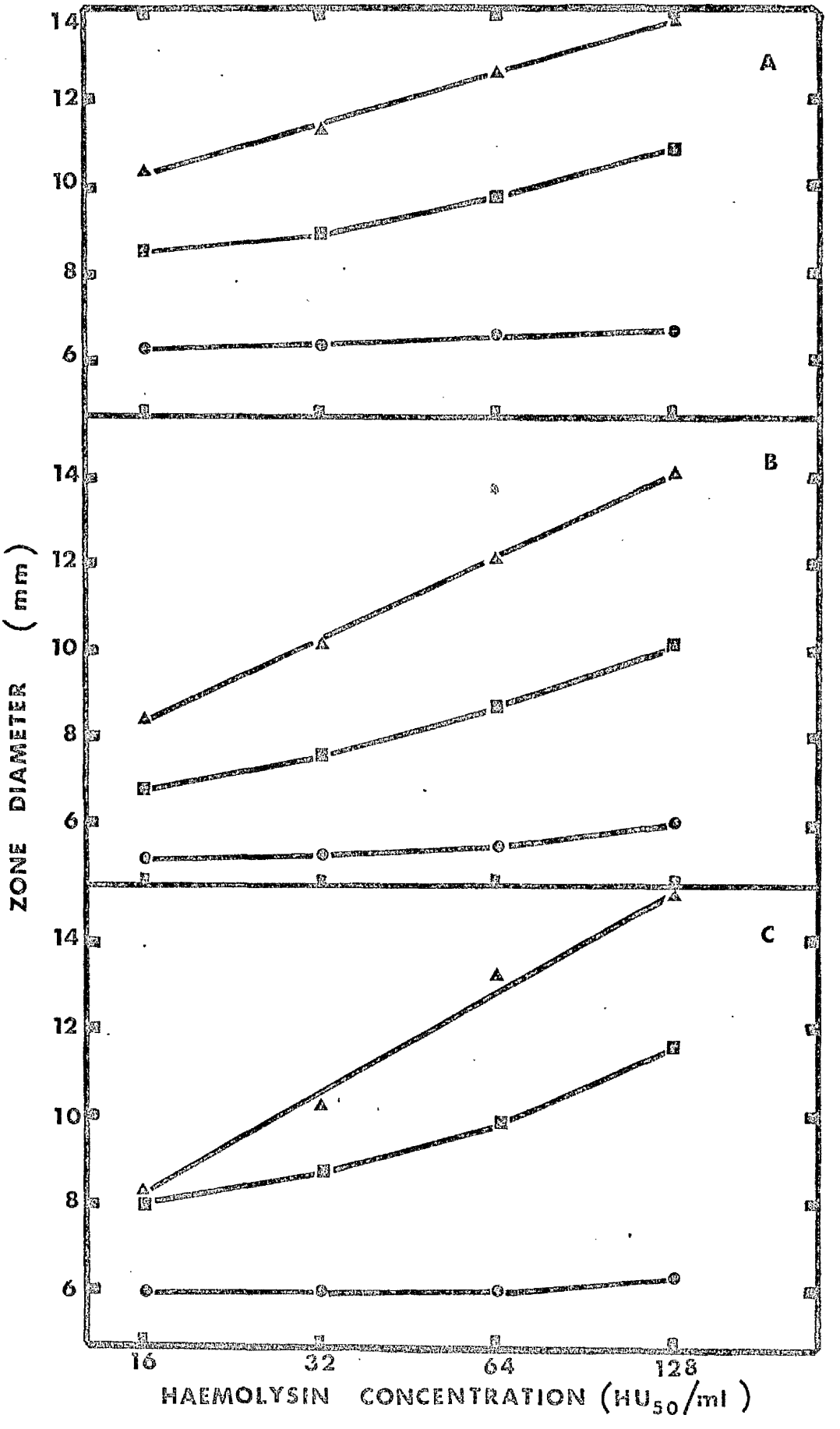
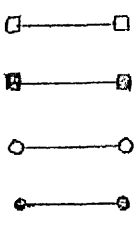

A
B
C
14
12
10
8
6
ZONE DIAMETER (mm)
16
32
64
128
HAEMOLYSIN CONCENTRATION (HU_{50}/ml)

Figure 9 The diameter of lysis zones produced by δ-haemolysin in blood agar plates of varying blood concentration

Delta-haemolysin (10 µl) was applied to 4 mm wells in cod or mackerel blood agar plates and the diameter of lysis zones measured after 48 hr at room temperature.

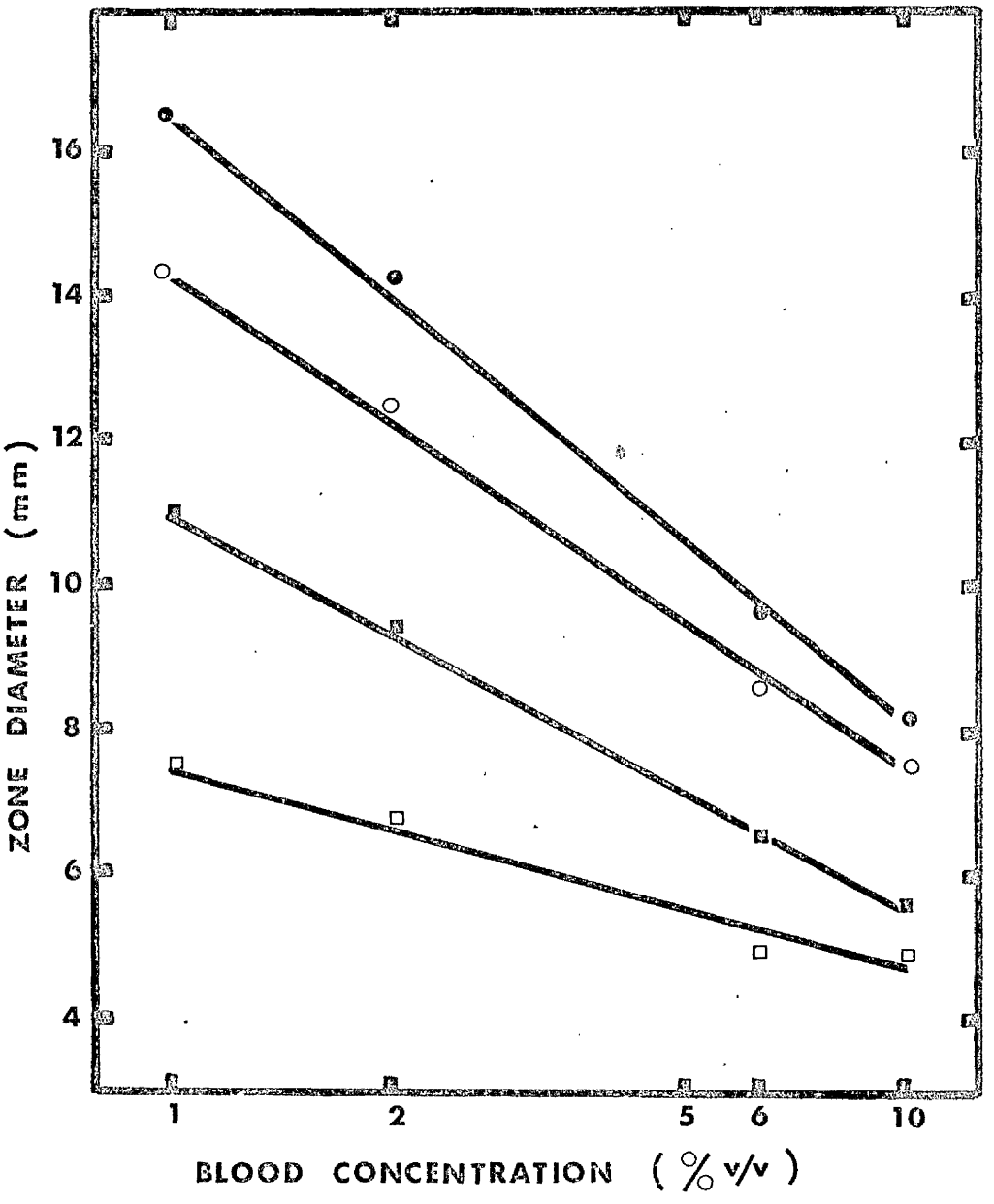


cod blood agar; 32 HU_{50}/ml δ-haemolysin

mackerel blood agar; 32 HU_{50}/ml δ-haemolysin

cod blood agar; 80 HU_{50}/ml δ-haemolysin

mackerel blood agar; 80 HU_{50}/ml δ-haemolysin


ZONE DIAMETER (mm)
16
14
12
10
8
6
4
1
2
5
6
10
BLOOD CONCENTRATION (% v/v)

after 48 hr incubation at room temperature and a graph of zone diameter against log haemolysin concentration plotted (Fig. 10). Each erythrocyte species showed a linear response over the δ-lysin concentration range used and the sensitivity of the three erythrocyte species was similar. The slopes of the lines varied widely and doubling the δ-haemolysin concentration resulted in an increase in zone diameter of 2.4, 1.9 and 1.3 mm for mackerel, human and cod erythrocytes respectively. The slope obtained with human cells was the same as that found by Kantor et al. (1972) for human blood agar incubated at 37°.

Although fish blood agar could be used satisfactorily for the assay of δ-haemolysin, the time and quantity of blood required did not justify its use in place of the tube dilution assay with erythrocytes in suspension.

Survey of Staphylococci for δ-haemolysin Production

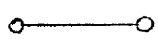
A preliminary survey of staphylococci was begun to explore the possible use of cod blood agar for detection of δ-haemolysin production by clinical isolates. The strains chosen for study, including five clinical isolates kindly provided by Miss S. McKay (designated SM), comprised S. aureus strains Wood 46 and SM 10 (producing α-haemolysin), SM 14 and SM 15 (producing β-haemolysin), JK 21 (producing epidermolysin) and strains SM 6 and SM 9 (weakly-toxinogenic). Strain NCTC 10345 was included for reference as a δ-haemolysin-producing strain.

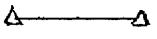
Growth of staphylococci on blood agar plates at 37°

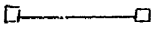
Staphylococcal isolates were initially cultured on mammalian blood agar plates to observe the haemolysis zones produced in conventional blood agar. Blood agar plates (containing 5% v/v human, rabbit, sheep or horse blood) were stab inoculated with eight staphylococcal strains and after incubation for 24 hr at 37° were examined and measured (Table 27).

Figure 10 Comparison of zones of lysis produced by δ-haemolysin in mackerel, cod and human blood agar

Delta-haemolysin (10 µl of solutions containing 16 - 128 HU_{50}/ml δ-haemolysin, assayed using cod erythrocytes) was applied to 4 mm diameter wells in 2% mackerel, cod or human blood agar and the diameter of lysis zones measured after 48 hr incubation at room temperature.


mackerel blood


cod blood agar


human blood agar

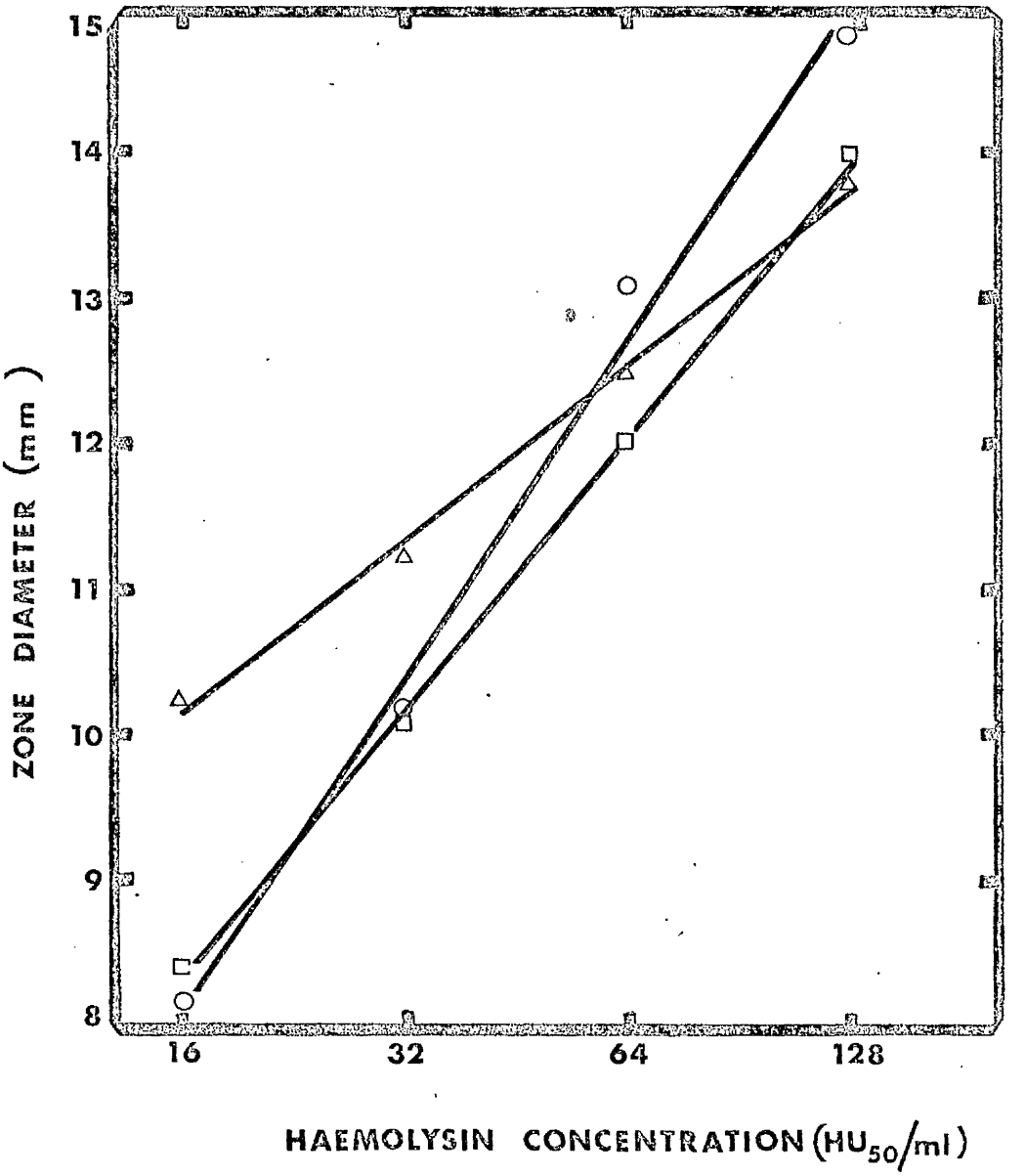

15
14
13
12
11
10
9
8
ZONE DIAMETER (mm)
16
32
64
128
HAEMOLYSIN CONCENTRATION (HU50/ml)

Table 27. Haemolysis zones produced in blood agar plates by eight strains of S. aureus

Size of haemolytic zone (mm, from colony edge to zone edge) produced in 5% blood agar plates of the following species of blood after incubation at 37° for 24 hrs.

Strain	Human	Horse	Sheep	Rabbit
NCTC 10345	0.4	0.5	0	0.3
Wood 46	0.5	0	1.8	0.4
SM 10	0	0	1.0	0.2
SM 14	0.8	1.5	2.3	0.9
SM 15	1.0	0	2.0	1.8
JK 21	1.2	2.0	2.0	1.3
SM 6	0.5	0.1	0	0.1
SM 9	0.5	0	1.4	0.8

On sheep blood agar, strains SM 14 and SM 15 produced large zones of partial lysis (approx. 10 mm radius) surrounding the central area of complete lysis (2 - 2.3 mm radius) which was characteristic of β-haemolysin production. Where the zone of partial lysis produced by strain SM 15 extended to the colony of strain SM 6 complete lysis of cells occurred near the SM 6 colony; such synergistic lysis (Williams & Harper, 1947) indicated that strain SM 6 probably produced δ-haemolysin in small amount and that strain SM 15 produced large amounts of β-haemolysin.

Strain Wood 46 behaved atypically in this experiment and zones of lysis were smaller than those usually attained; in further experiments, only colonies which yielded large haemolytic zones (>3 mm radius) in sheep blood agar were selected for experiment and these produced larger zones in rabbit blood agar.

Haemolytic zones produced by staphylococci in blood agar overlay plates

Cod blood deteriorated rapidly at 37^{o} and blood agar plates darkened so quickly that lysis zones could not be distinguished after overnight incubation. Staphylococci were therefore stab inoculated onto nutrient agar plates and cultured at 37^{o} for 24 hr. An overlay of 10 ml 5% blood agar was applied to each plate after which a further period of incubation at 6^{o} for 48 hr was given and the zones of lysis measured. S. aureus strains NCTC 10345, Wood 46, SM 15 and SM 6 were used in initial experiments with overlays of human, horse, sheep and rabbit blood. Four experiments were done on duplicate plates in each case and the mean size of zones of complete lysis is shown in Table 28.

Strain NCTC 10345 produced larger zones of lysis in human and rabbit blood agar overlays than on blood agar plates and strain Wood 46 typically produced large lysis zones in rabbit and sheep blood agar overlays.

Table 28. Haemolysis zones produced in blood agar overlay plates

Size of haemolytic zone in millimetres (colony edge to zone edge) produced in 5% blood agar overlays of the following species of blood.

Strain	Human	Horse	Sheep	Rabbit
NCTC 10345	1.6	0.5	0	2.2
Wood 46	1.9	2.6	3.9	6.9
SM 15	1.1	1.0	8.7	6.8
SM 6	0	0	0	0

Staphylococcal isolates were stab inoculated into nutrient agar plates, incubated for 24 hr at 37^{o}, overlaid with 5% blood agar and incubated again for 48 hr at 6^{o}. The results are the mean of four experiments each with two agar plates.

The δ-haemolysin producing strain SM 15 again produced very large lysis zones in sheep and rabbit blood agar and strain SM 6 failed to produce measurable lysis zones in any blood type.

When 5% cod blood agar was used as an overlay on nutrient plates previously inoculated with eight strains of S. aureus and cultured for 24 hr at 37° lysis zones were evident around all colonies. Zones of lysis in cod blood agar were in all cases clearly defined (Plate 2) and larger than comparable zones produced in human or horse blood agar (Table 29). Strain Wood 46 again produced smaller lysis zones than usual on sheep and rabbit blood agar and only strain SM 15 produced large lysis zones on sheep blood agar with a large surrounding zone of partial lysis which interacted with barely visible zones of partial lysis around colonies of strains SM 10 and SM 14 to give arcs of complete lysis (Plate 2).

Williams & Harper (1947) and Marks & Vaughan (1950) stated that the yield of δ-haemolysin was greatly increased by culture in an atmosphere containing carbon dioxide. Nutrient agar plates were therefore stab inoculated with the eight staphylococcal strains and incubated in an atmosphere of either air or 30% CO_2/70% air before application of the blood overlay. After 48 hr at 6° the zones of lysis produced in cod or human blood agar after culture in 30% CO_2 were smaller than those obtained by culture in air in all cases (Table 29). However, with sheep blood agar all zones on plates incubated in 30% CO_2 were greater or equal to the size of zones on plates incubated in air. The β-haemolysin producing strains SM 14 and SM 15 produced larger lysis zones on horse, sheep and rabbit blood agar after incubation in 30% CO_2.

Haemolysin titres of culture supernatants of staphylococci

The above experiment indicated that changes in one cultural condition

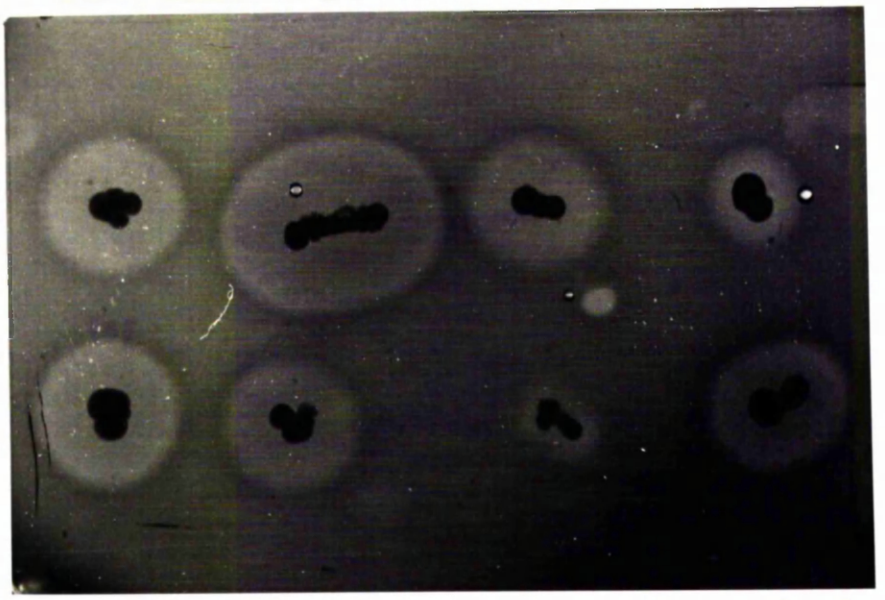
Plate 2. Haemolytic reaction of eight staphylococcal strains in blood-agar overlay plates

Staphylococcal strains were stab inoculated onto nutrient agar plates, incubated 24 hrs at 37°, overlaid with 10 ml 5% blood agar and reincubated for 24 hr at 4°.

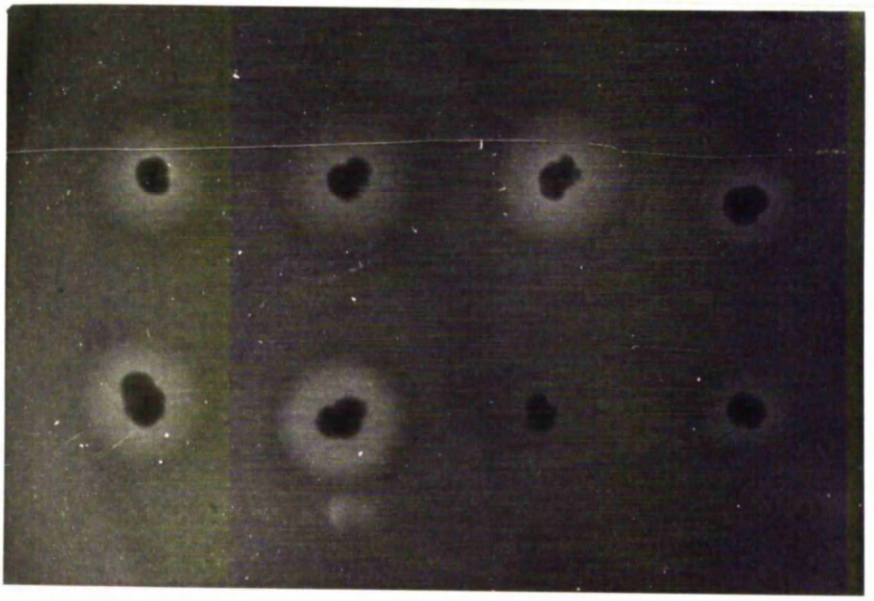
Staph. aureus Wood 46	SM 14	JK21	SM9	
SM 10	SM 15	SM 6	NCTC 10345	

a) COD
b) HUMAN
c) RABBIT
d) HORSE
e) SHEEP

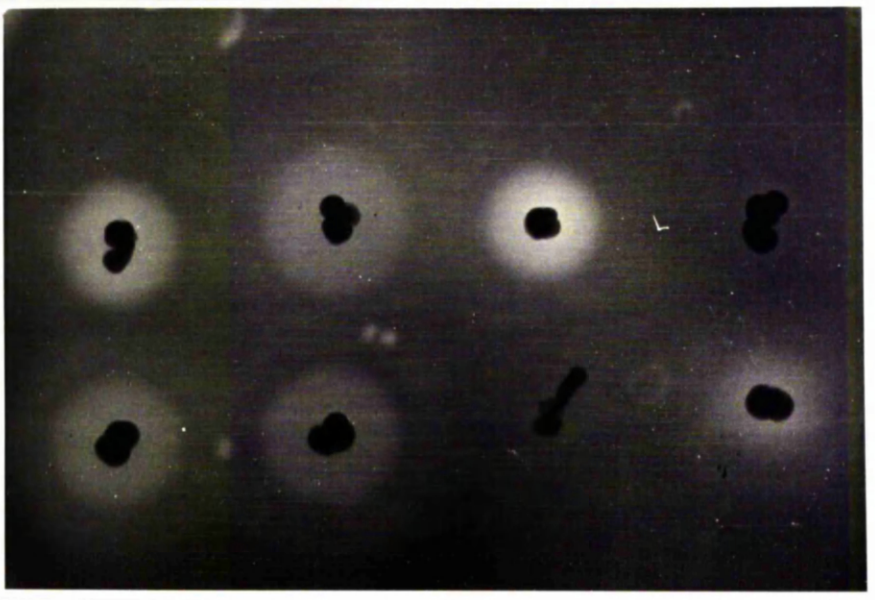
(a)



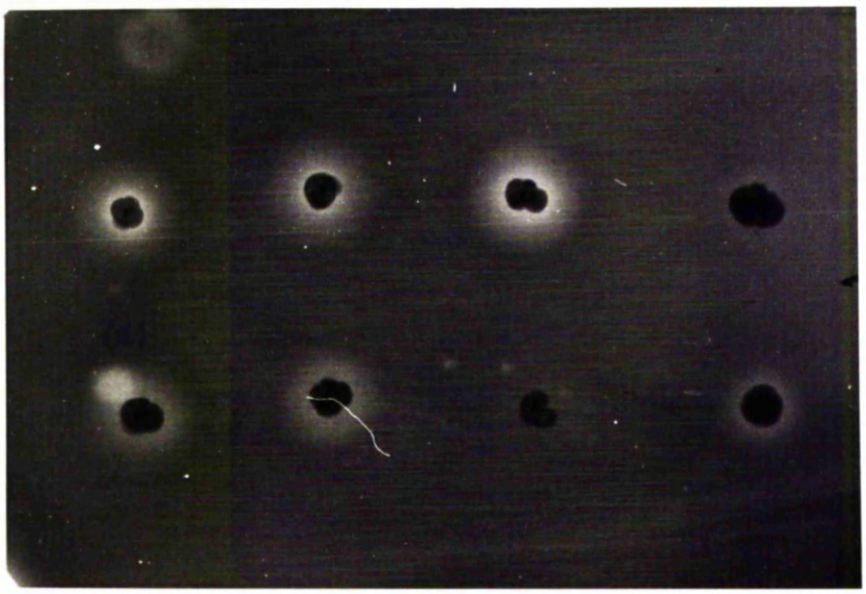
(b)



(c)



(d)



(e)

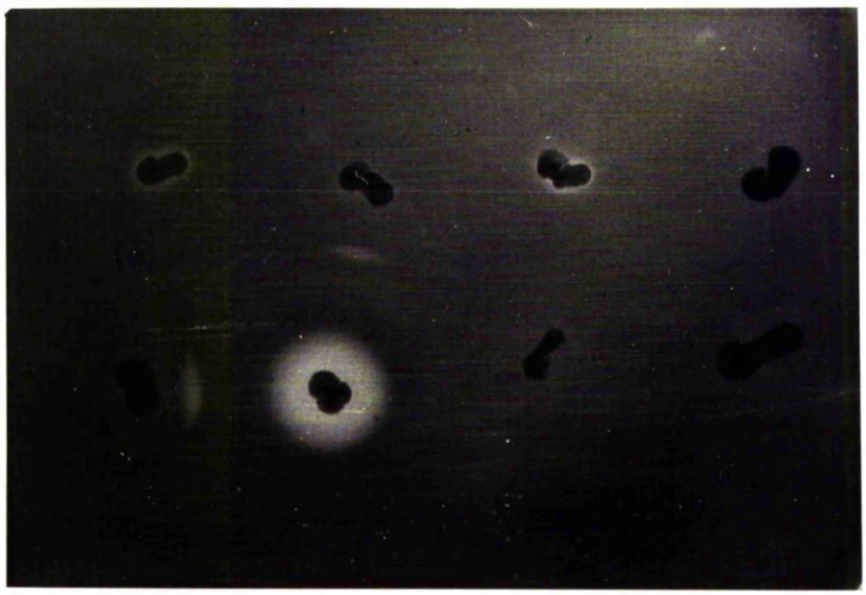


Table 29. Haemolytic zones produced by eight strains of S. aureus in blood agar overlay plates incubated in air or in 30% CO_2

Size of haemolysis zone (mm colony edge to zone edge) produced in blood agar overlays of following species of blood

Strain	Human		Horse		Sheep		Rabbit		Cod	
	air	air/CO_2	air	air/CO_2	air	air/CO_2	air	air/CO_2	air	air/CO_2
NCTC 10345	1.7	0.9	0.9	0.5	0	1.6	2.4	4.3	4.6	1.7
Wood 46	2.8	1.4	2.0	0.4	0.2	0.2	2.7	1.6	3.4	1.3
SM 10	2.3	0.7	1.4	1.4	0	2.1	2.5	5.3	4.3	1.3
SM 14	3.4	0.8	2.0	2.3	0	1.8	3.8	5.8	5.0	1.6
SM 15	2.8	1.9	1.3	3.7	7.6	9.3	3.6	8.0	4.1	1.1
JK 21	3.3	1.3	2.1	0.4	0.1	0.2	1.9	1.7	4.0	1.1
SM 6	0	0	0	0	0	0	0	0	1.2	0.2
SM 9	0.6	0	0	0	0	0	0	0	2.3	0

resulted in changes in the size of lysis zones in each type of blood agar which presumably reflected an altered balance of haemolysin production by each strain of S. aureus. To obtain optimum yield of a chosen haemolysin in broth culture requires consideration of medium composition, atmosphere, time and temperature of incubation appropriate for each strain. Five strains of S. aureus (NCTC 10345, Wood 46, SM 14, SM 15 and JK 21) were cultured in 100 ml Bernheimer's yeast extract diffusate medium for 18 hr using the cultural conditions previously described for strain NCTC 10345 (Results) and, after centrifugation (25,000 g for 10 min at 4^{o}) the haemolysin titres of culture supernatants were determined using a 1% suspension of human, horse, sheep, rabbit or cod erythrocytes. Samples of culture supernatant were also heated (100^{o}, 10 min) before titration to inactivate α-haemolysin (Table 30). Heating caused a significant reduction in titre (greater than 50%) only for supernatants of strains Wood 46 and SM 15 when titrated using sheep and rabbit erythrocytes. Haemolysin titres obtained using human, horse or cod erythrocytes were not significantly diminished by heating.

Isoelectric focusing of culture supernatant of S. aureus strain Wood 46

The haemolytic activity of the culture supernatant of S. aureus Wood 46 against rabbit and cod erythrocytes (Table 30) indicated the presence of α- and δ-haemolysins in the culture supernatant and in order to demonstrate this more conclusively and to exclude the presence of β-haemolysin the culture supernatant was analysed by isoelectric focusing. Ammonium sulphate (27 g) was added to 45 ml culture supernatant and the 85% saturated ammonium sulphate solution was stirred overnight and the precipitate collected by centrifugation. The precipitate was dissolved in 2 ml 1% glycine, dialysed overnight against 1% glycine and 1 ml was separated by isoelectric focusing in the Talbot isoelectro-focusing trough to a final potential of 1000V. The optical density, pH and

Table 30. Haemolysin titres of culture supernatants of five strains of S. aureus

Reciprocal haemolysin titre of culture supernatants assayed with the following erythrocytes.

Strain	Human		Horse		Sheep		Rabbit		Cod	
	C*	H	C	H	C	H	C	H	C	H
NCTC 10345	64	32	128	64	32	16	32	16	128	128
Wood 46	64	32	64	64	128	32	2048	256	128	64
SM 14	64	64	64	64	32	16	128	128	64	64
SM 15	32	16	16	16	64	16	128	32	16	8
JK 21	32	32	32	16	16	16	32	16	16	8

*C - unheated culture, supernatant

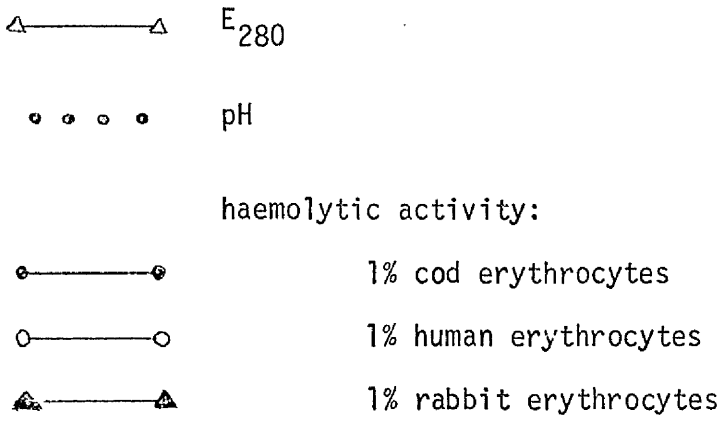
H - heated culture supernatant (100^{o}/10 min).

haemolysin titre of each fraction against cod, rabbit, human and sheep erythrocytes was determined. Two peaks of haemolytic activity were obtained (Fig. 11) with pI of 5.2 and 7.80. The haemolytic activity of the component of pI 5.2 was greatest against cod erythrocytes whereas the component of pI 7.8 was most active against rabbit erythrocytes (Table 31). Haemolysin titres against sheep erythrocytes were 16 - 32 fold less than against rabbit erythrocytes and did not increase after overnight incubation at 4^{o}.

The acidic haemolysin possessed an isoelectric point and haemolytic spectrum similar to that of δ-haemolysin and the basic haemolysin with preferential haemolytic activity for rabbit erythrocytes was focused within the pI range characteristic of α-haemolysin.

Figure 11 Isoelectric focusing of concentrated culture supernatant of S. aureus Wood 46

Culture supernatant from a broth culture of S. aureus Wood 46 was concentrated by ammonium sulphate precipitation, dialysed against 1% glycine and analysed by isoelectric focusing in a broad pH gradient (3.5 - 10). The E_{280}, pH and haemolytic activity of each fraction was assayed.



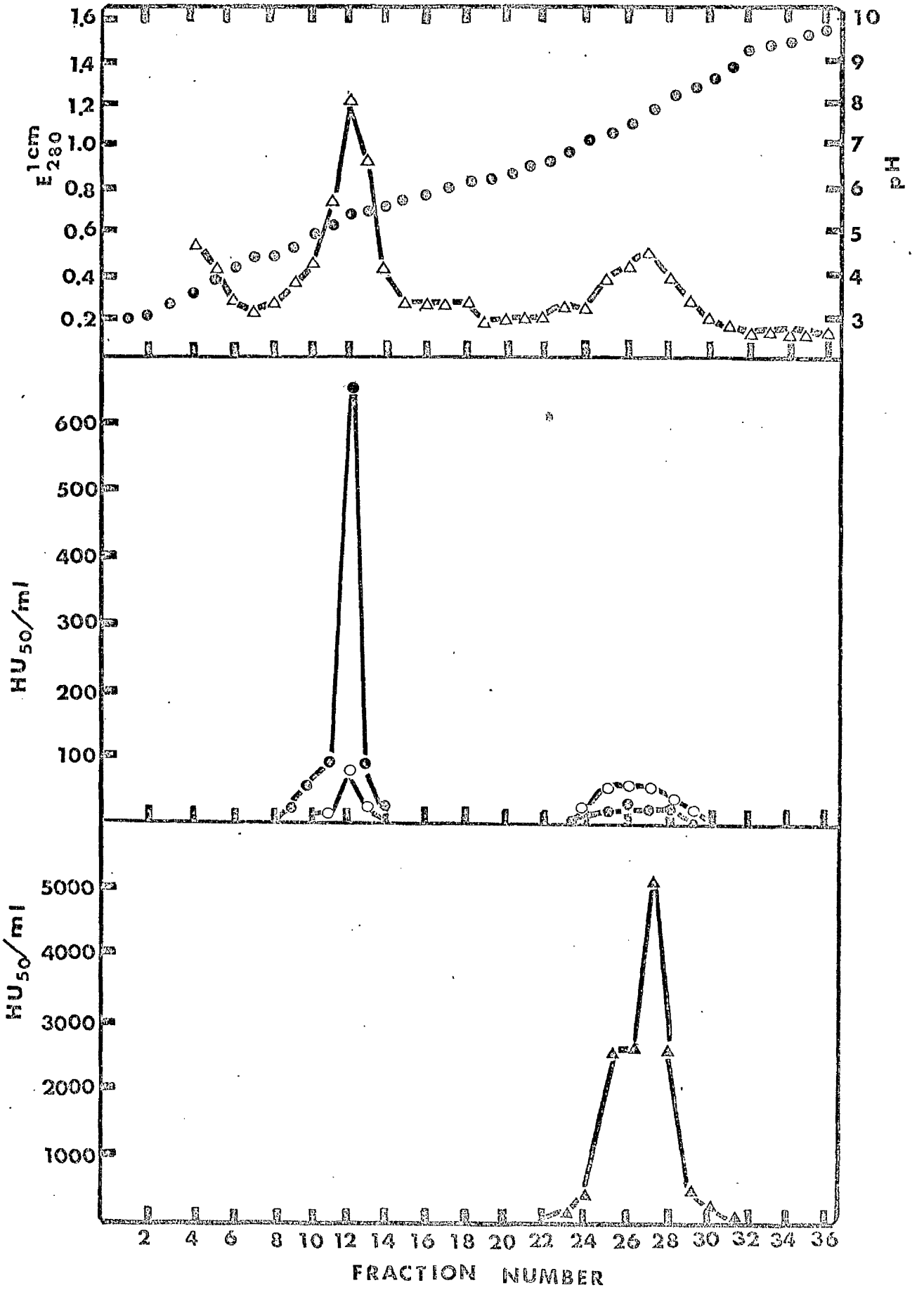

E^{1cm}_{280}
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
9
8
7
6
5
4
3
pH
HU_{50}/ml
600
500
400
300
200
100
HU_{50}/ml
5000
4000
3000
2000
1000
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
FRACTION NUMBER

Table 31. Haemolytic activity of peak fractions obtained by isoelectric focusing

Fraction	Haemolysin titre with erythrocytes from:				
	pI	Cod	Rabbit	Sheep	Human
12	5.2	640	40	<10	80
27	7.8	20	5120	320	40

*Cod erythrocytes were incubated at 15^{o} in CDS solution; rabbit and human erythrocytes at 37^{o} in Dulbecco A PBS and sheep erythrocytes at 37^{o} in Dulbecco A PBS containing Mg^{2+}.

DISCUSSION

The principal objective of this investigation was to assess the suitability of cod and other fish erythrocytes for the assay of staphylococcal δ-haemolysin. The unpublished studies of Birkbeck & Arbuthnott showed that staphylococcal α- and β-haemolysins had no significant effect on cod erythrocytes and that cod erythrocytes were more susceptible than mammalian erythrocytes to the action of δ-haemolysin.

One of the major difficulties envisaged in the use of fish erythrocytes in the laboratory was the procurement of regular supplies of fish blood and a major part of this study was therefore devoted to determining suitable conditions for the long-term storage of erythrocytes to avoid the necessity for frequent collection.

The development of the assay required purified δ-haemolysin and the production, purification and characterisation of δ-haemolysin was also a major part of the work. However, the prime objective was not a study of δ-haemolysin *per se* but the development of a more sensitive assay system which would eventually facilitate study of the properties of δ-haemolysin. Determination of the properties of purified δ-haemolysin was therefore regarded as secondary to assay development.

Preparation of δ-haemolysin

The staphylococcal strains initially surveyed for production of δ-haemolysin were chosen because of their production of haemolysins not neutralised by high titre anti α-haemolysin. Using rabbit erythrocytes as indicators for α-haemolysin and human erythrocytes as indicators for δ-haemolysin, strain NCTC 10345 was selected for further study due to the high human/rabbit erythrocyte titre ratio which reflected production of a large amount of δ-haemolysin and only a small quantity of α-haemolysin.

When δ-haemolysin was purified from crude culture supernatants of strain NCTC 10345 using hydroxylapatite, all haemolytic activity against cod erythrocytes was removed by hydroxylapatite although some haemolytic activity against rabbit erythrocytes remained (Table). This provided some evidence that a small quantity of α-haemolysin was produced by S. aureus NCTC 10345. Of the original haemolytic activity, 45% was recovered on elution with 1 M phosphate buffer and the final yield of δ-haemolysin was approximately 220 mg per litre of culture supernatant. The yield of purified δ-haemolysin was similar to that obtained by Kreger et al. (1971) and the total recovered activity (33,000 HU/litre supernatant tested using human erythrocytes) was in good agreement with the findings of these authors.

Properties of purified δ-haemolysin

Although one preparation of δ-haemolysin was freeze-dried from aqueous solution, after removal of the insoluble fraction described by Kreger et al. (1971), further preparations were concentrated by ultrafiltration and stored at -70^{o} as a concentrated solution. This appeared slightly yellow and remained stable for one year at -70^{o}. After thawing a slight precipitate was visible in solutions maintained at 4^{o} but no significant loss of activity occurred after centrifugation at 50,000 x g for 30 min to remove insoluble material. Adsorption to glass or plastic surfaces was not evident (Heatley, 1971) but Millipore filtration caused some loss of activity and solutions were not therefore filter-sterilised. The specific activity of the δ-haemolysin preparation used for the experiments described in this thesis was 750 HU/mg protein (0.8% cod erythrocytes) and 150 HU/mg (0.8% human erythrocytes) which was low compared to the preparations of Kreger et al. (1971) using a similar strain of S. aureus and similar purification procedure. Other preparations made in this laboratory (Birkbeck, personal communication)

and a sample provided by Dr. A. Kreger had specific activities comparable to that of Kreger et al. (1971). The relative haemolytic activity of δ-haemolysin using cod, human and rabbit erythrocytes was the same as for Kreger's preparation and the reasons for the lower specific activity were not investigated. It is possible that aggregation rather than denaturation of haemolysin occurred since haemolysin solutions heated briefly to 60^{o} or 100^{o} showed slightly increased haemolytic activity (Birkbeck, personal communication).

The ultraviolet absorption spectrum (Fig 1) showing a maximum at 271 nm with a small shoulder at 291 nm was very similar to those reported by Kayser (1966), Kreger et al. (1971) and Heatley (1971). The shoulder at 291 nm was consistently found and the ultraviolet spectrum was remarkably similar to that of the amino acid tryptophan, suggesting the absence of significant quantities of tyrosine in the preparation. Amino acid analyses of δ-haemolysin have not demonstrated the presence of tyrosine (Table 6) and from these analyses tryptophan would be expected to dominate the ultraviolet spectrum of δ-haemolysin.

Analysis of δ-haemolysin by polyacrylamide gel electrophoresis in acidic or alkaline buffers (Fig 2) yielded results comparable to those found by Kreger et al. (1971) and Heatley (1971) in that two bands were found in alkaline gels and a single diffuse band in acidic gels. The secondary band seen in alkaline gels was faint in comparison to the principal band and may have been due to contaminating protein or, if the model proposed by Kantor et al. (1972) is correct, a lower molecular weight component of δ-haemolysin. Kantor et al. (1972) detected haemolytic activity corresponding to the single stained protein band in polyacrylamide electrophoresis, but in the present study no residual haemolytic activity was detected even with the more sensitive cod erythrocyte assay.

Isoelectric point of δ-haemolysin

The published studies on isoelectric focusing of δ-haemolysin have been reviewed previously (p.). In this investigation, $(NH_4)_2SO_4$-precipitated crude haemolysins of Wood 46 and purified δ-haemolysin from strain NCTC 10345 were subjected to isoelectric focusing in the Valmé-type apparatus designed by Talbot & Caie (1975). This apparatus has certain advantages over columns in that precipitation did not disturb other protein zones as can occur in the LKB vertical column apparatus. Thus the fractions in the pH gradient could be collected directly thereby avoiding disturbance of the protein zones. However, because of the fixed number of fractions yielded by the trough apparatus, its potential resolution may be inferior to that of the vertical column apparatus.

Purified δ-haemolysin of strain NCTC 10345 was focused as a single peak (pI 4.45) which contained all of the 280 nm-absorbing material and haemolytic activity applied. The isoelectric focusing pattern for crude culture supernatant of strain Wood 46 (Fig 11) was not identical with that of McNiven et al. (1972) nor of Wadström (1968). However, it is of interest to note that the α-haemolytic fractions were focused in a similar pH range (pH 6.0 - 9.2) to those of McNiven et al. (1972) (α_A, α_B, α_C, α_D) and of Wadström (1968) (α_{Ia}, α_{Ib}, α_{II}), although discrete peaks were not observed in this pH range. Delta-haemolysin from strain Wood 46 focused with a pI of 5.2 and no cationic δ-haemolysin was detected.

In support of the values of pI 4.45 of purified δ-haemolysin, Wadström (1968) reported that δ-lytic activity was found only in fractions with pI 4.3 - 4.6 when he analysed a partially purified δ-lysin preparation of Bernheimer. Additionally, pI values of 5.0 and 4.65 for δ-lysin were reported by Kreger et al. (1971) and Kantor et al. (1972) respectively.

However in contrast to the reports of Kreger et al. (1971), Kantor et al. (1972) and McNiven et al. (1974), no cationic δ-haemolysin was found in the present study in either purified δ-haemolysin or crude culture supernatants from strain Wood 46. However since refocusing of the cationic component yielded both anionic and cationic forms of δ-haemolysin (Kreger et al., 1971) and electrofocusing in Tween 80 yielded only the anionic form (Kantor et al., 1972) it is likely that either total conversion of any cationic form to the anionic form had occurred in the present study or that the different isoelectric focusing apparatus caused the differences.

From the haemolytic spectrum, heat stability, neutralisation by serum and isoelectric point it was concluded that the preparation was indeed δ-haemolysin with properties broadly similar to those described by Kreger et al. (1971), Heatley (1971) and Kantor et al. (1972) and the distribution of protein and haemolytic activity on isoelectric focusing showed the preparation to be relatively homogeneous, with no evidence for contamination with the cationic α-, β- and γ-haemolysins. However, as stated previously, a critical evaluation of the homogeneity and other properties of the preparations was not within the scope of this thesis.

Storage of fish erythrocytes

Presumably the best environment for preservation of fish erythrocytes is one providing as nearly as possible the conditions experienced in vivo. Two main criteria, osmotic pressure and pH, will be discussed here. As expected from a survey of the literature, the osmolality of sera from various species of marine fish was higher than mammalian sera and varied from 359 to 501 milliosmoles/kg (Table 15). However the pH of the sera was relatively constant. CDS solution provided a pH similar to that of the sera tested although the osmolality was 20% lower than cod serum. Never-

theless CDS solution was considered satisfactory for several reasons: Firstly, Harden, Jones & Scholes (1974) showed that the measured osmolality of fish sera was influenced by the treatment of fish prior to withdrawal of blood and therefore the 'true' osmolality of sera is not certain. Secondly, Green & Hoffman (1953) showed that the optimum molarity of salt solutions for suspension of fish erythrocytes was less than that predicted by osmolality measurements. Thirdly, no morphological differences were observed between fresh or stored erythrocytes before or after washing in CDS solution (Plate 1). Finally, erythrocytes could be frozen in liquid nitrogen in CDS solution using DMSO as a protective additive for as long as five months (Table 17) with a yield up to 90% - 95% intact cells. This is similar to the values reported by Hodgins & Ridgway (1965) who used similar solutions to freeze trout and salmon erythrocytes although the time of storage was not given. CDS solution was therefore considered a satisfactory diluent although because of the varied osmolalities of sera of different fish species it may not be ideal for all.

Although glycerol was used as a cryoprotective agent in one preliminary experiment, dimethyl sulphoxide was used throughout the rest of this work because of the lower viscosity, easier removal during washing and the superior result obtained by Hodgins & Ridgway (1965) in storage of salmonid erythrocytes. The optimum DMSO concentration for storage of gadoid erythrocytes was 10 - 12.5% (Tables 16 - 18) and recoveries of 90 - 95% erythrocytes were obtained after five months storage under these conditions. Attempts to increase the volume of blood stored by addition of undiluted DMSO to citrated blood (to a final concentration of 10% DMSO) resulted in lysis of up to 28% of erythrocytes. Unbuffered DMSO was alkaline (pH 11.3) and on addition to blood caused a significant rise in temperature. It is probable that addition of undiluted DMSO caused localised extremes of pH and temperature which lysed the erythrocytes.

Addition of an equal volume of the nearly neutral pH solution of 15 - 30% DMSO in CDS was found to be optimal for preservation of most species of fish erythrocytes. The quantity of blood stored could be increased satisfactorily by removal of plasma from citrated blood before addition of DMSO without detriment to the recovery of erythrocytes (Tables 20 and 21). The maximum erythrocyte concentration used was 34% p.c.v. (equivalent to 68% p.c.v. before addition of CDS/DMSO) and higher concentrations were difficult to manipulate. However the most satisfactory means of storage was in pellets which allowed maximum use of storage space in the liquid nitrogen containers and greatest ease of recovery of any required quantity of blood.

Only with herring erythrocytes were satisfactory storage conditions not determined. The possible reasons for the high haemolysis were either the use of too high a concentration of DMSO or that the CDS solution had too low tonicity compared with that of the serum (Table 15), or both.

Doubling dilution assay of δ-haemolysin

In contrast to the staphylococcal α- and β-haemolysins, little variation was observed in the haemolytic activity of δ-haemolysin on fish erythrocytes when the incubation temperature and ionic composition of the buffer were altered. The δ-haemolysin titre was constant over the range 0^{o} - 37^{o} in phosphate-buffered or citrate-buffered saline of widely varied osmolality.

Delta-haemolysin was not activated (or destroyed) by mercapto-ethanol, observations compatible with the absence of cysteine in published amino acid analyses (Kreger et al., 1971; Heatley, 1971; Kantor et al.,

1971). Alteration in the erythrocyte concentration had a marked effect on haemolysin titres and the assay sensitivity could be increased 2-4 fold by reducing the erythrocyte concentration from 1.0 to 0.3%. However for visual estimation of end-points, erythrocyte concentrations of 0.5 to 1.0% were normally used.

Delta-haemolysin was also assayed by radial diffusion in blood agar plates as proposed by Kantor et al. (1972). With human erythrocytes, the response to δ--haemolysin at room temperature was as found by Kantor et al. (1972) and although a satisfactory response was obtained using fish blood, several disadvantages to the use of fish blood agar became apparent. The heat-lability of cod and mackerel erythrocytes, difficulty in avoiding contamination during withdrawal of blood from fish, the error in determining the zone diameters and time required (48 hours) to complete the assay led to this technique being abandoned for routine assay in favour of the more convenient doubling dilution assay in tubes.

Of the marine fish erythrocytes tested, mackerel were the most sensitive to the action of δ-haemolysin but since mackerel were not available throughout the year, erythrocytes of the gadoid species cod, saithe and haddock were used most frequently in this work. Such erythrocytes were less sensitive to δ-lysin but were more readily available. In contrast to the wide individual variation of rabbit erythrocytes in their sensitivity to staphylococcal α-haemolysin (Bernheimer & Schwartz, 1963), no significant variation was found in the response of cod and saithe erythrocytes to δ-haemolysin when twelve cod and five saithe were tested individually. It must be emphasised that extensive surveys of erythrocytes of other marine or freshwater fish for sensitivity to δ-haemolysin should also include sensitivity to α- and β-haemolysins as erythrocyte membranes of some fish may be sensitive to these haemolysins.

Mode of action of δ-haemolysin

Although this thesis was not directly concerned with a study of the mode of action of δ-haemolysin, the observations made during assays of the lysin merit discussion because of the conflicting views on mode of action. Bernheimer (1970) in a review of the mechanism of lysis of erythrocytes by cytolytic agents, postulated that enzymic and non-enzymic haemolysins would be expected to lyse a differing proportion of erythrocytes when a fixed quantity of haemolysin was added to varying concentrations of erythrocytes. For non-enzymatic lysins, such as saponin and sodium deoxycholate, percentage haemolysis should fall steeply with increasing cell concentration as these lysin molecules might be expected to act only once. On the other hand for an enzymatic lysin, the molecules might act on many cells sequentially, with the result that the percentage haemolysis would tend to remain constant with increasing cell concentration.

Addition of a fixed concentration of δ-haemolysin to varying concentrations of erythrocytes yielded a different response for human, mackerel and cod erythrocytes (Figure 6). The 'downward' curve obtained with human erythrocytes was similar to that shown by Bernheimer (1970), but mackerel and cod erythrocytes produced a linear response comparable to that of mellitin, sodium deoxycholate and staphylococcal β-haemolysin. Bernheimer (1970) found that the response of erythrocytes to staphylococcal β-haemolysin did not resemble that of another enzyme (Clostridium perfringens α-toxin) since the β-haemolysin was inhibited by the products of lysis. Such analyses are therefore of limited value as it is possible that a haemolysin with an enzymic mode of action could resemble a surface active haemolysin if inhibited by the products of lysis and, as stated by Bernheimer (1970) "There exist numerous studies of the kinetics of

haemolysis induced by cytolytic bacterial toxins but the results seldom if ever have provided an unequivocal answer to the question of whether a given lysin is or is not an enzyme."

Although no satisfactory conclusion was drawn from the above analyses, the temperature-independence of haemolysis of fish erythrocytes over the range 0^{o} - 37^{o} and lack of stimulation by divalent cations is more characteristic of a surface-active agent (Thelestam et al., 1974) than an enzyme. It should be noted that the action of δ-haemolysin on human erythrocytes is temperature-sensitive over the range 0^{o} - 37^{o} (Birkbeck, personal communication) although this may be due to phase transitions in membrane lipids (Oldfield & Chapman, 1972). Therefore no evidence was adduced to support the contention of Wiseman & Caird (1968) that δ-haemolysin is a phospholipase. However the experimental procedures of Wiseman & Caird (1968) were not followed and isolation of the plasma membranes of fish erythrocytes would be required before comparable experiments could be attempted.

Detection of δ-haemolysin production by staphylococci grown in blood agar plates

Although cod blood was too thermolabile to detect haemolysis zones around colonies of staphylococci grown at 37^{o} on cod blood agar plates, the technique of overlaying blood agar onto nutrient agar plates containing staphylococcal colonies yielded lysis zones of comparable or greater size to those detected in blood agar. Only a preliminary survey was attempted to compare the size of lysis zones in blood agar containing cod blood or mammalian blood. Some strains of S. aureus (Wood 46 and SM 14 in particular) showed a wide variation in zone sizes in different tests on the same type of blood agar.

On sheep blood agar overlay plates, strain SM 15 yielded zones of complete lysis surrounded by a large zone of partial lysis which was typical of β-haemolysin production. Synergistic lysis was observed only in sheep blood agar between strain SM 15 and strains SM 10, SM 14 (Plate 2) and SM 6. Assuming that synergism was due to the combined action of β-lysin produced by strain SM 15 and δ-haemolysin produced by other strains and that the distance between the colony edge and zone edge was a function of the quantity of δ-lysin produced, then strain SM 6 produced small amounts and strains SM 10 and SM 14 large amounts of this haemolysin. In cod blood agar, strain SM 6 produced very small zones and strains SM 10 and SM 14 produced large zones.

All staphylococcal strains incubated in air produced haemolytic zones in cod blood agar overlays and, with the exception of strains SM 15 and Wood 46 (on sheep blood agar and rabbit blood agar respectively), zones were larger than on other types of blood overlay. Zones in cod blood agar overlays were larger than in human or horse blood agar overlays in all cases.

Culture of the strains on nutrient agar in an atmosphere of 30% CO_2 led to a decrease in zone size in cod and human blood agar overlays with all strains and with horse blood agar, only the β-haemolysin producing strains SM 14 and SM 15 showed zones of increased size accompanied by a large increase in zone sizes on rabbit blood agar. Therefore the quantities of each haemolysin produced was markedly affected by alteration in cultural conditions but from the zone sizes in human, horse and cod blood agar there was no evidence for increased δ-haemolysin production (Williams & Harper, 1947).

Although only the δ-haemolysin has been shown to lyse cod erythrocytes, the possibility cannot be eliminated that other extracellular products may participate in lysis of cells and monospecific antiserum to the α-, β- and δ-haemolysins would be required to demonstrate the contribution of each in production of haemolysis zones.

When staphylococci were cultured in Bernheimer's yeast extract dialysate medium the resulting haemolysin titres were low and did not correspond to the zone sizes in blood agar overlays. Culture supernatant from the α-haemolysin producing strain, Wood 46, lysed rabbit erythrocytes to high titre and on isoelectricfocusing, the erythrocyte specificity and isoelectric point of haemolysin focused at pI 8.4 was comparable with that found by McNiven et al. (1973) for α-haemolysin. However for the β-haemolysin producing strains SM 14 and SM 15, more appropriate culture conditions such as growth on soft agar in an atmosphere containing CO_2 might be required for optimal β-haemolysin production (McKay, personal communication). Under these conditions the haemolysin spectrum should more closely reflect that produced in blood agar overlay plates.

Further work arising from this thesis

The development of suitable storage conditions for fish erythrocytes described in this thesis should permit an extension of the range of erythrocytes surveyed for suitable assays of other cytolytic agents. In one other instance Rennie (1973) found cod and haddock erythrocytes to be 1000 times less sensitive than sheep or rabbit erythrocytes to the action of Escherichia coli α-haemolysin. Mouse erythrocytes, the least sensitive of ten mammalian erythrocytes surveyed were still 200 times more sensitive than fish erythrocytes which were almost totally resistant to the action of E. coli α-haemolysin.

A secondary haemolysin of Bacillus thuringiensis was shown to be 32 times more active against saithe erythrocytes than against sheep erythrocytes which previously had been considered the most sensitive assay, based on mammalian erythrocytes (Pendleton, personal communication). For the principal haemolysin of B. thuringiensis (thuringiolysin) cod and saithe erythrocytes were 5 - 10 x less sensitive than dog erythrocytes and 5 - 10 x more sensitive than mouse erythrocytes.

An assay for S. aureus δ-haemolysin 4 - 16 times more sensitive than existing assays and not influenced by α- or β-haemolysins should facilitate the assay of δ-haemolysin even of relatively low specific activity in both crude and purified preparations. Despite the agreement of Kreger et al. (1971), Heatley (1971) and Kantor et al. (1972) with respect to many of the physico-chemical properties of δ-haemolysin, other properties are still disputed. Fackrell & Wiseman (1975) demonstrated that the δ-haemolysin preparation of Kreger contained α- and γ-haemolysins in sufficient quantities to yield precipitin lines on gel diffusion and proposed that this might account for many of the differences between their δ-haemolysin preparations. However published amino acid analyses of α-haemolysin show the presence of histidine, arginine, proline and tyrosine, none of which were demonstrated in amino acid analyses of δ-haemolysin (Table 6). Proline was found to be the N-terminal amino acid of Wiseman's δ-haemolysin in which γ-haemolysin was not detectable, but comparative serological analyses for α-haemolysin are not shown. Also the isoelectric point, heat stability, mode of action, immunogenicity, mode of neutralisation by serum and role in the pathogenicity of S. aureus have not yet been satisfactorily established. It is probable that differences exist between δ-haemolysin preparations from different strains and an improved

assay system should therefore be of use. In particular, if assay of δ-haemolysin is possible in crude preparations containing large quantities of α- and β-haemolysins, several strains of S. aureus could be assayed to investigate strain differences in properties of δ-lysin. Furthermore, cod erythrocytes would provide a more sensitive assay for δ-haemolysin contamination during the purification of α- and β-haemolysins.

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Appendix I

Media

Yeast diffusate medium (Bernheimer & Schwarz, 1963)

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

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

Yeast extract diffusate

Yeast extract (Difco)	200 g
Distilled water	500 ml

Yeast extract was dissolved in distilled water by steaming and after cooling was poured into a 50 cm length of 2.3/4" Visking dialysis tubing (Scientific Instrument Centre, London) previously soaked in 70% ethanol to minimise contamination. The dialysis sac was immersed in 1600 ml distilled water in a 5 l beaker and stirred for 48 hours at 4^{o}. 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.

Erythrocyte agar overlay medium

Base: (per 100 ml)

Tryptone soya agar (Oxoid)	4 g
Distilled water	100 ml

Sterilised by autoclaving for 15 min at 15 lb/in^2 and dispensed in 5 ml volumes into 5 cm petri dishes. The agar was allowed to harden and plates were stored at 4^o until required.

Erythrocyte overlay (per 100 ml)

a) (human, horse, rabbit or sheep blood)

Blood agar base (Oxoid No. 2)	4 g
Distilled water	100 ml

b) (fish blood)

Blood agar base (Oxoid No. 2)	4 g
Sodium chloride	5.8 g
Distilled water	100 ml

c)

Sodium azide	1 g
Distilled water to	100 ml

The agar bases (a or b) were sterilised by autoclaving for 15 min at 15 lb/in^2 and the 1% NaN_3 solution sterilised by membrane filtration. The agar was cooled to 45^o and packed, washed erythrocytes (5% v/v) and 1 ml of NaN_3 solution were added aseptically.

Appendix II

Buffers and Diluents

Citrate/Dextrose/Sodium chloride solution (CDS, Hodgkins & Ridgway, 1963)

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

The solution was sterilized by membrane filtration.

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

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

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

Potassium phosphate buffers

i) 1.0 M phosphate (pH 7.4) buffer.

Stock solns:

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

B: 1.0 M solution of potassium hydrogen orthophosphate (17.4 g K_2HPO_4 in 100 ml dist. 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) 0.4 M phosphate (pH 6.8) buffer.

Stock solutions were obtained by dilution of the above 1.0 M 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) 0.01 M phosphate (pH 6.8) was obtained by 1/40 dilution of the 0.4 M phosphate buffer.

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

A: 0.1 M sodium dihydrogen orthophosphate (13.9 g NaH_2PO_4/l in distilled water).

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

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

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

Appendix III

Preparation of hydroxylapatite (Tiselius et al., 1956)

Solutions

i) $CaCl_2$ 0.5 M — 2 litres

ii) Na_2HPO_4 0.5 M — 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 0.01 M 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 0.01 M buffer and then boiled twice in 0.001 M phosphate buffer for 15 min to convert the brushite to hydroxylapatite. The suspension was stored in 0.001 M phosphate buffer at 4^{o}.

Appendix IV

Reagents for Protein Estimation (Lowry et al., 1951)

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

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

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

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

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

Appendix V

Disc Gel Electrophoresis

Abbreviations

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

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

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

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

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

I. Stock solutions

A)	1 N Potassium hydroxide	48 ml
	Acetic acid (glacial)	17.2 ml
	TEMED	4.0 ml
	Distilled water to 100 ml	
B)	1 N Potassium hydroxide	48 ml
	Acetic acid (glacial)	2.87 ml
	TEMED	0.46 ml
	Distilled water to 100 ml	
C)	Acrylamide	30 g
	BIS	0.8 g
	Distilled water to 100 ml	
D)	Acrylamide	10 g
	BIS	2.5 g
	Distilled water to 100 ml	

E)	Riboflavin	4 mg
	Distilled water to 100 ml	
F)	Ammonium persulphate	0.28 g
	Distilled water to 100 ml	
G)	Sucrose (B.D.H., Analar)	40 g
	Distilled water to 100 ml	

Preparation of separating gel

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

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

II. Preparation of large pore solution (pH 6.8)

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

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

III. Tray buffer (pH 4.5)

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

The buffer was diluted 10 fold for use.

Staining solution

Amido black (1%) in 7% acetic acid

Destaining gel

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

Destaining solution

Acetic acid (7%) in distilled water.

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

I. Stock solutions

A)	1N HCl	48 ml
	Tris	36.6 g
	TEMED	0.23 ml
	Distilled water to 100 ml	
B)	1N HCl	48 ml
	Tris	5.98 g
	TEMED	0.46 ml
	Distilled water to 100 ml	

C) Acrylamide 28 g

Bis 0.735 g

Distilled water to 100 ml

D) Acrylamide 10 g

Bis 2.5 g

Distilled water to 100 ml

E) Riboflavin 4.0 mg

Distilled water to 100 ml

F) Ammonium persulphate 0.14 g

Distilled water to 100 ml

G) Sucrose 40 g

Distilled water to 100 ml

II. Preparation of separating gel and large pore solution

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

III. Tray buffer (pH 8.3)

Tris 6 g

Glycine 28.8 g

Distilled water to 1000 ml

The buffer was diluted 10 fold for use.

Staining solution, destaining gel and destaining solution

These were as described for the acid gel system.

Proceedings of the Society for General Microbiology 1974, 1, 55.

Sensitivity of Erythrocytes of Marine Fish to Staphylococcal Delta Haemolysin.

By T.H. Birkbeck, Lin-Po Chao and J.P. Arbuthnott (Department of Microbiology, University of Glasgow).

Staphylococcal δ-haemolysin lyses many erythrocyte species to low titre and assay is more difficult than for the more active α- and β-haemolysins. Conventionally horse or human erythrocytes are used for assay of δ-haemolysin although the more active α- and β-haemolysins may interfere at low concentration. Lysis of protoplasts (Bernheimer, A.W. et al. (1972) Infection and Immunity 6, 636) and release of radioactively labelled nucleosides from tissue culture cells (Thalestam, M., Mollby, R. and Wadstrom, T. (1974) Infection and Immunity, in the press) provide more sensitive assays (approximately 600 units/mg purified toxin) without interference by other haemolysins. For routine tests the haemolytic assay is simple and convenient.

We have found that erythrocytes from certain marine fish (cod, saithe, haddock and mackerel) were 4-16 times as sensitive as rabbit erythrocytes for assay of δ-haemolysin (1,200-5,000 HU/mg purified toxin). Blood obtained from the dorsal aorta of fish using a syringe and needle was collected into 3.8% trisodium citrate solution and was stable at 4^{o} for one week. To ensure availability of fish erythrocytes blood was stored in liquid nitrogen using 12.5% dimethyl sulphoxide as a cryoprotective agent (Hodgins, H.O. and Ridgway, G.J. (1964) Nature 201, 1336). In this way, blood from 8 cod (100 ml) was stored for several months with insignificant lysis during storage.

Fish erythrocytes were not lysed by purified α- and β-haemolysins and no synergistic action was found between β- and δ-haemolysins. In comparative assays the relative sensitivities of rabbit, human, cod and mackerel erythrocytes were 1 : 1.3 : 4 : 16 respectively.