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Production and properties of extracellular factors from *Aeromonas salmonicida*

by

Richard W. Titball, B.Sc.

A thesis submitted to the Council for National Academic Awards in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 1983

Research was conducted at Plymouth Polytechnic in collaboration with the Ministry of Agriculture, Fisheries and Food, Weymouth, England

Production and properties of extracellular factors from *Aeromonas salmonicida* - Richard W. Titball

ABSTRACT

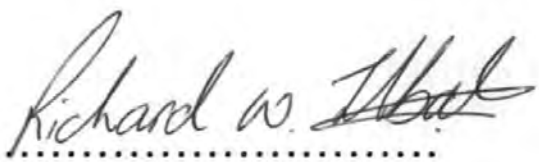
The production of extracellular products by *Aeromonas salmonicida*, *in vitro*, has been investigated. The results indicated that the bacterium produces at least two haemolytic activities *in vitro*. Unshaken cultural conditions favoured the production of a haemolysin with a broad spectrum of activity against various erythrocyte types (H-lysin), whilst shaken cultural conditions favoured the production of a haemolysin active against trout erythrocytes (T-lysin). The effects of growth medium type and culture conditions on the production of these haemolytic activities has been investigated.

The activity of the T-lysin appeared to be attributable to the combined effects of an activity which caused incomplete lysis of the erythrocytes (T_1 activity) and caseinase. The T_1 activity appears to be found in culture supernate associated with fragments of the bacterial cell wall or membrane resulting in apparent molecular heterogeneity.

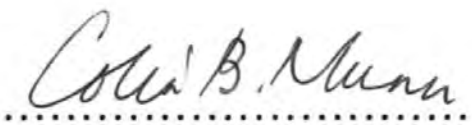
H-lysin activity appeared to be due to a single protein, which did not require a divalent cation for the expression of activity. The haemolysin was synthesised by the bacterium as an inactive precursor molecule (pro-H-lysin) which was cleaved by the bacterial protease to give the active haemolysin; other commercially available proteases were also able to effect this activation. An unidentified component of a variety of animal sera was also able to effect conversion of the pro-H-lysin to the active form, however, this conversion only occurred after the serum component had entered the bacterial cell. The H-lysin was purified 1770 fold using freeze fractionation, salt fractionation, ion exchange chromatography and gel filtration chromatography. The partially purified protein possessed erythrocyte lysing and glycerophospholipid:cholesterol acyltransferase activities, however it was not clear whether these activities were attributable to the same molecule. Investigation of the kinetics of erythrocyte lysis by the partially purified H-lysin suggested that the haemolysin possessed an enzymatic mode of action. *In vitro* the haemolysin was active against both rainbow trout leucocytes and tissue culture cells. However, *in vivo* the haemolysin had no obvious effect on rainbow trout.

DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record has been performed by myself, and that all sources of information have been specifically acknowledged.



Richard Titball



Colin B. Munn
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Abbreviations used

AOT	Agar overlay technique
ATEE	N'-acetyl-L-tyrosine-ethyl ester
MA	milliampere
BHK	Baby hampster kidney tissue culture cells
^{o}C	degrees centigrade
CDC	Centre for Disease Control, Atlanta, USA.
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid (sodium salt)
FAO	Food and Agriculture Organisation of the United Nations
Formol-PBS	10% v/v formalin in PBS
g	Acceleration due to gravity
g	gram
mg	milligram
µg	microgram
GCAT	Glycerophospholipid:cholesterol acyltransferase
hr	hour
Hu	Haemolytic unit
Hu/ml	Haemolytic units/ml
Iu	International unit
Kv	Killovolt
l	litre
ml	millilitre
µl	microlitre
cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre
M	Molar
mM	millimolar
µM	micromolar
min	minute
Mol.wt.	Molecular weight
NA	Not applicable
NK	Not known
NT	Not tested
NTG	N-methyl-N'-nitroso-N-nitrosoguanidine
NB No. 2	Nutrient broth Number 2 (Oxoid)

OD	Optical density
O/F	Oxidation/Fermentation of glucose
PBS	Phosphate buffered saline (pH 7.2)
PEG	Polyethylene glycol
PI	Isoelectric point
PMSF	phenylmethylsulphonylfluoride
%	Percent
Rf	Relative front
RNA	Ribonucleic acid
RNAase	Ribonuclease
TEM	Transmission electron microscopy
Tris	Tris (hydroxymethyl) aminomethane
TSA	Tryptone soya agar (Oxoid)
TSB	Tryptone soya broth (Oxoid)
TLC	Thin layer chromatography
UIBC	Unsaturated iron binding capacity
w/v	weight/volume
v/v	volume/volume

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1.0 INTRODUCTION

It is only in recent years that aquaculture has become truly commercialised. The increased costs of obtaining fish supplies from their natural environments along with the decline in some wild fish populations have probably accelerated the emergence of this new industry. Of the 6M tons of aquaculture production in 1975, 66% was fish, the remainder being molluscs, crustacea and seaweeds. The Food and Agriculture Organisation of the United Nations (F.A.O) estimated that aquaculture production could double by the year 2,000 with an eventual long term potential of 20 - 40M tons per year (F.A.O. Fisheries Circular 1977). Against this background the F.A.O. indicated the importance of research into fish breeding, feeding and disease control.

In western countries great expansion has been seen in the salmonid farming industry, the reared fish being used for both nutritional and sporting purposes. The constraints of such intensive rearing conditions have often resulted in transmissible diseases determining the success of such enterprises. Of the bacterial diseases of fish, those caused by Aeromonas salmonicida are perhaps the most important. A. salmonicida mediated diseases appear to occur worldwide (Herman 1968, McCarthy and Roberts1980) affecting most freshwater and some saltwater fish. Capable of killing entire fish populations, the disease is of greatest importance in those countries relying on intensively reared fish as an important food source. For example, in the Middle East, Far East and Asian countries, intensively reared fish have been used as food material for centuries. In Japan, fish protein provided 91% of the total animal protein in the diet (Rao 1962).

It is only in the last decade that this pathogen has received any detailed attention. An effective and reliable vaccine preparation is not available at this time and it is becoming apparent that a detailed approach to the host-pathogen relationship may provide valuable information concerning the disease process, which is poorly understood, in addition to indicating areas for subsequent vaccine development.

The study of the host-pathogen relationship is also important since it presents the opportunity to study the pathogenesis of a disease, in the natural host, in detail. Potential pathogenic mechanisms of human diseases have often been studied in vitro in great detail, however ethical problems mean that partially or wholly unsatisfactory unnatural hosts may be required to relate in vitro effects to in vivo situations. The study of the pathogenesis of A. salmonicida infections does not involve these limitations and the study of this disease may provide a 'model', indicating the potential importance of pathogenic mechanisms in other animal diseases.

The aims of this project were to examine the spectrum of extracellular products of A. salmonicida and to examine their interrelationships. In particular, the nature of haemolytic activity has been studied, and the potential contribution of haemolytic activity to the pathogenesis of furunculosis evaluated.

1.2.0 AEROMONAS SALMONICIDA - AN IMPORTANT FISH PATHOGEN

1.2.1. Properties of Aeromonas salmonicida.

In 1894 Emmerich and Weibel reported the isolation of a microorganism from a diseased trout, which they described as "Bacillus der Forellenseuche". Since this time bacteria have been isolated from a variety of fish suffering from furunculosis and have been referred to by a variety of names such as Bacterium salmonicida (Lehmannand Neuman 1896) and Bacterium salmonica (Chester 1897).

Griffin et al (1953) undertook a comprehensive study of the bacterium, concluding that B. salmonicida should be placed in the genus Aeromonas, a suggestion implemented in the 7th edition of Bergeys Manual of Determinative Bacteriology (Breed 1957). The bacterium, a short Gram negative rod, is a member of the Vibrionaceae. Important identification tests include non-motility and the ability of most strains to produce a melanin-like pigment on agar. The bacterium is unable to grow at 37^{o}C and ferments sugars producing acid from sucrose, xylose and a number of other sugars (McCarthy 1975).

Many workers have disagreed with the original classification. Eddy (1960) suggested that non-motility and inability to produce 2,3 butanediol from glucose precluded its inclusion in the genus Aeromonas.

Smith (1963) later suggested that the bacterium should be placed in the genus Necromonas, implemented by Cowan and Steel (1974) in the 2nd edition of "Manual for the Identification of Medical Bacteria". McCarthy later suggested that the bacterium should be placed in the genus Aeromonas (McCarthy 1977 b) and the dispute as to the correct generic classification of the bacterium is still unresolved at this time.

There is also disagreement concerning the nomenclature and characteristics of A. salmonicida subspecies. Strains capable of producing a melanin-like pigment are referred to as A. salmonicida subspecies salmonicida (Smith 1963, McCarthy 1977 b); non-pigmented strains are referred to as either subspecies achromogenes (Smith 1963) or subspecies masoucida (Kimura 1969). McCarthy, however, classifies non-pigmented strains as either subspecies achromogenes or nova, depending on the original species of host fish.

Other members of the genus Aeromonas are also fish pathogens. A. hydrophila, often an enteric human pathogen (Von Graevenitz and Mensch 1968), may cause disease in fish (Griffin 1953, Boulanger et al 1977) as may the less frequently encountered A. proteolytica (Griffin 1953, Foster and Hanna 1974) and A. liquefaciens (Griffin 1953, Kou 1973).

The location of the bacterium in the natural environment is open to speculation. The possibility of a free living life cycle for the bacterium has been considered. Cornick et al (1969) examined a variety of aquatic invertebrates and non-fish vertebrates, but failed to isolate the bacterium from them even during an active outbreak of furunculosis. Dubois-Darnaudpeys (1977) found the bacterium could survive in water for up to 50 days, whilst McCarthy (1977 a) suggested that survival in seawater of only one week was increased to seventeen days in freshwater.

Recently a number of workers have studied the possibility that carrier fish, showing no overt signs of infection, harbour the bacterium (McCarthy and Roberts 1980). Some workers have injected immunosuppressive agents into apparently healthy fish, resulting in some of the fish developing furunculosis (Bullock and Stuckey 1975, McCarthy 1977 a). Using this technique, brown trout from a variety of fish farms were found to have a carrier rate in the range 40-80%.

Jensen and Larsen (1980) suggested that carrier fish were only found during the summer months and that during the winter the bacterium may be located elsewhere in the environment.

1.2.2. Pathology of diseases caused by A. salmonicida

A wide variety of fish species are susceptible to infection by A. salmonicida. The first definitive isolation was from the brown trout (Salmo trutta L.), (Emmerich and Weibel 1894) and since that time there have been numerous reports of the isolation from other salmonids (Fuhrman 1909, Pittel 1910, Surback 1911, Mettam 1914, Fish 1937, Duff and Stewart 1933, Herman 1968, Kimura 1969, Hastein 1975, Miyazaki and Kubota 1975.) In salmonids the disease is commonly referred to as furunculosis. In view of the importance of salmonid culture the majority of published information concerns the disease in such salmonid species. Other species are infected; in carp (Cyprinus carpio L.), A. salmonicida infection is known as carp erythrodermatitis (Bootsma et al 1977) and is often caused by the achromogenic subspecies (1.1.2.). The bacterium has also been isolated from roach (Leueiscus rutilius L.), rudd (Scardinius erythrophthalmus L.), pike (Esox lucius L.), tench (Tinca tinca L.), eels (Anguilla anguilla L.) and other species (McCarthy 1975 , Bucke 1980).

Infections are most common in freshwater fish but Hastein (1975) reported that the disease occurred in the Atlantic salmon (Salmo salar L.) and Evelyn (1971) has described the disease in the strictly marine sable fish (Anaplopoma fimbria L.). McCarthy and Roberts (1980) experimentally infected the plaice (Pleuronectes platessa L.).

The gross symptoms of the disease vary according to the infected species. In salmonids, furunculosis is often characterised by the appearance of furuncles or boils, which often extend deep into the underlying muscle and contain a sanguinous fluid composed of bacteria, necrotic blood cells, necrotic elements of host tissue and tissue fluid. (Plehn 1911, Davis 1946, McCarthy and Roberts 1980). Such furuncles appear to represent a focus of infection. Prominent external symptoms also include haemorrhaging at fin bases, exophthalmia and lethargic behaviour. Histopathologically, the heart, spleen and kidney appear to be the predominant organs affected. Kidney tissue

may be totally liquefied in acute forms of the disease, with haemopoietic tissue destroyed (McCarthy and Roberts 1980). Changes in the spleen are centred around the ellipsoids where bacterial colonization results in degeneration. Splenic haematopoietic tissue may again be extensively affected. In the heart, epicardial colonization results in lesions with haemorrhaging and congestion of the sub-epicardial spaces. Large thrombi of erythrocytes, thrombocytes and bacteria are occasionally seen in coronary vessels. (Ferguson and McCarthy 1978). Furuncles are not apparent in A. salmoncida infections of carp (McCarthy and Roberts 1980), goldfish (Carassius auratus L.), (Mawdesley - Thomas 1969), or small mouthed bass (Micropterus dolomieui L.) (Le Tendre 1972) in which shallow skin ulcers are seen. Furuncles are often absent in peracute forms of the disease in salmonids when fish generally die rapidly. Death is the usual outcome of the disease although recovery from chronic infections is occasionally observed which may lead to persistent infections with the bacterium carried in a latent form (1.2.1.).

1.2.3. Prevention and cure of furunculosis

Good husbandry plays an important role in reducing disease. Eggs and fry should be obtained from reputable outlets and the farm itself should be preferably supplied with spring water (McCarthy and Roberts 1980). Attempts at selective breeding of salmonids for resistance to furunculosis (Ehlinger 1977) have been discouraging with increases in resistance often being offset against lower growth rates.

The ability to treat furunculosis infections has proved to be of benefit to fish culture. Sulphamerazine and oxytetracycline are common treatments (Herman 1970), often incorporated into feedstuffs (McCarthy and Roberts 1980), however drug resistance may dictate the use of other drugs. Post (1962) recommended the use of furazolidone whilst McCarthy et al (1974 a b c) found sulphamethylphenazole and trimethoprim in combination were highly effective. Recent work has demonstrated that oxolinic acid is an effective chemotherapeutic agent. This drug has been used in Japan for a number of years to control furunculosis (Endo et al 1973), and is currently under evaluation for use in England (Austin et al 1982).

Chemotherapy of furunculosis has a number of disadvantages. Legislative requirements and financial considerations mean that the spectrum of drugs suitable for use in fish destined for human consumption is limited whilst delay of therapy until clinical signs are apparent may mean that drug therapy is ineffective. Use of drugs often means that a suitable time must elapse for the elimination of drug from tissue following treatment, which may be of economic importance. Perhaps the most important objection to chemotherapy relates to the appearance of drug-resistant bacteria. Resistance to terramycin, supha-drugs (Novotny 1978), sulphamerazine (Post 1962), suphathiazole, tetracycline and chloramphenicol (Aoki et al 1971) has been reported. Resistance to these drugs appears to be plasmid mediated (Aoki et al 1971, Aoki 1974) however, such resistance has not been associated with oxolinic acid (Austin et al 1982). The emergence of multiply resistance A. salmonicida strains may result in chemotherapeutic drugs being used on a rotation policy to combat this problem.

The problems associated with chemotherapy have resulted in the possibility of vaccination against furunculosis receiving attention in recent years. A number of workers have attempted to vaccinate salmonids against furunculosis in the past, but results have been variable. Several oral vaccination trials have showed conference of protection against furunculosis (Duff 1942, Overholser 1968, Klontz and Anderson 1970, Smith et al 1980) while in other cases no protection against the disease was observed (Snieszko and Friddle 1949, Frost 1958, Spence et al 1965, Udey and Fryer 1978, Michel 1979). In some cases the failure may reflect the unsuitability of challenge techniques used as it may be important to select a challenge method resembling the natural route of infection (Paterson 1981). Some conclusions can be drawn from experimental work to date; the use of an adjuvant appears to enhance immunogenicity (Paterson 1981). Whilst injection of killed cells results in a good antibody response (Krantz et al 1963, Paterson and Fryer 1974, Fryer et al 1976, Udey and Fryer 1978), exposure of fish to similar preparations by oral routes did not elevate antibody levels (Antipa and Amend 1977, Udey and Fryer 1978, Smith et al 1980).

The stimulating antigen involved appears to be lipopolysaccharide of the cell wall (Paterson and Fryer 1974). However, since a number of workers have suggested A. salmonicida endotoxin is not important in the pathogenesis of disease (1.3.5) its importance in conferring protection against A. salmonicida must be questionable. The variability of results obtained to date along with the lack of identification of important factors in the pathogenesis of disease mean that an effective vaccine is as yet unavailable.

Recently Munn et al (1982) suggested that serum antibody titres are not a good indication of the degree of protection and that stimulation of the cell mediated arm of the immune response may be more important. The implication of this work is that evaluation of vaccination efficiency by virtue of increases in serum antibody levels may be misleading. This suggestion has also been made by other workers (Michel 1982) based on observations that, in a variety of vaccine trials, the degree of protection afforded to fish does not appear to correlate with serum antibody titres. Vaccines which stimulate the production of high levels of serum antibodies against A. salmonicida have often been shown to confer little or no protection against the disease (Krantz et al 1963, Klontz and Anderson 1970, Michel 1979).

It is against this background that recent work has involved a more rational approach to the host pathogen relationship , since a greater understanding of the bacterium may indicate important pathogenic mechanisms and the response of fish to such mechanisms.

1.3.0 PATHOGENIC MECHANISMS OF BACTERIA

1.3.1. The spectrum of pathogenic mechanisms

The vast majority of bacteria found in the environment are harmless or beneficial, whilst a few are capable of causing disease. The study of mechanisms by which such pathogens damage the host has received considerable attention over the last half century. Occasionally, the degree of pathogenicity of certain strains of a bacterium (virulence) can be attributed to one or a few factors.

However, as more diseases are studied in detail it is becoming clear that most diseases involve complex interactions between host and pathogen (Smith 1978). In many cases an array of mechanisms must be possessed by

the virulent bacterium to permit entry, growth and damage to the host (Mims 1976). In the subsequent sections the variety of pathogenic mechanisms possessed by bacteria will be discussed in more detail. The examination of pathogenic mechanisms of these comparatively well studied bacteria may indicate likely pathogenic mechanisms operating in A. salmonicida infections of fish.

Although pathogenicity is discussed as a number of individual stages, virulence can rarely be related to one specific stage but rather considered as a whole disease process.

1.3.2. Attachment and entry into the host

The initial stages of a disease may involve the pathogen gaining entry into the host body, and to achieve this the traversal of a host body surface is required. Even when the bacterium does not truly enter host tissues, such as is the case with some enteric pathogens, the pathogen may have to attach itself to host surfaces. The necessity for firm bacterial attachment to the host epithelial cells reflects the fact that such cells are often subjected to the flushing action of lumen contents found in the alimentary and urinogenital tracts, and the action of the mucociliary escalator in the respiratory tract.

The attachment of pathogens to host cells often involves specific attachment mechanisms. For example, enteropathogenic Escherichia coli possess pili for attachment to host intestinal surfaces and such pili are associated with virulence (McNeish et al 1975, Ørskov and Ørskov 1977, Gaastra and De Graaf 1982). Adherence mechanisms may be host specific with different antigenic properties. E. coli strains pathogenic to piglets possess K88 pili (Wilson and Hohmann 1974, Parry 1983) while K99 pili are possessed by strains pathogenic to calves or lambs (Ørskov et al 1975, Parry 1983). C.F. pili are possessed by human pathogenic strains (Evans et al 1978, Parry 1983).

Pili are thought to be important in the initial attachment of Neisseria gonorrhoeae to host cells (Ward and Watt 1975, Watt et al 1976). However, Swanson (1978) has suggested that certain outer membrane proteins may be important in pathogen-host cell interactions whilst electrostatic interactions may also play an important role (Watt and Ward 1980). There are numerous other examples of cell

surface structures thought to be important in attachment of bacteria to host cells.

The attachment of bacteria to cells represents a reciprocal process since the host cell must possess the correct receptors for attachment, the presence (or absence) of such receptors may be one of the most important ways in which host and tissue specificity of pathogens is determined (Mims 1976).

Whilst many bacterial pathogens do not truly enter host tissues others traverse epithelial surfaces and colonize host tissues. Some enteric pathogens are effectively phagocytosed by cells lining the intestinal tract (Takeuchi 1975, Mims 1976), others may enter the host by passing through intercellular spaces (Mims 1976). Prior physical damage may facilitate the entry of bacterium into the host. For example, Clostridium tetani gains entry into the host through wounds (Willis 1969) whilst Pseudomonas aeuruginosa may infect tissue damaged by burns (Nathan et al 1973); motility of this bacteria may also be important contributing to its invasive ability (McManus et al 1980, Craven and Montie 1981). Many bacterial extracellular products are potential invasive factors having roles as aggressins rather than toxins (1.3.5), however their contributions to the invasive ability of bacteria is in most cases unproven.

In the case of Aeromonas salmonicida the mode of entry of the bacterium into fish has received little attention. Infection via the gastrointestinal tract may occur; Plenn (1911) and Blake and Clarke (1931) infected brown trout by feeding them food containing viable bacteria while Klontz and Wood (1972) observed furunculosis in sablefish after the ingestion of infected coho salmon. Other workers have, however, failed to infect fish via this route (Krantz et al 1963, McCarthy 1977 a).

Surface factors may be important in the attachment of A. salmonicida to fish tissues and were implicated as virulence factors as early as 1939 by Duff, though his findings were apparently not recognised as being important for 40 years. His work indicated that virulent strains of bacterium in the goldfish were characterized by smooth glistening, convex and butyrous colonies (S forms) whilst avirulent

strains possessed convex, opaque and cream coloured colonies which could be pushed across an agar plate surface as an entire colony (R forms). The pathogenic nature of S forms was confirmed by Anderson (1972) in coho salmon. However, this relationship has been disputed by other workers. McCarthy and Rawle (1975) found S forms to be avirulent whilst R forms, characterised as possessing colonies which could be pushed across the surface of an agar plate without loss of integrity, were virulent. This observation was confirmed by Udey and Fryer (1978). It is not clear why the results obtained by Duff (1939) and Anderson (1972) are totally contradictory to the current views on the relationship of colony form to virulence, this discrepancy has not been commented on in the literature.

Udey and Fryer (1978) found that autoagglutinating R forms possessed a layer external to the cell wall, a feature absent in the S forms. The additional layer visible using electron microscopic techniques was, they suggested, a prerequisite for virulence. Other workers have looked at the additional, or A layer as it is now termed, in more detail. The A layer appears to cover the whole bacterial surface and consists of tetragonally arranged sub-units (Ishiguro et al 1981).

The A protein is also demonstrable using sodium dodecyl sulphate - polyacrylamide gel electrophoresis of outer membrane proteins from virulent strains of A. salmonicida. Using this technique the A protein appears as a major component with a molecular weight of 49,000. Cultivation of bacteria at elevated temperatures resulted in the concomitant loss of the A layer and decrease in virulence (Ishiguro et al 1981). The role of the A layer in attachment of bacteria to fish tissues has yet to be evaluated.

A. salmonicida strains have also been shown to possess adhesins allowing them to attach to a variety of eucaryotic cells in vivo (Trust et al 1980). In view of the fact that the A layer appears to mask these adhesins this result must be interpreted with caution (Ishiguro et al 1981). The A layer and surface adhesins may not be the only structures involved in the attachment of bacteria to fish

tissues. In studies of fin rot Schneider and Nicholson (1980) found that A. salmonicida was often the predominant member of the bacterial flora. Attachment to the fish lesion appeared to involve glyocalyx fibres extending from the bacterial surface.

These results suggest that a variety of surface factors may be important in the attachment of A. salmonicida to host tissues. The relative importance may be dependent on the host fish species involved, the form of the disease and the stage in the disease process.

The physiological status of fish populations may be important in altering the susceptibility of fish to A. salmonicida infections. Changes in the environmental conditions or adverse environmental conditions often result in the stressing of fish, predisposing such fish to infection. Le Tendre et al (1972) studied an outbreak of disease in small mouthed bass suggesting that stress and damage to fish after netting precipitated an outbreak of disease, whilst Bulkley (1969) suggested that the precipitating factor of disease in wild yellow bass (Morone missipiensis L.) was the rise in water temperature in spring; other changes in environmental conditons may precipitate disease.

It is frequently observed that mortalities approaching 50% may be expected following the transfer of Atlantic salmon from fresh to sea water (Palmer and Smith 1980). The high incidence of disease may be attributable to the effects of stress on the fish resulting in the release of latent infections (Drinan et al 1978).

1.3.3 Multiplication in vivo

The virulence of a particular strain of bacteria can sometimes be related to the inability of that strain to grow in vivo. Perhaps the best known example of this is the role of iron in the disease process. The vertebrate body contains the efficient iron chelating proteins transferrin and lactoferrin. In order to grow in vivo the bacterium may have to compete with such host systems for free iron, essential for bacterial growth (Weinberg 1978).

The bacterium may possess its own iron chelating system, competing with the host iron binding proteins, for limited free

iron. For example E. coli produces enterochelin and this siderophile appears to act in conjunction with an outer membrane protein receptor (the 81K protein) facilitating iron uptake (Miles and Khimji 1975, Ichihara and Mizushima 1977, Griffiths 1983). The 81K protein is inducible, appearing under iron limiting conditions. Some strains of E. coli may also possess a second, plasmid coded, iron chelating system involving the aerobactin siderophile and a 74K membrane protein receptor. This system is also inducible under iron limiting conditions (Williams 1979, Griffiths 1983).

Recent work with the fish pathogen Vibrio anguillarum has suggested that virulence in certain fish species is also determined by the presence of a plasmid coding for an iron chelating system (Crosa 1980). Under iron limiting conditions, virulent, plasmid possessing strains, were found to possess an outer membrane protein, absent in avirulent strains (Crosa and Hodges 1981). It is conceivable that the ability to obtain iron is related to the production of haemolytic factors by the bacterium (1.3.5.), which may provide access to iron in the form of haemoglobin. E. coli haemolysin is thought to have this role in invasive infections (Griffiths 1983, Waalwijk and De Graff 1983), however such a role for haemolytic factors from other bacteria has yet to be investigated.

Other bacterial products may aid multiplication in vivo. For example, tissue digesting enzymes may facilitate dispersal of the bacterium in the host, such as staphylococcal hyaluronidase which liquefies connective tissue (Schmidt 1965, Abramson 1972) whilst elastase produced by P. aeuruginosa (Pavlovskis and Wretlind 1979, Woods et al 1982) enhances virulence of the bacterium. Smith et al (1982) have suggested that collagenolytic activity of Vibrio vulnificus contributes to its invasiveness. Proteases, lipases, nucleases and other activities produced by various bacteria may play similar roles as aggressins rather than toxins (Mims 1976). Such enzymes may also conceivably provide essential nutrients for bacterial growth.

Invading bacteria may be disseminated through the host via the blood or lymphatic system and may then invade other organs. Such mechanisms may permit the bacterium to invade protected sites in the host where growth can occur unhindered, thus Salmonella typhi enters the host from the gut and is able to ultimately invade the liver and spleen (Bryan et al 1979). Such growth in vivo is obviously dependent on the bacterium effectively avoiding host defence mechanisms.

There is considerable evidence and information concerning the sites of multiplication of A. salmonicida in vivo. The initial focus of infection may occur in a number of tissues (McCarthy and Roberts 1980); from these initial sites the bacteria may be disseminated to other tissues resulting in a generalised septicaemia and death (1.2.2).

Recent work by Munn and Trust (1981) has indicated possible sites of multiplication in vivo. Virulent strains of A. salmonicida injected intramuscularly appeared to accumulate in phagocytic organs and multiply rapidly, while avirulent strains were rapidly cleared from these tissues. The authors suggested that extracellular products released by bacteria in these tissues may lead to tissue necrosis and death.

The association of plasmids with virulence or pathogenicity of A. salmonicida is unclear. Though the bacterium is known to possess a number of plasmids, loss of virulence is apparently not associated with plasmid loss (Ishiguro et al 1981). Although an iron chelating system in A. salmonicida has yet to be demonstrated, the necessity for such a system in infections of coho salmon (Oncorhynchus kisutch L.) by V. anguillarum (Crosa 1980) suggests that ia may be important to look for a similar system in A. salmonicida.

1.3.4. Avoidance of host defence mechanisms

The mere ability of micro-organisms to enter and grow within a host does not by itself mean that the bacterium will be pathogenic. It is now becoming apparent that probably all animals and plants

possess mechanisms capable of opposing the growth of the pathogen from the host.

The necessity for pathogens to avoid such host defence mechanisms has resulted in the evolution of a number of 'strategies'. After entry into the host,the bacterium may be confronted by one of the most important hostdefence mechanisms, the phagocytic cell, which presents a major threat to bacteria survival,especially during the early stages of infection.

The bacterium may be capable of avoiding ingestion by the phagocyte. A component external to the cell wall may prevent ingestion. The M protein of streptococci (Fox 1974, Peterson et al 1979) and the protein A of staphylococci (Peterson et al 1974, Quie et al 1981) appear to have this function. It is perhaps ironical to note however that the M protein, and probably other 'capsules', are responsible for attachment of bacteria to suitable host cells.

The cell wall itself may contain components capable of preventing ingestion; Salmonella typhimurium LPS (Stendahl and Edebo 1972 Valtonen 1977) and E. coli K antigens may act in this manner (Howard and Glynn 1971).

The exact mechanisms by which such capsules or cell wall components prevent ingestion is still,in most cases,unclear. They may function by preventing antibodies and other serum components coating the bacterium prior to attachment to the phagocyte (opsonization); alternatively they may mechanically prevent ingestion, (Quie et al 1981, Wilton 1981), or they may alter the bacterial cell surface hydrophobicity and charge preventing attachment (Van Oss 1978, Wadström et al 1978). Certain bacteria are effectively ingested but may resist intracellular killing by the phagocyte. Again,the bacterium may possess cell surface components capable of resisting attack by lysosomal enzymes after formation of the phagolysosome. The waxy surface of mycobacteria probably functions in this manner (Armstrong and Hart 1971). Mycobacterium tuberculosis also appears to be able to prevent

lysosomal fusion with the phagocytosed bacterium (Armstrong and Hart 1971).

Brucella abortus is apparently capable of resisting phagocyte killing mechanisms (Cheers and Pagram 1979) as is N. gonorrhoeae which appears to possess an outer membrane component responsible for such protection (Parsons et al 1981, Diaz and Heckels 1982).

The prevention of ingestion and digestion by phagocytes may occur in a totally different way. Bacterial toxins released from the bacterium may damage or kill phagocytes,either before bacterial ingestion,or after - when the phagocytic cell would provide a 'containment vessel' for the accumulation of toxins to a lethal level (Arbuthnott 1981). P. aeruginosa appears to produce such a leucocyte active factor which kills the phagocyte after ingestion of bacteria (Nonoyama et al 1979 a, Nonoyama et al 1979 b).

Survival of bacteria within phagocytes is often a key factor in the pathogenesis of disease. Many pathogenic mycobacteria multiply in macrophages (Evans and Levy 1972, Levy et al 1975).

It is conceivable that such long lived phagocytic cells may also provide sites for bacteria to survive resulting in a persistent infection. The successful pathogen may be able to interfere with the immune response; the complexity of the immune system means that a wide variety of strategies may be adopted by the microbe to evade the system. Only a few of the evasion mechanisms can be covered here.

Since the immune response depends on the recognition of foreign antigens,bacteria may conceal their antigenic nature. The surface antigens of Borrelia recurrentis appear to change periodically, avoiding the full onslaught of the immune response (Smith 1978). Other bacteria may possess surface antigens which evoke a poor response, perhaps because they mimic host antigens. Bacterial lipopolysaccharides, may react in this way (Mims 1976, Falcone and Campa 1981);similarly capsules may function in this manner (Schwab 1975, Mims 1976).

Other bacteria may multiply in sites which are inaccessible to the immune system such as glands, ducts and within cells such as macrophages.

B. abortus is able to persistently infect cows because it colonises mammary glands inaccessible to the immune system.

In conclusion, since the host possesses a wide variety of powerful anti-microbial systems, successful pathogens are able to evade these systems in an equally diverse manner.

A number of mechanisms may operate enabling A. salmonicida to avoid host defence mechanisms. The furunculosis committee (Mackie et al 1930, 1933, 1935) noted the lack of leucocyte infiltration into fish tissues injected with broth culture supernatant fluid from A. salmonicida, Griffin (1954) suggesting that this may be due to the production of a leucocidin, in vivo, destroying phagocytic cells. Fuller et al (1977) were the first workers to isolate such a factor (L.C.L.) from culture supernatant fluid and when injected, the L.C.L. caused a leucopenia in coho salmon. The authors also found that virulent strains produced more L.C.L. in vitro than avirulent strains. This observation was confirmed by Cipriano (1980) although it is not clear whether Cipriano's relatively impure preparation contained the same leucocytolytic activity. Recent work by Ellis et al (1981) has also indicated that crude A. salmonicida extracellular products contains factors active against rainbow trout (Salmo gairdneri L.) leucocytes and macrophages though again the relationship of the activity responsible to Fuller's or Cipriano's leucocidins is not known. Histopathological evidence for the role of a leucocidin in vivo is controversial. Klontz et al (1966) found that the injection of A. salmonicida cells into rainbow trout resulted in an initial cellular response followed by a leucopenia, while Ferguson and McCarthy (1978) suggested that leucocytolytic activity is not important because they found no evidence of impaired cellular responses in infected brown trout. In chronically infected fish, an increase in lymphoid cell numbers was seen which they attributed to the mitogenic effects of the bacterial lipopolysaccharide.

The A layer possessed by virulent strains of A. salmonicida may be important in the evasion of host defence mechanisms (1.3.2). The ability of the A layer to mask surface antigens and its poor immunogenic nature may prove to be of importance in the evasion of immune responses (Kay et al 1981), whilst its hydrophobic nature may affect interactions with phagocytic cells (Kay et al 1981). Certainly,the possession of the A layer promotes resistance to serum killing mechanisms (Munn et al 1982).

Recently Ellis (1982) has proposed that the melanin like pigment produced by A. salmonicida in vitro may be able to protect the bacterium from free radical damage either within or outside fish phagoeytic cells. Further evidence for this role of bacterial melanin is derived from the observation that fish phagocytes and lymphoid tissues are known to contain melanin (Agius 1980) which is thought to protect these tissues from auto-destruction (Ellis 1982).

The case for effective avoidance of host defence mechanisms is strengthened in view of the recent evidence that the bacterium can exist in a latent state in "carrier" fish (McCarthy 1977 a). The site of carriage is not known at present, though the kidney has been implicated (McCarthy and Roberts 1980) and the phagocytic cells represent another possibility.

1.3.5 The role of bacterial toxins in the pathogenesis of disease

Toxins have been implicated as factors in the pathogenesis of a large number of bacterial diseases. In some cases they have been demonstrated to be of overwhelming importance in pathogenicity. For example, the pathogenesis of diphtheria can be attributed almost completely to a toxin produced by the aetiological agent Corynebacterium diphtheriae (Murphy 1976). The toxin is produced only after infection of the bacterium with Corynebacteriophage β (Singer 1976) and causes cessation of protein synthesis in host cells by inactivating elongation factor 2 (Honjo et al 1968, Gill et al 1969). Many of the symptoms of cholera can be attributed to the production of a toxin by Vibrio cholerae. This toxin stimulates adenyl cyclase activity in intestinal cells leading to increased

cyclic AMP levels and hypersecretion of electrolytes and water (Finkelstein 1976, Richards and Douglas 1978). The pathogenesis of tetanus can be attributed to the production of a neurotoxin by Cl. tetani (Bizzini and Simpson 1976, Bizzini et al 1969); the toxin acts on central nervous system causing excitation of motor neurones and resulting in spastic paralysis (Bizzini 1979).

However, such clear cut examples of the roles of toxins in the pathogenesis of disease are rare. Many pathogenic bacteria produce a wide variety of toxic, and in some cases lethal, products in vitro though their roles in the disease process in vivo are often poorly understood. Many of the potential toxins in this group fall into the "cytolysin" group, defined by Alouf (1976) as "an activity capable of causing gross lysis of cells". However, this probably represents too limited a definition of the cytolysis since their effects against some cell types may be more subtle than gross cell lysis.

The majority of cytolytic activities are produced extracellularly by Gram positive bacteria. Cytolytic activities produced by Gram negative bacteria represent a poorly studied group. In most cases it is not clear whether individual cytolysins are true extracellular products or are released on autolysis of cells.

Almost all cytolysins have been shown to be proteins with molecular weights greater than 25,000, although some involve two components acting synergistically such as staphylococcal Y toxin (Taylor and Bernheimer 1974) and Streptococcus zymogenes haemolysin (Granato and Jackson 1971). Most cytolysins studied to date are active against erythrocytes (Alouf 1976) and this cell type provides an excellent model system for the study of their mode of action, but a wide variety of cell types may also be susceptible to the action of cytolysins (Alouf 1976, Stephen and Pietrowski 1981).

Streptolysin S, a cytolysin produced by various streptococci has been shown to be particularly active against certain tumour cells and may be an important chemotherapeutic agent as such (Okamoto 1976).

Cytolysins generally show no immunological relationship to one another with the exception of the thiol activated cytolysins produced by various Gram positive bacteria (Bernheimer 1976).

Lysis of cells may ocur by a number of mechanisms. Staphylococcal δ lysin (Thelestam and Möllby 1975, Rogolsky 1979) appears to have a detergent like action on membrane lipids and proteins.

Enzymatic action involving hydrolysis of lipids is displayed by staphylococcal sphingomyelinase (β lysin) and Clostridium perfringens phospholipase C (α toxin) (Bernheimer 1974, Wadström et al 1974). Staphylococcal α toxin is thought to be a proteolytic enzyme, cleaving membrane proteins and with resultant cell lysis (Wiseman and Caird 1970, 1972).

Finally, the cytolysin may bind to cell membrane components causing a rearrangement of membrane structure and subsequent lysis. Such a mechanism may be displayed by streptolysin O (Alouf 1976) and staphylococcal leucocidin (Woodin 1970).

Studies of mechanisms of lysis often involve the monitoring of rates of release of "marker" molecules of varying molecular weights. Such information may indicate the formation and size of functional holes produced by cytolytic action on cell membranes (Möllby and Wadström 1973, Thelestam amd Möllby 1975, Thelestam and Möllby 1979).

Generally the roles of cytolysins during the natural infection are poorly understood. Many, such as staphylococcal α toxin (Jeljaszewicz et al 1978) and thiol activated cytolysins (Smyth and Duncan 1978), are lethal when administered parenterally into mammals but production in vivo is not proven conclusively. Further, levels of toxin in body tissues during the natural infection may be too low to cause fatalities, promoting more subtle changes within cells. The difficulty of assigning particular roles to

individual toxins is exemplified by the vast array of cytolytic and non-cytolytic products of the staphylococci (Wadström et al 1974, Rogolsky 1979).

Many cytolysins may be important in vivo by damaging phagocytic cells. Virtually all cytolysins discovered to date appear to show some activity against phagocytes although the mechanism involved may be far more subtle in vivo than the gross lysis of cells observed in vitro with high concentrations of cytolysin. In many such cases it is likely that damage to the host will be attributable to a number of factors acting together rather than one toxin of overwhelming importance and only detailed study of individual factors can clarify the often complex picture in the disease process.

In the case of A. salmonicida, latency of the bacterium within fish may represent a balanced host -pathogen relationship, however, it is apparent that various factors may alter this balance favouring the pathogen when rapid multiplication in vivo is almost invariably associated with damage and death of the host.

Early workers made several observations concerning pathogenic mechanisms of A. salmonicida. The furunculosis committee (Mackie et al 1930, 1933, 1935) failed to demonstrate toxic activity in broth culture ultra-filtrates when injected into fish, however they noted the liquefaction of muscle at the injection site and the lack of leucocyte infiltration into this area. They suggested that pathogenicity may be attributable to the bacterium growing in the host's blood, interfering with the blood supply and leading to anoxic cell necrosis and death.

There is considerable evidence that a crude mixture of A. salmonicida extracellular products is toxic when injected into trout. Ellis et al (1981) found that ECP administered in this fashion was not only lethal to rainbow trout but also reproduced many of the symptoms associated with the disease.

The crude ECP was found to contain activity against trout erythrocytes, leucocytes, macrophages and rainbow trout gonad tissue culture cells in addition to proteolytic activity. Pol et

al (1980), working with carp , found that broth culture supernatant fluid was toxic and reproduced many symptoms of carp enythrodermatitis when injected into fish. Again, the factors responsible were found to be non-dialysable and inactivated by heat, low pH and formalin suggesting a proteinaceous nature.

Histopathological evidence does suggest that protease production is important in vivo. The degeneration of muscle tissue below furuncles (McCarthy and Roberts 1980) and the increased blood creatine (Field et al 1944), amino acid and nitrogen compound (Shieh and Maclean 1976) levels indicate protein breakdown. The protease(s) responsible may be of bacterial origin or alternatively may be released from host cells as a result of cytolytic activity (Ellis et al 1981). In contrast to these observations Ferguson and McCarthy (1978) found little evidence of tissue necrosis around bacterial colonies in naturally infected brown trout.

Several workers have partially purified the caseinase produced by A. salmonicida (Dahle 1971, Shieh and Maclean 1975, Sakai 1978, Sheeran and Smith 1981). Sheeran and Smith's work (1981) suggested that two proteases were produced by A. salmonicida, a factor apparently unknown by other workers.

The toxicity of the proteases to fish appears questionable. Sakai (1977, 1978) found 'purified' protease to be lethal to kokanee salmon (Oncorhynchus nerka L.) when injected. Sheeran et al (1981) failed to reproduce these results using their protease preparations and suggested that Sakai's protease may be contaminated with another lethal activity.

There is similar conflicting evidence for the role of cytolysins in the disease process.

Klontz et al (1966) found that a leucopenia occurred following injection of fish with A. salmonicida which may be attributable to the leucocidin studied by Fuller, Pilcher and Fryer (1977).

Various other workers have looked at blood parameter changes as evidence of haemolysis in vivo. Foda (1973) found decreased haematocrit and haemoglobin levels in the blood of moribund fish. Shieh and Maclean (1976) found similar changes and attributed them to either haemolysis of erythrocytes or haemorrhaging. McCarthy

and Roberts (1980) have found extensive haemosiderin deposits in the melanomacrophage centres of haemopoietic tissue during chronic furunculosis infections, and suggested this indicated extensive haemolysis in vivo. By contrast, Klontz et al (1966) found that erythrocyte counts, packed cell volumes and haemoglobin levels did not decrease in infected fish, whilst Munro et al (1980) found no evidence of haemolysis following injection of crude bacterial extracellular products.

Preliminary studies of the properties of the haemolytic activity were carried out by Karlson (1962) who noted that crude haemolytic activity was more active against trout erythrocytes than human, horse or guinea pig, whilst Nord et al (1975) found the bacterium to be haemolytic on human, horse, sheep and rabbit blood agar. Neither workers looked at the haemolytic activity in detail however.

Other workers have noted that A. salmonicida produces cytolytic activity against trout macrophages and rainbow trout gonad cells (Munro et al 1980) and against fathead minnow tissue culture cells (Anderson 1972). It is not clear, however, whether the haemolysin or leucocidin (Fuller, Pilcher and Fryer 1977) are responsible for these effects.

MacIntyre et al (1979) reported the production of a potentially toxic glycerophospholipid-acyltransferase by A. salmonicida though its role in pathogenicity does not appear to have been evaluated at this time.

The action of any of these potential toxic or invasive factors in vivo may be influenced by the fact that glyocalyx fibres extending from the bacterium to the host surface may effectively concentrate these factors to toxic or lethal levels (Munro 1982).

Endotoxin is known to be an important factor in the pathogenesis of disease in mammals (Milner et al 1970, Roantree 1970), but in fish it appears to have no effect even at high levels. Endotoxin extracted from a variety of bacterial species appears to have no effect when injected into fish (Berczi et al 1966, Wedeneyer and Ross 1969) Other workers looking at A. salmonicida endotoxin in particular had found no effect when injected into fish, although

the preparations were toxic to mice (Paterson and Fryer 1974, Pol et al 1980).

In conclusion, it can be seen that A. salmonicida produces a wide variety of extracellular products in vivo, which are potential toxins. In addition to those described above, the bacterium has been reported to produce DNAase, lipase, lecithinase and amylase in vivo (Nord et al 1975). Only the proteases have been studied in any detail and do not appear to be the sole factors responsible for disease symptoms. As found with many other bacterial diseases, the ability to damage the host may be multifactorial. Only a rational approach to the study of individual extracellular products can indicate their role in the disease process. The only exception to this statement appears to be endotoxin, which by all accounts appears to play a very limited role, if any, in the pathogenesis of furunculosis.

1.3.6 The Control of Disease

Though most bacterial diseases can be controlled using antibacterial drugs, vaccination against the etiological agent is a preferable alternative. In some cases, effective vaccines have been developed with little knowledge of the mechanisms of pathogenicity of nature of the protective antigen. In retrospect, however, the protective antigen has often been identified after the host-pathogen relationship has been studied in detail.

Vaccine preparations capable of protecting individuals against diphtheria (Griffith 1979) and tetanus (Rey et al 1979) rely on the ability of partially denatured toxin to evoke an immune response against the native toxin without any of the toxic properties of the native toxin being expressed (Toxoid preparations). Vaccination against these diseases is effective probably due to two reasons: firstly, the disease can be attributed to one bacterial product and secondly, that the product in question is easily toxoided, retaining immunological identity with the native toxin. In the case of cholera, an effective vaccine has eluded microbiologists for many years. Recently however, knowledge of the toxin structure (Finkelstein 1976) has resulted in considerable advances in this field. Finkelstein and Finkelstein

(1982) have demonstrated that an avirulent mutant producing only the B sub-unit of cholera toxin (non-toxic subunit) is capable of evoking a protective immune response against subsequent challenge by virulent bacteria. Other workers have also developed effective vaccines (Pierce et al 1982) and have suggested that the protective antigens involved are not only the toxoid but also other bacterial components such as the 'O' antigens. Klipstein et al (1982) have recently described a vaccine against enterotoxigenic E. coli using conjugated heat labile (LT) and heat stable (ST) enterotoxins. The non-toxic LT-ST conjugate appears to lose none of the antigenicity associated with native toxins. Various experimental vaccines against P. aeruginosa have been evaluated. Holder et al (1982) found that flagellar vaccine preparations were effective in mice following work suggesting that motility was important in the virulence of the bacterium (1.3.2.).

In the case of other diseases, pathogenencity appears to be multifactorial and it is difficult to identify the components required for an effective vaccine. Such a situation is seen with some P. aeruginosa vaccines where 'crude' vaccine preparations cause many side effects (Mellor and Miller 1979). Knowledge of pathogenic mechanisms and virulence determinants of this bacterium may facilitate vaccine production with fewer adverse reactions.

As discussed in earlier sections, A. salmonicida is an economically important fish pathogen. Although antibiotics are currently used to control the disease, the control of disease by vaccination is a long term goal. It is apparent from studies of vaccine development that a study of the host-pathogen relationship may lead to improved vaccines. In view of the variable results obtained with vaccine preparations developed by workers to date it is apparent that future work may involve prior knowledge of potential pathogenic mechanisms.

The aims of this project were to study in detail selected potential pathogenic mechanisms of A. salmonicida. In particular, the nature of haemolytic activities produced by the bacterium and their relationship to other extracellular products has been investigated.

CHAPTER 2 MATERIALS AND METHODS

2.1.0 CHEMICALS AND REAGENTS

Chemicals and reagents used in this project were obtained from BDH (Poole, Dorset) or the Sigma Chemical Company (London) unless otherwise stated.

2.2.0 BACTERIOLOGY

2.2.1 Bacterial strains

The sources of the strains of A. salmonicida used in this project are detailed in Table 1.

2.2.2 Storage of bacteria

Bacteria were stored using liquid nitrogen refrigeration with 10% w/v skim milk protectant, and subcultured weekly onto tryptone soya agar (TSA Oxoid). The bacteria were grown at 20°C or 25°C as required.

2.2.3 The identification of bacteria used in this project

The strains of bacteria used in this project were confirmed as A. salmonicida using biochemical tests in accordance with Bergey's Manual (1975) and McCarthy (1975) (Table 2).

2.2.4 The culture of bacteria in liquid growth media

Bacteria were normally grown in 100 ml volumes of liquid media contained in 250 ml Erlenmeyer flasks. The medium was inoculated with 1.0 ml of an 18 hour culture of the bacteria in tryptone soya broth. At the termination of each experiment the cultures were checked for purity and pigment production after the growth of a sample of the culture on TSA. Culture supernate samples were obtained after centrifugation (7,500 x g, 20 min, 4°C or 10,000 x g, 20 min, 4°C).

Where indicated, the cultures were shaken at 70 strokes/min (Grant shaking water bath) or 120 strokes/min (Tecam SB16, shaking water bath).

The bacteria were grown in a variety of liquid growth media. Peptone broth contained peptone (Oxoid, 10 g/l) NaCl (5 g/l) and K_2HPO_4 (5 g/l). Lab lemco broth contained lab lemco powder in place of the peptone. Yeast extract (Oxoid, 2 g/l) was added to these.

Table 1 The strains of *A. salmonicida* used in this project

Laboratory Strain Number	Donated by	Original host	LD_{50}	Comments
25/77	Dr. B. Austin[1]	Rainbow trout	NK	
84/77	Dr. B. Austin[1]	Rainbow trout	NK	
84/78	Dr. B. Austin[1]	Sea trout	NK	
CM 30	Dr. C. Munn[2]	Salmon	NK	
10/76	Dr. B. Austin[1]	NK	NK	
113/76	Dr. B. Austin[3]	Brook trout	NK	Type strain NCMB 833 ATCC 14174
114/76	Dr. B. Austin[3]	Salmon	NK	NCMB 834
119/76	Dr. B. Austin[4]	Salmon	NK	Suggested neotype NCMB 1102
449	Dr. T. Trust[5]	Rainbow trout	8×10^3 [6]	
449/3	Dr. T. Trust	NA	1×10^8 [6]	A layer [7] negative mutant of 449
449/3/R	Dr. T. Trust	NA	NK	A layer and [7] LPS negative mutant of 449
450	Dr. T. Trust[8]	Rainbow trout	2×10^4 [6]	
450/3	Dr. T. Trust	NA	2×10^8 [6]	A layer [7] negative mutant of 450
450/3/R	Dr. T. Trust	NA	NK	A layer and [7] LPS negative mutant of 450

Table 1 cont'd...

451	Dr. T. Trust[5]	Rainbow trout	1×10^4	
451/3	Dr. T. Trust	NA	1×10^8	A layer negative mutant of 451
451/3/R	Dr. T. Trust	NA	NK	A layer and LPS negative mutant of 451

NK = Not known
NA = Not applicable

[1] Dr. B. Austin, Ministry of Agriculture Fisheries and Food, Fish Disease Laboratory, Weymouth, England.

[2] Isolated in this laboratory.

[3] Originally isolated by Dr. H. Ewing, CDC, Atlanta, USA.

[4] Originally isolated by Isabel Smith, Wales,

[5] Originally isolated by Dr. C. Michel, Laboratoire d'Ichtyopathologie, INRA, Thiveral-Grignon, France, strain TG 36/75 donated by Dr. T. Trust, University of Victoria, Canada.

[6] Determined in Rainbow trout (Ishiguro et al 1981)

[7] Reference: Ishiguro et al 1981.

[8] Originally isolated by Dr. C. Michel, Laboratoire d'Ichtyopathologie, INRA, Thiveral-Grignon, France, strain TG 72/78 donated by Dr. T. Trust, University of Victoria, Canada.

[9] Originally isolated by Dr. C. Michel, Laboratoire d'Ichtyopathologie, INRA, Thiveral-Grignon, France, strain TG 51/79 donated by Dr. T. Trust,University of Victoria, Canada.

Table 2 Biochemical tests used to identify strains of *A. salmonicida*

Biochemical test	Result
Gram stain	Gram -ve
Pigment production	+
Motility	-
Cytochrome oxidase	+
Catalase	+
Growth at 37°C	-
Urease production	-
O/F test	F
Gelatin liquefaction	+
Starch hydrolysis	+
Acid from sucrose	-
Acid from xylose	-
Ornithine decarboxylase	-
Arginine hydrolysis	+

broths in some experiments. Furunculosis broth consisted of tryptone (Oxoid 10 g/l), yeast extract (5 g/l), L-tyrosine (1 g/l) and NaCl (5 g/l). Nutrient broth, nutrient broth No. 2 (NB No. 2) and brain heart infusion were supplied complete by Oxoid. Shieh and Reddy medium (Shieh and Reddy 1972) contained $MgSO_4.7H_2O$ (0.05%), K_2HPO_4 (0.1%), NaCl (0.5%), $FeSO_4.7H_2O$ (0.001%), DL-alanine (0.05%), L-asparginine (0.05%), Na-glutamate (0.01%), DL- serine (0.01%), L-methionine (0.01%), DL-isoleucine (0.005%), L-cysteine HCl (0.001%), arginine (0.1%) and L-tyrosine (0.1% w/v).

Nutrient broth No. 2 was supplemented with 1% (w/v) yeast ribonucleate, 5% (v/v) glycerol, 1% (w/v) glucose, 0.5% (w/v) bovine serum albumin or 2% (w/v) haemoglobin as required. Thermolabile additives were membrane filtered (Millipore, 0.45 μm, 25 mm) before addition to autoclaved media.

The pH of all media was adjusted to 7.5 before autoclaving (121°C, 15 min). Anaerobic conditions were achieved by placing the flask in an anaerobic jar and flushing the jar with nitrogen. The jar was also flushed daily with nitrogen. Samples were withdrawn through a clampable pipe passing through the lid of the anaerobic jar.

Bacteria were also grown inside dialysis tubing (6 mm diameter) suspended in 100 ml of NB No. 2. The dialysis tubing was filled with 1 ml of PBS and placed in the flask with the ends protruding through the cotton wool bung, capped with foil. After autoclaving, the bacteria (1 ml, 18 hr culture in TSB) were innoculated into the tubing using a sterile pasteur pipette.

Fermenter cultures of the bacteria were grown in 6l volumes of NB No. 2 contained within a 10 L fermenter vessel (LH engineering, LHE 1/100 fermenter).

2.2.5 Production of extracellular products using the agar overlay technique

The agar overlay technique was, in some experiments, used to obtain extracellular products from A. salmonicida. Tryptone soya agar (250 ml) was poured into sterile 30 x 30 cm glass assay plates under an ultraviolet hood. When set, the agar was overlaid with a

sheet of cellophane (British Cellophane Company, 325 PV cellophane) which had been autoclaved between moist sheets of tissue paper.

Five ml of inoculum (2.2.4) was spread over the surface of the cellophane and the plate incubated for 72 hours at 25°C. Growth was scraped off the cellophane using a sterile glass slide and streaked onto a plate of TSA to check culture purity. The cellophane was washed with 2-3 ml of phosphate buffered saline (PBS, 0.1M, pH 7.2) and the pooled material made up to 9 ml with PBS. After gentle shaking with glass beads (15 min, room temperature) and centrifugation (7,500 x g, 20 min, 4°C) the supernatant fluid was dialysed against PBS (18 hr, 4°C, 2 x 1 l, +0.02% NaN_3) and frozen until required.

2.2.6 Enumeration of bacteria

Viable bacteria were counted using the method of Miles and Misra (1938) or using a spiral plater (Spiral Systems, Bethesda USA). Dilutions were made in peptone water (1% w/v peptone, 5 g/l NaCl) and the bacteria grown on TSA at 25°C.

2.2.7 Determination of iron levels in liquid growth media

Free iron levels in NB No. 2, after the addition of transferrin (Sigma, iron free) were determined using Sigma diagnostic kit number 65. The method used was based on the total iron binding assay but was modified to permit the determination of free iron levels in media containing transferrin as detailed below; Two ml of UIBC buffer (pH 8.1) was pipetted into clean cuvettes, a further 1.0 ml of glass distilled water was added to 'blank' cuvettes whilst to the 'standard' cuvettes a further addition of 0.5 ml of glass distilled water and 0.5 ml of iron standard solution was made. The sample cuvettes contained UIBC buffer (2 ml), glass distilled water (0.5 ml) and test growth medium sample (0.5 ml). After determination of the optical density of the standard and test samples against the blank sample (560 nm, Initial A_{560}), 50 µl of iron colour reagent was added. The cuvette contents were mixed and after incubation (37°C, 10 min) the resultant magenta colour was measured at 560 nm

(Final A_{560}). The intensity of the magenta colour was proportional to the quantity of free iron.

The quantity of free iron in the test samples was determined using the equation:

$$\text{Free iron } (\mu g/ml) = \frac{\text{Final } A_{560} \text{ test samples} - \text{Initial } A_{560} \text{ test samples}}{\text{Final } A_{560} \text{ standard sample} - \text{Initial } A_{560} \text{ standard sample}} \times 500$$

2.2.8 Extraction of haemolytic activity from A. salmonicida cells

To study the possible intracellular location of haemolytic activity, 500 ml volumes of broth cultures (NB No. 2) were centrifuged (7,500 x g, 20 min, 4°C), the pelleted cells washed three times in PBS and the cells resuspended to 42 ml in PBS. The cell suspension was divided into five 8 ml volumes and the suspensions treated in a variety of ways; cells were disrupted by sonication (MSE sonicator, 18 μm peak, large probe, 30 secs, on ice) or using lysozyme (225 μg/ml) and ethylenediaminetetraacetate (EDTA, sodium salt, 4 mM) in tris (hydroxymethyl) aminomethane - HCl buffer (tris-HCl, 40 mM, pH 8.2) according to the method of Garrard (1971). In some experiments, sucrose (0.5 M) was added to the mixture to maintain integrity of the cytoplasmic membrane, and allow only material from the periplasmic space to be extracted. In these experiments, resultant sphaeroplast formation was confirmed using phase contrast microscopy.

Cells obtained by the agar overlay technique (1 plate) were also treated as detailed above to release haemolytic activity.

2.2.9 Detection of the presence of the A layer

The presence of the A layer, associated with virulence of A. salmonicida (Ishiguro et al 1981), was determined for the strains of A. salmonicida used in this project. The bacteria were grown at 20°C and after centrifugation (7,500 x g, 20 min, 4°C) the pelleted cells were resuspended in PBS (pH 7.2) or TSB.

Autoagglutination of the cell suspension was indicative of the presence of the A layer.

For some strains of A. salmonicida the presence or absence of the A layer was determined using electron microscopy. Bacteria (24 hr cultures, 20°C in TSB) were placed on carbon stabilised, formvar coated copper grids and negatively stained using 2% phosphotungstic acid (pH 7.0). The grids were observed using a Philips 300 TEM microscope at 80 kV and photographed using electron image plate film (Kodak).

2.3.0 AMMONIUM SULPHATE PRECIPITATION AND PROTEIN ASSAY TECHNIQUES

2.3.1 Ammonium sulphate precipitation

Ammonium sulphate solution (saturated at 0°C, BDH low in heavy metals) was adjusted to pH 8.0 by the addition of a small volume of ammonium hydroxide solution. Ammonium sulphate solution and culture supernatant fluid were cooled to 0°C (on ice) and a suitable volume of the ammonium sulphate added slowly to the culture supernate to give 40% saturation. After slowly mixing for 1 hour precipitated material was collected by centrifugation (10,000 x g, 20 min, 0°C) and the volume of remaining supernate determined. Ammonium sulphate solution was added to this supernate to give 60% saturation and after mixing (1.5 hr, 0°C) the precipitated material collected by centrifugation. The pellet was dissolved in a small volume of PBS (0°C) and dialysed against PBS (0°C) overnight.

In some experiments, EDTA was added to the ammonium sulphate solution before use (Sigma, sodium salt, 40 mM).

2.3.2 Protein determinations

The protein content of samples was normally determined by the method of Lowry et al (1951) with the final colour intensity read at 500 nm using a Cecil CE303 spectrophotometer. When samples were in tris buffer or contained ammonium sulphate the samples were dialysed extensively against distilled water before assay.

When the sample volume was considered too low to permit the use of the Lowry protein assay the protein content of the sample

was determined by finding the optical density (OD) of the sample at 260 nm and 280 nm. The protein concentration was calculated using the formula of Kalkar and Shafran (1947):

$$\text{Protein (mg/ml)} = 1.45 \times OD_{280} - 0.74 \times OD_{260}$$

2.4.0 DETECTION OF EXTRACELLULAR PRODUCTS

2.4.1 Detection of extracellular products on solid media

Bacteria were inoculated onto TSA (4/3 strength) containing a variety of substrates for extracellular products of A. salmonicida (Table 3). After growth (20°C or 25°C), the radii of zones of digestion were determined using a vernier caliper.

In experiements when ammonium sulphate was added to the growth medium 9.75 ml volumes of TSA (4/3 strength) were aseptically pipetted into sterile test tubes, in a water bath at 50°C. To some tubes, 1.125 ml of membrane filtered 40% ammonium sulphate solution was added (BDH, low in heavy metals) with the further addition of 0.375 ml of sterile distilled water. To other tubes 1.5 ml of sterile distilled water was added (control growth medium). The tubes were vortex mixed during the addition processes. The tubes received the appropriate volume of substrate (Table 3), were vortex mixed, and the agar poured into sterile petri dishes.

In some experiments 1.33% agar (Oxoid No. 1) in 4/3 strength PBS was used in place of the TSA. The further addition of 0.02% NaN_3 was made to these tubes and after the plates had set, 4 mm wells were cut in the agar. Supernate samples were placed in the wells in these plates and after incubation (24 hr, 25°C) zones of digestion were measured using a vernier caliper.

Tributyrin agar was used as supplied (Oxoid). Ammonium sulphate or sodium azide were added directly to this agar as required.

Casein and gelatin digestion zones could not be visualised directly. Plates of casein agar were flooded with acetic acid (3%) and plates of gelatin agar were flooded with tannic acid (5%) to enable the zones of digestion to be measured.

Table 3 The use of various solid media to detect extracellular products.

Substrate added to growth medium	Final substrate concentration	Extracellular product activities detected using this medium
Horse erythrocytes (washed)	5% v/v	Haemolysis of erythrocytes
Trout erythrocytes (washed)	7% v/v	Haemolysis of erythrocytes
Sodium caseinate (Kodak Eastman) +	1% w/v	Digestion of casein by proteases
Gelatin (BDH)	0.4% w/v	Digestion of gelatin by proteases
Egg yolk emulsion (Oxoid)	30% v/v	Proteolytic activity (diffuse digestion), lipases, Lecithinases
Tributyrin agar (used as supplied, Oxoid)		Lecithinases

2.4.2 Detection of haemolytic activity

Blood from a variety of animals was collected freshly, using heparin as an anticoagulant. The blood was washed three times in PBS, with centrifugation at 3,000 x g for 10 minutes at room termpature. To maintain isotonicity, eel and dogfish blood were washed using appropriate PBS (eel 1.05% NaCl, dogfish 2.2% NaCl + 2.9% urea, pH 7.2). Horse blood (Difco, defibrinated) was washed (x 3, PBS) and resuspended in PBS so that when mixed in the ratio 1 : 1 with sodium lauryl sulphate (SLS, 0.025% w/v) the optical density (OD) at 540 nm against a blank of PBS and SLS (1 : 1) was 0.8 units (Cecil CE 303 sepctrophotometer). This preparation was found to contain, on average, 9.75×10^7 cells/ml. Other blood types were resuspended in appropriate PBS to give 9.75×10^7 cells/ml.

To determine haemolytic activity, samples (0.1 ml) were diluted in twofold steps in appropriate PBS (0.1 ml) in a microtitre tray. After the addition of erythrocyte suspension to each well (0.1 ml), and incubation (37°C, 1 hr), the haemolytic titre, expressed as haemolytic units/ml (Hu/ml), was obtained from the reciprocal of the dilution that gave a visually estimated end-point of 50% haemolysis.

2.4.3 Detection of haemolytic activity using the microelisa reader

In work concerning purified H-lysin, the 50% end point of haemolysis, in the microtitre tray assay (2.4.2) was determined using a microelisa reader (Dynatech, MR590 minireader) at 490 nm. The haemolytic activity was then calculated using the formula:

$$\text{Apparent haemolytic activity (Hu/ml)} = \frac{OD_{490} \text{ of unlysed cells} - OD_{490} \text{ of cells with approximately 50\% haemolysis}}{OD_{490} \text{ of unlysed cells} - OD_{490} \text{ of completely lysed cells}} \times 2$$

However, in view of the fact that changes in OD at 490 nm were not directly proportional to the number of lysed cells a calibration curve was constructed to determine the actual haemolytic activity.

Horse erythrocyte suspension was centrifuged and resuspended to half the original volume in distilled water. After mixing and lysis of the cells, the lysate was made up to the full original volume in double strength PBS. This lysate was mixed with intact horse erythrocyte suspension in various ratios and 0.1 ml volumes of the mixtures placed into the wells in a microtitre tray. After the further addition of PBS (0.1 ml) and incubation (1 hr, 37°C) the OD at 490 nm of the well contents was determined using the microelisa reader. From these results a calibration curve was constructed (Fig. 1). The actual haemolytic activity of a sample was determined from this graph, with knowledge of the apparent haemolytic activity (equation 1). To determine the full haemolytic activity in the original sample, the actual haemolytic activity (Hu/ml) was multiplied by the dilution factor involved in the well with 50% haemolysis.

In experiments when other concentrations of horse erythrocytes were used appropriate calibration curves were used to calculate the haemolytic activity of the sample.

2.4.4 The use of a recording spectrophotometer to study the kinetics of haemolysis

A recording spectrophotometer (Pye Unicam SP 1800) was used to study the kinetics of horse erythrocyte lysis by H-lysin. The reaction temperature was maintained at a constant level in these experiments using a water-jacketed cuvette holder. Water was pumped through the cuvette holder at the appropriate temperature. The temperature of the water jacket was determined using a thermometer placed in a water filled cuvette in the cuvette holder.

The blank cuvette, in these experiments, contained horse erythrocyte suspension (0.6 ml) and PBS (0.6 ml). The instrument was zeroed before use and the blank cuvette placed in the position normally occupied by a sample cuvette; the sample cuvette, containing only horse erythrocyte suspension (0.6 ml) was placed into the position normally occupied by a blank cuvette. The reaction was started by the addition of H-lysin (0.6 ml) to the sample cuvette, with mixing. The optical density of the sample cuvette was

monitored at 590 nm, a wavelength which was previously shown to result in the maximum difference between lysed and unlysed cells. Changes in optical density were recorded using a chart recorder at 2 min/cm (Pye Unicam AR25). At approximately 5 minute intervals, the contents of both the blank and sample cuvettes were mixed using plastic paddles.

The resultant curves could not be interpreted directly because the optical density in the sample cuvette was not directly proportional to the number of lysed cells. A calibration curve was constructed (2.4.3) and the curves redrawn from selected points to indicate the true percentage lysis of the erythrocytes (Fig. 2).

2.4.5 Detection of caseinase activity

Caseinase activity in liquid samples was determined using a protease assay kit (Bio-Rad) in accordance with the manufacturer's instructions. Samples (10 µl) were placed into 4 mm wells cut in the gel. After incubation (18 hr, 25°C), caseinase activity was determined by measuring the radius of the zone of casein digestion with a vernier caliper. Caseinase activity (units/ml) was determined from a standard curve (Fig. 3), constructed using the method described by Sandvik (1962). Papain (1 mg/ml in PBS) was used as a positive control in these assays.

2.4.6 Detection of glycerophospholipid:cholesterol acyltransferase activity

Glycerophospholipid:cholesterol acyltransferase (GCAT) activity was detected using human erythrocyte membranes as the enzyme substrate, by the method of MacIntyre et al (1979). Outdated human transfusion blood (80 ml) was washed three times (PBS) with removal of the buffy layer after each centrifugation (3,500 x g, 15 min, 22°C). The erythrocytes were lysed by gently aspirating them in 10 mM tris-HCl buffer (pH 7.4). The erythrocyte membranes were collected by centrifugation (36,000 x g, 20 min, 4°C) and the pelleted material repeatedly washed (tris-HCl buffer) and centrifuged

Figure 1 Calibration curve for the estimation of haemolytic activity using the microelisa reader.

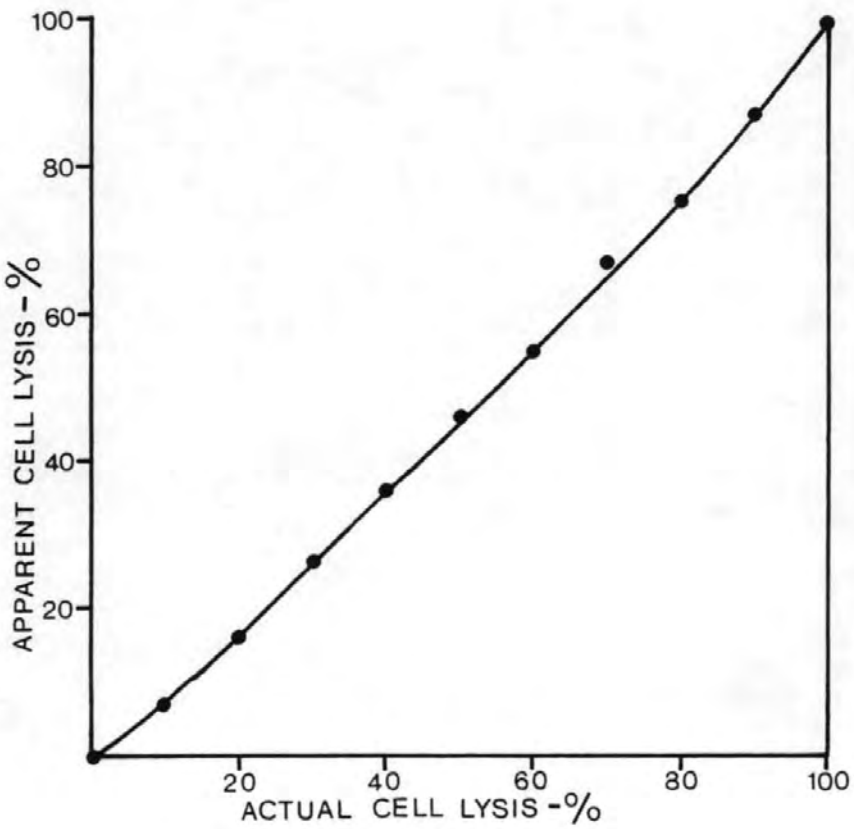


Figure 2 Calibration curve for the estimation of percentage lysis using the recording spectophotometer

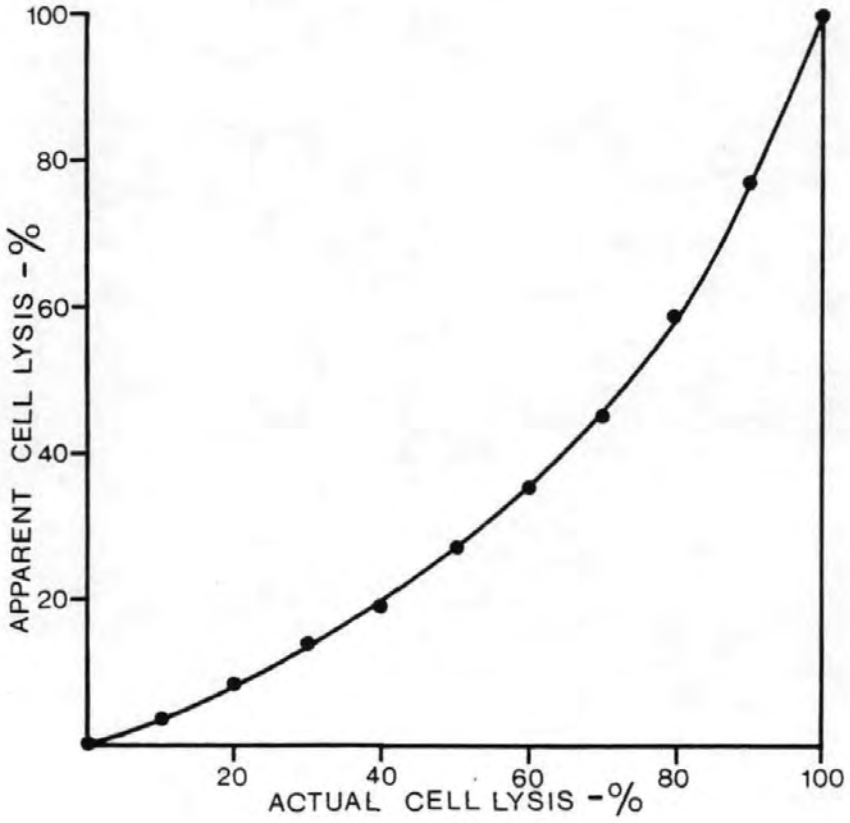
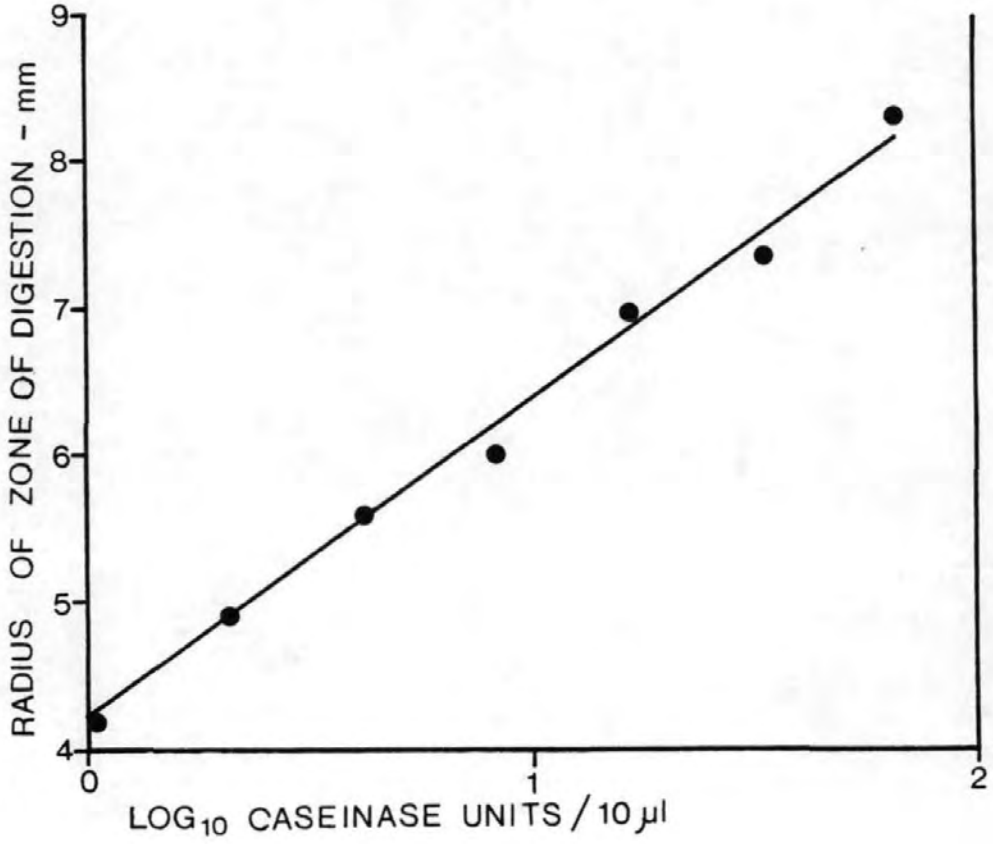


Figure 3 Calibration curve for the estimation of caseinase activity



until no more haemolgobin was visually detected in the supernatant fluid. The membranes were resuspended in 20 ml of 20 mM tris-HCl buffer (pH 7.4) and frozen (-20°C) as 1 ml aliquots until required. Using the method of Lowry et al (2.3.2) the protein content of the membrane suspension was found to be 0.425 mg/ml.

Samples to be tested for GCAT activity were mixed (0.2 ml) with membranes (0.2 ml), incubated (15 min, 37°C) and the reaction terminated by the addition of 2.5 ml of chloroform/methanol (2 : 1). After vortex mixing and centrifugation (3,000 x g, 5 min), the lower phase was removed and dried under nitrogen. Dried material was resuspended to 50 µl in chloroform and 25 µl aliquots spotted onto thin layer chromatography plates (Silica gel MN, Merck). The plates were developed using petroleum ether: ether:acetic acid (90 : 10 : 1) and the separated components visualised using iodine vapour.

Cholesterol oleate (2 mg/ml), cholesterol (4 mg/ml) and palmitic acid (4 mg/ml) were run concomitantly with the test samples. Control samples wereerythrocyte membranes only and culture supernatant only treated to extract the lipids as detailed above.

2.4.7 Detection of pigment production by A. salmonicida

The optical density (410 nm) of 0.1 ml volumes of culture supernate placed into microtitre tray wells, was determined using a microelisa reader (Dynatech, MR 590 minireader).

2.5.0 ELECTROPHORETIC TECHNIQUES

2.5.1 The elctrophoretic system

Proteins were separated using a vertical slab electrophoretic system according to the method of Laemmli (1970). A discontinuous electrophoretic system was employed using polyacrylamide gels (16 x 14 cm) with an acrylamide:bisacrylamide ratio of 37.5 : 1. The proteins, in sample buffer (10% glycerol and 0.01% bromophenol blue in 0.0625M Tris-HCl buffer pH 6.8) were applied (20 µl) to stacking gels (4.5% acrylamide) and electrophresed under constant

current conditions (10 mA, Pharmacia ECPS 3000/150 power supply) for 75 minutes. The stacked proteins were separated in 12.5% acrylamide separation gels at 20 mA until the tracking dye front was within 1-2 cm of the end of the gel.

When the gels were to be overlaid to detect extracellular products (2.5.2) the electrophoretic system was pre-cooled and run in a refrigerator at 4°C. Under these conditions the gel temperature did not rise above 10°C during the experiment.

2.5.2 Zymogram techniques

In some experiments, a zymogram technique was used to study the electrophoretic separation of extracellular products. In these experiments the electrophoretic system was placed in a refrigerator at 4°C. Under these conditions, the gel slab temperature did not rise above 10°C. After electrophoresis, (2.5.1) the gel slab was immersed in a bath of PBS (room temperature) for 5 minutes and the gel sliced lengthways to give individual gel tracks. Some tracks were stained to locate proteins (2.5.3) whilst others were overlaid to detect extracellular products. The individual gel tracks were placed onto a clean glass plate which had strips of Plasticine attached to it in the form of a rectangle, slightly larger than the dimensions of the gel track. The Plasticine well containing the gel track was filled with PBS agar containing an appropriate substrate (2.4.1) (3.5ml). The gels were incubated at 25°C and examined at intervals up to 24 hours for evidence of digestion of the substrate. The relative front (Rf) values of these zones of digestion were determined. Zones of casein digestion were visualised after incubating the gel (1 hr, 25°C) and flooding with acetic acid (3% v/v). Gelatin overlays were incubated for 75 minutes (25°C) and flooded with mercuric chloride (1% w/v) to detect zones of digestion.

2.5.3 Detection of proteins after electrophoresis

Proteins in gels after electrophoresis were detected by staining the gel with Coomasie brilliant blue R (Sigma 5 g/l) dissolved in acetic acid: ethanol: distilled water (1 : 3 : 6).

After staining overnight (room temperature) the gel was destained in an acetic acid: ethanol: distilled water (1 : 3 : 6) mixture until individual protein bands were visible. The Rf values of these bands were determined . In some experiments, the stained gel track was scanned using a densitometer (Pye Unicam SP1800) to determine the intensity of the protein bands. Photographs of gels were taken using an orange filter.

2.5.4 Detection of glycoproteins and lipoproteins after electrophoresis

Gel tracks were stained to locate glycoproteins using a periodic acid Schiff stain. The gels were fixed in 7% acetic acid (28 hr, room temperature) and immersed in 0.2% periodic acid for 45 minutes at 4°C. Following this, the gel was immersed in Schiff reagent (BDH) for 45 minutes at 4°C. The gel was destained in 10% acetic acid until the red glycoprotein bands were visible.

Lipoproteins were detected using two techniques. In some experiments, lipids present in the sample to be electrophoresed were pre-stained by mixing 50 µl of sample with 25 µl of 1% lipid crimson in diethylene glycol (filtered before use). Lipids were also post-stained by immersing the gel tracks in a solution of sudan black, staining overnight (room temperature with shaking) and examining the gel for stained material. The Sudanblack (500 mg) was dissolved in 20 ml of acetone and acetic acid (15 ml)/distilled water (80 ml) added. The mixture was mixed (30 min, room temperature) and centrifuged (3,000 x g, 10 min) before use. Gels were destained using acetic acid: acetone: distilled water (15 : 20 : 65).

In these experiments using glycoprotein and lipoprotein stains, samples of human serum (mixed 1 : 1 with sample buffer) were also electrophoresed and stained to determine the efficiency of the stains.

2.6.0 CHROMATOGRAPHIC TECHNIQUES

2.6.1 Gel filtration chromatography

Gel filtration chromatography was performed in a cold room at

4°C unless otherwise stated. The columns were packed with suitable medium in accordance with the manufacturer's instructions. Samples were applied to the gel bed surface under the eluting buffer, using a 1 ml syringe attached to a 5 cm length of teflon tubing. The columns were eluted using either a constant head reservoir or a pump at the appropriate flow rate (Pharmacia). The optical density of the column eluant was continuously monitored (280 nm) using either an LKB Uvicord S linked to a chopper bar recorder (LKB) or using a Pharmacia dual path monitor linked to a flat bed chart recorder (Pharmacia). Eluant fractions were collected using an LKB Ultrorac or a Pharmacia Frac 100 fraction collector.

2.6.2 Molecular weight standardisation of gel filtration columns

When required, the columns used for gel filtration chromatography were calibrated with respect to molecular weight (Andrews 1965). The standard proteins bovine serum albumin (Sigma, fraction V , 6.21 mg/ml, molecular weight 66,000), ovalbumin (Sigma Grade V, 4.0 mg/ml, molecular weight 45,000) and cytochrome C (Sigma, type VI 1.38 mg/ml, molecular weight 12,500. Blue dextran (Pharmacia, 0.69 mg/ml) was added to these proteins to indicate the location of the void volume of the column. Where possible,the mixed protein standards (0.5 ml) were run concomitantly with the sample, however, when the sample proteins would have obscured the locations of the standard proteins in the column eluant the column was firstly standardised using the standard protein mixture. Knowledge of the elution volumes of these proteins then enabled the molecular weights of the sample proteins to be determined.

2.6.3 Ion exchange chromatography

Ion exchange chromatography was carried out at 4°C. The sample was applied to the ion exchange solumn (Wright 3.1 cm x 2.9 cm) via a flow adaptor. The columns were eluted using a linear gradient of NaCl (0-0.4 M) in tris-HCl buffer (0.0i M, pH 7.1). The NaCl gradient was generated using two 100 ml beakers linked by a tube. At the start of the experiment one beaker contained

starting buffer (50 ml, no NaCl) whilst the other contained final buffer (50 ml, + 0.4 M NaCl). The contents of the starting buffer beaker were mixed using a magnetic stirrer. The columns were eluted using a pump (Watson-Marlow or Pharmacia) which was placed between the column and the beakers. The column eluant was monitored, and eluant fractions collected, as described previously (2.6.1). After use,the columns were regenerated using 2M NaCl solution (50 ml) and washed with tris-HCl buffer until no NaCl was detected in the column eluant (determined using refractometry).

2.7.0 TECHNIQUES USED TO STUDY THE INTERRELATIONSHIPS OF EXTRACELLULAR PRODUCTS

2.7.1 Inhibition of enzyme activities using phenylmethylsulfonylfluride (PMSF)

PMSF (0.1 M in n-propanol) was added to culture supernate samples in the ratio 1 part PMSF solution to 99 parts supernate. The samples were incubated for 2 hours (25°C) before testing for enzyme activities. In experiments using PMSF, appropriate controls were included, n-propanol (1% v/v, final concentration) was added to supernate samples with incubation (2 hr, 25°C).

2.7.2 The removal of H-lysin from culture supernate

Shaken culture supernatant fluid (72 hr, 25°C) contained low levels of H-lysin activity (3.2.14). Residual H-lysin activity in these samples was removed by membrane filtration of the culture supernate (5.2.3). Culture supernate (50 ml) was passed through 2.5 cm membrane filters (Millipore, 0.45 µm) twice and the eluant fluid tested for H-lysin activity before use (2.4.1 and 2.4.2). Experiments indicated that caseinase activity was not lost after membrane filtration.

2.7.3 Mutagenesis of *A. salmonicida*

Cultures (18 hr) of *A. salmonicida* strain 25/77 in tryptone soya broth (10 ml) were centrifuged (6,000 x g, 20 min, 20°C). The cell pellet was resuspended in citrate buffer (0.1M, pH 5.5) containing N-methyl-N'-nitroso-N-nitrosoguanidine (NTG, 50 µg/ml)

and incubated for 35 minutes at 25°C. This treatment resulted in a kill rate of 98%. After treatment, the cells were washed three times in PBS (0.1M, pH 7.2), resuspended in 10 ml of fresh medium, and incubated for 6 hours at 25°C. The number of viable bacteria was determined at this stage using a spread plate method (0.1 ml volumes on TSA) and the remaining broth culture was stored at 4°C overnight. After determination of the number of viable cells in the culture, the dilution necessary to give 30 colonies per plate was calculated. In addition, a further twofold dilution was effected to compensate for bacterial growth at 4°C.

The appropriate dilution of culture was spread (0.1 ml) over the surface of TSA + dried skim milk (2.5% w/v) or TSA + sodium caseinate (Eastman Kodak, 1% w/v). After incubation (48 hr, 25°C) colonies which were not surrounded by zones of casein digestion were picked off and streaked onto fresh growth medium. Pure cultures of caseinase negative mutants were confirmed as A. salmonicida using key biochemical tests (McCarthy 1975) and were stored under liquid nitrogen refrigeration.

In some experiments NTG treated bacteria were grown on TSA + washed horse erythrocytes (5% v/v). Approximately 2×10^4 bacteria were screened using this growth medium, however no haemolysin negative mutants were isolated.

2.7.4 Activation of pro-H -lysin using chymotrypsin

In many experiments involving pro-H-lysin, activation was achieved using chymotrypsin (BDH, 15,000 ATEE units/mg). Samples containing pro-H-lysin were incubated with chymotrypsin (10 µg/ml final concentration) for 10 minutes at 25°C and the reaction terminated by the further addition of PMSF (1 mM final concentration). After incubation (2 hr, 25°C), the samples were tested for H-lysin activity using the microtitre tray assay system (2.4.2).

2.8.0 ANTISERUM AGAINST A CRUDE PREPARATION OF H-LYSIN

Ammonium sulphate precipitated (40-60% saturation) unshaken broth culture supernate was used to raise an antiserum against crude A. salmonicida extracellular products. The material was mixed with

Freund's complete adjuvant (1 : 1 ratio) and 1.0 ml of the mixture (78 µg protein) injected into two Wistar rats using a multiple site intradermal route (0.1 ml/site) on the dorsal surface of the animal. The animals received a second immunisation (1.0 ml) 15 days later using a concentrated extracellular product sample mixed with adjuvant (300 µg protein).

After a further 14 days the rats were sacrificed and blood obtained by cardiac puncture. The blood was allowed to coagulate (1 hr, 25°C and 18 hr, 4°C) before removal of the serum. The serum from both rats was pooled, heated (56°C, 30 min) to destroy complement and stored at -20°C until required.

2.9.0 TECHNIQUES USED TO STUDY THE H-LYSIN

2.9.1 The preparation of horse erythrocyte membranes

Standardised horse erythrocyte suspension (40 ml) (2.4.2) was centrifuged (3,500 x g, 10 min, 20°C) and the cell pellet resuspended in phosphate buffered 0.025M, 0.22% w/v NaCl) and agitated gently. The lysed cells were centrifuged at 36,000 x g (30 min, 20°C) and the pellet resuspended in PBS (20 ml). After resuspension and centrifugation (36,000 x g, 30 min, 20°C), the pellet was resuspended in PBS (4 ml) and stored at 4°C. Membranes were used within 24 hours. One erythrocyte ghost equivalent was designated as the volume of membrane suspension which was originally derived from 0.1 ml of standardised horse erythrocyte suspension.

2.9.2 Enzyme treatment of horse erythrocyte membranes

Horse erythrocyte membranes were treated with a variety of enzymes; membranes (0.3 ml, 20 erythrocyte ghost equivalents) were mixed with an equal volume of chymotrypsin (BDH, 15,000 ATEE units/mg, 250 µg/ml in PBS) or papain (Sigma, type IV, 19 units/mg, 250 µg/ml in PBS) and incubated for 30 minutes at 25°C. Similar volumes of membranes were also mixed with phospholipase C (Sigma, type X, 250 units/mg, 4 units/ml in 10 mM tris-HCl + 10 mM $CaCl_2$ + 1 mg/ml BSA, pH 7.3) or lipase (Sigma, type VII, protease and α amylase free, 660 units/mg, 500 µg/ml in tris/$CaCl_2$/BSA buffer) for 60 minutes at 25°C. Neuraminidase (Sigma, type VI chromatographically

purified, 0.5 units in PBS) was mixed with an equal volume of membranes (0.3 ml) and incubated for 60 minutes at 37°C. Appropriate controls were membranes incubated with PBS at 25°C or 37°C.

After incubation, the samples were centrifuged (10,000 x g, 15 min, 4°C) and the pellet washed three times with PBS (0.8 ml). After the final centrifugation, the pellet was resuspended to 0.3 ml in PBS (+ 1 mg/ml BSA), mixed with H-lysin (0.3 ml) and incubated (30 min, 30°C). After centrifugation (10,000 x g, 15 min, 4°C) the supernatant fluid was tested for H-lysin activity.

2.9.3 Preparation of trout leucocytes

Rainbow trout blood was drawn from the caudal sinus and centrifuged in sterile centrifuge tubes (3,000 x g, 10 min, room temperature) and the buffy layer removed aseptically. Buffy layer material was mixed with an equal volume of sterile PBS and carefully layered over the surface of 6 ml of lymphoprep (Nyegaard) in a sterile conical centrifuge tube. After centrifugation (3,000 x g, 15 min, room temperature) the separated leucocytes were aseptically removed and resuspended in tissue culture medium (2.9.4). The cells were washed three times in tissue culture medium with centrifugation at 200 x g (15 min, room temperature). After the final centrifugation, the cells were resuspended in tissue culture medium to 9.4×10^6 cells/ml.

2.9.4 Rainbow trout gonad cells

Rainbow trout gonad cells (Wolf and Quimby 1962) (RTG - 2) were maintained at 20°C and grown in BHK medium (Flow, Glasgow modification) supplemented with $NaHCO_3$ (7.5% w/v), L-glutamine (200 mM), newborn calf serum (Flow, 10% v/v) tryptose phosphate broth (Flow, 10% v/v), tris-HCl buffer (19 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

2.9.5 Membrane filtration of H-lysin

When sterile preparations of H-lysin were required, the sample was membrane filtered (Millipore, 0.45 µm, 25 mm). Before

use,the membrane filter was washed with BSA solution (2 ml, 10 mg/ml) in PBS) and PBS (10 ml). The membrane filtered H-lysin was streaked onto TSA to check sterility.

2.10.0 FISH MAINTENANCE

Rainbow trout were obtained from the Tavistock Trout Company and were maintained in 600 l tanks containing recirculating, filtered, fresh water at 13^{o}C.

CHAPTER 3. THE PRODUCTION OF *A. SALMONICIDA* EXTRACELLULAR PRODUCTS *IN VITRO*

3.1.0 INTRODUCTION

Extracellular products from Gram positive bacteria have been relatively well studied. In comparison, Gram negative extracellular products form a poorly studied group of potential toxins or aggressins. The investigation of production of these activities *in vitro* is important for a number of reasons:-

Such studies can provide information concerning the nature of factors controlling the synthesis and release of Gram negative bacterial extracellular products. When considering potential toxins of aggressins these studies may be related (with caution) to *in vivo* disease situations.

Investigation of the spectrum of extracellular products from virulent and avirulent strains of the bacterium may indicate potential virulence factors or differences in the mechanisms controlling production.

Finally, such studies form an essential basis for further studies concerning the purification and properties of individual extracellular products. The success of such investigations is often dependant on cultural conditions for the bacterium selectively favouring the production of the activity being investigated.

The experiments in this section of the project were designed to fulfil the criteria outlined above, in particular the production of haemolytic activity *in vitro* was investigated.

3.2.0 RESULTS

3.2.1 The Production of Extracellular products on solid growth media

A. salmonicida strain 25/77 was grown on solid media incorporating a variety of enzyme substrates to indicate the variety of extracellular products of the bacterium. Zones of digestion were observed around stab inoculated colonies on tryptone soya agar (T S A)+ egg yolk emulsion (10% v/v),T S A + gelatin (0.4% w/v), T S A + sodium caseinate (1% w/v) and on tributyrin agar (2.4.1).

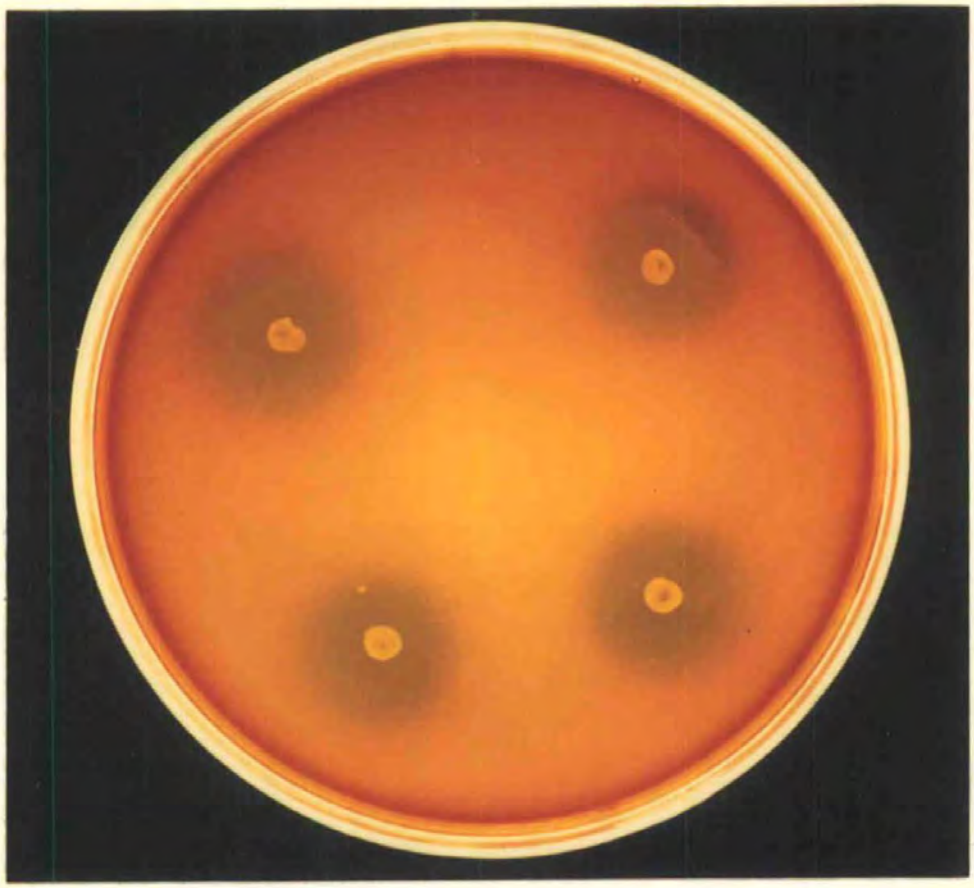
Haemolysis was observed on T S A + horse blood (5% v/v) and on T S A + trout blood (7% v/v) though the appearance of haemolytic zones

was different.

The form of haemolytic zones on horse blood agar is shown in Plate 1 and Figure 4. Colonies were surrounded by a zone of clear haemolysis, which was bordered by an area of apparently incomplete haemolysis; outside this zone a narrow bright red 'halo' was observed. On trout blood agar (Plate 2 and Figure 5) the colony was surrounded by an area of clear haemolysis which was in turn surrounded by an opaque orange coloured zone. The orange zone was bordered by a thin white zone only clearly visible in obliquely transmitted light. Haemolytic zone sizes were measured as the radius of the zone between the colony edge and the red halo (horse blood) or the outer edge of the orange zone (trout blood). When grown on T S A + gelatin two zone forms were visible after flooding the plate with tannic acid (2.4.1). An inner clear zone was surrounded by a cloudy zone (Plate 3). Growth on T S A + egg yolk emulsion also revealed the presence of two zones of digestion. Surrounding the bacterial colony was a zone of clear digestion of the egg yolk, this zone was surrounded by a hazy zone of digestion, visible only on close examination of the plate (Plate 4).

Plate 1. The form of zones of haemolysis surrounding colonies of A. salmonicida (strain 25/77) on T S A + horse erythrocytes.

Plate 2. The form of zones of haemolysis surrounding colonies of A. salmonicida (strain 25/77) on T S A + trout erythrocytes.



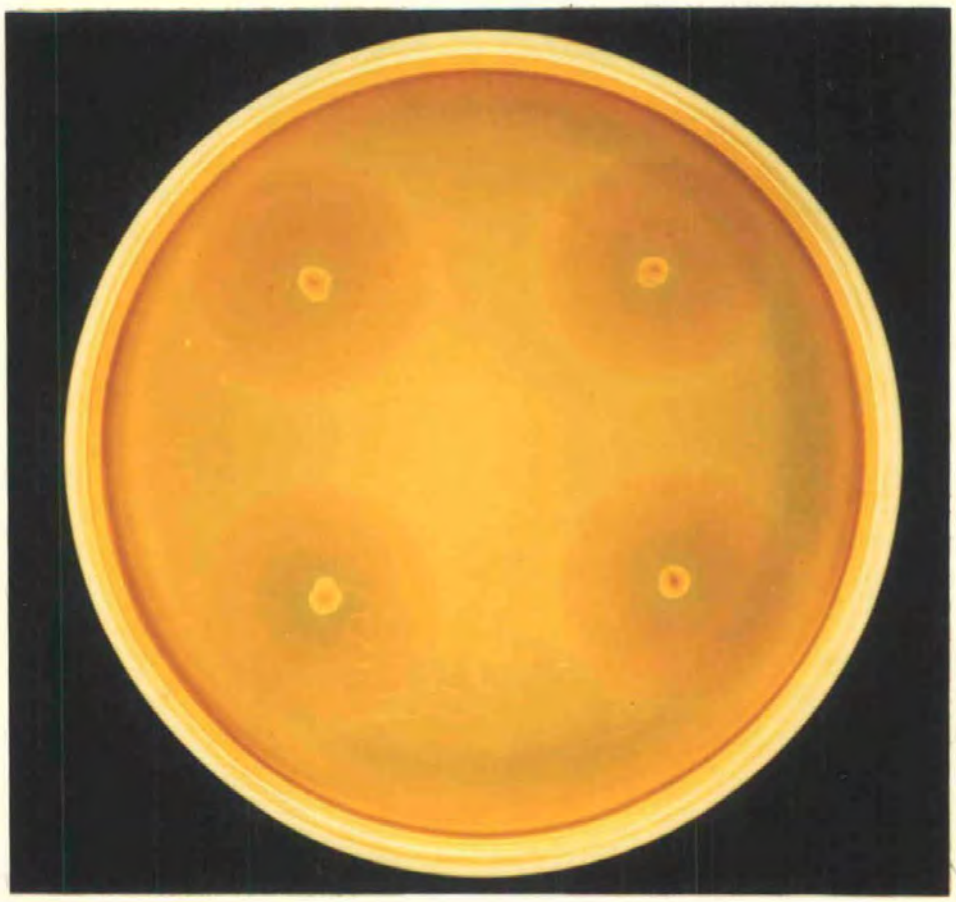
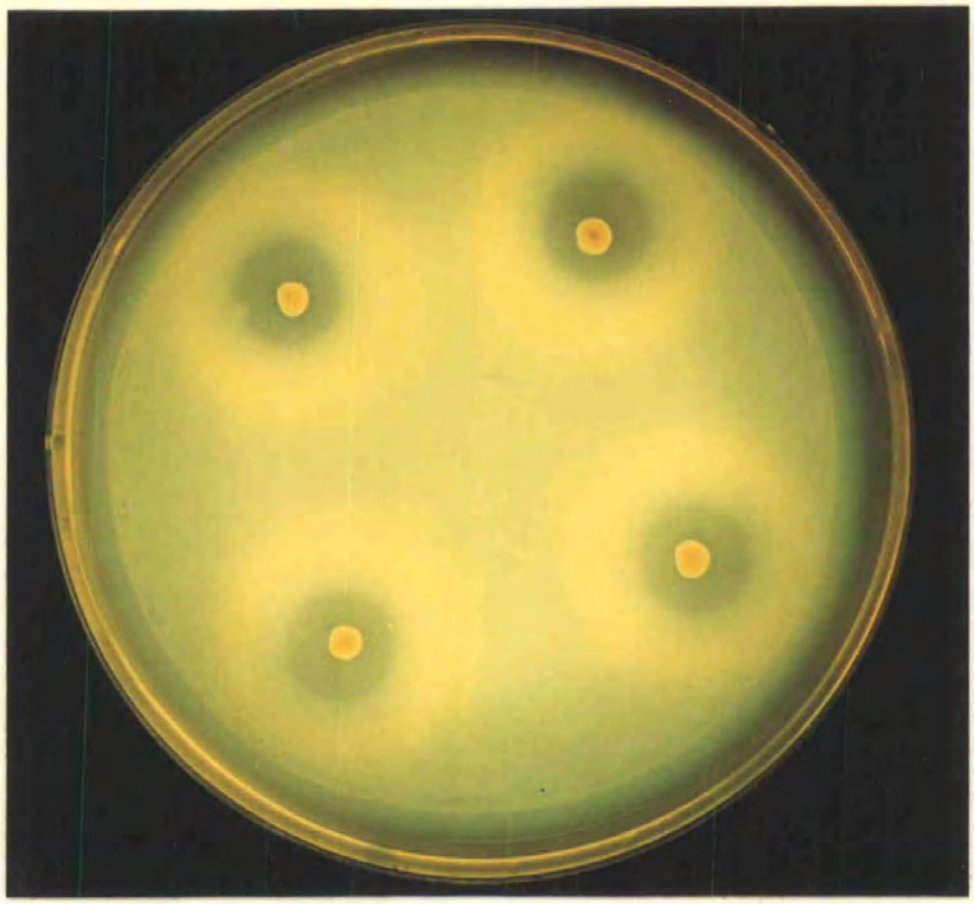


Plate 3 The form of zones of digestion surrounding colonies of A. salmonicida (strain 25/77 on T S A + gelatin.

Plate 4 The form of zones of digestion surrounding colonies of A. salmonicida (strain 25/77) on T S A + egg yolk emulsion.



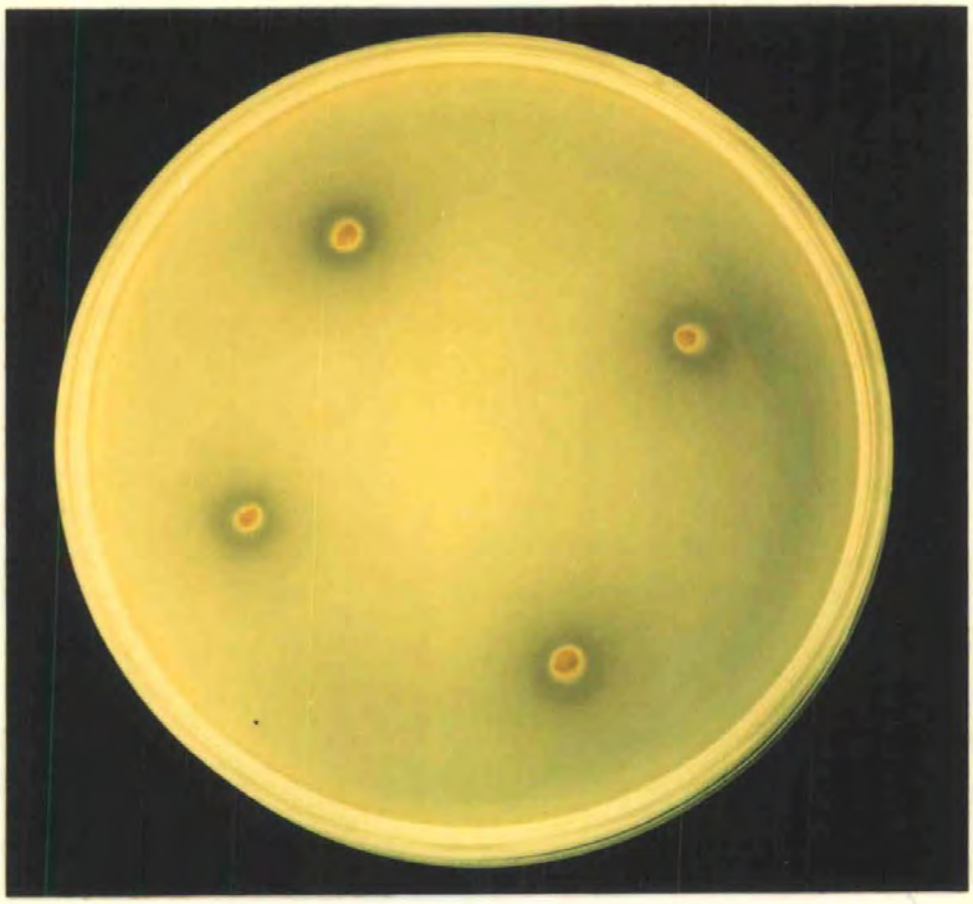


Figure 4 The form of zones of haemolysis surrounding colonies of A. salmonicida (strain 25/77) on T S A + horse erythrocytes.

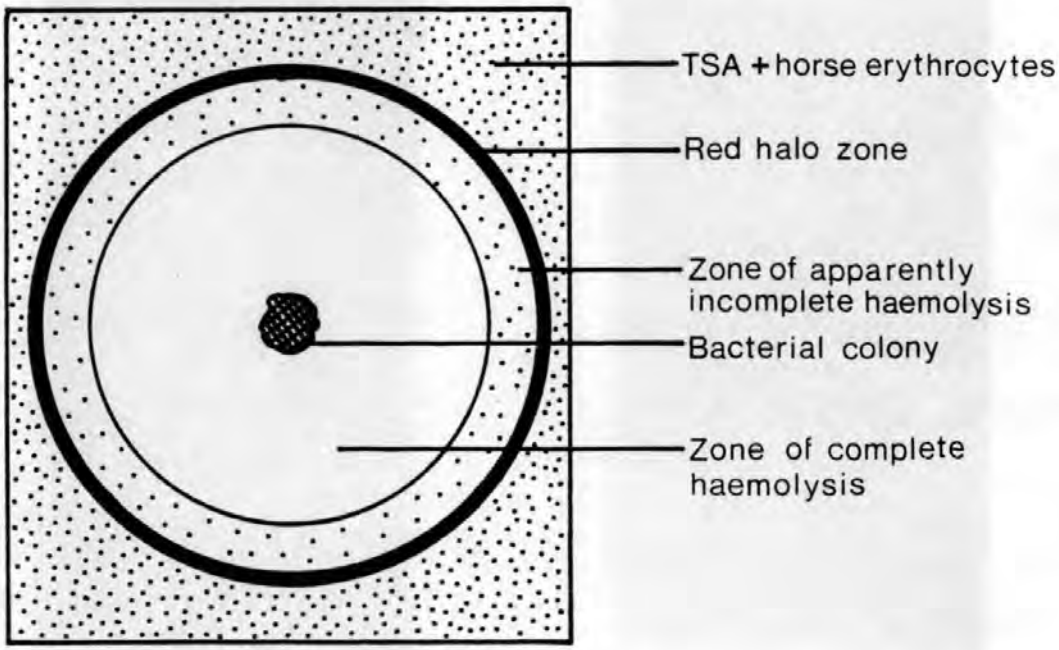
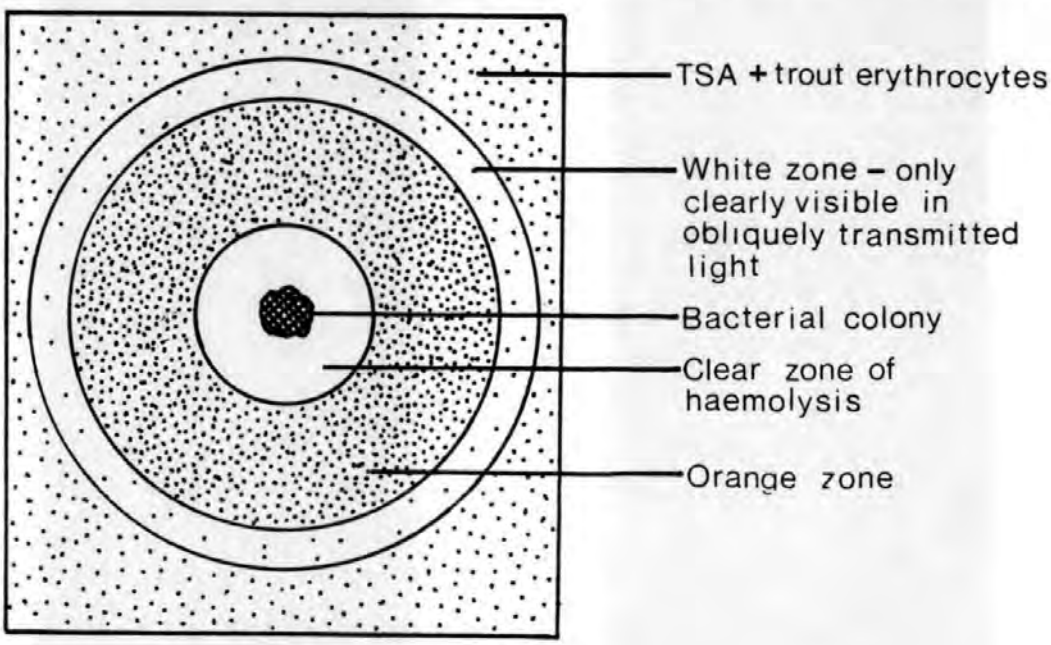


Figure 5 The form of zones of haemolysis surrounding colonies of A. salmoncida (strain 25/77) on T S A + trout erythrocytes.



3.2.2. Initial tests for haemolysin production by A. salmonicida

Initial experiments were designed to evaluate the sensitivity of easily obtainable erythrocyte types to haemolytic activity. Four strains of A. salmonicida were stab inoculated on to tryptone soya agar (T S A) + horse, rabbit or rat blood (5% v/v). Zones of haemolysis were measured after 24, 40 and 60 hours of growth at 25°C. The results (Table 4) indicated that horse blood was the most sensitive to haemolysis whilst rabbit blood was the least sensitive. Of the four strains tested,strain 25/77 was the most haemolytic on horse blood agar and was chosen for further work. Similar zone forms (3.2.1) were seen on horse, rat and rabbit blood agars and with all four strains tested.

3.2.3. Production of haemolytic activity in shaken and unshaken cultures

Many bacteria produce more than one haemolysin, which may be differentiated on the sensitivity of different types of erythrocytes. Since the production of such haemolysins is often selectively favoured by different cultural conditions a series of experiments were undertaken to investigate the effect of cultural conditions on haemolysin productions. Strain 25/77 was the only strain used in these experiments.

Supernatant fluid from unshaken (96 hr, 25°C, Nutrient broth No. 2) or shaken (72 hr, 25°C, Nutrient broth No. 2) cultures was tested against a variety of mammalian, fish and reptilian erythrocytes diluted to give suspensions of similar numbers of cells (2.4.2).

Results (Table 5) indicated that supernates from unshaken cultures contained haemolytic activity against a wide variety of erythrocytes, horse red blood cells being the most sensitive. Shaken cultures contained activity against trout erythrocytes only.

3.2.4 Production of haemolytic activity by the agar overlay technique

The agar overlay technique, involving the growth of bacteria on a sheet of cellophane overlayed on T S A (2.2.5),was tested for the

Table 4 The sensitivity of erythrocytes in a solid growth medium to haemolysis by A. salmonicida

A. salmonicida strain	erythrocyte type	Size of zone of haemolysis (mm) Time - hours 24 hr	40 hr	60 hr
CM30	Horse	0	2.9	5.6
CM30	Rat	0.75	1.2	1.3
CM30	Rabbit	0	0.75	0.75
25/77	Horse	1.9	2.8	6.1
25/77	Rat	1.1	1.6	1.9
25/77	Rabbit	0.8	0.9	1.0
84/78	Horse	0	2.7	5.6
84/78	Rat	0	1.5	1.8
84/78	Rabbit	0	1.0	0.9
84/77	Horse	0	2.9	5.8
84/77	Rat	0.6	0.9	1.2
84/77	Rabbit	0	1.1	1.1

Zone sizes are expressed as the radius between the colony edge and the outer edge of the clear zone of haemolysis

Table 5 The sensitivity of erythrocytes from various species to lysis by 72 hr culture supernatant fluid

Erythrocyte suspension tested	Haemolytic activity detected (Hu/ml)	
	Unshaken culture	Shaken culture
Horse	32	0
Pig	24	0
Rabbit	16	0
Eel	16	0
Rat	8	0
Mouse	8	0
Trout	7	128
Cow	4	0
Guinea pig	1	0
Dogfish	1	0
Sheep	0	0
Frog	0	0

Erythrocytes were washed and resuspended to 9.75×10^7 cells/ml before testing.

production of haemolytic activity. Seventy two hour cultures ($25^{o}C$) grown in this manner contained limited activity against horse erythrocytes (2Hu/ml) but high activity against trout erythrocytes (1024 Hu/ml). During haemolysin assays against trout erythrocytes it was noticed that the 50% end point of haemolysis was followed by a number of wells containing apparently partially lysed cells (4.2.1).

3.2.5 Haemolysin production under anaerobic conditions

It was thought that the low activity against horse erythrocytes seen in shaken and AOT cultures might reflect the effect of oxygen tension on haemolysin production. When cultures were grown under unshaken anaerobic conditions (N_2 only) activity against horse erythrocytes was not detected up to 144 hours of growth at $25^{o}C$. Activity against trout erythrocytes was not tested.

3.2.6 Haemolysin production in Fermenter vessel cultures

The use of fermenter cultures was investigated since these may have provided closely controlled conditions leading to high yields and large volumes of crude haemolytic activity for further studies. When bacteria were grown under such conditions (5l Nutrient broth No. 2, 100 r p m stirring speed 250 r p m , no aeration) no activity against horse erythrocytes was detected at 24 hour intervals up to 168 hours. When aeration was used 1l/min) limited activity against horse erythrocytes was seen (maximum 2 Hu/ml at 48 hr). The use of corn oil (6 ml) as an antifoam agent did not affect haemolysin production.

3.2.7 Haemolysin production within dialysis tubing

The growth of bacteria within dialysis tubing may increase the haemolysin production by providing access to a large supply of low molecular weight nutrients whilst effectively concentrating extracellular products of high molecular weight.

Cultures of A. salmonicida grown in this fashion in nutrient broth No. 2 (2.2.4) produced similar levels of haemolytic activity

against horse erythrocytes (64 Hu/ml) to those found in unshaken control cultures (64 Hu/ml).

3.2.8 Attempts to increase haemolysin production by increasing cell densities

Haemolytic activity was produced predominantly during the stationary phase of growth (3.2.13 and 3.2.14). An experiment was devised to harvest cells before the stationary phase and concentrate these cells, possibly leading to increased haemolytic activity on subsequent incubation.

Cells were harvested after either 20 hr growth in shaken cultures (70 r.p.m., 25°C) or 24 hr growth in unshaken cultures and after centriguation (7,500 x g, 20 min, 25°C) resuspended in phosphate buffered saline (PBS pH 7.2) or in fresh culture medium to 1/10th of their original volume. The cell concentrates were incubated (25°C) and tested at 24 hr intervals for haemolytic activity against horse erythrocytes (2.4.2).

The results (Table 6) indicated that activity in such cultures did not show the ten fold increase over control (unconcentrated) cultures, which may have been expected. In cultures of ten fold concentrated shaken culture cells (concentrated in Nutrient broth No. 2) the final haemolysin titres was similar to that seen in unshaken control cultures (26 Hu/ml). In all other concentrated cultures the haemolysin titres were lower than those in control cultures.

3.2.9 The effect of growth medium type of the production of haemolytic activity

The effect of different liquid growth media on the production of haemolytic activity against horse erythrocytes (unshaken cultures) or trout erythrocytes (shaken cultures) was investigated (2.2.4).

The results (Table 7) indicated that Nutrient broth No. 2 cultures (NB No. 2) resulted in the highest activity against horse erythrocytes (unshaken cultures) and trout erythrocytes (shaken cultures). Generally the patterns of production of haemolytic

Table 6 The effect of concentration of stationary phase cells on haemolytic activity against horse erythrocytes

Culture treatment	Haemolytic activity (Hu/ml) detected Time - hours			
	20 hr	24 hr	48 hr	72 hr
Unshaken culture (Control)	N.T.	N.T.	N.T.	16
Shaken culture (Control)	N.T.	N.T.	N.T.	1
Unshaken culture cells resuspended to 1/10th original volume in:- NB No.2	N.T.	0	4	6
PBS	N.T.	0	0.5	1.5
Shaken culture cells resuspended to 1/10th original volume in:- NB No.2	4	N.T.	7	16
PBS	3	N.T.	5	6

Cells were harvested at 20 hr (shaken cultures) or 24 hr (unshaken cultures) by centrifugation and resuspended to 1/10th of the original culture volume in the medium shown. Supernatant fluid was tested at intervals for haemolytic activity.

activity, with respect to time, were similar in all the broth types tested. Some deviations from the typical pattern of production were observed; in unshaken cultures haemolytic activity against horse erythrocytes was higher in NB No. 2 + bovine serum albumin (BSA) at 24 hr (16 Hu/ml) than in NB No. 2 only cultures (3 Hu/ml), however maximum levels were similar (64 Hu/ml at 72 hr). In nutrient broth, and NB No. 2 + yeast extract, haemolytic activity against horse erythrocytes was higher at 24 hr (8 Hu/ml, 11 Hu/ml) than in NB No. 2 only cultures (3 Hu/ml), however the final haemolytic activity was highest in NB No. 2 only cultures.

Activity against trout erythrocytes in shaken cultures was high at 24 hr, rising slowly after this time until the termination of the experiment (72 hr). Deviation from this pattern of production was observed in NB No. 2 + glycerol shaken cultures, after a maximum activity at 24 hr (3 Hu/ml) a decline was observed on further incubation (No activity at 72 hr).

3.2.10 The effect of growth medium type of preformed haemolytic activity

It was thought that differences in the maximum haemolytic activity found when the growth medium type was altered may be explicable in terms of the effect of broth components on the expression of haemolytic activity.

To test this possibility, culture supernatant fluid containing haemolytic activity against horse erythrocytes (96 hr, 25°C unshaken, NB No. 2) or activity against trout erythrocytes (72 hr, 25°C, shaken in NB No. 2) was diluted out in a microtitre tray using various broth types as diluents. Samples were then assayed for haemolytic activity as detailed previously (2.4.2).

The results indicated that, generally, the broth type did not affect the haemolysin titre, some exceptions to this rule were observed; the addition of BSA to NB No. 2 enhanced haemolytic activity against horse erythrocytes (100% increase) whilst depressing activity against trout erythrocytes (75% decrease). The

Table 7 The effect of growth medium on the production of haemolytic activity.

Growth medium tested	Haemolytic activity detected (Hu/ml)					
	Against horse erythrocytes (unshaken cultures)			Against trout erythrocytes (shaken cultures)		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Tryptone soya broth	0	4	4	8	14	16
Furunculosis broth	4	4	4	16	32	32
Peptone broth	2	3	3	32	48	32
Peptone + yeast extract broth	4	3	4	64	64	48
Lab lemco broth	4	4	4	6	32	7
Lab lemco + yeast extract broth	4	4	4	16	32	16
Brain heart infusion	0	3	3	64	64	64
Nutrient broth	8	16	8	16	5	4
Nutrient broth No. 2 (NB No. 2)	3	18	64	64	96	128
NB No. 2 + BSA	16	32	64	8	32	32
NB No. 2 + glycerol	0	32	64	3	1	0
NB No. 2 + yeast RNA	0	4	8	16	16	32
NB No. 2 + yeast extract	11	24	32	8	32	16
NB No. 2 + glucose	0	0	1	1	1	0
NB No. 2 + haemoglobin	2	2	8	16	32	16
Shieh & Reddy medium	0	0	0	0	0	0

addition of haemoglobin to NB No. 2 had no effect on activity against horse erythrocytes unless the haemolysin and haemoglobin were pre-incubated (18 hr, 4°C, + 0.02% NaN_3 to prevent bacterial growth). After this treatment 75% loss of activity was seen in comparison with control samples incubated with PBS only.

3.2.11 The effect of zinc ions on haemolysin production

Differences in metal ion concentrations have been shown to affect the production of many extracellular products. Zinc ions have often been shown to elicit this effect. Raised zinc levels were tested for their ability to promote haemolysin production by A. salmonicida.

Zinc ions were added to NB No. 2 as zinc sulphate to give zinc concentrations in the range 1μM to 1 mM zinc. 0.1 ml aliquots of these solutions were placed into sterile microtitre trays and 0.1 ml of a culture of A. salmonicida added to the wells (strain 25/77, 18 hr, 10^2 diluted). After incubation (25°C, 72 hr), the optical density (OD) at 490 nm of the well contents was determined using a microelisa reader. In addition, well contents were removed, centrifuged (10,000 x g, 20 min, 4°C) and the supernate tested for haemolytic activity against horse erythrocytes (2.4.2).

The results (Table 8) indicated that zinc had no effect on growth or haemolytic activity (expressed as the mean of 4 determinations) over the range tested. Control experiments indicated that zinc had no effect on preformed haemolytic activity or OD 490 nm over the range tested.

3.2.12 The effect of iron levels on the production of haemolytic activity

The effects of raised and lowered iron levels on the production of haemolytic activity against horse erythrocytes was evaluated using a method similar to that used to evaluate the effect of zinc on haemolysin production (3.2.11). Iron was added

as ferrous sulphate to give final concentrations in the range 1 μM to 100 μM. Iron levels in broth were lowered by the addition of transferrin (Sigma, iron free, 0.25 to 10 mg/ml added transferrin). Free iron levels in broths containing transferrin were also determined (2.2.7). Strain 25/77 (A layer -ve), the virulent strain 451, and the avirulent strain 451/3 (2.2.1) were tested in this system.

The results indicated that the addition of transferrin reduced the level of free iron in the broth (Table 9). After 72 hr (20°C) the growth of all strains was reduced, though complete inhibition of growth was not observed (Table 9 and Figure 6). Production of haemolytic activity against horse erythrocytes appeared to reflect the cell density over the range of increased and decreased iron levels tested. No marked stimulation of haemolysin production was observed. Control experiments indicated that added ferrous sulphate or transferrin had no effect on the optical density of sterile broth. The activity of preformed haemolytic activity was also unaffected by the presence of transferrin or ferrous sulphate.

3.2.13 The effect of incubation temperature on the production of haemolytic activity against horse erythrocytes

The effect of temperature on the production of haemolytic activity against horse erythrocytes by A. salmonicida over the range 15 - 30°C was determined. Samples from unshaken cultures in NB No. 2 were centrifuged (10,000 x g, 20 min, 4°C) and tested for haemolytic activity (2.4.2) at 24 hr intervals up to 96 hr.

The results (Table 10) indicated that maximum activity against horse erythrocytes was observed at 25°C after 96 hr. After 24 hr growth activity was also highest in cultures at 25°C. Haemolytic activity in cultures maintained at 30°C showed a decline between 72 hr and 96 hr a feature that was not apparent in cultures maintained at other temperatures.

Table 8 The effect of raised zinc levels in broth cultures of strain 25/77 on the production of haemolytic activity against horse erythrocytes

Zinc ion concentration	Optical density of culture (490 nm)	Haemolytic activity against horse erythrocytes (Hu/ml)
Control - no added zinc	0.335	24
1 μM added zinc	0.325	32
5 μM added zinc	0.343	32
10 μM added zinc	0.333	32
50 μM added zinc	0.325	26
100 μM added zinc	0.328	24
500 μM added zinc	0.335	24
1 mM added zinc	0.338	24

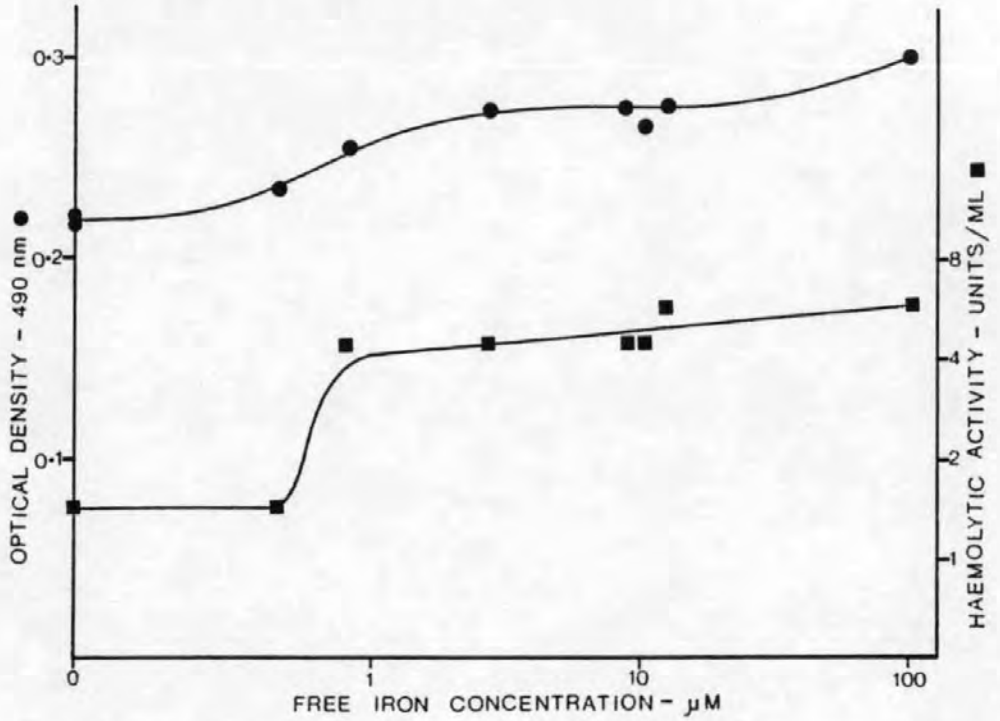
Cultures were grown for 72 hour (25°C) before testing.

Table 9 The effect of iron levels in broth on the growth and production of haemolytic activity against horse erythrocytes by strains 25/77, 451 and 451/3.

Transferrin added (mg/ml)	Ferrous sulphate added (μM)	Free iron level in broth (μM)	Optical density of cultures (490 nm)			Haemolytic activity against horse erythrocytes (Hu/ml)		
			25/77	451	451/3	25/77	451	451/3
	100	119.6	0.3	0.16	0.27	6	9	6
	10	19.6	0.275	0.14	0.25	6	9	6
	1	10.6	0.265	0.16	0.24	4.5	9	6
Control - broth only		9.6	0.275	0.13	0.275	4.5	9	4.5
0.25		4.8	0.275	0.125	0.25	4.5	4.5	3
0.5		0.91	0.255	0.12	0.26	4.5	6	3
2.5		0.68	0.235	0.09	0.215	1.5	1.5	1.5
5		0	0.22	0.075	0.23	1.5	1.5	1.5
10		0	0.225	0.07	0.135	1.5	1.5	1.5

Cultures were grown for 72 hours (20°C)

Figure 6 The effect of raised and lowered broth iron levels on the growth and production of haemolytic activity against horse erythrocytes by strain 25/77



Cultures were tested after 72 hr growth (20°C)

Table 10 The effect of temperature on the production of haemolytic activity against horse erythrocytes

Temperature (°C)	Haemolytic activity detected (Hu/ml) Time - hours			
	24 hr	48 hr	72 hr	96 hr
15	0	4	8	8
20	4	8	10	16
25	8	12	16	32
30	0	12	16	2

3.2.14 The time course for haemolysin, caseinase and pigment production in shaken broth cultures

Having established a set of cultural conditions which, in broth, resulted in maximal haemolysin production, it was decided to investigate the time course for the production of these activities. Shaken cultures,which appeared to favour production of haemolytic activity against trout erythrocytes,were initially investigated. The production of caseinase activity, pigment and pH changes in the culture were also investigated to facilitate the study of interrelationships between the release of various extracellular products.

The results (Figure 7) indicated that pigment, caseinase and haemolytic activity were first detected at the end of the logarithic phase of growth. All of these extracellular products appeared to accumulate throughout the stationary phase of growth. A peak in haemolytic activity against horse erythrocytes was observed after 33 hr growth (32 Hu/ml) which was followed by a rapid decline in activity. After 72 hr growth,haemolytic activity against horse erythrocytes was low (0.5 Hu/ml).

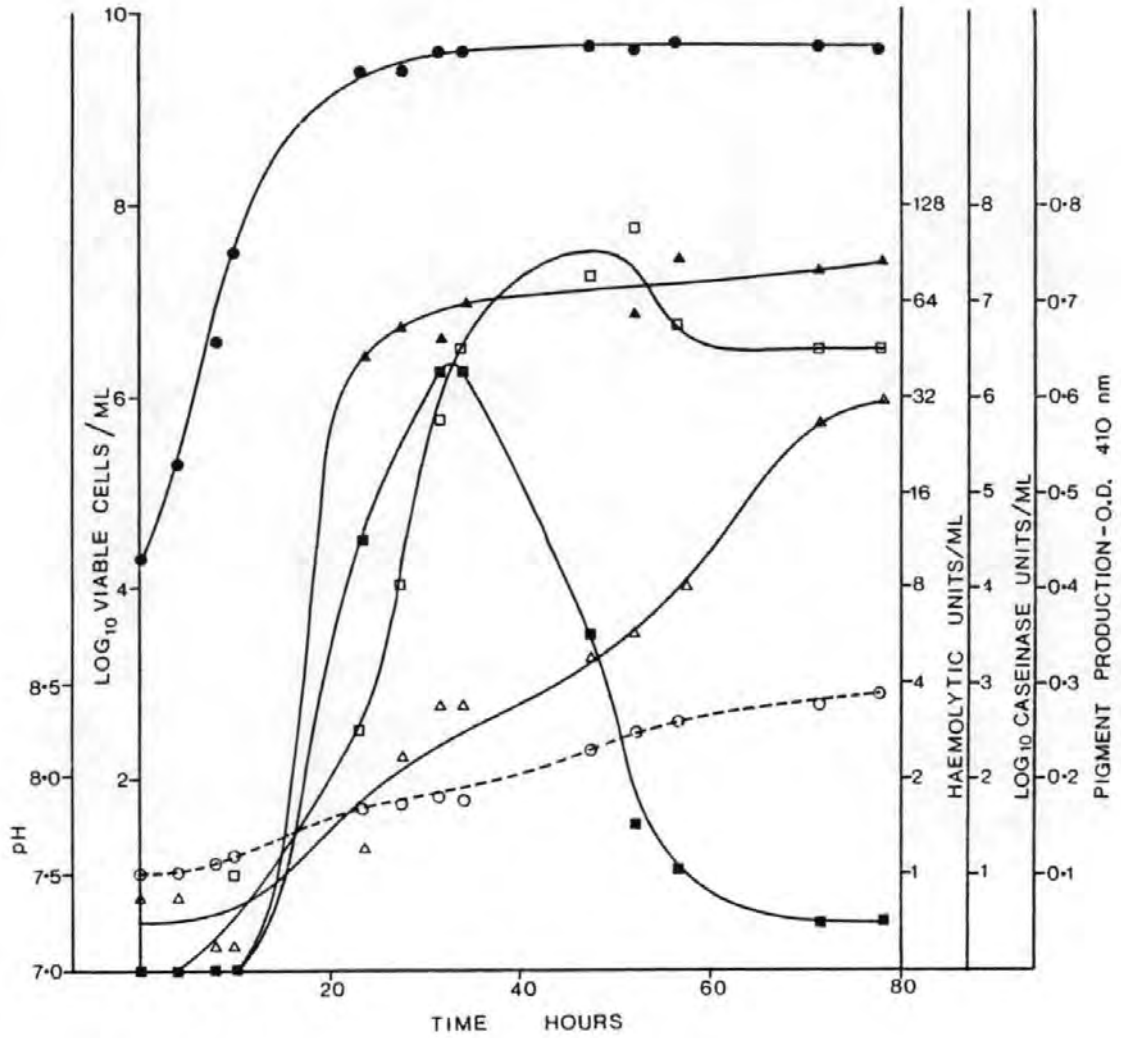
Haemolytic activity against trout erythrocytes was maximal after 50 hr growth (128 Hu/ml) and showed a limited decline in activity after this time.

Both caseinase and pigment production appeared to rise steadily throughout the stationary phase of growth whilst the pH of the culture appeared to rise, from an initial value of 7.5, throughout the logarithmic and stationary phases of growth. After 72 hr growth the pH of the culture was 8.4.

3.2.15 The time course for haemolysin, caseinase and pigment production in unshaken cultures

The time course for the production of haemolysin, caseinase and pigment production in unshaken cultures was also investigated; these cultural conditions appeared to favour the production of

Figure 7 The production of A. salmonicida extracellular products (strain 25/77) in shaken cultures at 25°C.



● = Viable cells (colony forming units)/ml
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (Units/ml)
△ = Pigment production (OD 410 nm)
○ = pH of culture supernate

haemolytic activity against horse erythrocytes.

The results (Figure 8) indicated that haemolytic and caseinase activities were first detected at the end of the logarithmic phase of growth thereafter rising throughout the stationary phase of growth. No peak in haemolytic activity was observed at any stage in the growth cycle.

Caseinase activity and haemolytic activity against trout erythrocytes were both low in unshaken cultures; however, haemolytic activity against horse erythrocytes was higher after 96 hr growth (64 Hu/ml) than at any time in shaken cultures.

Pigment production was below detectable levels in unshaken cultures whilst the pH of the cultures showed little change over the course of the experiment.

3.2.16 The effect of shaken conditions on preformed haemolytic activity against horse erythrocytes

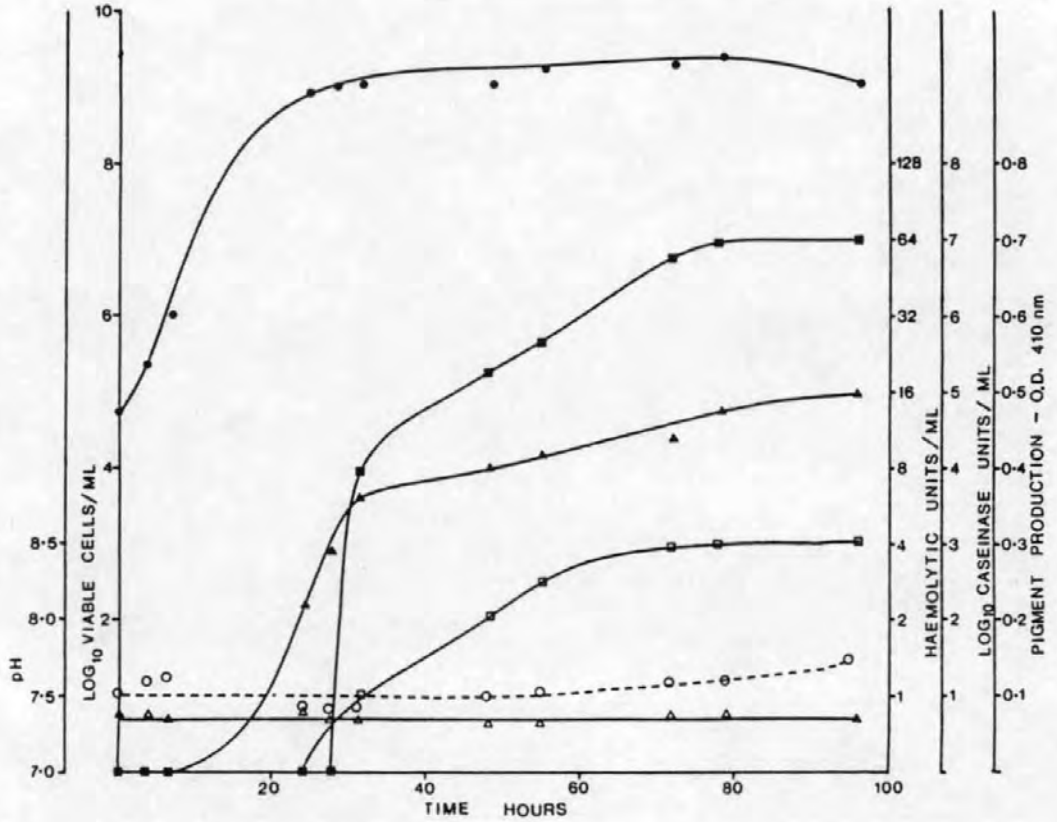
The nature of the results concerning the production of haemolytic activity against horse erythrocytes in shaken cultures suggested that after the peak in activity observed at 33 hr the haemolysin was being denatured. To test this possibility supernate samples from 96 hr unshaken cultures were subjected to unshaken or shaken conditions (90 rpm). Sodium azide was added to the samples to inhibit bacterial growth (0.02% w/v).

The results (Figure 9) indicated that, when shaken, haemolytic activity against horse erythrocytes declined with a half life of 90 mins. No change in haemolytic activity against trout erythrocytes was observed. Samples maintained under unshaken conditions showed no change in haemolytic activity against horse or trout erythrocytes over the course of the experiment (24 hr).

Sodium thioglycollate (0.5% w/v) did not prevent the decline in haemolytic activity against horse erythrocytes in shaken samples though BSA (1% w/v) had a marked stabilising effect.

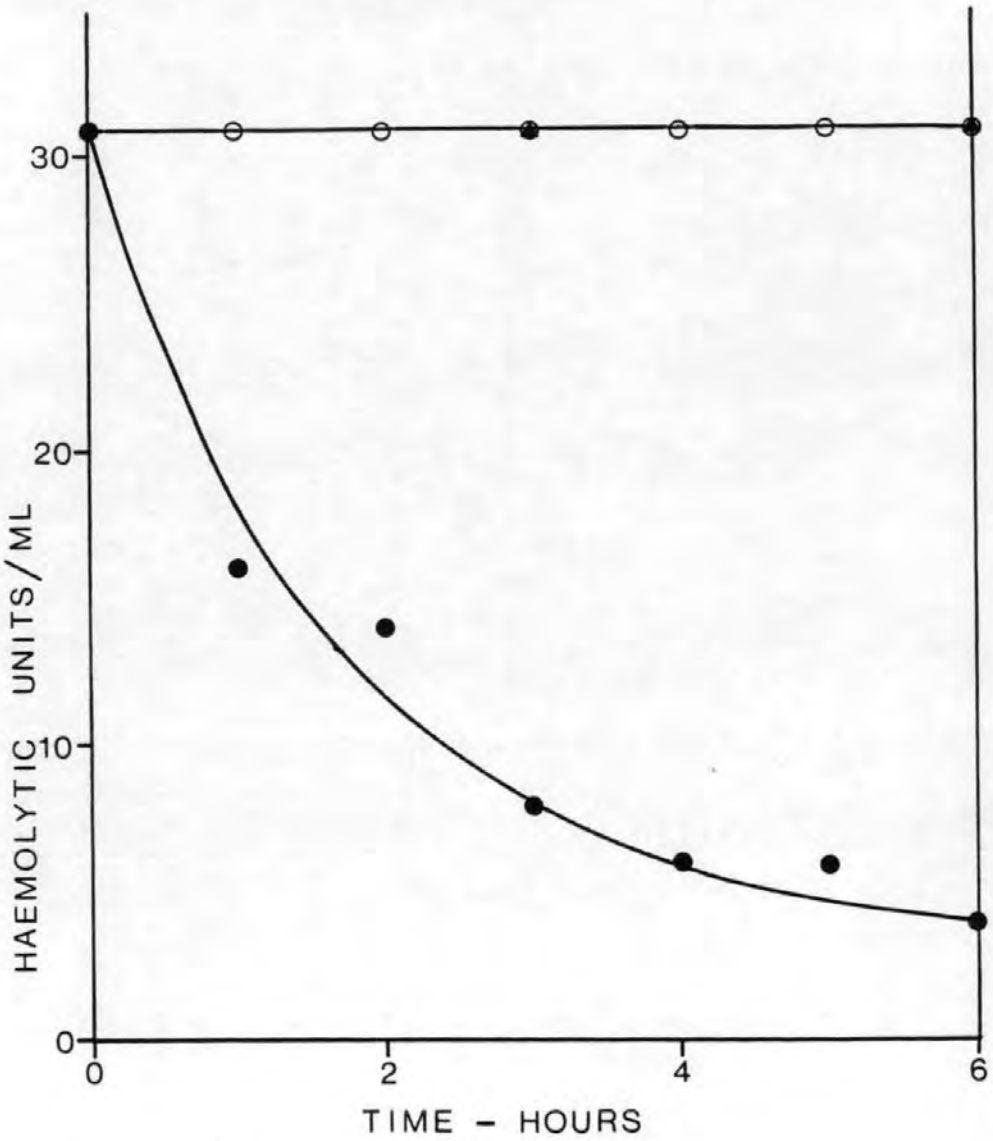
The prior addition of BSA (1% w/v) to cultures of A. salmonicida grown under shaken conditions did not increase the production of haemolytic activity against horse erythrocytes at 75 hr when

Figure 8 The production of A. salmonicida extracellular products (strain 25/77) in unshaken cultures at 25°C.



● = Viable cells (colony forming units) /ml
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (Units/ml)
△ = Pigment production (O.D. 410 nm)
○ = pH of culture supernate

Figure 9 The effect of shaken conditions on the stability of preformed haemolytic activity against horse erythrocytes



● = Haemolytic activity against horse erythrocytes when shaken
▲ = Haemolytic activity against horse erythrocytes in unshaken control samples
○ = Haemolytic activity against horse erythrocytes when shaken + 1% BSA.

Unshaken culture supernate (32 Hu/ml + 0.02% NaN_3) was maintained under shaken (90 rpm) or unshaken conditions and haemolytic activity determined at intervals.

compared with control cultures (No BSA).

3.2.17 Investigation of the possible intracellular location of haemolytic activity

In view of the fact that haemolytic activity in broth culture supernate was first detected at the end of the logarithmic growth phase, and increases in supernate haemolytic activity were observed throughout part of all of the stationary phase of growth (3.2.14 and 3.2.15), it was decided to investigate the possible intracellular location of haemolytic activity. Cells obtained by centrifugation of shaken cultures (20 hr 25°C), unshaken cultures (24 hr or 48 hr, 25°C) or AOT cultures (72 hr 25°C) were washed (40 mM tris-HCl, pH 8.2) and treated using a variety of techniques to completely lyse the cells or allow the escape of materials located in the periplasmic space (2.2.8). Control cells were treated with tris-HCl buffer (40 mM, pH 8.2). The treated cells were centrifuged (7,500 x g, 20 min, 4°C) and the supernatant fluid dialysed against PBS (2 l , 18 hr, 4°C, +0.02% NaN_3). The non-dialysable material was tested for haemolytic activity against horse erythrocytes, trout erythrocytes (2.4.2) and caseinase activity (2.4.5). In addition the optical density at 280 nm of the supernates was determined as an indication of the quantity of material released from the cells by the extraction processes.

The results (Table 11) indicated that although all the extraction processes released ultra-violet absorbing material very little haemolytic activity or caseinase activity was detected.

The pelleted material remaining after centrifugation of the cells treated by sonication was resuspended in PBS (5 ml), whilst pelleted material remaining after treatment with ethylenediamine-tetraacetic acid (EDTA), lysozyme and sucrose (2.2.8) was resuspended in distilled water (2.5 ml) and mixed with an equal volume of PBS (x 2 concentrated). When these samples were tested no caseinase or haemolytic activity against horse erythrocytes was detected.

Table 11 Investigation of the possible intracellular location of haemolytic activities

Source of cells	Cell treatment	Viable cell concentration per treatment	Supernatant fluid after cell treatment: Optical density (280 nm)	Supernatant fluid after cell treatment: Haemolytic activity detected: Horse erythrocytes	Supernatant fluid after cell treatment: Haemolytic activity detected: Trout erythrocytes: Complete lysis	Supernatant fluid after cell treatment: Haemolytic activity detected: Trout erythrocytes: Incomplete lysis	Cell pellet after treatment: Haemolytic activity detected: Trout erythrocytes: Complete lysis	Cell pellet after treatment: Haemolytic activity detected: Trout erythrocytes: Incomplete lysis
Unshaken 24 hr	CONTROL	4.8×10^9	0.11	0	0	0	N.D.	N.D.
Unshaken 24 hr	TEL	4.8×10^9	0.2	0	0	0	N.D.	N.D.
Unshaken 24 hr	TELS	4.8×10^9	0.18	0	0	0	0	2
Unshaken 24 hr	SONIC	4.8×10^9	2.66	0	0	0	0	0
Unshaken 48 hr	CONTROL	4.8×10^9	0.16	0	1	<1*	N.D.	N.D.
Unshaken 48 hr	TEL	4.8×10^9	0.24	0	0	0	N.D.	N.D.
Unshaken 48 hr	TELS	4.8×10^9	0.25	0	0	0	0	3
Unshaken 48 hr	SONIC	4.8×10^9	3.04	0	2	<2*	0	0
Shaken 20 hr	CONTROL	2.2×10^{10}	0.41	0	2	2*	N.D.	N.D.
Shaken 20 hr	TEL	2.2×10^{10}	1.1	0	0	0	N.D.	N.D.
Shaken 20 hr	TELS	2.2×10^{10}	0.9	0	0	0	0	4
Shaken 20 hr	SONIC	2.2×10^{10}	4.16	0	2	<2*	0	0
AOT 72 hr	CONTROL	4.8×10^9	0.52	0	6	<6*	N.D.	N.D.
AOT 72 hr	TEL	4.8×10^9	0.73	0	8	<8*	N.D.	N.D.
AOT 72 hr	TELS	4.8×10^9	0.47	0	4	<4*	0	192
AOT 72 hr	SONIC	4.8×10^9	4.68	0	48	<48*	0	6

Haemolytic activity is expressed as Hu/ml. TEL = treatment with EDTA + lysozyme. TELS = treatment with EDTA + lysozyme + sucrose. SONIC = sonication of cells. * = Complete lysis masks any incomplete lysis.
N.D. = Not determined.

None of the samples caused complete lysis of trout erythrocytes but some samples appeared to cause incomplete lysis of these cells in the microtitre assay system (2.4.2).

It was thought that haemolytic activity may have been present within the cells in an inactive form. The cell extract supernates were incubated with culture supernatant fluid (shaken 44 hr, 25°C or unshaken 96 hr, 25°C) or with chymotrypsin solution (4.4.5). When these samples were tested for haemolytic activity no increase was observed in comparison with control samples.

3.2.18 The production of extracellular products on solid growth media by virulent and avirulent strains of A. salmonicida

Having investigated the effect of cultural conditions on the production of extracellular products by A. salmonicida it was decided to investigate the possibility that a correlation existed between virulence and the production of selected extracellular products. Initial work involved the study of haemolysin and caseinase production by a variety of virulent and avirulent strains of A. salmonicida . The bacteria were stab inoculated onto TSA + washed horse blood (5% v/v), TSA + washed trout blood (7% v/v) and TSA + sodium caseinate (1% w/v). Virulence of A. salmonicida is associated with the presence of the A-layer (1.3.2) external to the cell wall.

Strains possessing the A-layer were identified by emulsifying bacteria in a small volume of PBS and observing subsequent auto-agglutination (2.2.9). The presence and absence of the A-layer was determined for strains CM30 and 25/77 respectively, using electron microscopy (Plates 5 and 6) (2.2.9).

The results (Figure 10) indicated that all strains tested produced haemolysis of horse erythrocytes, trout erythrocytes and zones of digestion of casein. After 72 hr growth (25°C) the levels of production of these activities appeared to be related for all

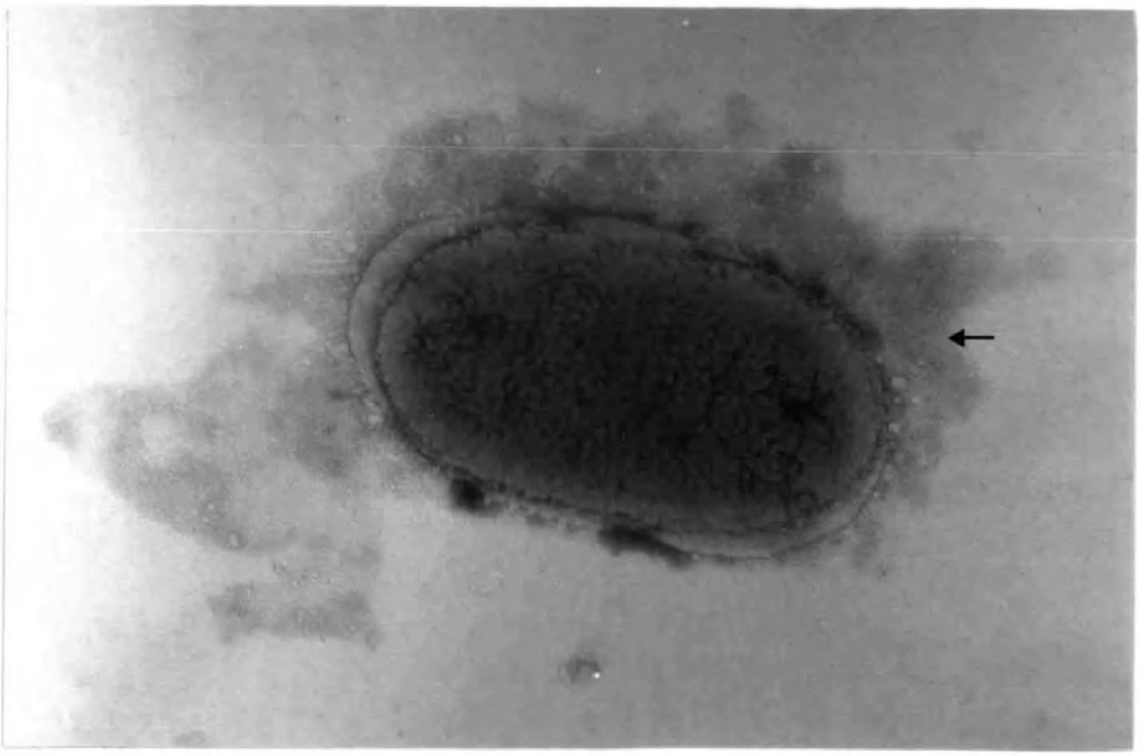
Plate 5 Electron microscopic examination of A. salmonicida strain CM30 showing the presence of the A-layer

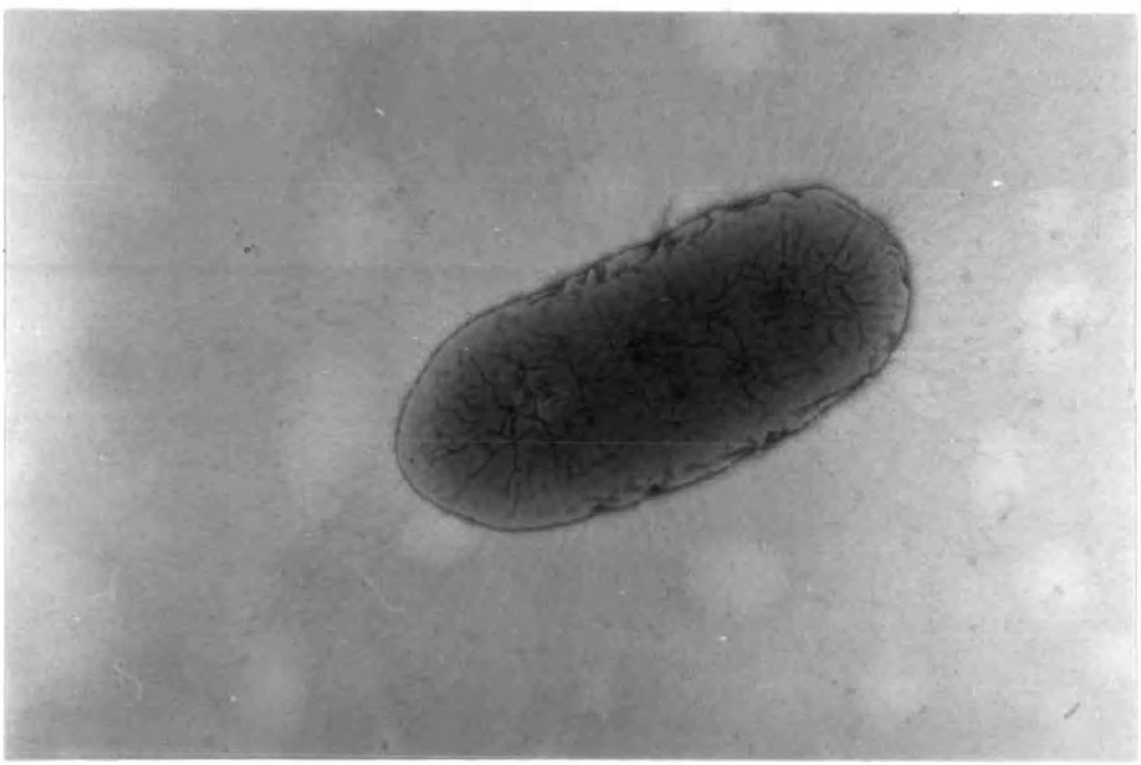
The A-layer surrounding the bacterial cell is indicated by an arrow.

Magnification 5.3×10^4

Plate 6 Electron microscopic examination of A. salmonicida strain 25/77 showing the absence of the A-layer

Magnification 5.3×10^4





strains tested. The form of the zones of haemolysis (3.2.1) was similar with all the strains.

The results suggested that there was no correlation between the possession of the A-layer and the production of the extracellular products studied.

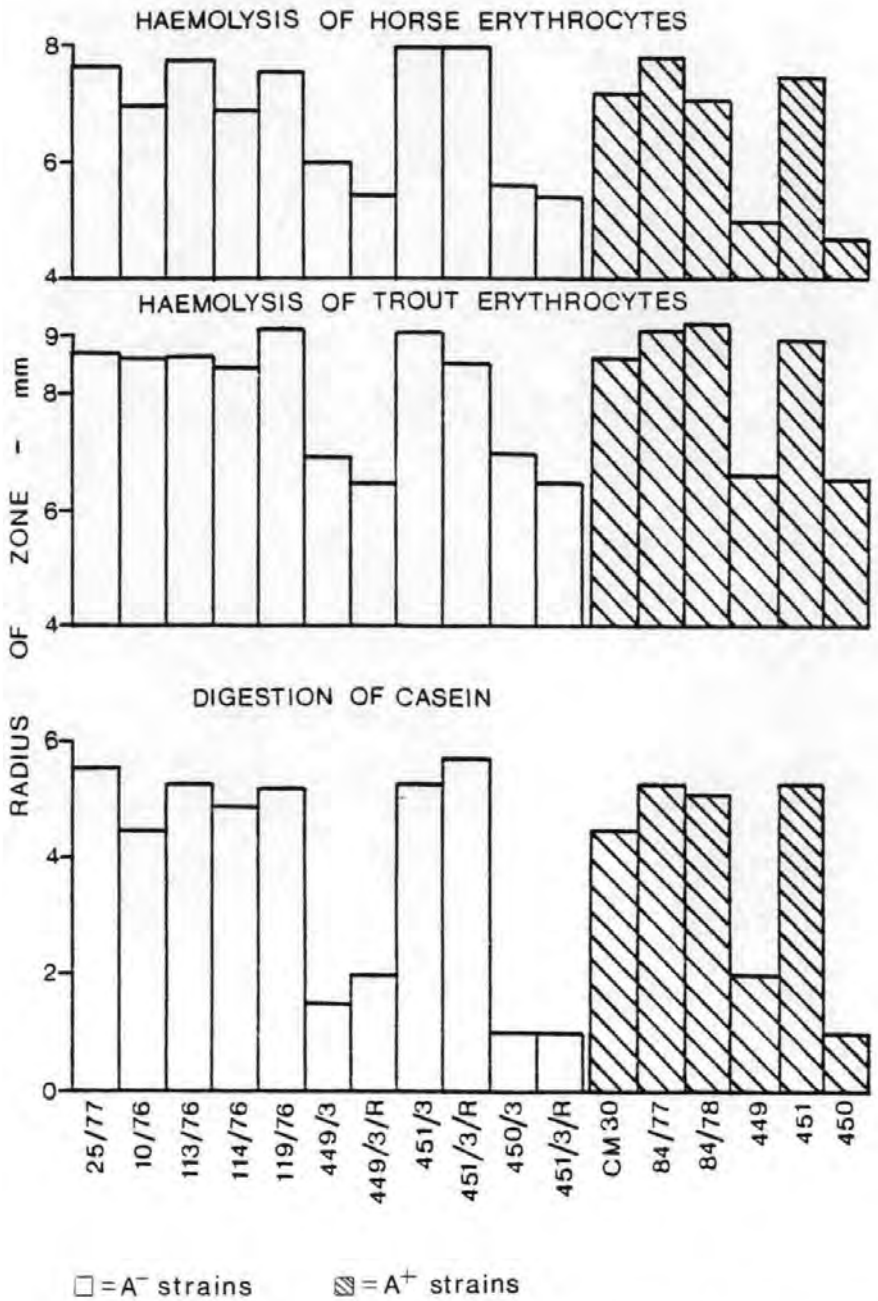
3.2.19 The production of extracellular products in liquid media by virulent and avirulent strains of A. salmonicida

It was thought that the study of growth characteristics and patterns of production of extracellular products in liquid media may have provided a more detailed approach to the study of in vitro differences between virulent (A-layer positive, A^+) and avirulent (A-layer negative, A^-) strains of A. salmonicida. Growth of three A^+ and three A^- strains in unshaken cultures was investigated; unshaken cultures were previously found to favour production of haemolytic activity against horse erythrocytes (3.2.15). The bacteria were grown at 20°C, rather than 25°C because cultivation of bacteria at temperatures above 22°C has recently been reported to result in the loss of the A-layer from virulent strains (1.3.2).

The bacteria were grown in 100 ml volumes on NB No. 2 in 250 ml Erlenmeyer flasks and viable counts determined at intervals using a spiral plater. Culture supernatant fluid samples were tested for haemolytic activity against horse erythrocytes, activity against trout erythrocytes (2.4.2) and caseinaseactivity (2.4.5). Pigment production was not evaluated since this was shown to be below detectable levels in unshaken cultures (3.2.15). The A^+ strains CM30, 449 and 451 and the A^- 25/77, 449/3, and 451/3 were tested. Strains 449/3 and 451/3 were derived from their parent, virulent, strains 449 and 451 respectively (2.2.1).

The results (Figures 11a, 11b, 11c, 11d and Table 12) indicated that there was no difference in growth rates between A^+ and A^- strains under these cultural conditions. Haemolytic activity against horse erythrocytes and activity against trout erythrocytes were first detected at the end of the logarithmic phase of growth in cultures of 451/3 and remained at plateau

Figure 10 The production of haemolytic and caseinase activities by virulent and avirulent strains of A. salmonicida on solid growth medium



□ = A⁻ strains ▨ = A⁺ strains

Haemolytic activity on horse blood + TSA is expressed as the radius between the colony edge and the red halo.

Haemolytic activity on trout blood + TSA is expressed as the radius between the colony edge and the outer edge of the orange zone.

Caseinase activity is expressed as the radius between the colony edge and the edge of the zone of digestion.

levels thereafter. Caseinase activity from this strain was first detected before the end of the logarithmic phase of growth (14 hr) (Figure b). Strains 451 and 25/77 gave similar results to those obtained for 451/3 (Figures 11a, 11d). However, in strain CM30 haemolytic activities against horse or trout erythrocytes were first detected during the stationary phase of growth (33 hr) (Figure 11c). Strains 449 and 449/3 produced no detectable haemolytic activity or caseinase activity during the experiment.

These results are summarised in the form of a table (Table 12).

3.2.20 The production of extracellular products in liquid media, under shaken conditions, by virulent and avirulent strains of A. salmonicida

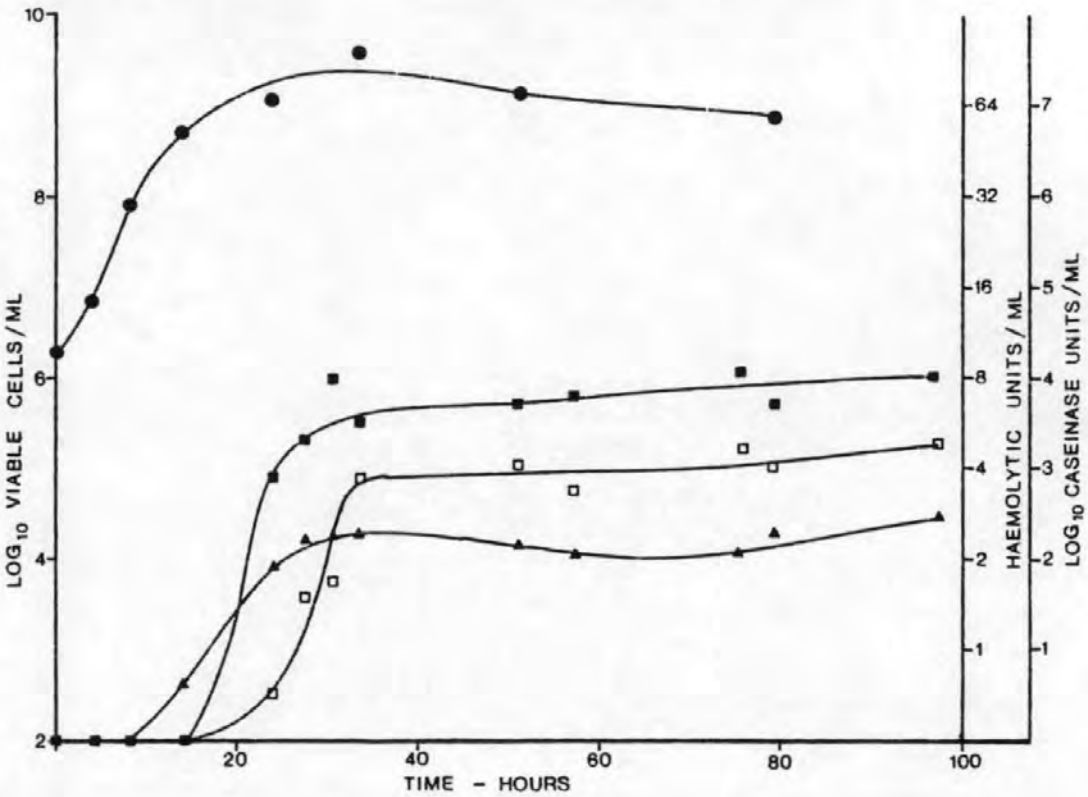
In view of the fact that shaken culture conditions are known to favour the production of haemolytic activity against trout erythrocytes, caseinase activity and pigment by A. salmonicida (3.2.14) it was decided to investigate the growth and extracellular products of A^+ and A^- strains of A. salmonicida in shaken cultures.

The bacteria were grown as described previously (3.2.19), however the culture flasks were shaken at 120 rpm.

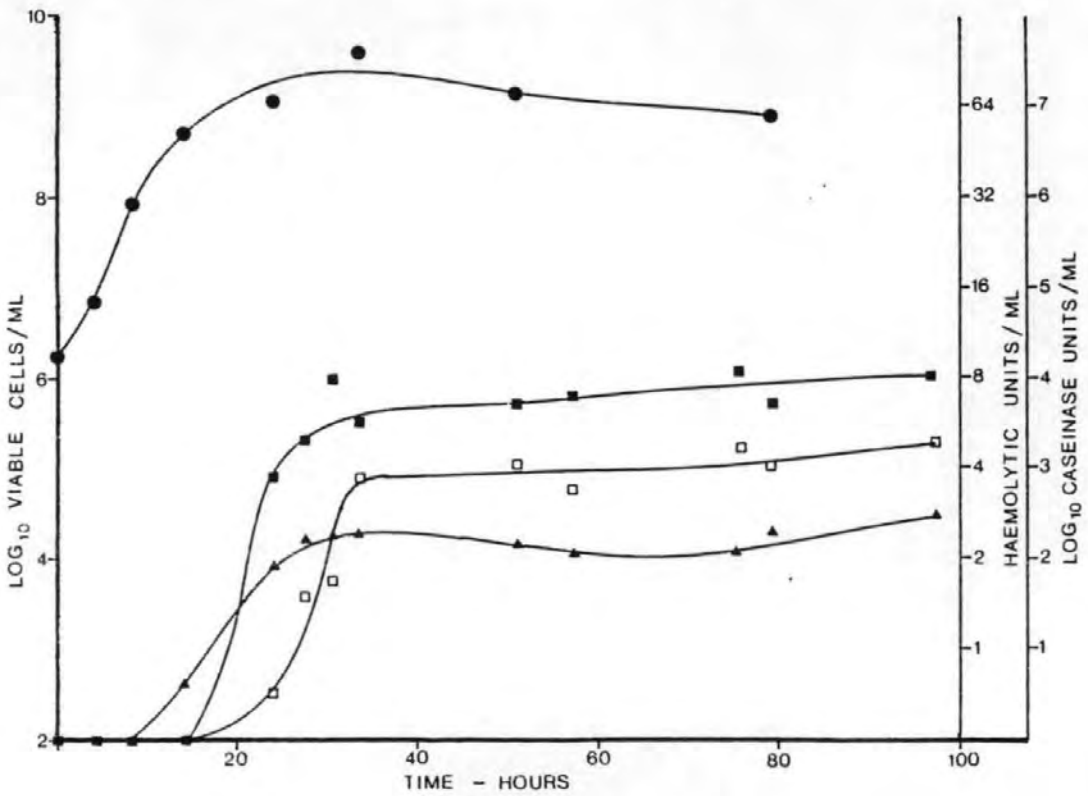
The results (Figures 12a, 12b, 12c, 12d, 12e, 12f and Table 13) indicated that there was little difference in the growth rates of A^+ and A^- strains under these cultural conditions. In cultures of the A^- strain 451/3, haemolytic activity against horse erythrocytes was first detected towards the end of the logarithmic phase of growth reaching a peak at 24 hr and thereafter declining rapidly to negligible levels (Figure 12b). In the A^+ strain 451 haemolytic activity against horse erythrocytes was first detected at 28 hr reaching a maximum level at 32 hr and thereafter declining rapidly (Figure 12a). A similar pattern was observed with other strains, the A^- strains producing haemolytic activity

Figure 11 The production of extracellular products by A-layer positive and A-layer negative strains of A. salmonicida in unshaken cultures at 20°C.

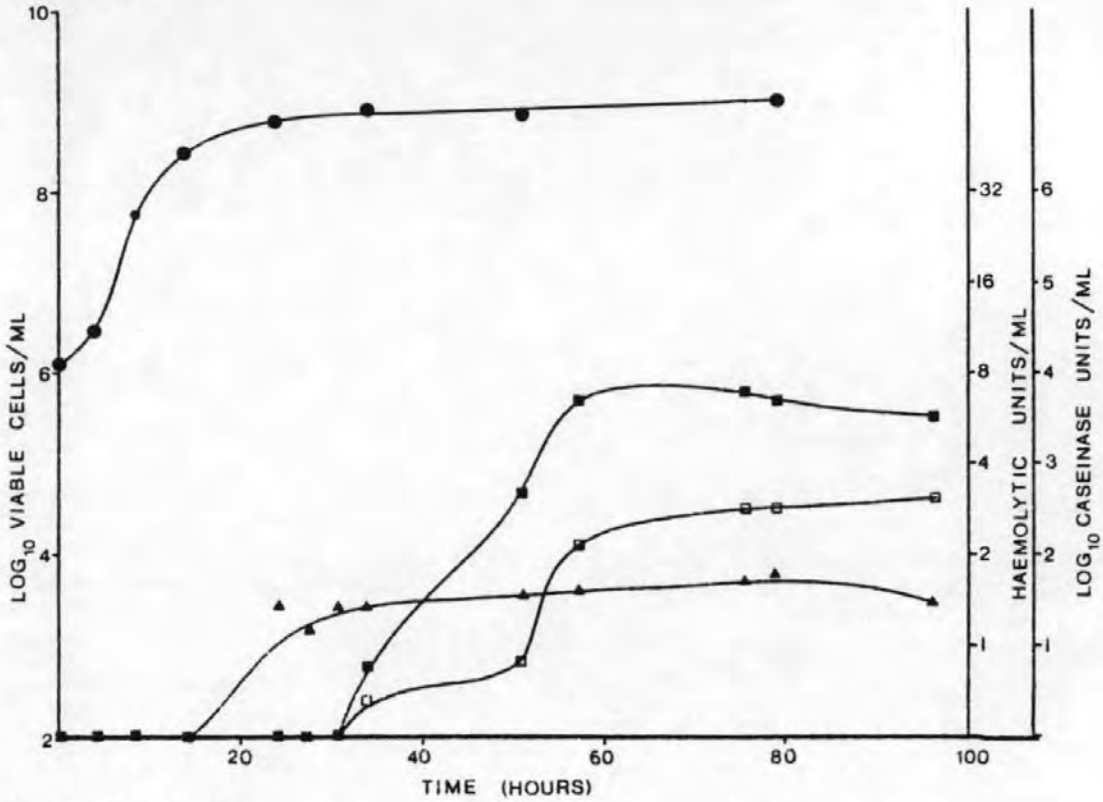
(a) Strain 451



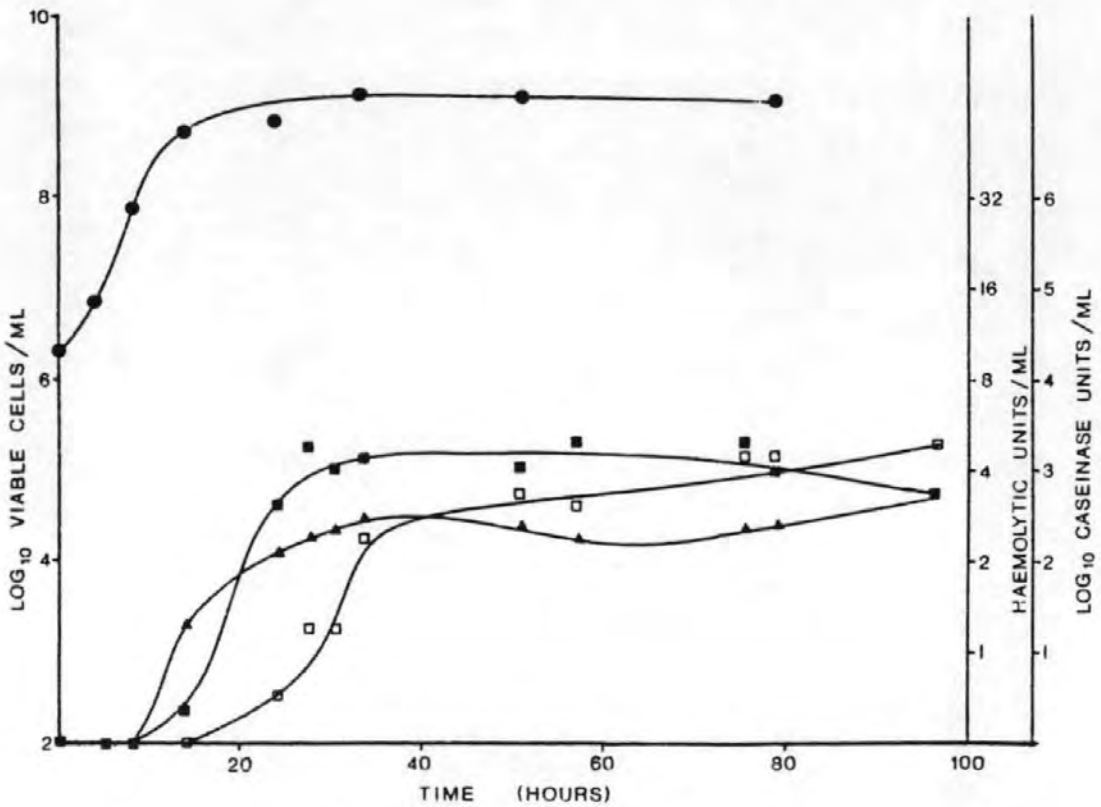
(b) Strain 451/3



(c) Strain CM30



(d) Strain 25/77



● = Viable cells / ml (colony forming units/ml)

■ = Haemolytic activity against horse erythrocytes (Hu/ml)

□ = Haemolytic activity against trout erythrocytes (Hu/ml)

▲ = Caseinase activity (units/ml)

Table 12 The production of extracellular activities by A^+ and A^- strains of *A. salmonicida* in unshaken culture at 20°C

	25/77	CM30	449/3	449	451/3	451
Presence of A layer	-	+	-	+	-	+
Mean generation time (hr)	1.53	1.36	1.40	1.53	1.24	1.40
	Age of culture at which activity was first detected					
H-lysin	14	33	-	-	24	24
T-lysin	24	33	-	-	24	24
Caseinase	14	24	-	-	14	14
Pigment[1]	-	-	-	-	-	-
	Maximum activity detected					
H-lysin[2]	6	7	0	0	6	8
T-lysin[2]	5	3	0	0	6	5
Caseinase[3]	620	70	0	0	880	290
Pigment[4]	0	0	0	0	0	0

[1] Absorbance at 410 nm < 0.05

[2] Haemolytic units/ml

[3] Units/ml

[4] Absorbance at 410 nm

against horse erythrocytes several hours earlier than the A^+ strains (Figures 12c, 12d, 12e, 12f). The production of haemolytic activity against horse erythrocytes by strain 449 was very delayed, activity being first detectable after 72 hr growth (Figure 12e).

Production of haemolytic activity against trout erythrocytes by strain 451/3 was first detected after 20 hr growth, increasing steadily after this time. Caseinase activity was also first detected at this time but did not increase appreciably on further incubation (Figure 12b). In cultures of 451, haemolytic activity against trout erythrocytes and caseinase activities were similar to those observed in cultures of 451/3. A similar pattern of production was observed with the other strains tested, with activities first detected much earlier in cultures of A^- than A^+ strains. Haemolytic activity against trout erythrocytes and caseinase production were of low levels and delayed in release in cultures of the A^+ strain 449 (Figure 12e).

Levels of pigment in the culture supernates increased steadily from the end of the logarithmic phase of growth with strain 451/3 (Figure 12b) though the time of first detection was delayed in culture supernates of 451 (Figure 12a). The delay of pigment production was also apparent in other A^+ strains when compared with A^- strains.

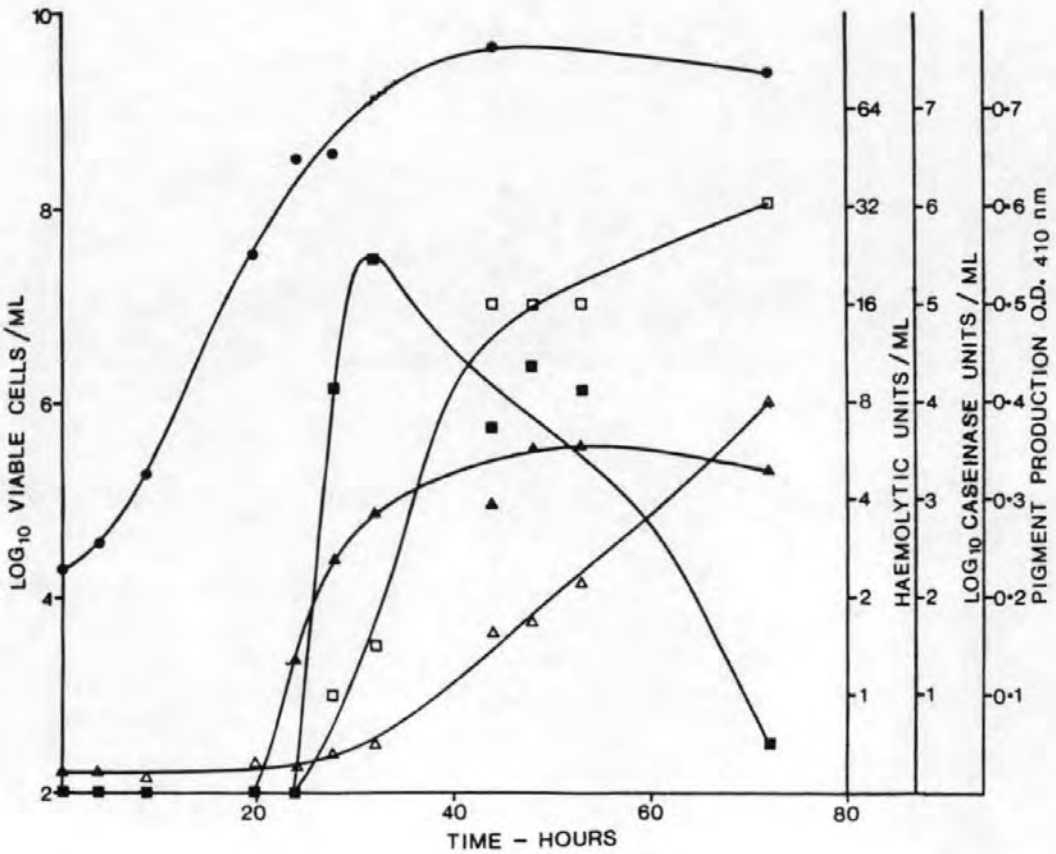
These results are summarised in the form of a table (Table 13).

3.2.21 The comparison of extracellular products from A^+ and A^- strains of *A. salmonicida*

The results obtained suggested that there were no differences in the spectrum or quantities of selected extracellular products released by A-layer positive, (A^+) and A-layer negative strains (A^-) of *A. salmonicida* in broth culture or on solid growth medium (3.2.18, 3.2.19 and 3.2.20). It was decided to investigate the spectrum of extracellular products released by an A^+ strain (CM30)

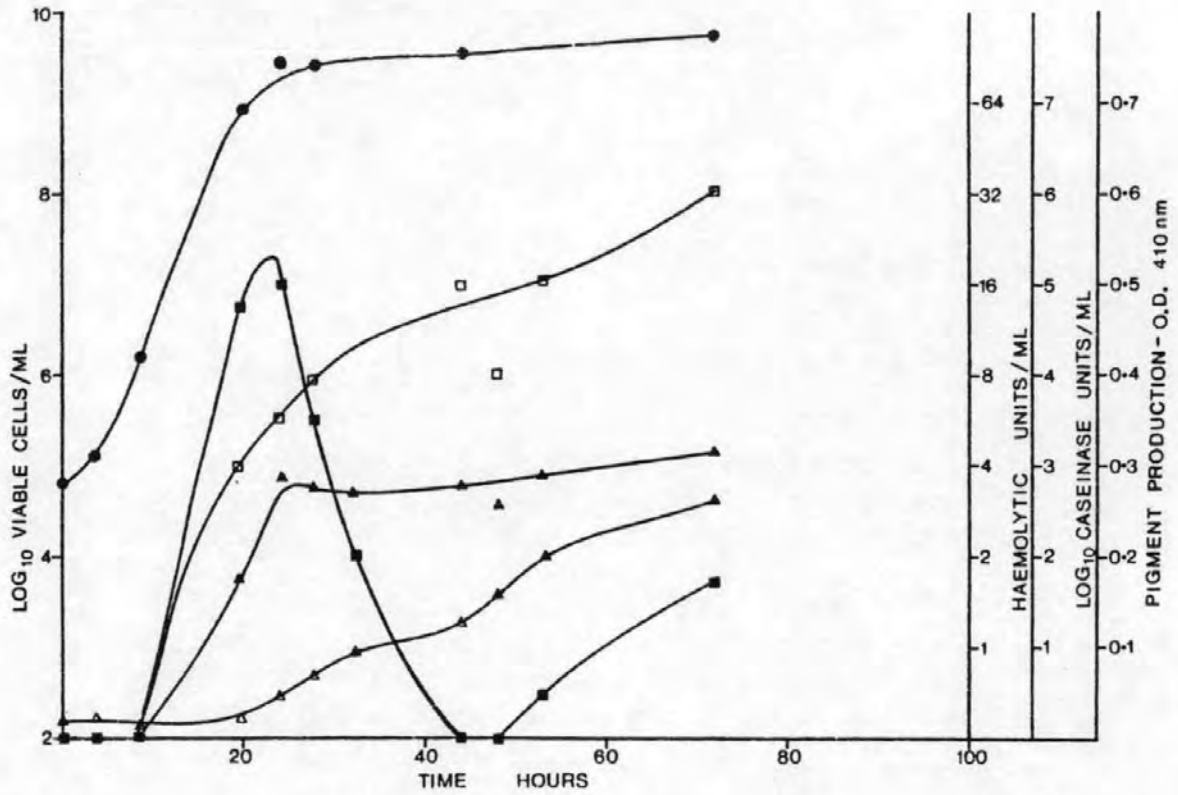
Figure 12 The production of extracellular products by A layer positive and A-layer negative strains of A. salmonicida in shaken cultures at 20°C.

(a) Strain 451



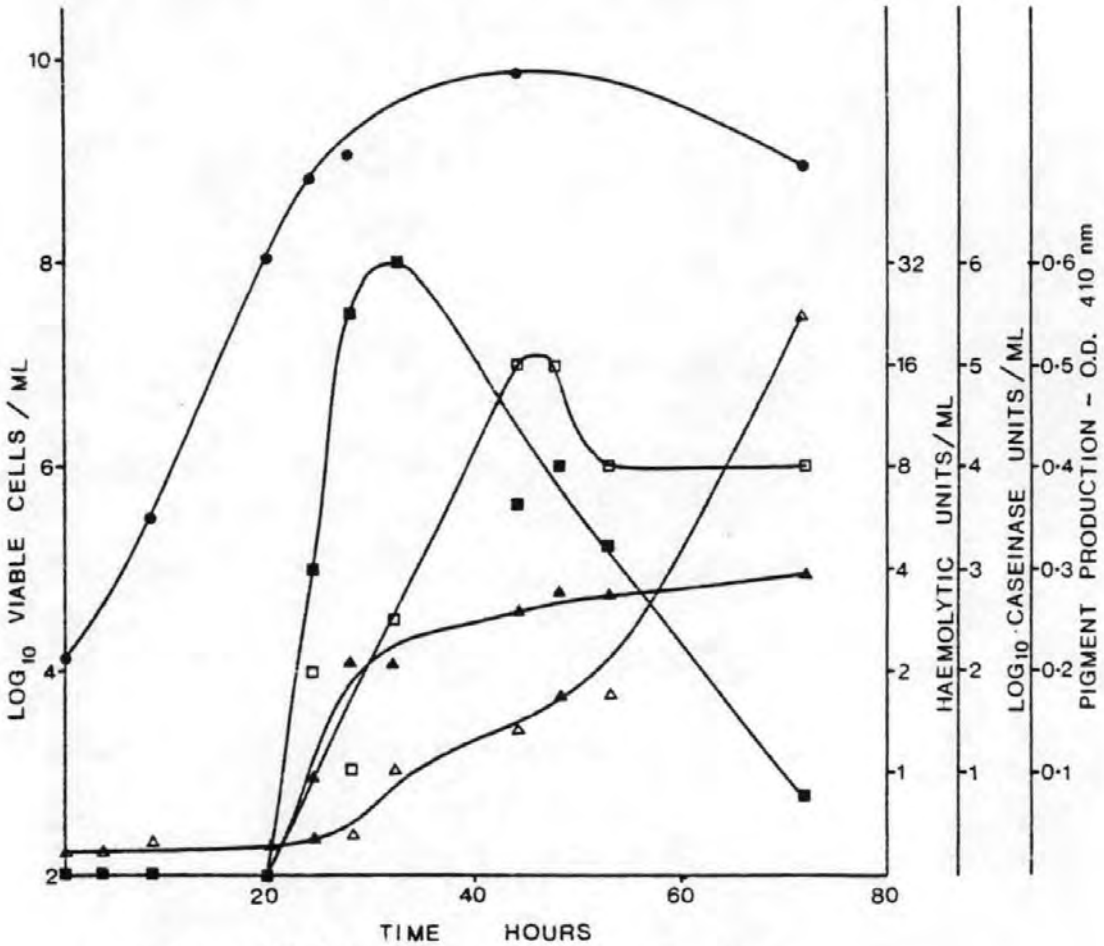
● = Viable cells/ml (colony forming units/ml)
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (units/ml)
△ = Pigment production (OD 410 nm)

(b) Strain 451/3



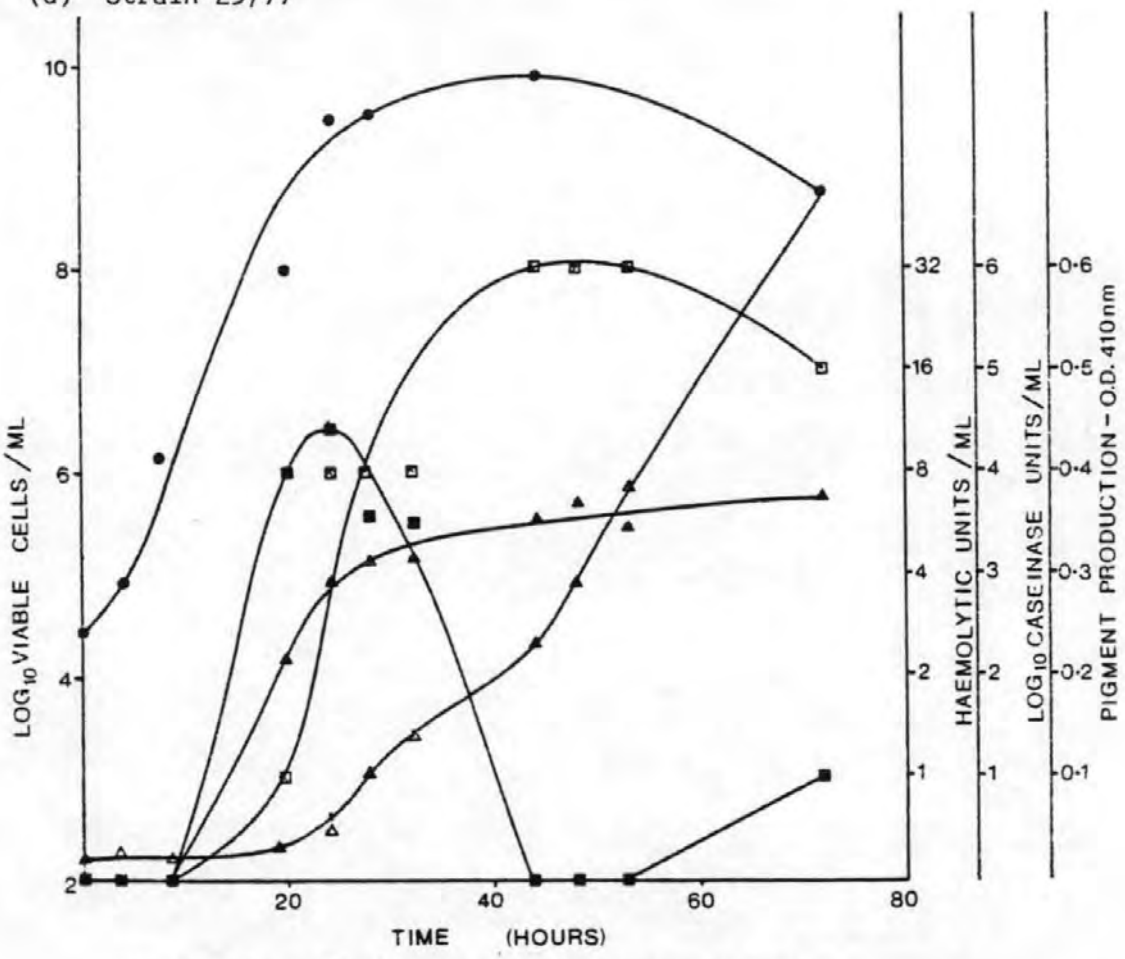
● = Viable cells/ml (colony forming units/ml)
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (units/ml)
△ = Pigment production (OD 410 nm)

(c) Strain CM 30



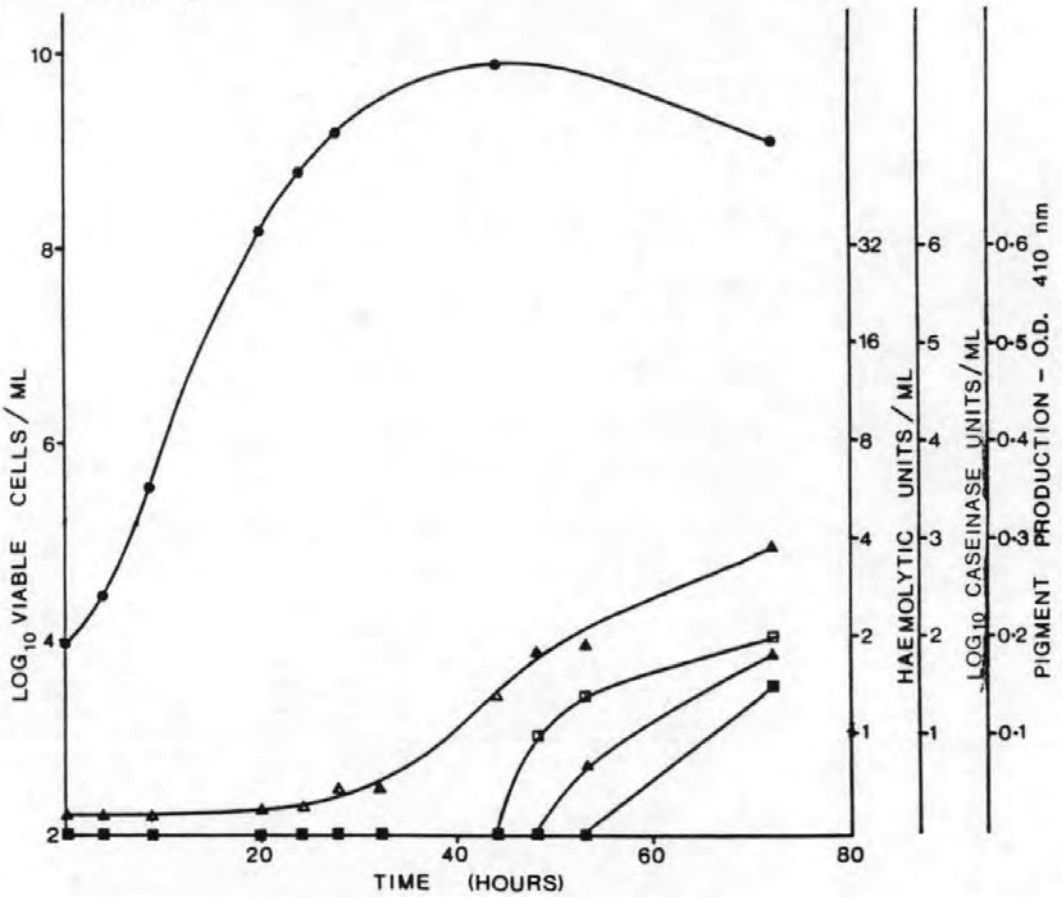
● = Viable cells/ml (colony forming units/ml)
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (units/ml)
△ = Pigment production (OD 410 nm)

(d) Strain 25/77



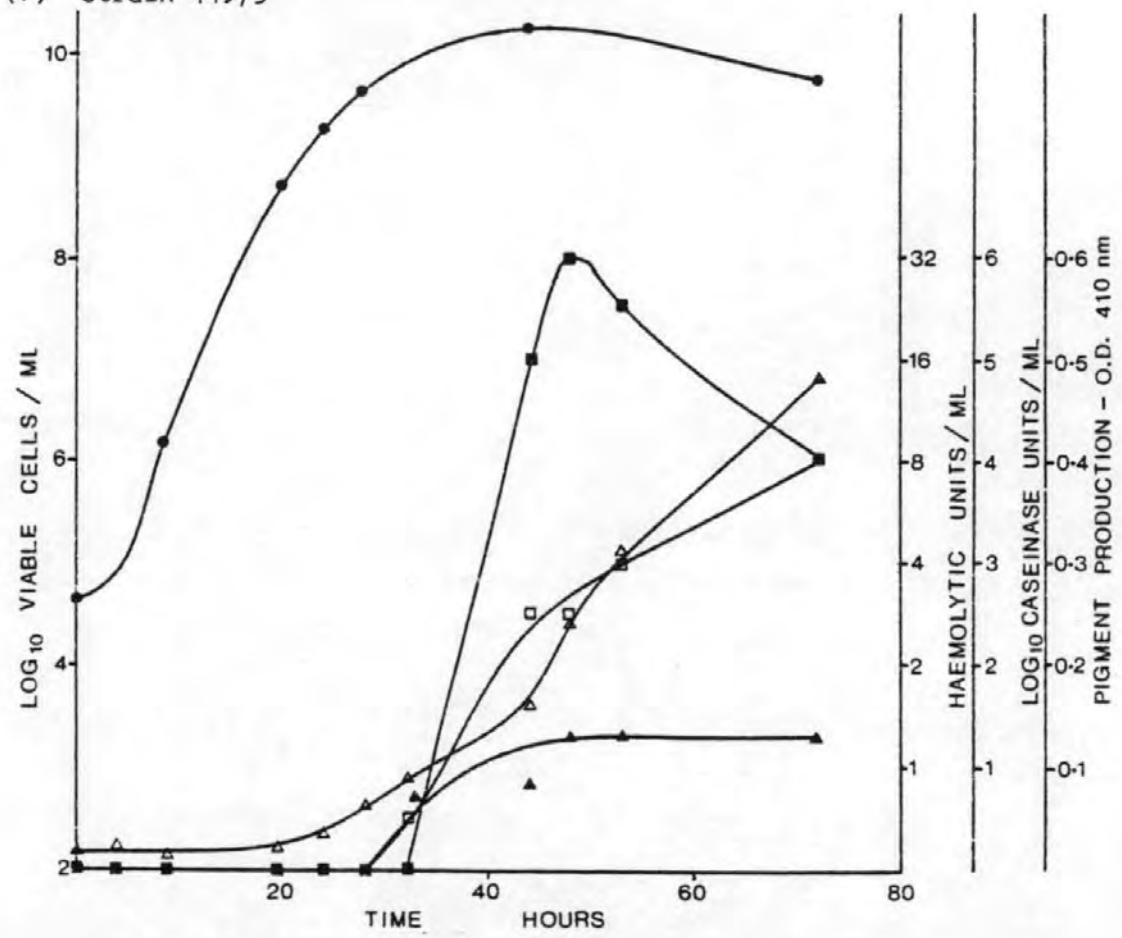
● = Viable cells/ml (colony forming units/ml)
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (units/ml)
△ = Pigment production (OD 410 nm)

(e) Strain 449



● = Viable cells/ml (colony forming units/ml)
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (units/ml)
△ = Pigment production (OD 410 nm)

(f) Strain 449/3



● = Viable cells/ml (colony forming units/ml)
■ = Haemolytic activity against horse erythrocytes (Hu/ml)
□ = Haemolytic activity against trout erythrocytes (Hu/ml)
▲ = Caseinase activity (units/ml)
△ = Pigment production (OD 410 nm)

Table 13 The production of extracellular activities by A^+ and A^- strains of A. salmonicida in shaken cultures at 20°C.

	25/77	CM30	449/3	449	451/3	451
Presence of A layer	-	+	-	+	-	+
Mean generation time (hr)	1.36	1.07	1.37	1.44	1.28	1.18
	Age of culture (hr) at which activity first detected					
H-lysin	20	24	44	72	20	28
T-lysin	20	24	32	48	20	28
Caseinase	20	24	53	53	20	24
Pigment[1]	25	29	27	33	25	33
	Maximum activity detected					
H-lysin[2]	12	32	32	2	16	24
T-lysin[2]	32	16	8[5]	2	32	32[5]
Caseinase[3]	5700	800	11	72	1370	3600
Pigment[4]	0.68[5]	0.55[5]	0.49[5]	0.30[5]	0.27[5]	0.40[5]

[1] Absorbance at 410 nm 0.05

[2] Haemolytic units/ml

[3] Units/ml

[4] Absorbance at 410 nm

[5] Activity still rising at termination of experiment

and an A^- strain (25/77) in greater detail using an electrophoretic technique. Extracellular products from strains CM30 and 25/77 were obtained by the agar overlay technique (2.2.5). The samples were concentrated by lyophilisation to give similar protein concentrations (5.2 mg/ml) and 20 µl samples separated in a slab gel electrophoretic system (T = 7.5%)

Extracellular products in broth cultures of CM30 and 25/77 were also separated by electrophoresis after ammonium sulphate precipitation (40-60% saturation) (2.3.1) and lyophilisation (3.4 mg/ml protein).

The results (Plate 5) indicated that there were no visible differences in the spectrum or quantity of extracellular products from CM30 and 25/77 in broth culture supernate samples. In particular, CM30 samples appeared to possess three bands which were absent from 25/77 samples (arrowed). Several bands in the 25/77 sample were more intense, one of these (Rf = 0.195, arrowed) appeared to correspond with the main band of caseinase activity (4.2.3).

Plate 7 The comparison of extracellular products from an A^+ and an A^- strain of A. salmonicida

Track number	Sample
1	Strain CM30 AOT extracellular products (2.6 mg/ml)
2	Strain CM30 AOT extracellular products (5.2 mg/ml)
3	Strain 25/77 AOT extracellular products (2.6 mg/ml)
4	Strain 25/77 AOT extracellular products (5.2 mg/ml)
5	Blank well
6	Strain CM30 broth culture extracellular products (3.4 mg/ml)
7	Strain 25/77 broth culture extracellular products (3.4 mg/ml)

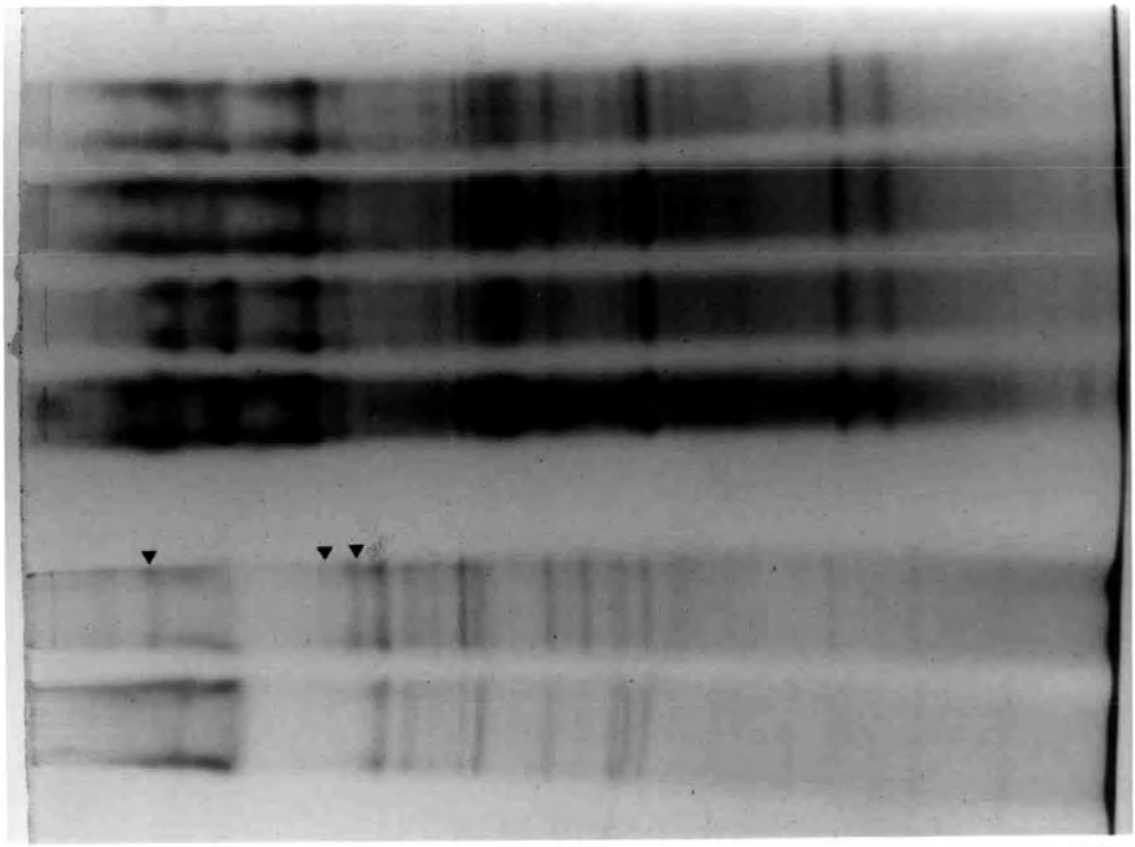
TD = Tracking dye front

Strain CM30 = A layer positive (A^+)

Strain 25/77 = A layer negative (A^-)

AOT = Agar overlay technique

Broth culture extracellular products were precipitated using ammonium sulphate before testing.


TD

3.3.0 DISCUSSION

A. salmonicida appears to produce a variety of enzymes and other extracellular products in vitro. Previous workers have reported that the bacterium produces haemolytic activity (Karlsson 1962, Nord et al 1975, Ellis et al 1981), however, this is the first report that two haemolysins are produced by the bacterium. Activity against trout erythrocytes (T-lysin) was detected maximally in shaken cultures, however, no activity against other erythrocyte types was detected. In unshaken cultures, haemolytic activity against a wide variety of erythrocyte types was detected, horse cells appearing to be the most sensitive to lysis and this activity was termed the H-lysin. Differences in the spectra of susceptible erythrocytes to different haemolysins has been observed with a variety of other bacterial species (Alouf 1976), however, fish erythrocytes have rarely been tested. One notable exception involves S. aureus δ lysin which is more active against fish than mammalian erythrocytes (Chao and Birkbeck 1978), the increased sensitivity possibly reflecting the increased numbers of haemolysin receptors on the fish erythrocytes.

Investigation of the time course for extracellular product formation in unshaken cultures confirmed that these cultural conditions selectively favoured H-lysin production, with only low levels of caseinase, T-lysin and pigment detected. In shaken cultures high levels of caseinase, T-lysin and pigment were produced. H-lysin was also detected in these cultures during the early stationary phase of growth but the activity declined rapidly on further incubation suggesting that the H-lysin was being denatured. Changes in culture pH, which were more marked in shaken cultures, were not thought to be responsible for this effect because H-lysin degradation was observed whilst the pH was within the range for optimum H-lysin stability (5.2.5). The increased caseinase production in shaken cultures, leading to digestion of the H-lysin, presents a possible explanation for the results obtained, however, the finding that pre-formed H-lysin was susceptible to foaming denaturation suggested that this was the reason for the decline in activity in shaken cultures. Denaturation

of S. aureus enzymes has been described in stirred fermenter cultures, due to foaming denaturation (Arvidson et al 1971). BSA added to preformed shaken H-lysin stabilised the haemolysin, tending to confirm that foaming denaturation was responsible for H-lysin instability. Oxygen lability of the H-lysin, a feasible explanation for the results in view of the low activity in shaken and AOT cultures, is unlikely in view of the observation that sodium thioglycollate did not stabilise preformed H-lysin, neither did anaerobic cultural conditions promote H-lysin production. In view of these conclusions it is not clear why the addition of BSA to shaken cultures did not enhance H-lysin production. This result may reflect the fact that the BSA was digested by the caseinase negating its protective effect. Unshaken cultural conditions have been found to favour A. proteolytica haemolysin production (Foster and Hanna 1974) whilst A. hydrophila haemolysins are found in shaken cultures (Wretlind et al 1973, Bernheimer and Avigad 1974).

The nutritional requirements for the production of various bacterial extracellular products have in some cases been elucidated in detail. For example, L-trytophan has been found to stimulate Kanagawa haemolysin production by Vibrio parahaemolyticus (Cherwonogrodzky and Clark 1982). In the case of A. salmonicida, no T or H-lysin was detected in cultures in a simple, defined growth medium. Both T and H-lysins were detected in cultures grown in a variety of complex growth media, the patterns of haemolysin production being generally similar in different media. Maximal H and T-lysin activity was observed in nutrient broth No. 2 cultures (NB No. 2). Yeast ribonucleate has been found to stimulate the production of A. hydrophila haemolysin II (Bernheimer and Avigad 1974), however, no similar effect was observed with A. salmonicida haemolysins in this project. The addition of some compounds to NB No. 2 appeared to adversely affect haemolysin production. The effect of glucose addition may have been to produce acidic cultural conditions, which have been found to adversely affect haemolysin stability (5.2.5). The effects of other additions may reflect the effect of these additions on the pre-formed

haemolysins. Haemoglobin was found to inhibit H-lysin activity, probably because of the inhibitory effect of contaminating erythrocyte membrane components on the H-lysin.

The components present in NB No. 2 promoting haemolysin production are not known, the differences in production between different culture medium types may reflect the ability of the medium to support bacterial growth rather than the presence of a component that specifically stimulates haemolysin production. Measurement of bacterial growth in different media was not attempted in this project because pigment production (variable in different media) affects the results of any method using the parameter of culture optical density as an indication of bacterial growth. More complex growth measurement techniques, such as viable count determinations, would have required a prohibitive amount of equipment.

Metal ion concentrations have been shown to affect the production of a variety of bacterial toxins. High iron levels depress the production of Clostridium perfringens α toxin and a similar effect has been reported for tetanus toxin (van-Heyningen 1970). Low iron levels appeared to depress both growth and H-lysin production by A. salmonicida whilst the reverse effect was observed at high iron concentrations. Both virulent and avirulent strains were affected in this manner. A. hydrophila protease production has been reported to be depressed by the presence of zinc ions in the culture medium with increases in haemolysin production (Allan and Stevenson 1981), however a similar effect was not observed with A. salmonicida in this project.

Culture incubation temperature affected H-lysin production by A. salmonicida. Maximum H-lysin production was observed at 25°C. Decreased H-lysin production below this temperature may be due to decreased bacterial growth. At 30°C the decline in H-lysin activity as the culture aged may reflect the thermal instability of the H-lysin (6.2.11).

The extracellular products of A. salmonicida that were studied in this project appeared in the culture supernate during the late logarithmic and stationary growth phases. A similar pattern of

production has been observed for a variety of other gram negative bacteria. In the case of Pseudomonads many of the extracellular products are secondary metabolites (Leissinger and Margraff 1979) and this possibility may explain the pattern of production of some or all A. salmonicida extracellular products. Alternatively, the extracellular products may be produced during the logarithmic phase of growth and activated or released during the stationary growth phase. Bacteria harvested at the end of the logarithmic phase of growth, concentrated to above normal cell densities and incubated in PBS appeared to release very little H-lysin suggesting that the H-lysin is not formed within the cell during the logarithmic growth phase. It is likely that the extracellular products are released on cell death or that the cell wall undergoes permeability changes as the cell ages. The possibility that the cell wall permeability changes at the end of the logarithmic phase of growth has been demonstrated for the gram negative bacterium Salmonella typhimurium (Lindsay et al 1973), the authors suggesting that changes in the lipopolysaccharide allowed the release of alkaline phosphatase. Because the stationary phase of growth in reality reflects equilibrium between cell division and cell death this would result in the extracellular products being produced throughout the stationary phase of growth. Attempts to extract H-lysin from A. salmonicida cells at various stages in the growth cycle were unsuccessful. This may reflect the fact that the lysin was present in the cells in the form of an inactive precursor and/or the fact that H-lysin was exported from the cells shortly after synthesis. Alternatively,because the stationary growth phase of a culture contains individual cells which are of various ages,only a proportion of the cells at a given time would contain H-lysin,which may be below detectable levels. Later work in this project demonstrated that the H-lysin was apparently synthesised as a precursor molecule, activated by proteolytic cleavage however, treatment of cell extracts with proteases did not result in the appearance of H-lysin suggesting that the intracellular precursor theory does not explain the results obtained. Attempts to extract haemolytic activity from A. hydrophila cells by Wretlind et al (1973)

were also reported to be unsuccessful.

Limited T-lysin activity was extracted from cells suggesting that active T-lysin may be found within cells. Sonicated AOT cells released relatively high levels of T-lysin, the remaining cell pellet containing limited amounts of an activity which caused incomplete lysis of cells (T_1 activity, 4.2.1). Bacterial cells treated with EDTA, lysozyme and sucrose showed the reverse pattern with limited T-lysin released but high levels of T_1 activity associated with the cell pellet. This result may reflect the association of the T_1 activity with cell wall components, an association which was disrupted by sonication.

The experiment failed to indicate likely sites of extracellular product formation However, it is interesting to note in this context that Aurstad and Dahle (1972), using electron microscopic techniques, found melanin like granules in the periplasmic space of A. salmonicida.

When a variety of A layer positive (A^+) and A layer negative strains (A^-) strains of A. salmonicida were grown on solid media no correlation was observed between levels of H-lysin, T-lysin or caseinase production and possession of the A layer. Karlsson (1962) also found little difference between haemolysin production by six strains of A. salmonicida. This result was confirmed by broth culture studies of extracellular product formation by A^+ and A^- strains. However, it is difficult to relate this observation to the in vivo situation when host conditions may influence the production of these activities. When extracellular products from an A^+ and an A^- strain were examined electrophoretically some differences were observed between broth culture extracellular products, several proteins being present in the A^+ sample which were absent in A^- samples. It is not known whether these proteins are important as virulence determinants or whether they merely reflect interstrain variations. One of the additional bands may represent the leucocidin described by Fuller et al (1977) which they found to be produced in greater quantities by virulent A. salmonicida strains. In view of the fact that no differences were observed in the electrophoretic profiles

of AOT extracellular products from the A^+ and A^- strain, the importance of the additional A^+ extracellular products is difficult to evaluate. Karlsson (1964) reported that there were no differences in the antigens from 12 strains of A. salmonicida after testing sonicated cell extracts against an antiserum, using a double diffusion technique.

Although there was no apparent difference in the levels of extracellular products produced by A^+ and A^- strains of A. salmonicida, the results obtained indicated that the onset of extracellular product release was delayed in A^+ cultures. The observation that the A layer acts as a barrier to the release of extracellular products is in agreement with the finding that the A layer prevents access of macromolecules to the outer membrane (Kay et al 1981). The delayed release of extracellular products may reflect changes in the integrity of the A layer at the beginning of the stationary phase of growth. As discussed previously a similar phenomenon involving S. typhimurium LPS has been reported (Lindsay et al 1973). It is not clear why the effect was not observed in unshaken cultures of A. salmonicida, it may reflect fundamental differences in A layer structure or integrity under these cultural conditions. The observation that the A layer, possessed by virulent strains of A. salmonicida, delays the release of extracellular products appears paradoxical. The importance of the A layer as a virulence determinant may outweigh the disadvantage of later release of aggressins by the bacterium, however; it is possible that the delayed release of aggressins is not disadvantageous to the bacterium. It may only be after the initial focus of infection has been established that dissemination of the bacterium within the host body or outside the host body to infect new hosts is important. Until the roles of various extracellular products in the pathogenesis of disease are established this possibility cannot be fully evaluated.

There are a number of areas which merit further attention. A detailed study of the effects of growth medium components on T and H-lysin production may prove particularly valuable for future

purification studies. It is not clear whether haemolysin production is inducible or whether yields can be increased further. In this respect it may be useful to investigate the effect of addition of various nutrients to a simple growth medium which does not support haemolysin production. Although iron levels do not appear to affect haemolysin production the incidental finding that bacteria are able to grow when there was no free iron available suggests that A. salmonicida possesses an iron chelating system which merits investigation.

The results obtained suggest that H-lysin, T-lysin and caseinase are not virulence determinants for the bacterium. These extracellular products may function as aggressins in vivo. However, before this possibility can be investigated it is important to establish that such extracellular products are produced by the bacterium in vivo. This goal may not be achievable until the individual factors have been purified and studied in vitro in greater detail. Of the extracellular products described in this section of the project, the H-lysin appears to have received little or no attention; the T-lysin may also merit further attention in view of a recent report indicating that haemolytic activity against trout erythrocytes is important in the pathogenesis of A. hydrophila mediated disease of trout (Allan and Stevenson 1981). It is not clear whether this haemolytic activity is similar to A. salmonicida T-lysin or whether it is a separate entity to the A. hydrophila haemolysins I and II described by other workers (Wretlind et al 1973, Bernheimer et al 1975, Thelestam et al 1981).

Of particular interest is the possibility that the A layer and/or cell wall act as permeability barriers whose properties change with cell age. Electron microscopic and outer membrane protein labelling techniques applied to cultures of various ages may indicate changes in the A layer integrity as the cell ages.

CHAPTER 4. THE INTERRELATIONSHIPS OF A. SALMONICIDA EXTRACELLULAR PRODUCTS

4.1.0 INTRODUCTION

Extracellular products of some bacteria have been shown to be inter-related. For example, such a situation is observed with S. aureus PV leucocidin, where leucocytolytic activity is attributable to two components acting synergistically, the components being inactive individually. In other cases,one bacterial product may be responsible for a variety of effects observed in vivo and in vitro.

In view of the diversity of extracellular products released by A. salmonicida, and the limited information concerning these activities, it was decided to investigate potential interactions between individual activities. In particular, the relationship between proteolytic and haemolytic activities was investigated in this section of the project.

4.2.0 THE SEPARATION OF EXTRACELLULAR PRODUCTS

4.2.1 The separation of extracellular products by gel filtration chromatography

Initial work involved the study of patterns of separation of extracellular products by virtue of molecular size differences, using gel filtration chromatography.

Material obtained after ammonium suphate precipitation of unshaken culture supernate (2.3.1) was lyophilised, resuspended in distilled water (4 fold concentration effected) and applied to a column of sepharose 6B (1·7 cm x 30 cm). The column was eluted with phosphate buffered saline (PBS) and the fractions collected (3.7 ml) tested for H-lysin, T-lysin and caseinase activity.

The results indicated that these activities could be separated using this technique (Fig. 13). The T-lysin was separated into two components; a large peak of activity tailing back from the void volume contained an activity which when tested in the microtitre assay against trout erythrocytes (2.4.2) caused the well contents to become more translucent

in appearance without becoming completely clear. No concomitant activity against horse erythrocytes was detected in these fractions. This activity was termed the T_1 activity.

Eluted after the T_1 activity was an activity which caused complete clearing of microtitre tray wells containing trout erythrocytes. This activity was considered to be similar to the T-lysin in crude culture supernate. No concomitant activity against horse erythrocytes was detected in these fractions.

The H-lysin was the last activity to be eluted from the column, between the H-lysin and T-lysin activities caseinase activity was detected.

The results also indicated that the caseinase activity extended into the T-lysin peak. By extrapolation, the T_1 activity also appeared to extend into the T-lysin peak. It was not possible to confirm this latter suggestion since fractions containing T-lysin 'masked' any T_1 activity.

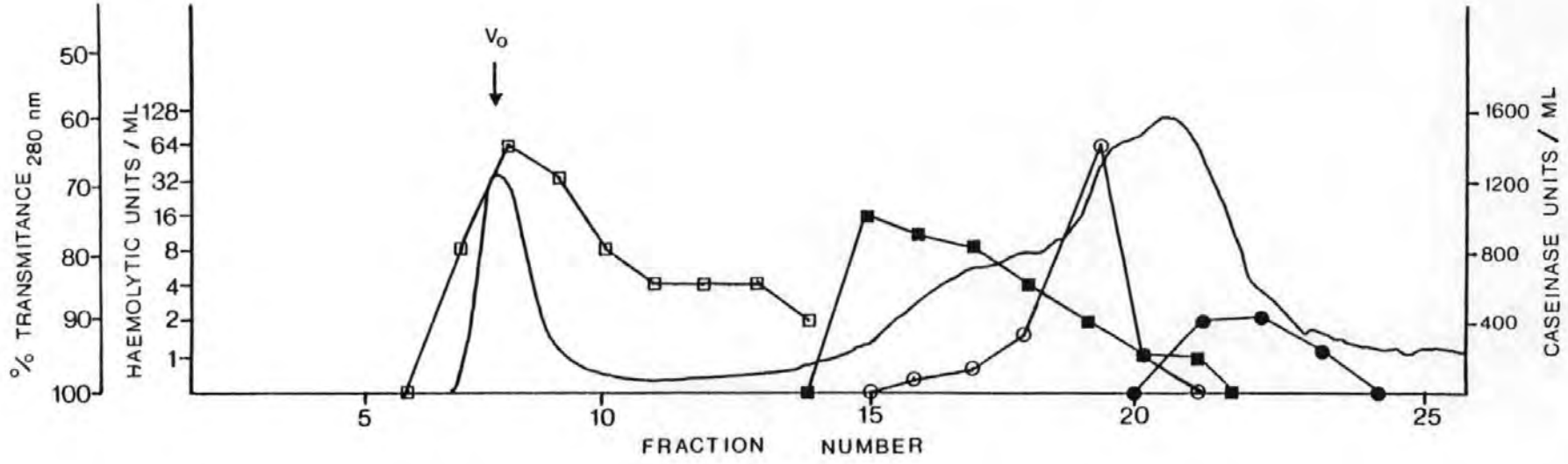
To test the possibility that T-lysin activity reflected the combined effects of T_1 and caseinase activities, fractions containing T_1 activity (fractions 8 + 9) or caseinase activity (fractions 18 + 19) were mixed together and tested for haemolytic activity. The results (Fig. 14) indicated that whilst T_1 and caseinase containing fractions possessed no T-lysin activity individually, when mixed together T-lysin was detected (8 Hu/ml).

In a separate experiment, fractions containing H-lysin (fractions 22 + 23) were mixed with T_1, caseinase or T-lysin containing fractions. No change in T-lysin or H-lysin titres was seen under these conditions.

4.2.2 Electrophoretic analysis of extracellular products obtained using the agar overlay technique

In view of the results obtained after gel filtration of extracellular products it was considered that the use of an electrophoretic technique would facilitate a more detailed study of extracellular product identities. Work was initially performed using extracellular product obtained using the agar overlay technique (AOT); this preparation was rich in T-lysin and caseinase activity (3.2.4).

Figure 13 The separation of ammonium sulphate precipitated broth culture extracellular products using gel filtration chromatography.



— = Optical density (280 nm)

□ = T_1 activity (Hu/ml)

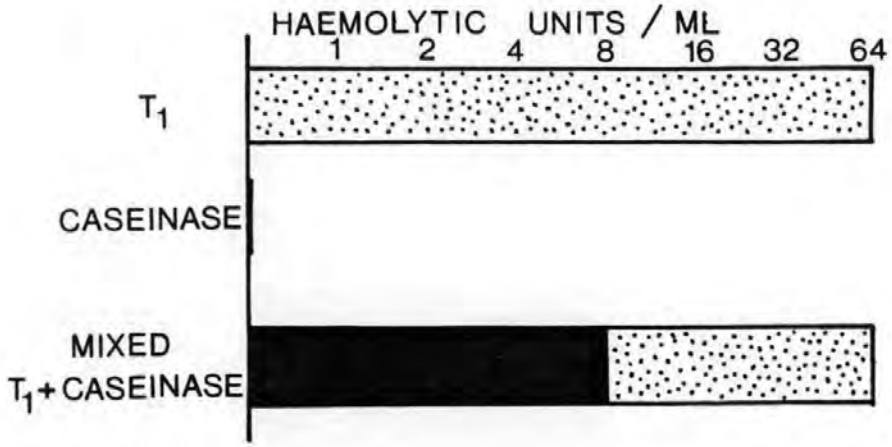
■ = T-lysin activity (Hu/ml)

● = H-lysin activity (Hu/ml)

○ = Caseinase activity (Units/ml)

V_o = Void volume

Figure 14 The effect of mixing T_1 activity and caseinase activity on the haemolysis of trout erythrocytes.



T_1 activity and caseinase obtained after gel filtration chromatography of ammonium sulphate precipitated extracellular products were mixed and tested for haemolytic activity against trout erythrocytes.

▨ = Partial haemolysis of trout crythrocytes

■ = Complete haemolysis of trout erythrocytes

The extracellular products were lyophilised and resuspended in distilled water (2.32 mg protein/ml). 20 µl aliquots of the material were pipetted into the wells of a polyacrylamide slab gel (T = 7.5%) and electrophoresed under constant current conditions at 4°C (2.5.1). After electrophoresis, the gel was sliced into individual tracks and the tracks overlaid with horse erythrocyte, trout erythrocyte, egg yolk, casein, gelatin or tributyrin agars (2.5.2). After incubation, the Rf values of zones of digestion were determined. One gel track was stained to locate protein bands and a densitometer trace of the stained gel obtained (2.5.3).

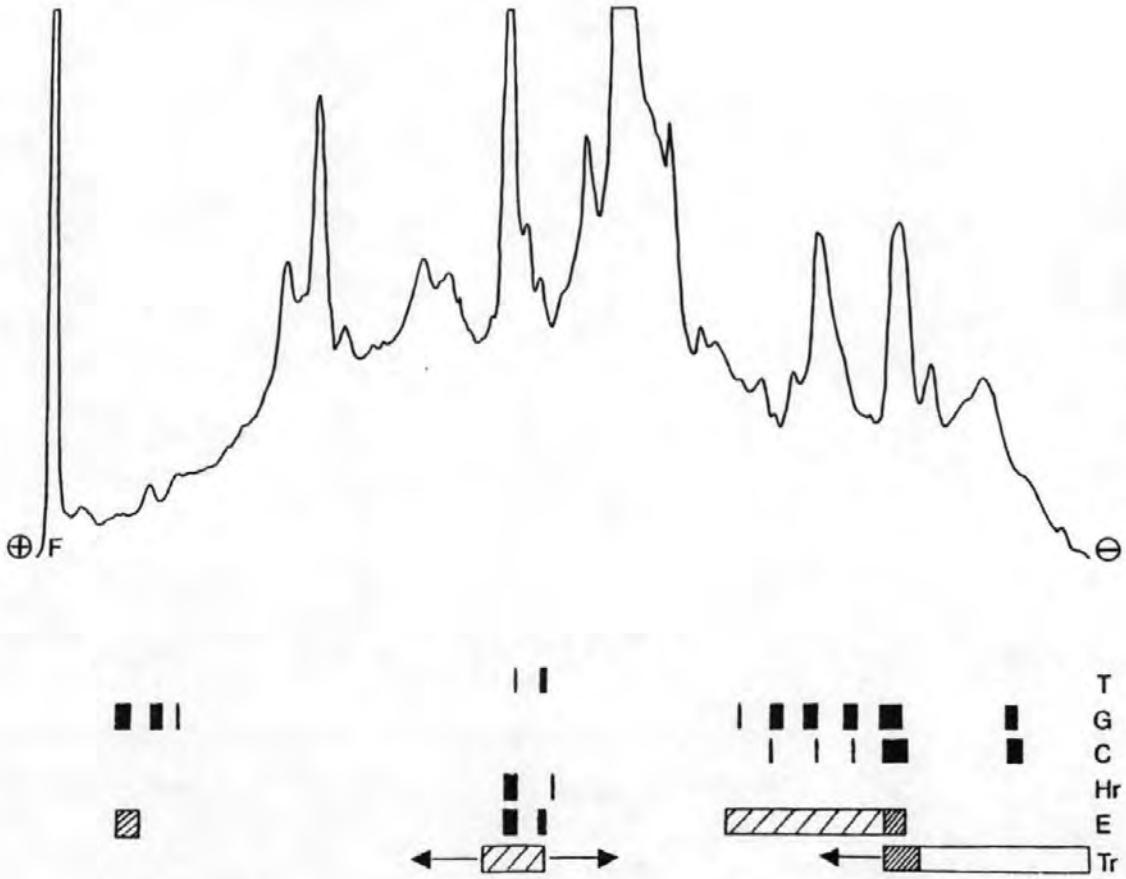
The results (Fig. 15) indicated that 37 protein bands were visible Nine zones of gelatin digestion were observed, five of these zones also appeared to possess caseinase activity and these five zones were located closely together. Three zones of gelatinase activity appeared as a group located close to the tracking dye front and showed no caseinase activity.

Activity against trout erythrocytes was not located as a discrete band but appeared as a diffuse track of activity with maximal activity coinciding with the main caseinase activity digestion zone. Prolonged incubation of trout erythrocyte overlays resulted in the lysis of cells along the whole length of the gel track.

Overlays with egg yolk agar revealed a diffuse zone of digestion coinciding with the main zone of caseinase digestion in addition to two zones of complete digestion at Rf values of approximately 0.6. These zones showed no concomitant caseinase or gelatinase activity though limited and diffuse trout erythrocyte lysis was seen at this location. Tributyrin agar overlays revealed two zones of digestion corresponding to the zones of complete digestion seen with egg yolk agar overlays.

Overlays of horse erythrocyte agar indicated that limited activity against this substrate coincided with the tributyrin agar digestion zones and the zones of complete egg yolk agar digestion.

Figure 15 The separation of extracellular products obtained by the agar overlay technique, using a zymogram technique.



Extracellular products were separated in a polyacrylamide gel (7.5%) and gel tracks overlaid with various substrates to detect individual extracellular products.

T = tributyrin agar. G = gelatin agar. C = casein agar.
Hr = horse erythrocyte agar. E = egg yolk agar.
Tr = trout erythrocyte agar

Hatching indicates visually estimated activity

4.2.3 Electrophoretic analysis of broth culture extracellular products

Extracellular products obtained by the agar overlay technique had previously been found to contain very low levels of H-lysin activity (3.2.4). It was therefore considered that electrophoretic analysis of unshaken broth culture extracellular products, containing relatively high levels of H-lysin activity (3.2.15) may have indicated the electrophoretic identity of the H-lysin.

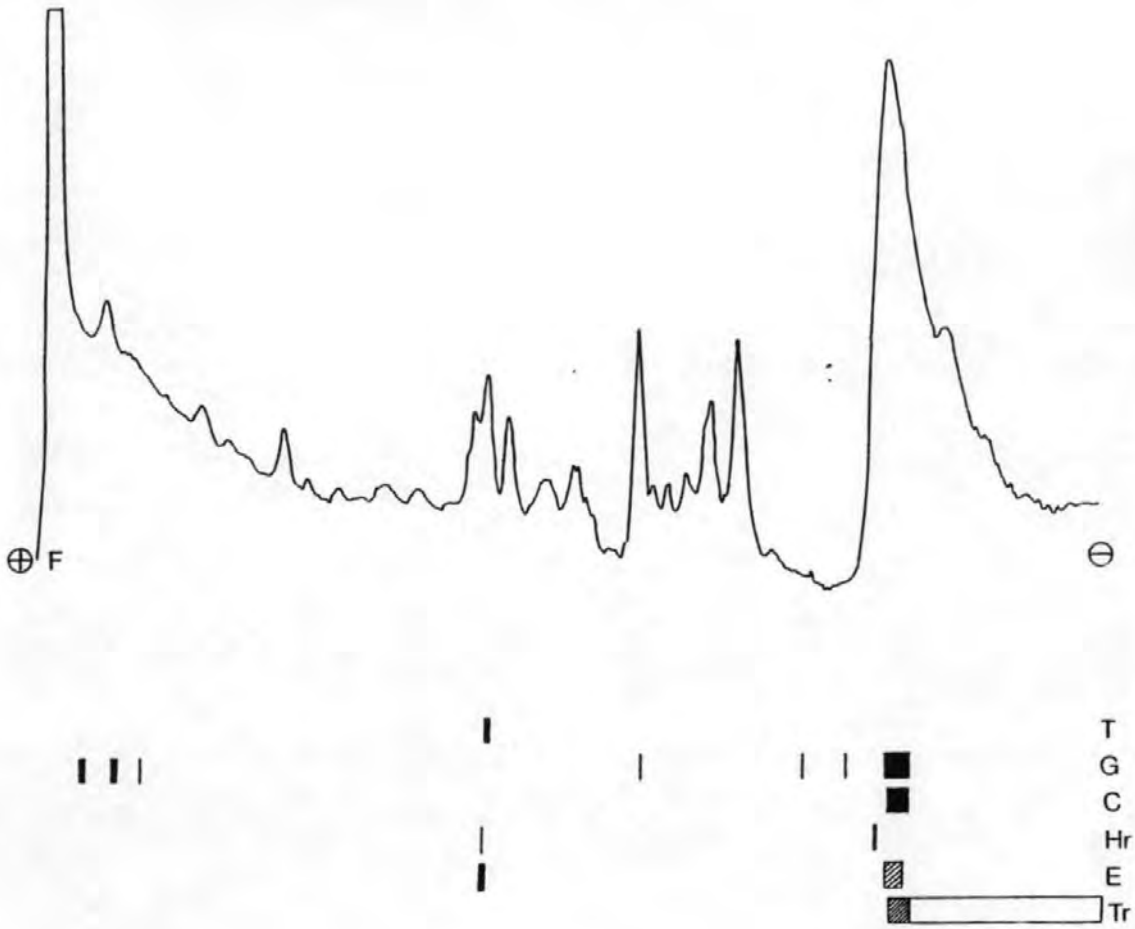
Initial experiments indicated that, even after extensive dialysis of crude unshaken broth culture supernate (against 2 x 1 l distilled water), this material could not be separated using electrophoretic techniques. The culture supernate was therefore treated with ammonium sulphate to precipitate haemolytic activity (40-60% saturation) (2.3.1), dialysed (2 x 1l PBS, $4^{o}C$, 18 hr) and lyophilised. After resuspension to 1/10th of its original volume (distilled water) the material was dialysed against distilled water ($0^{o}C$, 6 hr, 2 x 1l) and the non-dialysable material again lyophilised. This preparation was resuspended in electrophoresis sample buffer to 1/100th of the starting volume. The concentrated material (5.6 mg/ml) was electrophoresed and overlaid to detect extracellular products as detailed previously (4.2.2).

The results (Fig. 16) indicated that gel tracks stained to detect proteins indicated the presence of 21 distinct bands. Overlays with gelatin agar revealed 7 zones of digestion, the major digestion zone coincided with the only zone of caseinase digestion detected. A group of 3 zones of gelatin digestion was observed close to the tracking dye front.

Overlaying tracks with trout erythrocytes revealed a diffuse band of cell lysis extending from the top of the gel with maximal activity coinciding with the caseinase activity digestion zone.

Egg yolk agar overlays revealed the presence of a diffuse band of activity at a similar Rf value to the caseinase digestion zone. A narrow zone of complete digestion was also observed with an Rf value of approximately 0.6; this zone appeared to coincide with the only zone of tributyrin agar digestion observed.

Figure 16 The separation of ammonium sulphate precipitated, unshaken broth culture extracellular products using a zymogram technique.



Extracellular products were separated in a polyacrylamide gel (7.5%) and gel tracks overlaid with various substrates to detect individual extracellular products.

T = tributyrin agar. G = gelatin agar. C = casein agar. Hr = horse erythrocyte agar. E = egg yolk agar. Tr = trout erythrocyte agar.

hatching indicates visually estimated activity

Overlaying a gel track with horse erythrocyte agar revealed a narrow zone of activity with a similar Rf value to the zones of tributyrin and egg yolk agar digestion. In addition, a larger zone of horse erythrocyte lysis was observed at an Rf value of 0.22. This zone did not appear to coincide with any of the caseinase or gelatin digestion zones.

4.3.0 STUDIES CONCERNING A. SALMONICIDA T-LYSIN

4.3.1 Investigation of the nature of electrophoretically separated T lysin

The diffuse band of T-lysin activity, followed ultimately by the complete lysis of the gel track, observed after electrophoretic analysis of the T-lysin suggested that the activity was either heterogenous with respect to molecular weight and/or charge. Alternatively, the techniques used to separate or detect the T-lysin may have been unsuitable. To investigate these possibilities a number of experiments were performed.

Samples of AOT extracellular products were electrophoresed as detailed previously (4.2.2) and the gel tracks treated using a variety of different techniques; tracks containing sample buffer only were overlaid with trout erythrocyte agar. These tracks showed no lysis of the erythrocytes on incubation.

In an experiment to investigate the possibility that the polyacrylamide gel was binding the T-lysin, preventing effective separation, extracellular product samples (2 ml, 32 Hu/ml T-lysin) were mixed with a polyacrylamide gel filtration medium (1 ml packed Bio-Gel P.100). After incubation (90 min, 4°C) the supernate was tested for T-lysin activity. No loss of activity was detected in comparison with control samples (2 ml extracellular products + 1 ml P B S).

Reduction of the quantity of material used for electrophoretic separation (20 µl, 116 µg/ml or 58 µg/ml) did not affect the form of the T-lysin separation, though the time taken for the lysis of overlaid trout erythrocytes was increased.

Attempts to stain gel tracks of electrophoresed extracellular products for the presence of glycoproteins (2.5.4) or lipoproteins (2.5.4) did not reveal the presence of these materials associated with T-lysin activity. Pre-staining extracellular products for lipids (2.5.4) also failed to indicate any correlation. Samples of human serum separated electrophoretically (2.5.4) showed 14 bands when stained for glycoproteins and 1 band when stained for lipoproteins using either the pre-stain or post-stain technique.

In order to investigate the possibility that the T-lysin was associated with acetyl-amino-polysaccarides, leading to apparent molecular heterogeneity, samples of extracellular products were treated with lysozyme. Samples (4.64 mg/ml protein) were incubated with lysozyme (140 µg/ml in sample buffer, 90 min, 4°C) prior to electrophoretic analysis. Overlays with trout erythrocytes after electrophoresis showed similar patterns of lysis with both lysozyme-treated and control (no lysozyme) tracks. Tracks of electrophoresed lysozyme only showed no lysis of trout erythrocytes.

The poor separation of the T-lysin in polyacrylamide gel electrophoretic system may have reflected the heterogenous molecular weight properties of this activity (4.2.1). In view of the fact that separation in polyacrylamide gels is partially dependant on molecular weight differences (Lunney et al 1971) it was decided to investigate the electrophoretic mobility of this activity in a polyacrylamide gel with limited ability to differentiate molecules of various molecular weights. Samples of extracellular products containing T-lysin, were electrophoresed in a low concentration polyacrylamide gel (T = 5%). After overlaying these gels with trout erythrocyte agar (2.5.2) or casein agar (2.5.2) zones of activity against these substrates were observed at similar Rf values (1 hr incubation, 25°C). Further incubation resulted in the lysis of trout erythrocytes along the whole length of the gel track.

4.3.2 The repression of caseinase production by *A. salmonicida*

The results obtained suggested that caseinase activity was required for the full expression of T-lysin activity. Liu et al (1969) previously reported that *A. salmonicida* caseinase production could be repressed if ammonium sulphate was added to the growth medium. This technique was utilised to investigate the role of caseinase in the expression of T-lysin activity.

Ammonium sulphate (BDH heavy metal free) was added to TSA containing sodium caseinate (1% w/v) to give final concentrations of 2%, 2.5%, 3% and 3.5% ammonium sulphate (w/v).

Stab inocula of *A. salmonicida* strain 25/77 were grown on this medium (72 hr, 25°C) and examined for caseinase production (2.4.1). The results suggested that 3% ammonium sulphate completely repressed caseinase production (Plate 8). Growth was, however, also retarded using this technique. Ammonium sulphate (3% w/v) was added to egg yolk, tributyrin, gelatin, trout erythrocyte and horse erythrocyte agars (2.4.1) and the bacterium stab inoculated onto these plates. After growth for 72 hours (25°C), plates were examined.

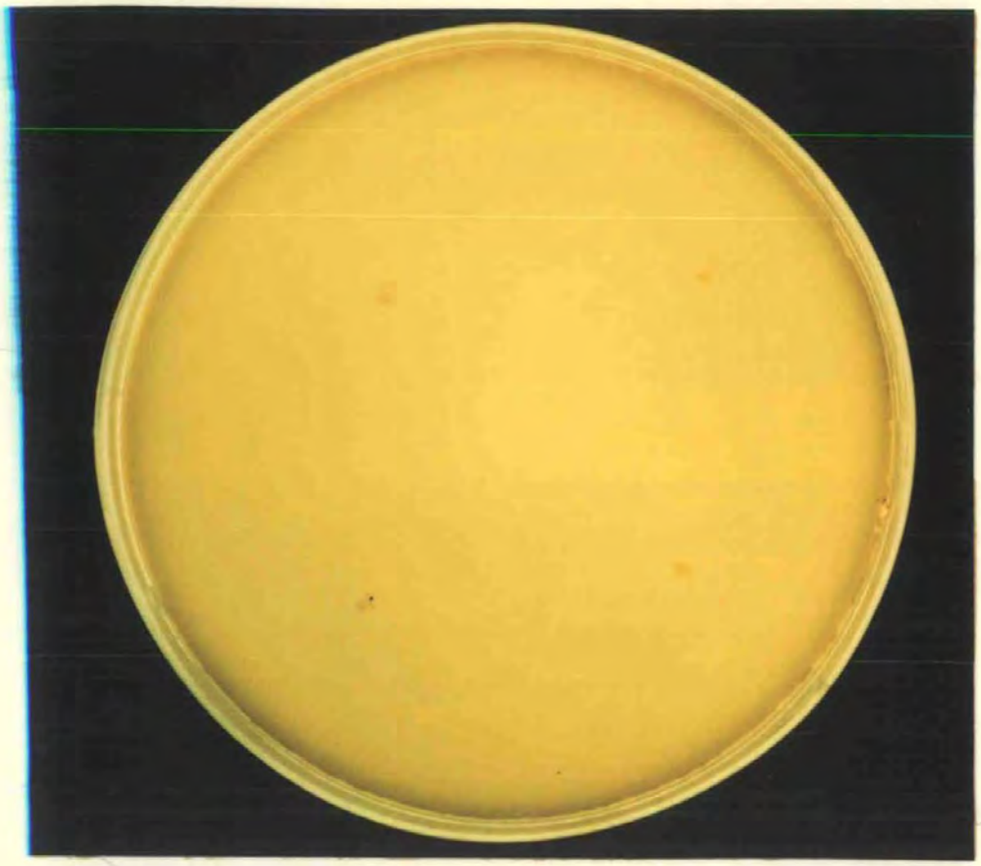
The results indicated that gelatinase activity was still detectable though loss of the clear zone of digestion was noted (3.2.1) (Plate 9). Growth on egg yolk agar revealed the loss of the hazy zone of digestion though the zone of complete digestion was still visible (3.2.1)(Plate 10).

Growth on trout erythrocyte agar indicated that the clear zone of lysis surrounding colonies on agar without ammonium sulphate was not visible when ammonium sulphate was added to the medium. The "orange" and "white" zones surrounding colonies were visible in medium with or without ammonium sulphate (Plate 11) (3.2.1).

Zones of lysis around colonies on horse erythrocyte agar (+3% ammonium sulphate) also showed a zone of partial lysis surrounding the colony, distinguishable from the clear zone (horse erythrocyte agar without ammonium sulphate) only on close examination of the plates (Plate 12) (3.2.1).

Plate 8 The effect of ammonium sulphate (3% w/v) on the production of caseinase by A. salmonicida

Plate 9 The effect of ammonium sulphate (3% w/v) on the production of gelatinase activity by A. salmonicida



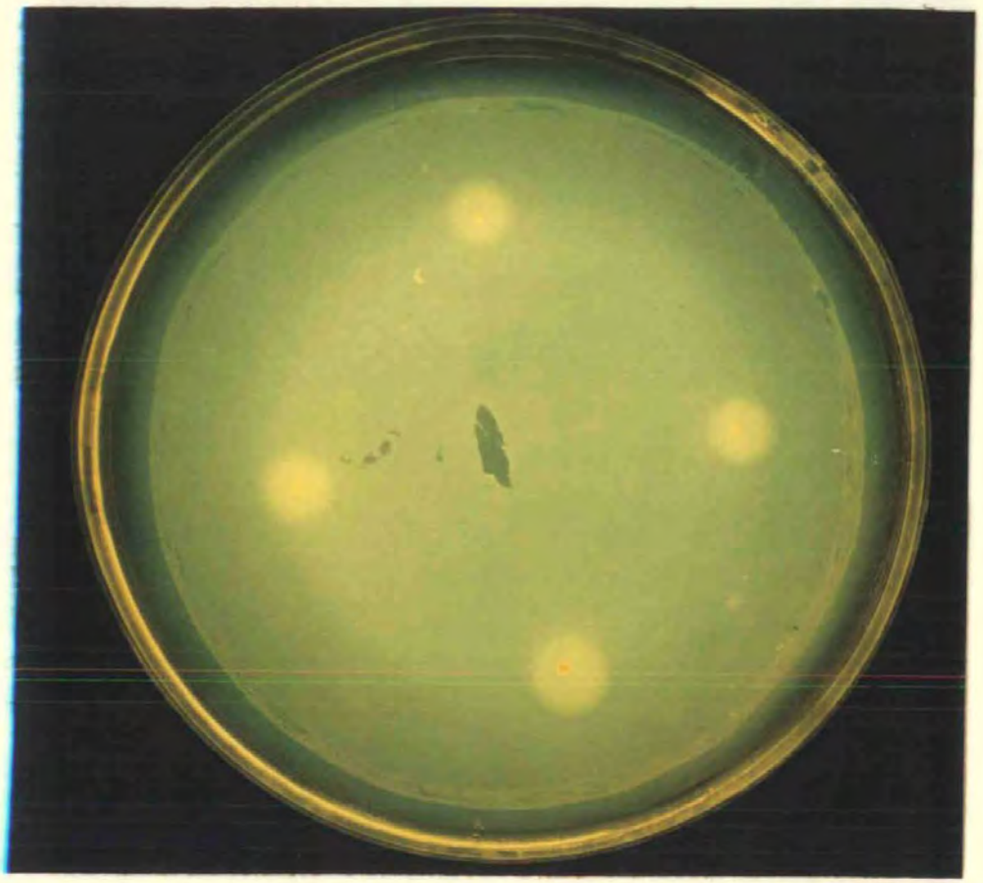
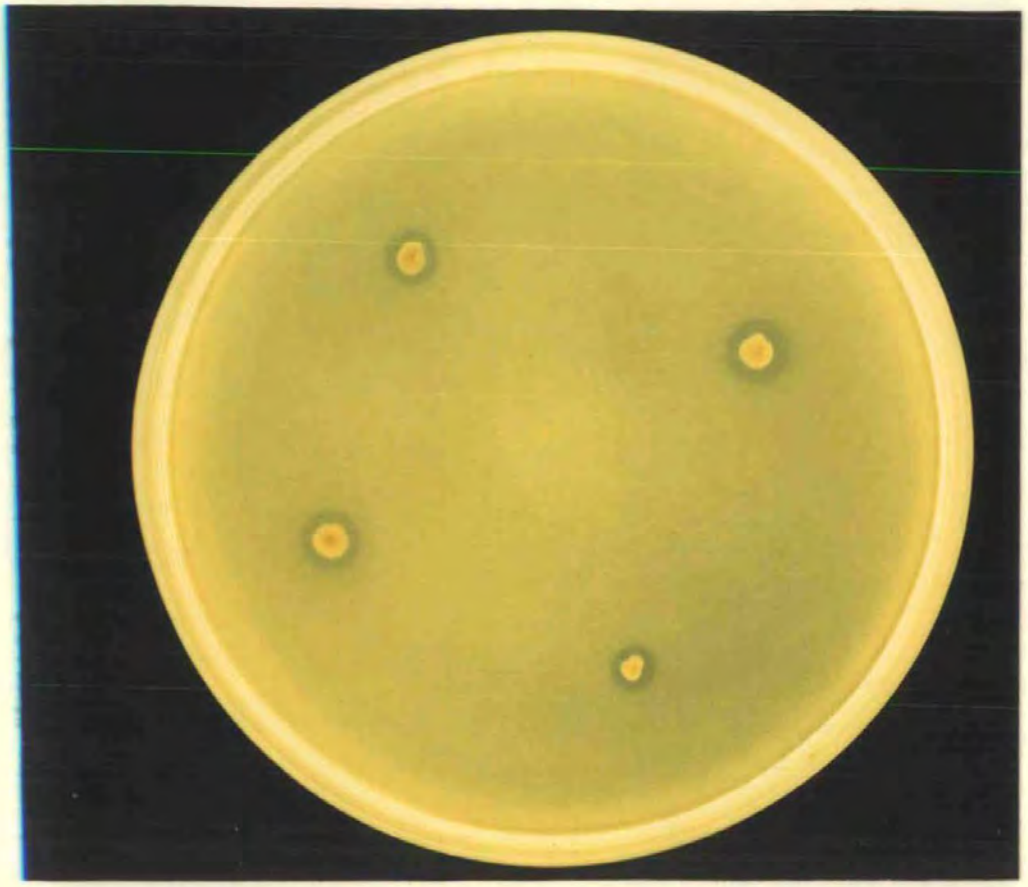


Plate 10 The effect of ammonium sulphate (3% w/v) on the production of egg yolk digesting activity by A. salmonicida

Plate 11 The effect of ammonium sulphate (3% w/v) on the production of haemolytic activity against trout erythrocytes by A. salmonicida



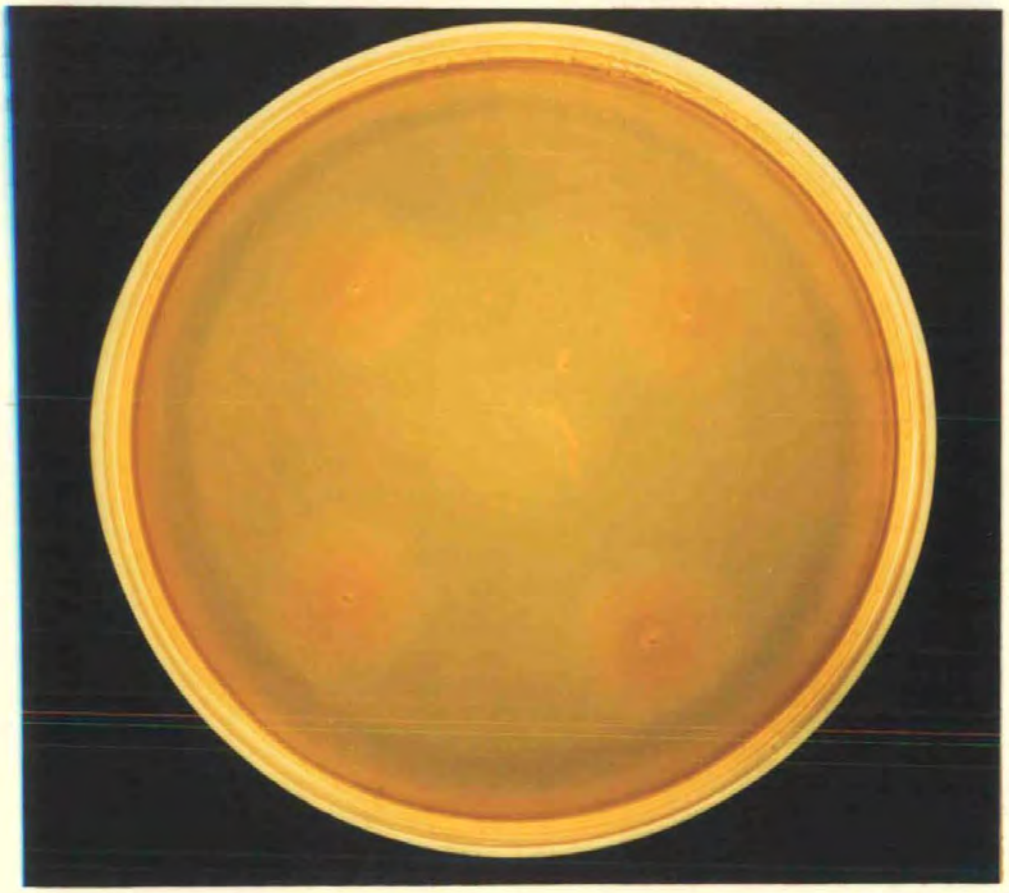
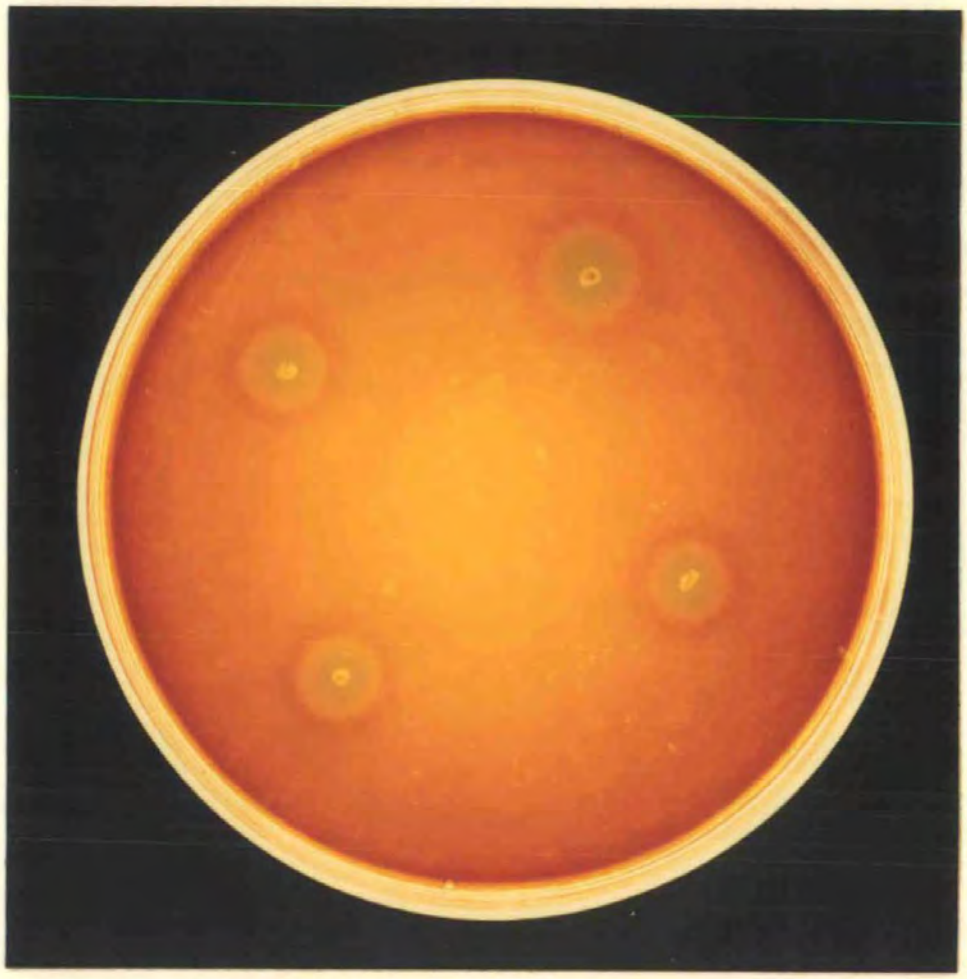


Plate 12 The effect of ammonium sulphate (3% w/v) on the production of haemolytic activity against horse erythrocytes by A. salmonicida



Zones of digestion on tributyrin agar were unaffected by the addition of ammonium sulphate.

Control experiments indicated that the addition of ammonium sulphate (3% w/v) to supernate samples did not affect the activity of preformed caseinase (3,700 units/ml). The addition of ammonium sulphate (3% w/v final concentration) to the microtitre tray haemolysin assays indicated that preformed T-lysin activity was unaffected (16 Hu/ml) though H-lysin activity (16 Hu/ml) was enhanced in the presence of the ammonium sulphate (48 Hu/ml).

4.3.3. Investigation of the relationships of haemolytic activity using caseinase-negative mutants

The results obtained in previous experiments all indicated that A. salmonicida caseinase was involved in T-lysin activity. It was decided that studies using caseinase negative mutants of A. salmonicida would confirm this observation.

Two caseinase negative strains of bacteria were obtained after mutagenesis (2.7.3). These strains were confirmed as A. salmonicida using biochemical tests (2.2.3) and were termed Cl^- and $C2^-$. Cl^- and $C2^-$ were grown on TSA containing a variety of substrates for extracellular product activities (3.2.1) (72 hr, 25°C)

The results (Table 14) indicated that the mutants possessed no caseinase activity (Plate 13) though gelatinase activity was still detectable.

Growth on trout erythrocyte agar revealed loss of the clear zone of haemolysis, the orange and white zones(3.2.1) were however unaffected (Plate 14).

Zones of digestion of tributyrin agar were similar with both wild type and $Cl^-/C2^-$ strains.

Growth on washed horse erythrocyte agar revealed the unexpected result that complete loss of the clear zone of haemolysis was observed. After prolonged incubation (72 hr, 25°C) the zone of partial haemolysis and the red halo were however observed (3.2.1) (Plate 18).

Strains $C1^-$ and $C2^-$ were grown in nutrient broth No. 2 with or without shaking (3.2.3) and the supernates tested for H-lysin and T-lysin activity. The results indicated that no H-lysin was detectable, using the microtitre tray haemolysin assay (2.4.2). Using a similar assay technique no T-lysin activity was detectable against trout erythrocytes, however, partial haemolysis of cells, characteristic of the T_1 activity was detected (8Hu/ml).

4.3.4 The effects of T_1 activity and T-lysin on trout erythrocytes

The T- lysin caused complete lysis and clearing of trout erythrocytes in microtitre tray wells whilst the T_1 activity appeared to cause partial lysis of the cells (4.2.1). To investigate this effect T_1 activity and T-lysin separated by gel filtration chromatography (4.2.1) were mixed, after suitable dilution, with trout erythrocyte suspensions (9.75×10^7 cells/ml in PBS). Samples of the mixture (40 µl) were spread over an area of a clean glass side and a coverslip placed over the slide. After incubation (37^oC, 1 hr), the slides were examined using light microscopy (Zeiss photomicroscope, green filter, x80 magnification). The results (Plate 15) indicated that cells treated with T_1 activity (4 Hu/ml) became crenated in appearance with a decrease in size, however the cells were intact. Cells treated with T-lysin (4 Hu/ml) (results not shown) were apparently completely lysed and only small particles of cell debris were visible.

In a later experiment, trout erythrocyte suspensions were mixed with T_1 activity (32 Hu/ml) or T-lysin (32 Hu/ml) (0.5 ml + 0.5 ml) in a microcentrifuge tube (Eppendorf), incubated (1 hr, 37^oC) and centrifuged (10,000 x g, 10 min. 4^oC).

Examination of centrifuge tubes revealed the presence of a pellet in T_1 treated tubes, the supernate in these tubes contained haemoglobin (OD 540 nm = 0.51 units, Pye Unicam SP1800). Tubes containing T-lysin + trout erythrocytes contained no pellet after centrifugation. The supernate contained haemoglobin (OD 540 nm = 0.55 units); in addition the supernate was of a viscous nature in comparison with T_1 treated supernate.

Table 14 Comparison of extracellular product activities from wild type and caseinase negative mutant bacteria.

Activity	Wild type strain (25/77)	Caseinase negative mutant strains ($C1^-$)	($C2^-$)
Casein digestion	+	-	-
Gelatin digestion	+ +	+	+
Egg yolk digestion	Hazy and clear zones of activity	Clear zone of digestion only	
Tributyrin digestion	+	+	+
Trout erythrocyte lysis	+	Loss of clear zone of haemolysis. Orange and white zones apparent.	
Horse erythrocyte lysis	+	Loss of clear zone of haemolysis. Zones of partial haemolysis and red halo visible on prolonged incubation of cultures.	

Bacteria were grown using a stab inoculation technique on TSA + substrate (2.4.1) for up to 75 hr (25^oC).
When grown on egg yolk agar loss of the hazy zone of digestion was observed though the clear zone of digestion was similar to the wild type digestion zone.

Plate 13 The growth of the caseinase negative mutant Cl^- and the wild type strain 25/77 on TSA + sodium caseinate.

Strain 25/77

Strain Cl^- Strain Cl^-

Strain 25/77

Plate 14 The growth of the caseinase negative mutant Cl^- and the wild type strain 25/77 on TSA + washed trout blood.

Strain 25/77

Strain Cl^- Strain Cl^-

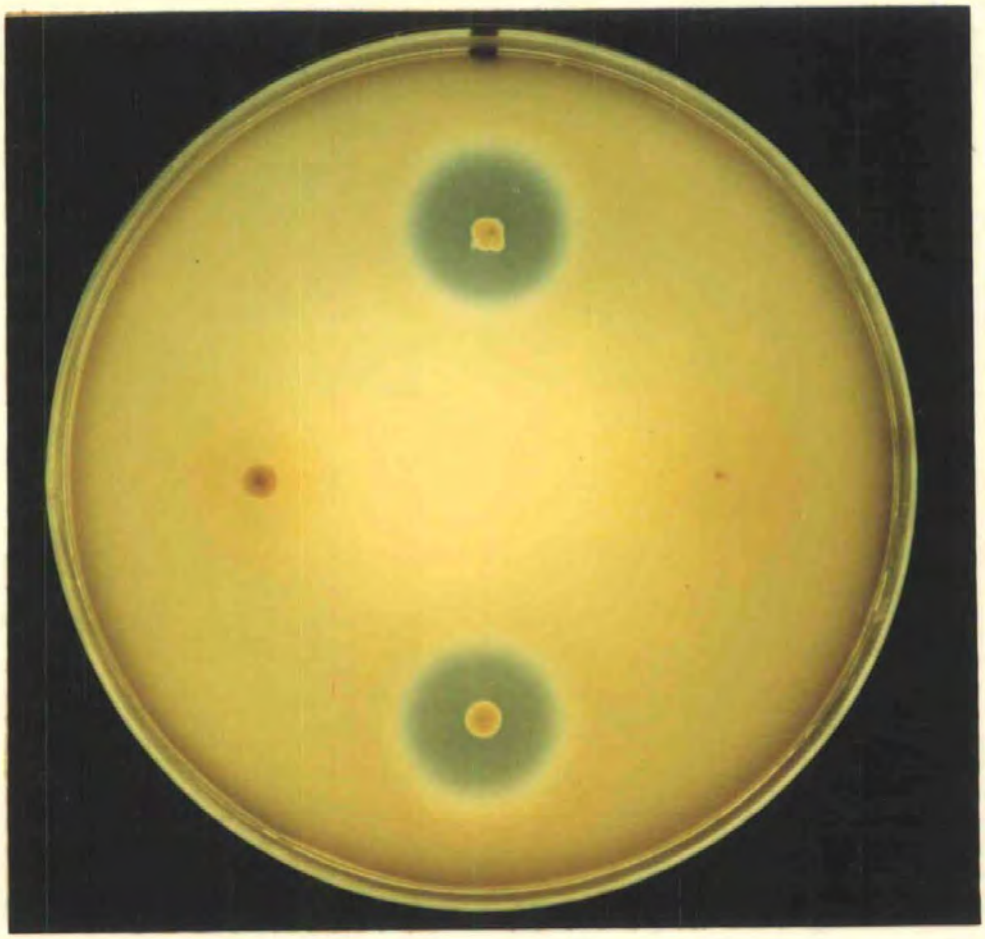
Strain 25/77

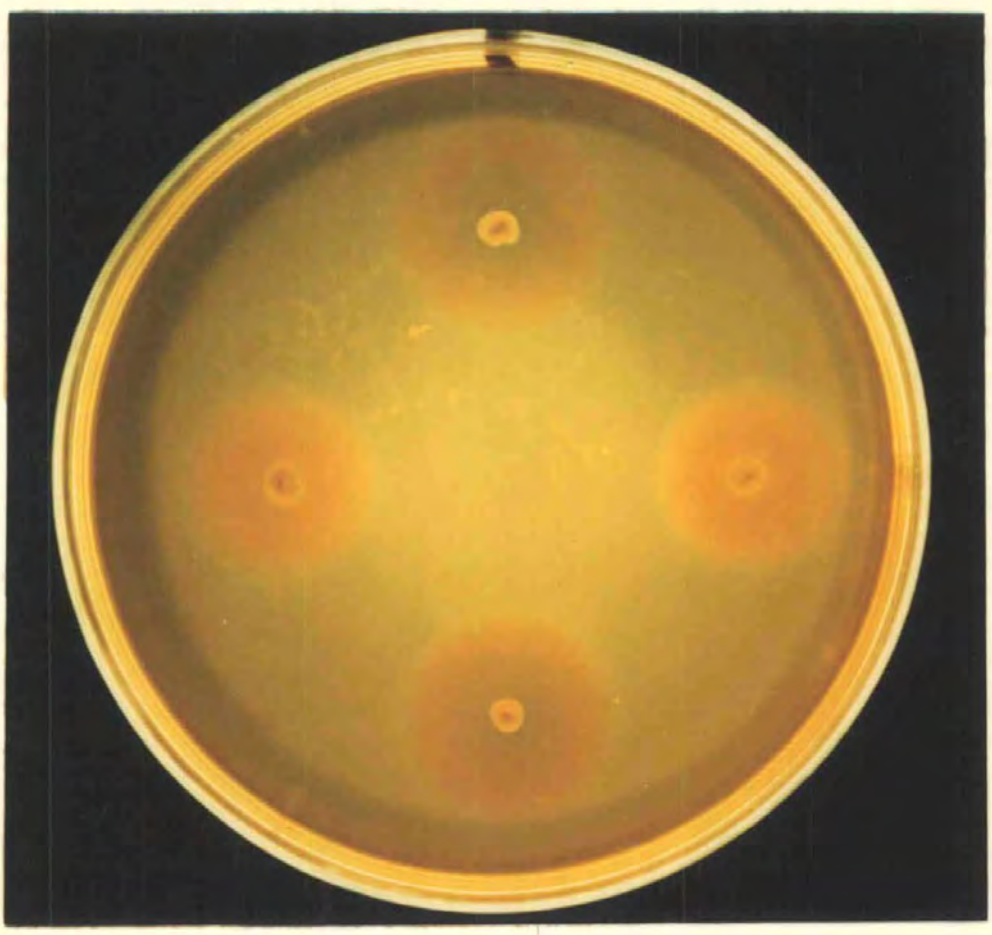
Plate 15a The appearance of trout erythrocytes in PBS visualised using light microscopy

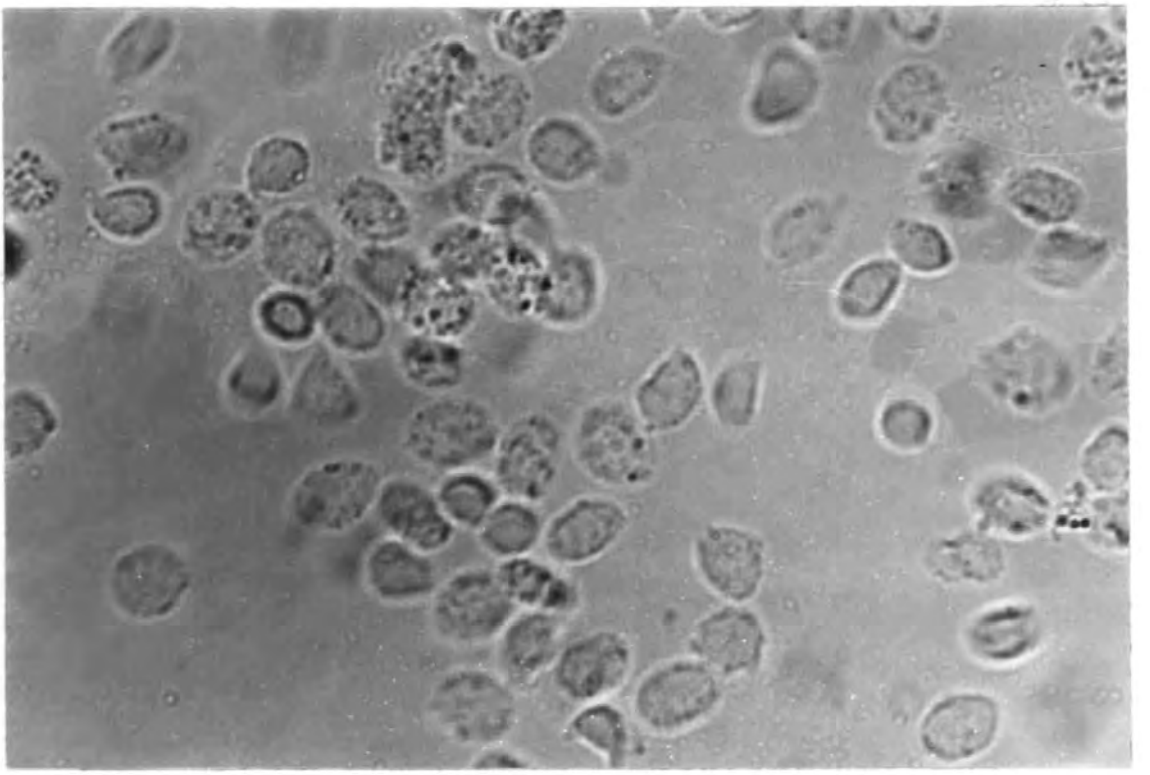
Magnification x 80

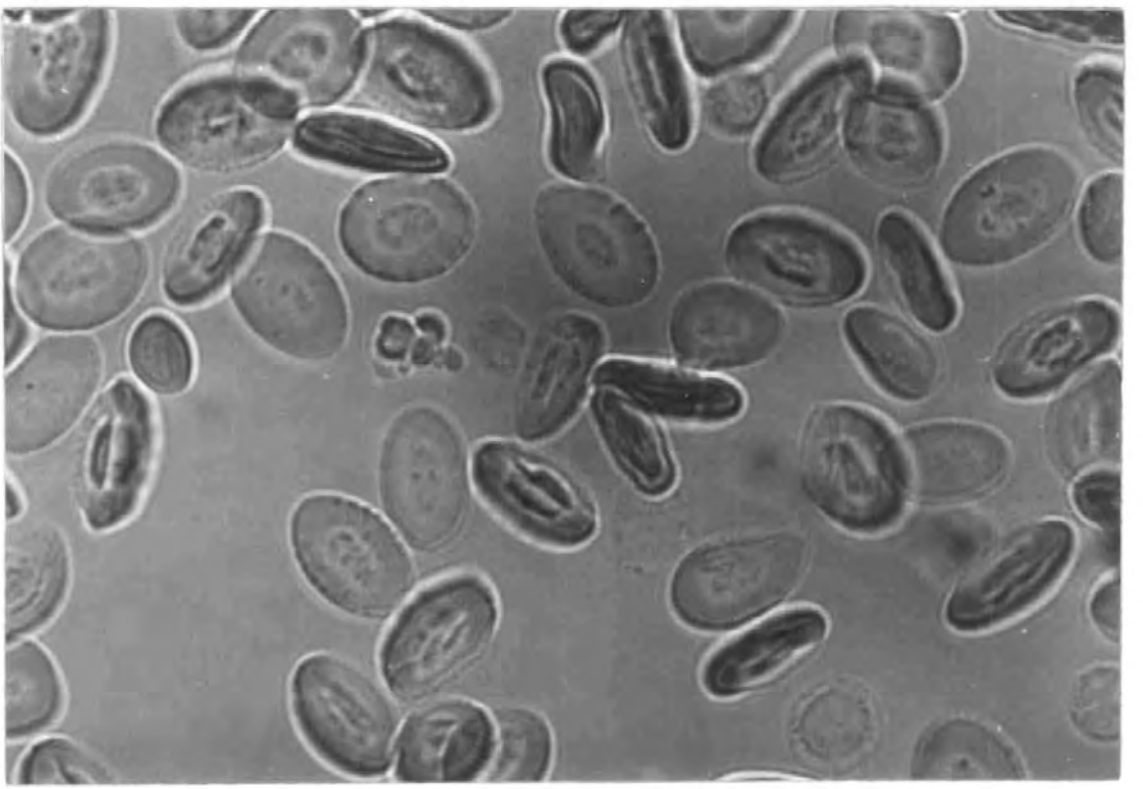
Plate 15b The appearance of trout erythrocytes treated with T_1 activity (4 Hu/ml) visualised using light microscopy

Magnification x 80









The optical density (260 nm) of T_1 treated cell supernate was found to be 1.71 units whilst T-lysin treated cell supernate had an optical density of 1.98 units (Pye Unicam SP 1800 spectrophotometer).

4.3.5 The effect of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) on extracellular product activities

PMSF has been shown to inhibit A. salmonicida caseinase (Sheeran and Smith 1981). It was decided to investigate the effect of this inhibitor on a variety of extracellular products. Agar overlay technique(2.2.5) or broth culture supernate (unshaken, 96 hr 25°C) extracellular products were treated with PMSF (2.7.1) (1 mM final concentration), incubated (2 hr 25°C) and tested for caseinase, gelatinase, T-lysin and H-lysin activities.

Samples(10 µl) were placed into wells (4 mm) in plates of TSA containing appropriate substrate (+0.02% NaN_3) (2.4.1.) and incubated (24 hr 25°C). The results (Table 15) indicated that caseinase activity was completely inhibited by PMSF (Plate 16).

The zones of digestion around wells in TSA + gelatin plates were also affected, the innermost clear zone of gelatin digestion was not apparent after PMSF treatment. The outer cloudy zone of activity was not affected by PMSF treatment,extending to the well edges in the absence of the clear zone. Control samples (+ n-propanol) in place of PMSF in n-propanol showed no loss of caseinase or T-lysin activity.

Zones surrounding wells in TSA + trout erythrocytes were affected by PMSF treatment. The innermost zone of complete haemolysis was not visible after PMSF treatment, though the orange and white zones were unaffected (3.2.1) (plate 17). Zones surrounding wells in TSA + horse erythrocytes showed no changes after PMSF treatment.

The effect of PMSF treatment on the T and H-lysins was also investigated using the microtitre tray assay (2.4.2). After PMSF treatment of the T-lysin (128 Hu/ml) the microtitre tray wells appeared to contain partially lysed cells, similar in appearance to cells treated with the T_1 activity (4.2.1). H-lysin activity (16 Hu/ml) was unaffected by PMSF treatment.

Table 15 The effect of the protease inhibitor PMSF on the activity of various extracellular products of *A. salmonicida*

Activity detected	Appearance of zones surrounding wells in a solid detection medium		Haemolytic activity detected (microtitre tray assay)	
	- PMSF	+PMSF	Hu/ml - PMSF	+PMSF
Caseinase	Clear zones of casein digestion	No zones	-	-
Gelatinase	Inner zone of clear digestion, outer zone cloudy in appearance	Cloudy zone visible only	-	-
T-lysin	Inner zone of complete haemolysis, outer zone orange in appearance. White 'halo' zone	Orange and halo zones visible only	-	-
H-lysin	Inner zone of complete haemolysis, outer zone of partial haemolysis. Red halo zone.	All zones visible	-	-
T-lysin	-	-	128 Hu/ml T-lysin	128 Hu/ml T_1 activity
H-lysin	-	-	16 Hu/ml	16 Hu/ml

PMSF (1 mM final concentration) was added to the samples with incubation (2 hr 25°C) before testing

Plate 16 The effect of PMSF treatment on caseinase activity.

Shaken culture supernate

Shaken culture supernate + PMSF

Shaken culture supernate + propanol

Shaken culture supernate (2nd sample)

PMSF + PBS

Plate 17 the effect of PMSF treatment on T-lysin activity

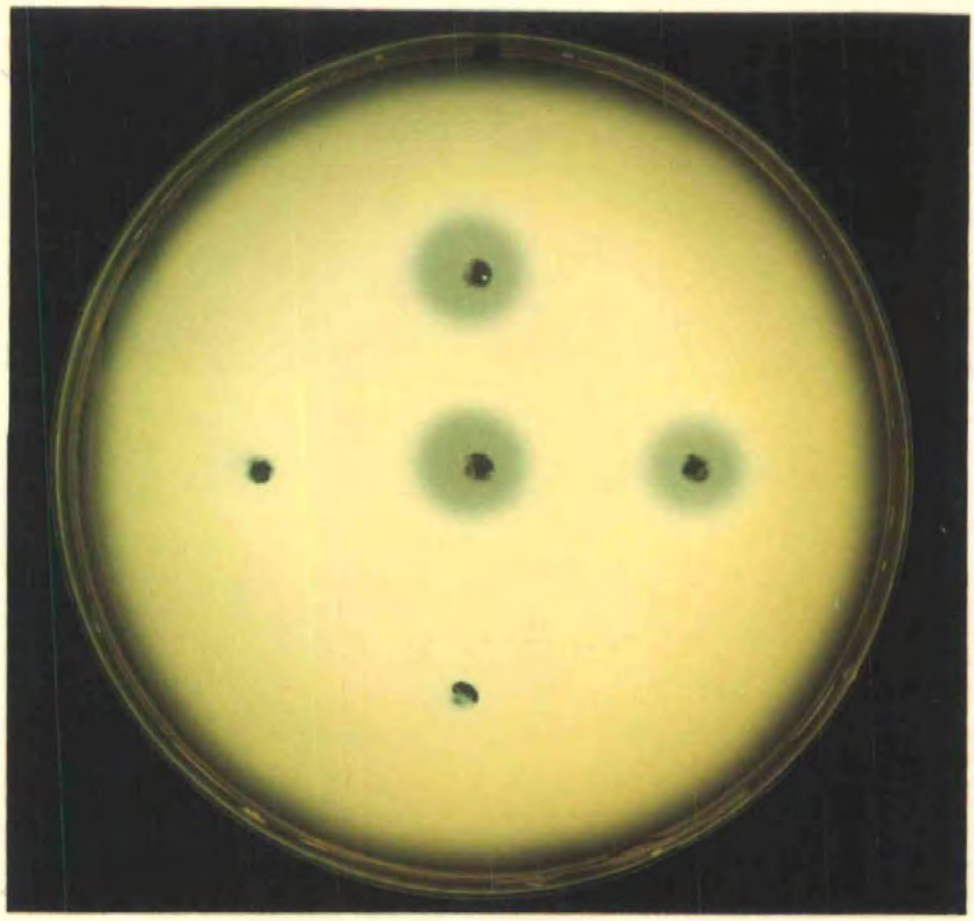
Shaken culture supernate

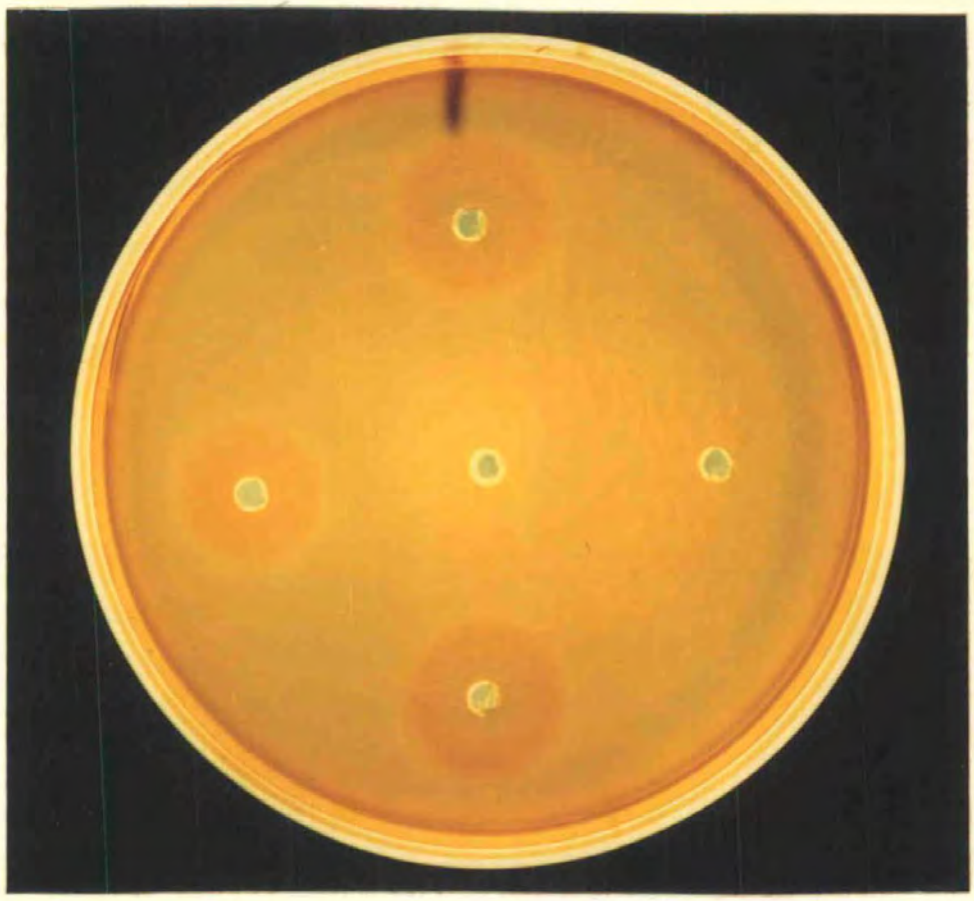
Shaken culture supernate + PMSF

PMSF

PBS ONLY

Shaken culture supernate + propanol





4.3.6 The effect of proteases on the expression of T_1 activity

The results obtained indicated that autogenous caseinase was involved in the expression of T-lysin. It was of interest to investigate the possibility that other, commercially available, proteases could mimic the effect of autogenous caseinase. Samples containing T_1 activity (Cl^- shaken culture supernate, 72 hr 25°C, 0.1 ml) were mixed with δchymotrypsin solution (2 µg/ml to 2 mg/ml, 15,000 ATEE units/mg, 0.1 ml) and activity against trout erythrocytes determined using the microtitre tray assay (2.4.2).

The results (Figure 17) indicated that whilst Cl^- supernate + PBS (0.1 ml + 0.1 ml) contained 8 Hu/ml of T_1 activity, the presence of chymotrypsin resulted in the appearance of T-lysin. 1 µg/ml chymotrypsin resulted in the appearance of 8 Hu/ml of T-lysin, 1 mg/ml chymotrypsin resulted in the appearance of 64 Hu/ml of T-lysin.

During this experiment it was also noticed that the T_1 activity titre was also increased by the addition of chymotrypsin. 1 µg/ml chymotrypsin resulted in a T_1 activity titre of 64 Hu/ml whilst 1 mg/ml chymotrypsin resulted in a T_1 activity titre of 256 Hu/ml.

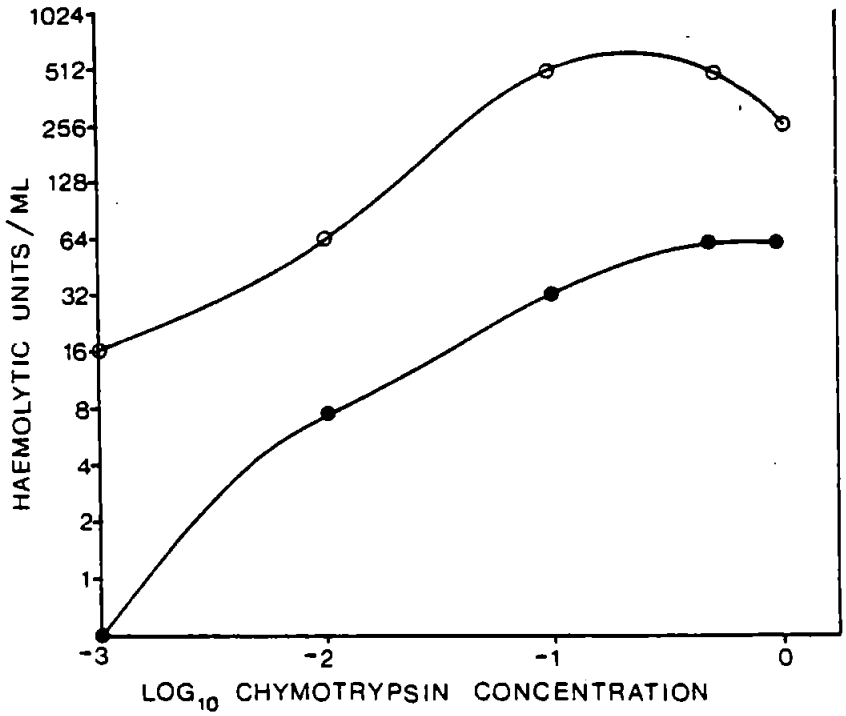
Control experiments indicated that chymotrypsin (1 µg/ml to 1 mg/ml in PBS had no visible effect on trout erythrocytes in the absence of T_1 activity.

4.4.0 STUDIES CONCERNING A. SALMONICIDA H-LYSIN

4.4.1 Investigation of the role of autogenous caseinase in the expression of H-lysin activity

The results obtained confirmed that autogenous caseinase was required for the complete lysis of trout erythrocytes. However, the loss of H-lysin activity from caseinase negative mutants (4.3.3) was unexpected (Plate 18). In view of the fact that H-lysin and caseinase activities appeared to be due to separate entities it was thought that caseinase activity may have been involved in the production of active H-lysin.

Figure 17 The effect of chymotrypsin concentration on the expression of T_1 activity.



○ = T_1 activity detected (Hu/ml)

● = T-lysin activity detected (Hu/ml)

Supernate containing T_1 activity was mixed with chymotrypsin and the mixture tested for haemolytic activity against trout erythrocytes.

Initial experiments to test this possibility involved mixing unshaken broth culture supernate from Cl^- cultures (96 hr 25°C, 5 µl) with wild type shaken culture supernate (strain 25/77 72 hr 25°C, 5 µl). The latter supernate contained low levels of H-lysin activity (2 Hu/ml) but high levels of caseinase activity (3.2.14); residual H-lysin activity in this preparation was removed by membrane filtration of the supernate (Millipore, 0.45 µm, 2.5 cm) (2.7.2). The mixed samples were placed into wells (4 mm) in washed horse erythrocyte agar plates (5% v/v horse blood + 0.02% NaN_3) and incubated (24 hr 25°C).

The results indicated that whilst Cl^- or wild type supernates produced no zones of haemolysis on their own, when mixed together zones of haemolysis typical of H-lysin activity were observed.

4.4.2 The activation of an inactive form of H-lysin by *A. salmonicida* caseinase

The results obtained suggested that *A. salmonicida* caseinase was involved in the activation of a precursor form of H-lysin. This possibility was investigated further.

PMSF is known to inhibit *A. salmonicida* caseinase though it has no effect on preformed H-lysin activity (4.3.5). It was therefore decided to use this inhibitor to confirm the hypothesis outlined above. Cl^- supernate (0.5 ml) (4.4.1) was mixed with wild type (H-lysin depleted) supernate (0.5 ml) (2.7.2) The mixture was incubated (1 hr 25°C) and the reaction terminated by the addition of 0.1 M PMSF solution (1 mM final concentration) with further incubation (2 hr 25°C).

The results (Table 16) indicated that whilst Cl^- or wild type supernates possessed no H-lysin activity individually, when mixed together active H-lysin was detected (16 Hu/ml). The addition of PMSF to the reaction mixture after incubation had no effect on the formed H-lysin activity. Treatment of wild type supernate with PMSF (1 mM 2 hr 25°C) before it was mixed with Cl^- supernate resulted in the non-appearance of H-lysin activity.

The samples tested were also placed into wells (10 µl/well, 4 mm wells) in washed horse erythrocyte agar plates (4.4.1), incubated

Plate 18 Zones of haemolysis surrounding colonies of caseinase negative mutants (Cl^-) and wild type strains of A. salmonicida grown on washed horse erythrocyte agar.

Wild type strain (25/77)

Caseinase negative mutant (Cl^-)

Caseinase negative mutant

Wild type strain

Plate 19 Zones of haemolysis surrounding wells in washed horse erythrocyte agar containing culture supernatant fluid from caseinase negative mutant (Cl^-) and wild type cultures of A. salmonicida

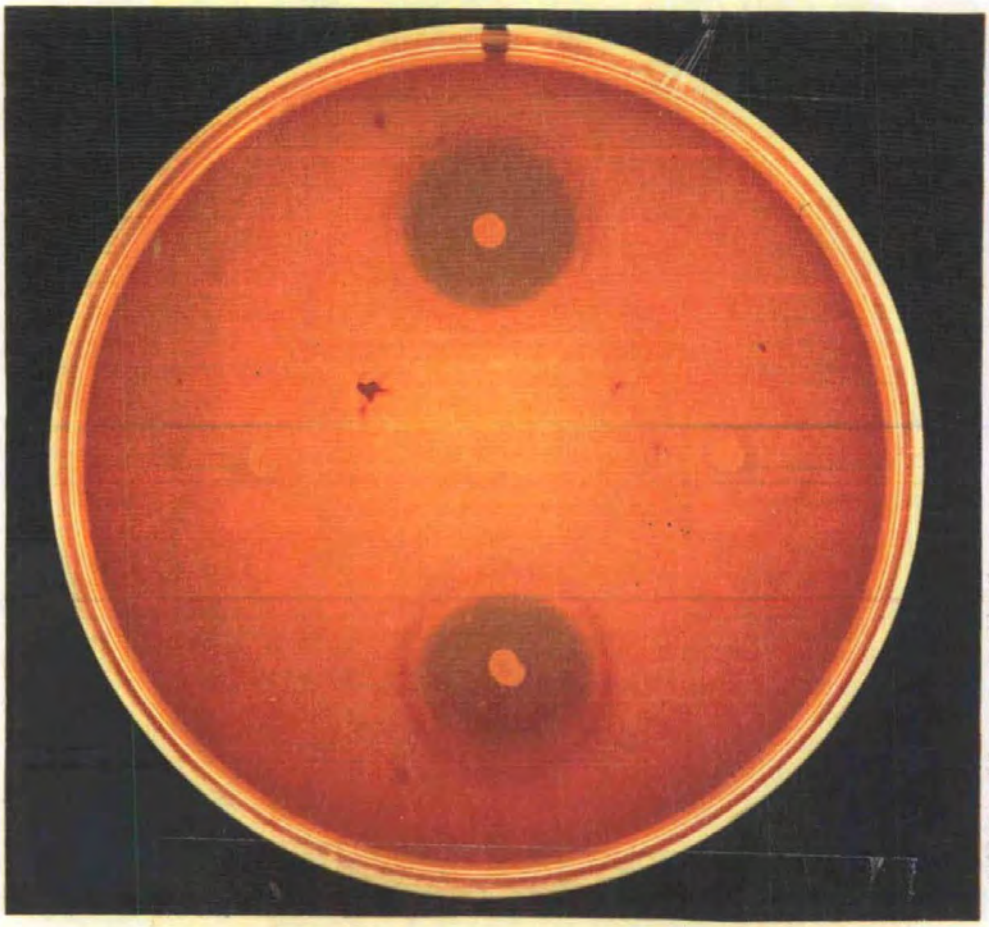
Cl^- supernate + wild type supernate

Cl^- supernate only

Wild type supernate

Cl^- supernate + wild type supernate Post-treated with PMSF

PMSF pre-treated wild type supernate + Cl^- supernate



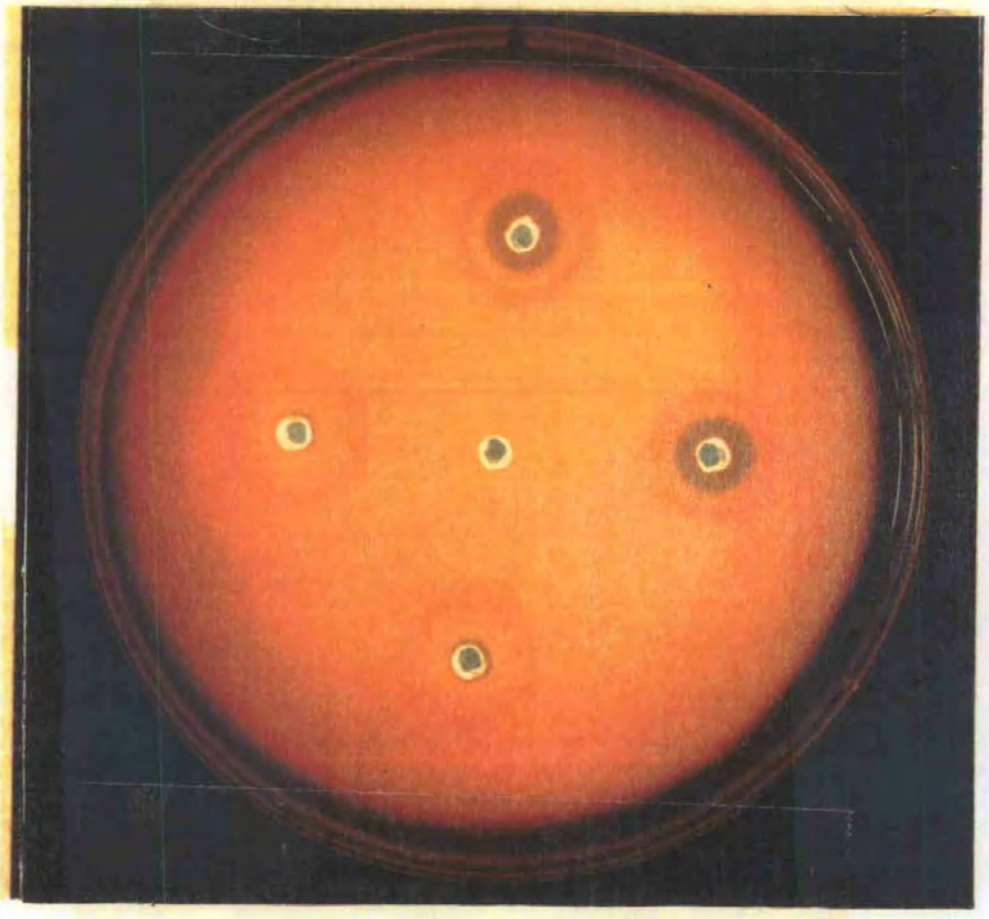


Table 16 The activation of precursor H-lysin from the caseinase negative mutant (Cl^-) by wild type culture supernate.

Mixture tested	H-lysin activity detected (Hu/ml)
Cl^- supernate	0
Wild type supernate	0
Cl^- supernate + wild type supernate	16
Cl^- supernate + wild type supernate, PMSF added after incubation together	16
Cl^- supernate + wild type supernate, PMSF added to wild type supernate before incubation with Cl^- supernate	0

Cl^- supernate was mixed with wild type culture supernate (H-lysin depleted, 145 U/ml caseinase activity) and incubated (1 hr 25°C). In some cases PMSF was added to the mixture with further incubation (2 hr 25°C). In the samples indicated,the wild type supernate was treated with PMSF before mixing with Cl^- supernate.

and examined for zones of haemolysis. The results (Plate 19) reflected the results obtained when the samples were tested using the microtitre assay system.

4.4.3 The time course for the activation of Cl^- precursor H-lysin by autogenous caseinase

The time course for the activation of precursor H-lysin (Cl^- supernate) (4.4.1) by autogenous caseinase (wild type supernate) (4.4.1) was investigated. Cl^- supernate (0.3 ml) was mixed with wild type supernate (0.3 ml), the mixture incubated for various times (25°C) and the reaction terminated by the addition of PMSF (1 mM) (2.7.1).

The results (Figure 18) indicated that when the samples were tested for H-lysin activity (2.4.2) maximum activity was observed after 15 min. incubation of Cl^- and wild type supernate together (8 Hu/ml). No increase or decline in H-lysin activity was detected after up to 1 hr incubation of the supernates.

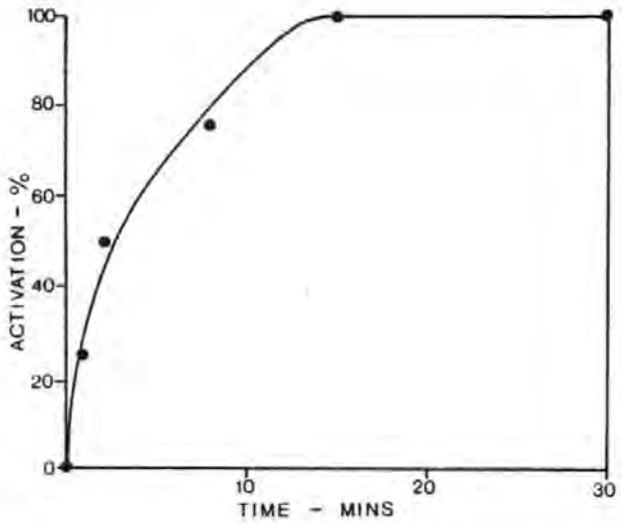
4.4.4 The activation of Cl^- precursor H-lysin by a variety of proteolytic enzymes

In view of the results obtained, suggesting that autogenous caseinase was involved in the activation of precursor H-lysin, it was decided to investigate the abilities of some commercially available proteases to activate the precursor H-lysin.

δ Chymotrypsin (BDH, 15,000 ATEE units/mg) and trypsin (Sigma, 0.54 Anson units/g) were incubated (1 ml, 200 µg/ml or 20 µg/ml in PBS with Cl^- unshaken culture supernate (1 ml) (4.4.1) for 10 min. (25°C). The reaction was terminated by the addition of PMSF (1 mM) (2.7.1). A. salmonicida caseinase (4.4.1) (470 U/ml) was tested under similar conditions. Control samples containing Cl^- supernate (1 ml) + PBS (1 ml) were also treated in a similar manner. The H-lysin activity in the samples was determined using the microtitre tray assay (2.4.2).

The results (Table 17) indicated that both chymotrypsin and trypsin were capable of activating Cl^- precursor H-lysin. Both

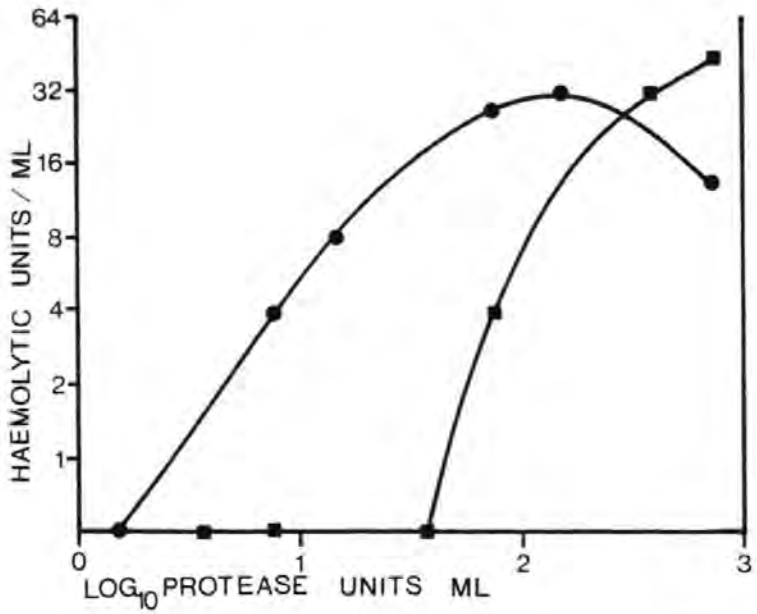
Figure 18 The time course for the activation of precursor H-lysin by autogenous caseinase.



● = H-lysin activity detected

Precursor H-lysin (Cl^- culture supernate) was mixed with autogenous caseinase (wild type culture supernate, H-lysin depleted), incubated (25°C) and the reaction terminated by the addition of PMSF (1 mM).

Figure 19 The effect of chymotrypsin and autogenous caseinase concentration on precursor H-lysin activation.



■ = H-lysin activity after incubation with autogenous caseinase
● = H-lysin activity after incubation with chymotrypsin

Precursor H-lysin (Cl^- culture supernate) was mixed with chymotrypsin or autogenous caseinase (wild type shaken culture supernate, H-lysin depleted), incubated (10 min, 25°C) and the reaction terminated by the addition of PMSF.

Table 17 The ability of proteases to activate a precursor form of H-lysin from Cl^-, a protease negative mutant of *A. salmonicida*.

Mixture tested	H-lysin detected (Hu/ml)
Cl^- supernate + PBS	0
Crude *A. salmonicida* caseinase	0
Cl^- supernate + *A. salmonicida* caseinase	48
Cl^- supernate + chymotrypsin (100 µg/ml)	2
Cl^- supernate + chymotrypsin (10 µg/ml)	32
Cl^- supernate + trypsin (100 µg/ml)	24
Cl^- supernate + trypsin (10 µg/ml)	32
Chymotrypsin only (100 µg/ml)	0
Trypsin only (100 µg/ml)	0

Reactions involving Cl^- supernate (10 min, 25°C) were terminated by the addition of PMSF (1 mM) with further incubation (2 hr, 25°C).

Samples containing chymotrypsin or trypsin only were also treated with PMSF prior to testing for haemolysis of horse erythrocytes.

proteases showed maximal activation at 10 µg/ml concentration (chymotrypsin 32 Hu/ml, trypsin 32 Hu/ml). At 100 µg/ml concentrations, less H-lysin activity was detected (chymotrypsin 2 Hu/ml, trypsin 24 Hu/ml). Neither enzyme was as effective as A. salmonicida caseinase in activating the H-lysin (48 Hu/ml).

Neither chymotrypsin nor trypsin showed any capacity to lyse horse erythrocytes either before or after treatment with PMSF.

The protease solutions used in this experiment were tested for caseinase activity (2.4.5) before the addition of PMSF (chymotrypsin 100 µg/ml = 2100 U/ml, 10 µg/ml = 190 U/ml; trypsin 100 µg/ml = 125 U/ml, 10 µg/ml = 11 U/ml). After the addition of PMSF to these solutions no caseinase activity was detected.

4.4.5 Comparison of the effect of chymotrypsin and autogenous caseinase concentration on the activation of Cl^- precursor H-lysin.

It was decided to investigate the effects of chymotrypsin and autogenous caseinase (wild type shaken culture supernate, H-lysin depleted) (4.4.1) on the activation of Cl^- precursor H-lysin (4.4.1). Chymotrypsin dissolved in PBS (pH 7.2) was diluted to give a variety of concentrations (0.2 µg/ml to 200 µg/ml). Autogenous caseinase was also diluted in PBS to give a variety of concentrations. The caseinase activity of these protease solutions was determined (2.4.5). The proteases were mixed (0.2 ml) with Cl^- supernate (0.2 ml) and incubated (10 min, 25°C). Reactions were terminated by the addition of PMSF (2.7.1).

The results (Fig. 19) indicated that when tested for H-lysin activity (2.4.2) a maximum of 48 Hu/ml was detected after incubation of Cl^- supernate with autogenous caseinase (750 U/ml) and a maximum of 32 Hu/ml after incubation with chymotrypsin (150 U/ml). At higher chymotrypsin concentrations a decline in the active H-lysin formed was observed, a feature not apparent with reactions involving autogenous caseinase. At low protease concentrations (7.5 U/ml) chymotrypsin was more effective in activating the precursor H-lysin than the autogenous caseinase.

4.4.6 The separation of precursor and active H-lysin

The results obtained indicated that the H-lysin was converted from an inactive (precursor) form to an active form by the action of a variety of proteases. It was thought that this reaction might involve a change in molecular weight.

Cl^- supernate (unshaken, 96 hr, 25°C, 8 x 100 ml) was treated with ammonium sulphate (40 - 60% saturation) (2.3.1) and the precipitated material dialysed against PBS (0.1 M + 0.85% NaCl, 18 hr, 0°C, 0.01M + 0.085% NaCl, 6 hr, 0°C). Non-dialysable material (18 ml) was centrifuged (10,000 x g, 20 min, 4°C), concentrated 20 fold by lyophilisation and mixed with an equal volume of purified H-lysin (0.25 ml) (6.2.5). The mixture was applied to a gel filtration chromatography column (Ultrogel ACA 54, 1.5 x 28 cm) and eluted using PBS. Fractions (2.8 ml) were tested for H-lysin activity before and after treatment with chymotrypsin (10 µg/ml) (2.7.4). The optical density of the column eluant was continuously monitored (280 nm)(2.6.1).

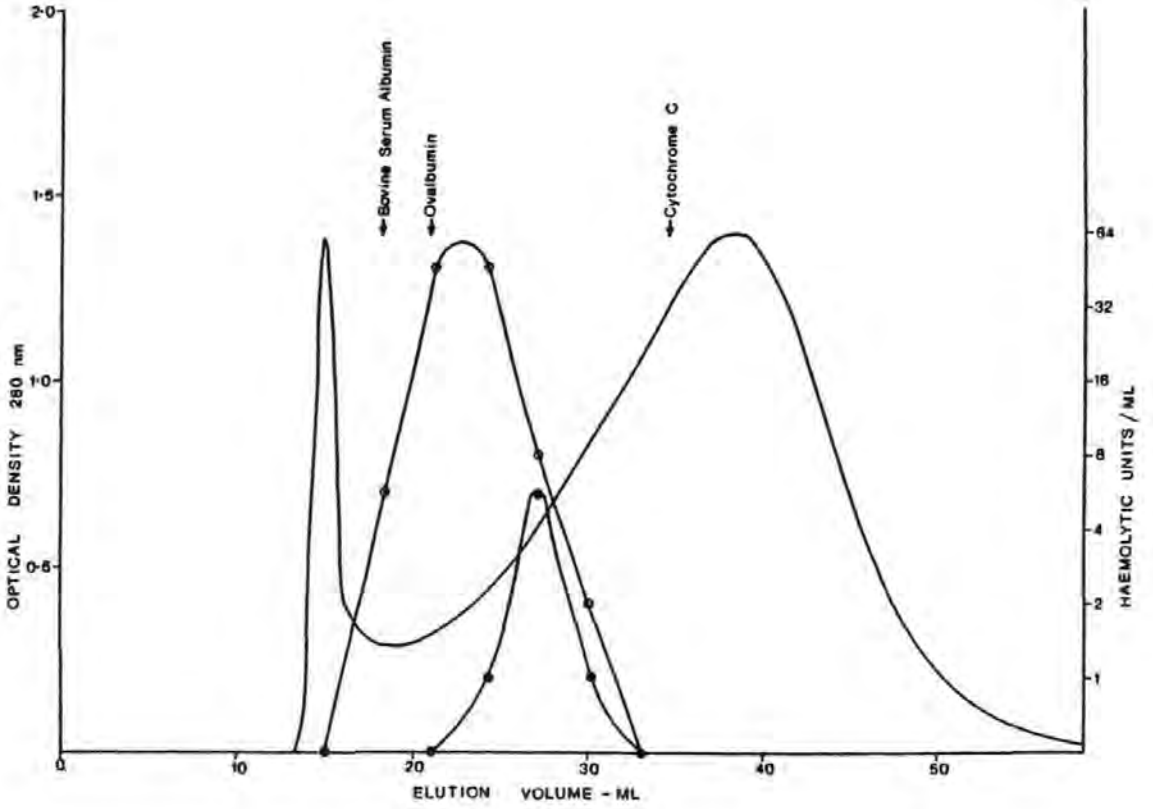
The results (Fig. 20) indicated that precursor and active H-lysin could be separated using this technique. A comparison of the elution volumes of precursor and active H-lysin with the elution volumes of proteins of known molecular weights (2.6.2) indicated that the precursor H-lysin had a molecular weight of 42,300 whilst the active H-lysin possessed a molecular weight of 27,500.

4.4.7 The effect of serum on H-lysin production by caseinase negative mutants of A. salmonicida

During experiments with the caseinase negative mutants Cl^- and $C2^-$, it was noticed that whilst no haemolysis was apparent when the bacteria were grown on TSA + washed horse blood, growth on TSA + unwashed horse blood resulted in normal zones of haemolysis (3.2.1). Similar results were observed if horse serum (3.3% v/v) was incorporated into washed horse erythrocyte TSA plates.

Dogfish or trout serum (heated 45°C, 20 min) added to TSA +

Figure 20 The separation of precursor and active H-lysin by gel filtration chromatography.



● = H-lysin activity in column eluant.

○ = H-lysin activity detected after incubation of eluant fractions with chymotrypsin (10 µg/ml, 10 min, 25°C) (precursor H-lysin)

— = Optical density of column eluant (280 nm)

Material obtained after ammonium sulphate precipitation of Cl^- culture supernate was concentrated (20 fold) and mixed with an equal volume of purified H-lysin (0.25 ml). The column was eluted with PBS.

The elution volumes of a number of proteins of known molecular weight was also determined; Bovine serum albumin (66,000) ovalbumin (45,000) cytochrome C (12,500).

washed horse erythrocyte plates also resulted in the appearance of normal zones of haemolysis around colonies of Cl^-.

4.4.8 The effect of horse serum concentration on the production of H-lysin by Cl^-

It was decided to investigate the effect of horse serum concentration on the production of H-lysin by Cl^- and 25/77 strains of A. salmonicida. Horse serum (Wellcome donor horse serum) was added to TSA + horse erythrocyte plates to give final concentrations in the range 0.0035% to 33% v/v. Stab inoculated bacteria were grown for 75 hours (25°C) and zones of haemolysis measured (3.2.1).

The results (Fig. 21) indicated that although the wild type strain (25/77) produced H-lysin at low serum concentrations (0.0035 to 0.07% v/v) no zones of haemolysis were observed around colonies of the caseinase negative mutant strain (Cl^-). At higher serum concentrations haemolysis around colonies of Cl^- was observed with maximal haemolytic zone sizes observed at 0.7 to 3.3% v/v added serum. Concentrations of serum above 3.3% v/v resulted in smaller zones of haemolysis around colonies of Cl^- a feature that was also apparent with strain 25/77.

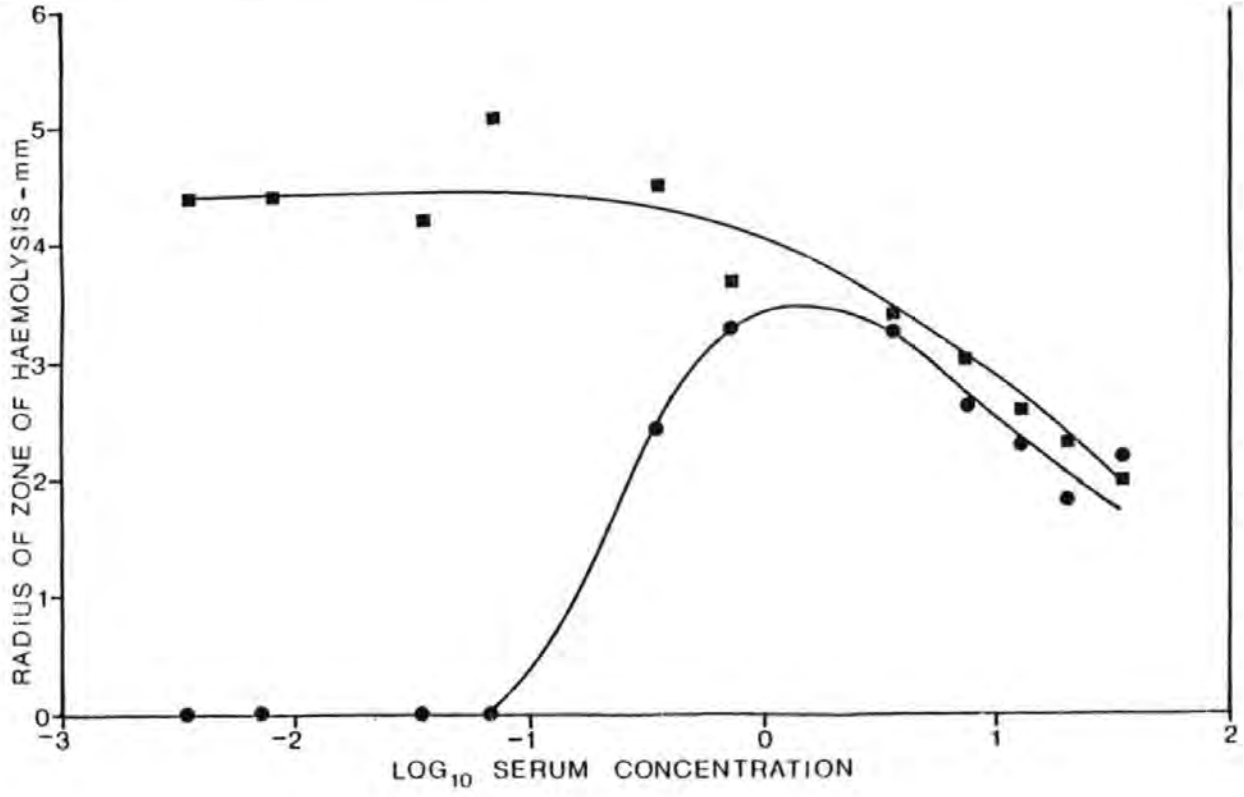
4.4.9 The activation of preformed, precursor H-lysin, by horse serum

It was of interest to know whether the serum was activating the precursor H-lysin after release from the bacterial cell or whether the effect was dependant on the cells growing in the presence of serum.

Supernate samples containing precursor H-lysin (1 ml, unshaken culture supernate, 96 hr, 25°C) were incubated with serum (0.1%, 1% and 9% v/v, 1 hr, 25°C) then tested for H-lysin activity).

The results indicated that when tested in the microtitre tray assay (2.4.2) no active H-lysin was detected. Control experiments indicated that the activity of H-lysin from wild type culture supernates (32 Hu/ml) was unaffected by serum at 0.1% and 1% v/v though

Figure 21 The effect of horse serum concentration on H-lysin production by Cl^- and wild type strains of A. salmonicida



● = H-lysin production by Cl^- strain
■ = H-lysin production by wild type strain

Bacteria were stab inoculated onto TSA + washed horse blood which had been supplemented with various amounts of horse serum. Zones of haemolysis were measured after 24 hour growth (25°C).

at higher concentrations (9% v/v) some loss of activity was detected (8 Hu/ml).

Samples (10 µl) from Cl^- or 25/77 culture supernates were also placed into wells (4 mm) in TSA + washed horse erythrocyte plates (+0.02% NaN_3). Some plates contained horse serum (1.7% v/v). After incubation (24 hr, 25°C) zones of haemolysis were examined.

The results indicated that the sizes (3.7 mm) and forms (3.2.1) of zones of haemolysis around wells containing 25/77 supernate were unaffected by the presence of serum. No zones were visible around wells containing Cl^- supernate in plates without serum, however in plates containing serum partial haemolysis of erythrocytes around the wells was observed (3.7 mm zone). The clear zone of haemolysis was not visible though the red halo and zone of partial haemolysis, extending to the well edge, were visible (3.2.1).

4.4.10 The effect of serum on caseinase and T-lysin production

The results obtained suggested that active H-lysin was produced by caseinase negative mutants of A. salmonicida when the bacteria were grown in the presence of serum. A possible explanation for this observation was that the serum caused the mutants to produce active caseinase which was then able to activate the precursor H-lysin (4.4.2). To test this possibility Cl^-, (caseinase negative mutant) was grown on TSA + sodium caseinate (1% w/v) containing serum (1.7% v/v). The results indicated that no digestion of casein was apparent around colonies of the bacterium.

The bacteria were also grown on TSA + washed trout erythrocytes (7% v/v) containing horse serum (heated 56°C, 30 min) because these mutants had also been shown to produce incomplete zones of haemolysis on trout erythrocyte agar (4.3.3). The results of this experiment indicated that added horse serum had no effect on the form of zones of incomplete haemolysis around colonies of Cl^-.

4.4.11 The effect of EDTA and PMSF on the capacity of serum to activate the precursor H-lysin

The results obtained suggested that limited activation of

precursor H-lysin could occur outside the bacterial cell. It was decided to utilise this system to investigate the effect of pre-treatment of the serum with EDTA or PMSF on the capacity of the serum to elicit this effect. The bacteria could not be grown on agar containing these compounds because of their associated toxicity.

PMSF (1 mM), or EDTA (5 mM) were incorporated into TSA + washed horse erythrocyte plates (+0.02% NaN_3, +1.7% v/v horse serum). Culture supernate samples from Cl^- or 25/77 unshaken cultures (96 hr, 25°C) were placed into wells (4 mm) in these plates and incubated (24 hr, 25°C). Zones of haemolysis were measured and compared with control sample zones (supernate samples in plates without PMSF, EDTA or serum).

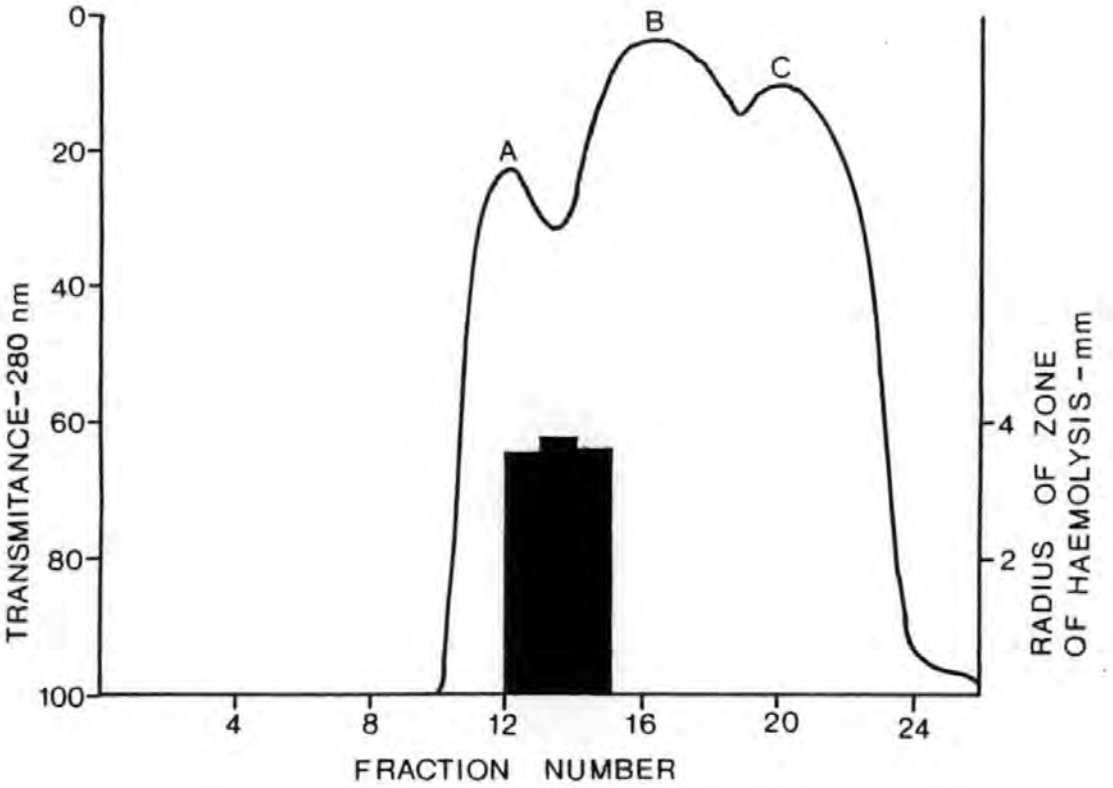
The results indicated that the ability of serum to partially activate the H-lysin from Cl^- supernates was inhibited by the addition of EDTA to the plates. PMSF had no inhibiting ability. Control experiments indicated that preformed H-lysin (25/77 supernate) was not affected by these treatments.

4.4.12 Separation of the H-lysin activation factor from serum by gel filtration chromatography

The results obtained suggested that a factor in horse serum was capable of activating precursor H-lysin from protease-negative mutants of A. salmonicida. It was decided to attempt to identify this component by investigating its elution pattern after gel filtration chromatography of serum. Horse serum (4 ml) was applied to a column of Sephadex G-200 (85 x 1.5 cm) and eluted using PBS (pH 7.2). Twentyfour fractions (4.1 ml) were collected, sterilised by membrane filtration (0.45 µm) and the fractions added to TSA + washed horse erythrocyte plates at 6.8% v/v.

The caseinase-negative mutant Cl^- was stab inoculated onto one set of these plates and, using another set (+0.02% NaN_3), supernates from unshaken broth cultures of the mutant were placed into wells (4mm). After incubation (24 hr, 25°C), the component responsible for the activation of precursor H-lysin was found to be located at the end of the serum macroglobulin peak (Fig. 22).

Figure 22 The separation of horse serum using gel filtration chromatography and detection of the H-lysin activation factor



— = OD 280 nm of column eluant

■ = The diameter of the zone of haemolysis observed when strain Cl^- was grown on TSA + washed horse blood + serum fraction.

Plate 20 The detection of GCAT activity in culture supernates of *A. salmonicida*

Track 1 = Shaken culture - membrane filtered (0 Hu/ml H-lysin 16 Hu/ml T-lysin)

Track 2 = Unshaken culture - membrane filtered (0 Hu/ml H-lysin, 4 Hu/ml T-lysin)

Track 3 = Erythrocyte membranes only

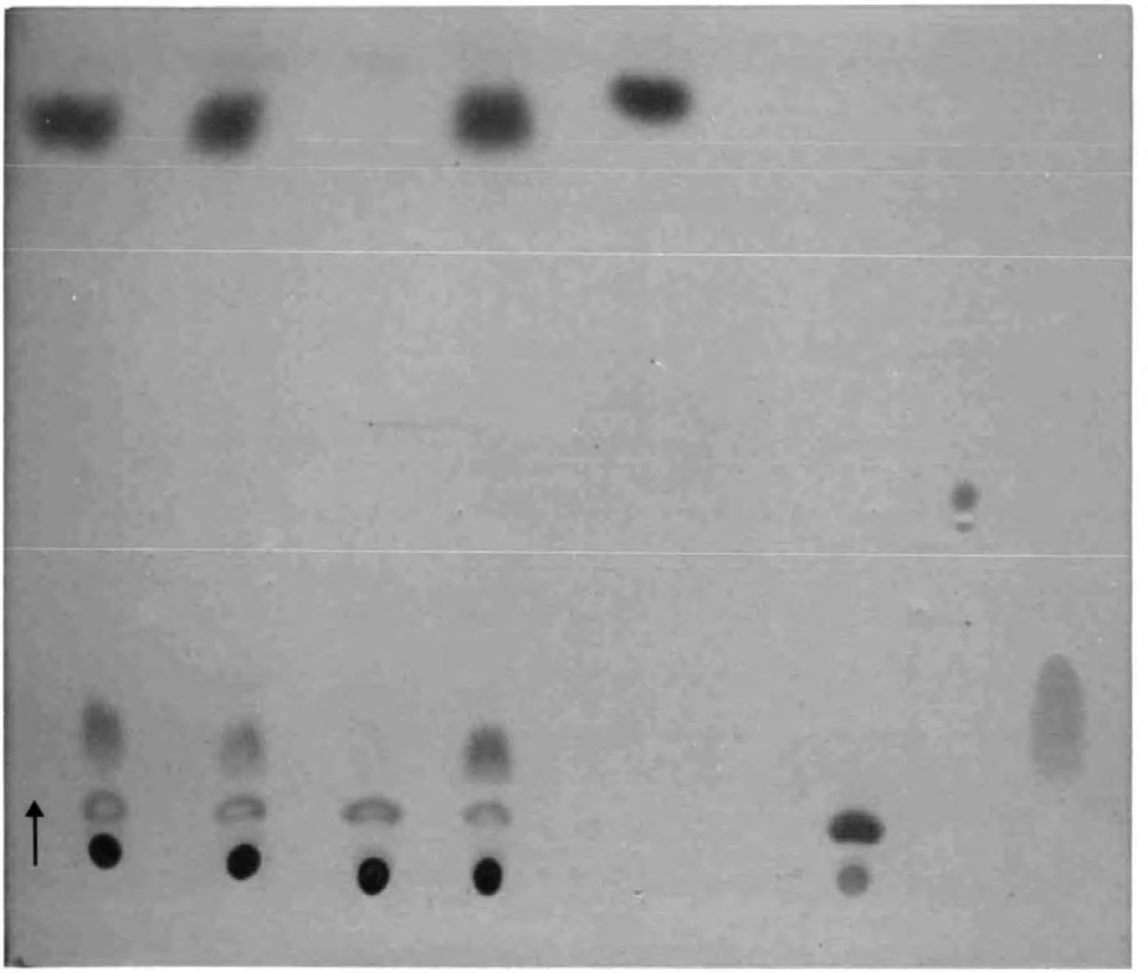
Track 4 = Unshaken culture (64 Hu/ml H-lysin, 6 Hu/ml T-lysin)

Track 5 = TLC standard - cholesterol ester

Track 6 = TLC standard - cholesterol

Track 7 = TLC standard - palmitic acid

→ = Direction of separation



4.5.0 THE PRODUCTION OF GLYCEROPHOSPHOLIPID:CHOLESTEROL ACYLTRANSFERASE BY *A. SALMONICIDA*

4.5.1 The production of glycerophospholipid:cholesterol acyltransferase (GCAT) by wild type and caseinase-negative mutants of *A. salmonicida*

As *A. salmonicida* has been reported to produce a potentially haemolytic GCAT enzyme, it was of interest to elucidate the relationship of this activity to the H-lysin, T-lysin and T_1 activities produced by this bacterium. GCAT activity was detected using human erythrocyte membranes as enzyme substrate. The reaction products were separated using thin layer chromatography (TLC) and cholesterol ester production was considered to reflect the presence of GCAT (2.4.6.). The production of cholesterol ester was evaluated visually.

Shaken (72 hr) cultures of wild type strain (25/77) and protease-negative mutant (Cl^-) were both found to contain GCAT activity. Unshaken cultures of strain 25/77 were found to contain limited GCAT activity whilst GCAT was detected only in shaken cultures of Cl^-.

The results of a typical assay to detect GCAT activity are shown in plate 20. In preliminary experiments,culture supernate, in the absence of membranes,was demonstrated to contain no material that was detected after TLC.

4.6.0 DISCUSSION

Extracellular products from *A. salmonicida* can be separated from one another by virtue of molecular weight or charge differences.

Electrophoretically separated caseinase appeared as a number of discrete bands of activity, though only one peak of activity was detected after gel filtration chromatrography. The appearance of six caseinase bands after electrophoretic separation has not been reported by other workers. It is unlikely that the bands represent individual gene products because caseinase-negative mutant bacteria were relatively easily obtained,whereas concomitant mutations at

six loci would be a rare event. It is possible that the individual enzymes are part of a single regulon when a single mutation may result in the concomitant loss of active caseinase production, however close grouping of the bands of activity suggests that the electrophoretic forms observed are either true isozymes or multiple forms of one enzyme. Multiple forms of proteases have been described for other bacteria. Young and Broadbent (1982) reported that V. cholerae, PMSF-inhibited protease appeared as two forms after isoelectric focusing, a feature that was also observed with the EDTA-inhibited protease from this bacterium. Similar multiple forms have been reported for V. parahaemolyticus proteases (Iuchiand Tanaka 1982). In general, multiple forms of bacterial enzymes appears to be a common phenomenon (Wadstrom 1978).

Gelatinase activity from A. salmonicida appeared as a number of bands after electrophoretic separation; some of these bands were coincident with the caseinase activity bands, reflecting the ability of A. salmonicida caseinase to digest gelatin (Sheeran et al 1981). An additional group of three hands with gelatin-only digesting activity was also observed which may be due to the separate gelatinase enzyme described by Sheeran and Smith (1982). These workers found that the gelatinase activity separated into three forms after ion exchange chromatography, a feature that they attributed to autodigestion products of the enzyme.

The existence of separate caseinase and gelatinase enzymes is further confirmed by other experiments performed in this project. Repression of caseinase production, or the growth of caseinase-negative mutants revealed that gelatin digestion was still observed.

The results obtained in this section of the project provide considerable evidence that the T-lysin reflects the combined effects of A. salmonicida caseinase and an activity which causes incomplete trout erythrocyte lysis - the T_1 activity. The T_1 activity may have been responsible for the incomplete lysis of trout erythrocytes in haemolysin assays reported by Munro et al (1980) though these workers did not investigate the reason for this effect.

Trout erythrocytes treated with T-lysin lysed completely, whereas those treated with T_1 activity, although 'leaking' some haemoglobin,

appeared to retain some cell membrane integrity. Supernatant fluid from T-lysin-treated cells was viscous and contained greater amounts of material absorbing ultra-violet light at 260 nm than T_1-treated cell supernate. This may indicate that the membranes of T_1-treated cells are not damaged sufficiently to allow the leakage of DNA from the nucleated trout erythrocytes.

The serine protease, chymotrypsin, was able to mimic the effect of autogenous caseinase and a synergistic effect was observed between T_1 activity and chymotrypsin was observed.

These results can be related to the form of haemolytic zones in agar containing trout erythrocytes. The zone of complete cell lysis would appear to reflect full T-lysin activity whilst the orange zone may be due to the action of T_1 activity on the cells.

The crude T_1 activity appears to possess some unusual biophysical properties being heterogenous with respect to both molecular weight and charge. Gel filtration chromatography experiments indicated that a large proportion of the T_1 activity molecules possessed molecular weights of greater than 4×10^6 daltons. This result, in conjunction with the finding that T_1 activity was found within the cell associated with the cell wall (3.3.0) suggests that the T_1 activity found in culture supernate is associated with fragments of cell wall or membrane. GCAT activity from A. salmonicida behaves in a similar manner to the T_1 activity, exhibiting molecular weight heterogeneity. In this case the enzyme is thought to be associated with outer membrane 'blebs' which are released from the bacterial cell (MacIntyre et al 1979). A similar phenomenon has been described for S. typhimurium alkaline phosphatase which is thought to associate with LPS (Lindsay et al 1973) and Cl. tetani haemolysin which may associate with membrane fragments (Mitsui et al 1982).

The effects of T_1 activity and T-lysin on trout erythrocytes at a membrane level are not clear. The T_1 activity may possess an enzymatic mode of action hydrolysing a membrane component which is absent in mammalian erythrocytes. Alternatively, the T_1 activity may act on the membrane by creating pores. This mode of action would explain the cell shrinking which was apparent after T_1 treatment

because of the osmotic imbalance which would be created. This hypothesis would also explain why the T_1 activity is found associated with fragments of bacterial membranes since such a pore forming protein would be expected to have a high affinity for cell membranes. Buckley et al (1982) have suggested that some pore forming bacterial haemolysins may be modified bacterial cell membrane porins. The possibility that the T_1 activity is such a protein requires further attention. If the T_1 activity possess a porin like activity then it is not clear how haemoglobin release occurs since the pores would presumably be too small to allow haemoglobin release without gross cell lysis. In this context it may be important to note that fish erythrocytes are much more resistant to hyperosmotic lysis than mammalian cells, hence an osmotic imbalance may be tolerated with only partial disruption of the membrane. T_1 activity-mediated membrane damage may expose membrane proteins in the cell membrane to proteolytic cleavage leading to complete dissolution of the membrane structure. Alternatively, the damaged membranes may allow the entry of proteases into the erythrocyte, the proteases hyrolysing proteins on the inner surface of the cell membrane which are absent on the outer surface.

A similar hypothesis has been proposed for the action of S. aureus α toxin and nuclease on tissue cultured cells (Korbecki and Jeljaszewicz 1964), in this case the α toxin is thought to damage the cell membrane allowing entry of the nuclease into the cell with subsequent cell death. It is interesting to note that in this case, the nuclease, like A. salmonicida caseinase, appeared to possess no cytotoxic activity on its own. There are also other reports of synergistic effects between S. aureus δ lysin and α toxin (Thelestam et al 1973).

Such observations tend to underline the possibility that A. salmonicida exerts toxic effects on the host by means of a variety of, rather than an individual, extracellular products.

The results obtained in this section of the project also indicated that A. salmonicida caseinase was involved in the formation of active H-lysin. This hypothesis arose from the observation that caseinase

negative mutants appeared to produce little or no active H-lysin. One explanation for this observation is that the caseinase was responsible for lysis of horse erythrocytes. This is unlikely in view of the fact that selective inhibition of pre-formed caseinase by PMSF was not accompanied by the loss of pre-formed H-lysin activity. The results may also be explained if the caseinase-negative mutants were also mutated at the H-lysin locus. This also seems unlikely in view of the similar properties of two independently isolated caseinase-negative mutants, neither of which produced active H-lysin. A remaining possibility explaining the concomitant loss of H-lysin and caseinase is that the caseinase is involved in the activation of the H-lysin. This hypothesis is supported by the observation that caseinase from wild type culture supernate, when mixed with caseinase-negative mutant culture supernate, resulted in the appearance of active H-lysin. Other serine proteases were also able to mimic the effect of autogenous caseinase, resulting in the conversion of an inactive form of the H-lysin (pro- H-lysin) to the active form. Autogenous caseinase appeared to be the most effective activator, of the proteases tested; this may reflect the fact that at high chymotrypsin and trypsin concentrations pro- H-lysin activation was offset by increased digestion of the formed H-lysin. The role of proteases in the activation of pro-H-lysin was confirmed by the observation that activation was accompanied by a molecular weight decrease.

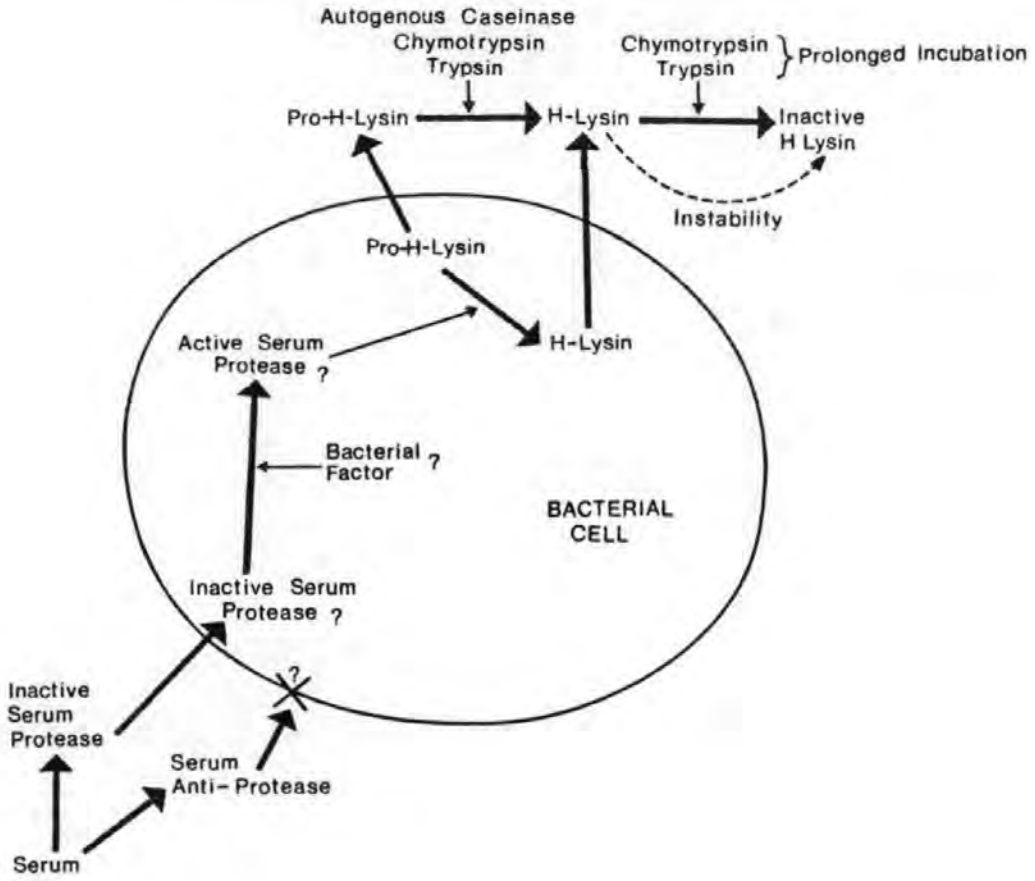
The phenomenon observed appears to have been rarely described as a mechanism for the activation of extracellular products from other bacteria, one notable exception being the suggested role of Clostridium botulinum protease in the activation of neurotoxin (Das Gupta and Sugiyama 1972). Activation of S. aureus α toxin is also thought to occur although in this case the protease responsible appears to be of target cell rather than bacterial origin (Wiseman and Caird 1972, Wiseman et al 1975).

Ammonium sulphate added to the growth medium was able to repress caseinase production by A. salmonicida, though the results of these experiments in relation to the above hypothesis require careful interpretation. Ammonium sulphate added to liquid growth medium resulted in the formation of pro- H-lysin only by the wild type bacterium, detected after chymotrypsin activation in the microtitre

tray assay. On solid growth medium,containing horse erythrocytes and ammonium sulphate,the zones of haemolysis around colonies bore little resemblance to the zones observed around caseinase-negative mutant bacteria-which may have been expected. The zones of haemolysis around caseinase-repressed bacteria resembled those seen around unrepressed bacteria, however close examination revealed that not only were the zones reduced in size but the clear zone of cell lysis was indistinct. A number of factors may lead to these results, which were apparently incompatible with the caseinase activation hypothesis: precursor forms of enzymes often possess limited activity of the active enzyme form (Neurath 1975). The limited erythrocyte-lysing activity of the pro- H-lysin may be enhanced by the presence of ammonium sulphate. The fact that activity was detected on solid media but not in the microtitre tray assay may reflect differences in the sensitivity of these two methods for detecting low concentrations of pro- H or H-lysin activities.

Serum also appeared to be able to mimic the effect of autogenous caseinase,resulting in normal H-lysin production when caseinase negative mutants were grown in the presence of a variety of types of serum. This observation may reflect the fact that serum contains proteases in a zymogen form (Neurath 1975). The bacterium only appeared to be able to activate these proteases successfully within the bacterial cell. The inability of culture supernate to activate the proteases may indicate that the activating factor required is found only within the bacterial cell. Alternatively, the serum protease may be activated by the culture supernate but rapidly inactivated by serum anti-proteases (Heimburger 1975). This result would suggest that the bacterial cell wall although allowing entry of the serum zymogen proteases is able to prevent entry of these anti-proteases. A similar system has been described for some influenza virus infected tissue culture cells where the serum protease plasmin appears to cleave the viral haemagglutinnin after entry into the cell (Korant 1975, Lazarowitz and Choppin 1975).

The complex system controlling pro- H-lysin is detailed below:



The findings reported here concerning the H and T-lysins of A. salmonicida have important implications concerning the interpretation of studies by other workers. Sakai (1978) found that culture supernate from caseinase-negative mutant strains of A. salmonicida was not cytotoxic to tissue culture cells. This loss of cytotoxicity may have reflected the loss of H-lysin or full T-lysin activity rather than indicating the cytotoxic nature of the caseinase as Sakai suggested.

There are a variety of areas which merit further investigation. The action of T_1 and caseinase activities on trout erythrocytes at the biochemical level is of interest, though the lack of knowledge concerning fish erythrocyte membrane structure may hamper this

investigation. Testing a variety of fish erythrocytes for sensitivity to lysis followed by examination of the membrane composition may indicate the presence of components which are absent, or present in low quantities, in mammalian erythrocytes.

The role of caseinase in the activation of H-lysin has been elucidated in some detail. The effect of serum on the activation of pro- H-lysin requires further attention to confirm the hypothesis outlined.

Perhaps one of the most important general observations in this chapter is the complex pattern of inter-relationships which may occur between A. salmonicida extracellular products. This is particularly relevant to future studies concerning the roles of individual extracellular products in the pathogenesis of furunculosis, either by direct administration of the individual extracellular products or by the use of mutants. In these cases the results must be interpreted carefully with due consideration for the possibility that the toxic factor, which is thought to be produced by the bacterium (Ellis et al 1981, Pol et al 1980), may in fact be due to a number of extracellular products which are non-toxic individually but which exert a toxic effect when combined.

CHAPTER 5 THE PROPERTIES OF CRUDE PREPARATIONS OF T AND H-LYSIN ACTIVITIES

5.1.0 INTRODUCTION

Studies concerning the properties of crude preparations of bacterial extracellular products can often provide valuable information pertaining to the biochemical nature of these activities. Such studies are also an essential pre-requisite for further purification studies because they may indicate conditions which provide maximal stability of the extracellular product. The properties of a crude bacterial extracellular product are often influenced by its environment. Interaction of growth medium components or other bacterial products with the activity being studied may distort its apparent properties. For these reasons investigations concerning the properties of crude extracellular products should be interpreted with caution.

In this section of the project some properties of crude preparations of T and H-lysins were evaluated. The experiments were designed, primarily, to indicate the possible nature of these activities. In addition the effects of various environmental conditions which may have been encountered during purification studies were evaluated.

5.2.0 RESULTS

5.2.1 The thermal stability of T and H-lysins from A. salmonicida

The thermal stability of the T and H-lysins was investigated for a number of reasons. Such studies may indicate the possible biochemical nature of these activities - proteins generally being thermally unstable. These investigations may also indicate thermal conditions which should be avoided to maintain activity of the haemolysins.

Supernate samples containing T-lysin (shaken, 72 hr, 25°C) or H-lysin (unshaken, 96 hr, 25°C) were heated to various temperatures for 5, 10 or 15 minutes, cooled, and tested for H or T-lysin activity.

The results indicated that after 5 minutes at 48°C no H-lysin activity (16 Hu/ml) was detected, T-lysin activity was unaffected by

this treatment (8 Hu/ml). After heating samples to 56°C a similar result was obtained. Heating T-lysin samples (64 Hu/ml) to 63°C resulted in some loss of activity after 5 minutes incubation (3 Hu/ml) whilst after 10 minutes at this temperature no activity was detected.

5.2.2 Initial experiments concerning the optimum temperature for H-lysin and T-lysin activity

It was of importance to determine the optimum incubation temperature for the assay of H-lysin and T-lysin in the microtitre tray assay (2.4.2).

Supernatant fluids containing H-lysin (unshaken, 96 hr, 25°C) or T-lysin (shaken, 72 hr, 25°C) were tested for haemolytic activity after incubation at various temperatures for 1 hour.

The results indicated that H-lysin activity was similar at 37°C and 44°C (32 Hu/ml). At 25°C, activity detected was 16 Hu/ml, whilst at 13°C 4 Hu/ml were detected. After incubation at 4°C, no activity was detected.

T-lysin activity was 64 Hu/ml at 37°C, 8 Hu/ml at 25°C and 1 Hu/ml at 4°C. Samples containing T_1 activity (obtained by gel filtration chromatography) (4.2.1) were found to have 256 Hu/ml of activity at 37°C, 16 Hu/ml at 25°C and 1 Hu/ml of activity at 4°C.

In a separate experiment, supernates containing H or T-lysin, and samples of T_1 activity, were assayed for activity at 37°C (1 hr) then cooled to 4°C. No increase in haemolytic activity was observed in comparison with samples incubated at 37°C only. Trout erythrocytes treated with T_1 activity did not lyse completely after cooling to 4°C (4.2.1)

5.2.3 The effect of membrane filtration on haemolytic activity

Crude extracellular products from many bacterial species are often rendered cell free by membrane filtration of culture supernatant fluid. It was therefore decided to investigate the effect of membrane filtration on A. salmonicida haemolytic activity. Two ml volumes of shaken or unshaken supernatant fluid were passed through membrane filters (Millipore, 0.45 μm, 25 mm) and the eluant fluid assayed for

T and H-lysin respectively. The results indicated that H-lysin activity (16 Hu/ml) was lost after this treatment whilst T-lysin activity (12 Hu/ml) was unaffected.

Membrane filtration of larger volumes (7 ml) of supernatant fluid containing H-lysin (32 Hu/ml) resulted in the eventual appearance of H-lysin in the eluant (Fig. 23). The prior addition of BSA (1% w/v) to culture supernate prevented the loss of H-lysin activity.

Membrane filters pre-washed with BSA and PBS (5 ml, 1% w/v BSA, 2 x 5 ml PBS) showed no capacity to affect filtered H-lysin activity.

Neither BSA (1% w/v in PBS) nor high salt concentrations (3M NaCl or 3M $(NH_4)_2 SO_4$ in PBS) were effective in eluting the "lost" H-lysin activity from membrane filters.

5.2.4 The effect of dialysis on haemolytic activity

In view of the importance of dialysis steps in protein purification regimes, it was decided to investigate the effect of dialysis on H-and T-lysin activity. Supernatant fluid samples containing H-lysin (2 ml) or T-lysin were dialysed against phosphate buffered saline (PBS + 0.02% NaN_3, 18 hr, 4°C) and the non-dialysable material tested for haemolytic activity compared with similar, frozen control samples. To compensate for changes in volume of the supernate fluid during dialysis the volume of the control samples was increased proportionately by the addition of PBS.

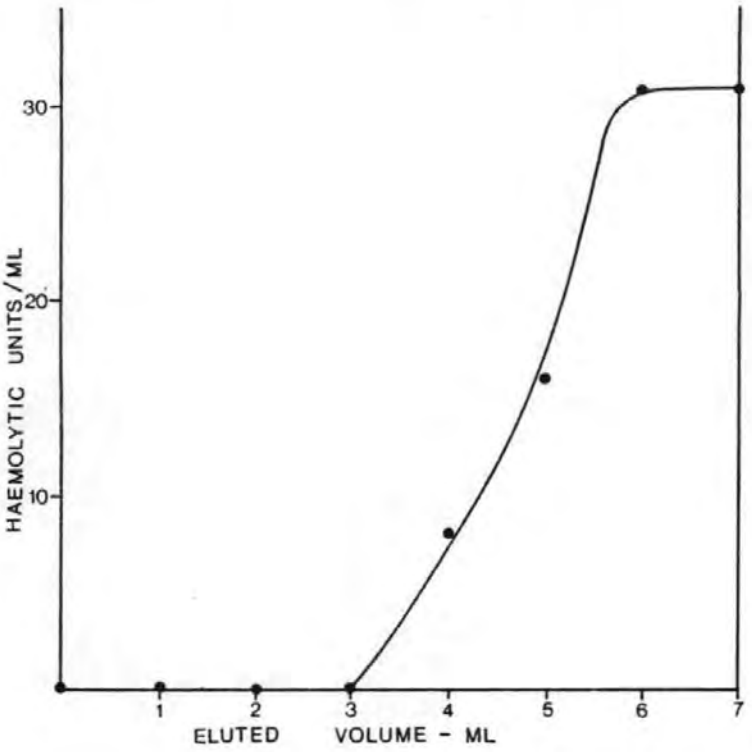
The results indicated that the H-lysin activity (32 Hu/ml) was similar in dialysed and control samples.

T-lysin activity was also unaffected by dialysis, similar activity was found in dialysed and control samples (128 Hu/ml).

5.2.5 The effect of pH on the stability of haemolytic activities

In view of the fact that changes in environmental pH conditions are known to affect the stability of most biological molecules, it was decided to investigate the effect of pH on H and T-lysin stability. The pH of supernate samples (5 ml), containing 0.02% NaN_3 to inhibit bacterial growth, were altered to give pH's in the range 4 - 10.

Figure 23 The effect of membrane filtration on H-lysin activity



● = H-lysin activity (Hu/ml)

Unshaken (96 hr) culture supernate was passed through a membrane filter and the eluant tested for H-lysin activity.

The pH was altered by careful, slow addition of 1M NaOH or 1M HCl . The volume of NaOH or HCl was considered to have an insignificant effect on the volume of the supernatant fluid. After incubation for 48 hours at 4°C, the pH of samples was adjusted to 7.2 using 1M NaOH or 1M HCl . Samples were assayed for haemolytic activity.

The results indicated that H-lysin was stable over the range pH 7 - 9 (Fig. 24) whilst T-lysin was most stable at pH 7 (Fig. 25).

5.2.6 The effect of buffers on haemolytic activity

In view of the finding that H-lysin was most stable at pH 8.0 it was decided to investigate the effects of various buffer solutions on H-lysin activity at pH 8.0. Unshaken culture supernatant fluid (96 hr, 25°C) containing 0.02% NaN_3 was concentrated by dialysis against polyethylene glycol (30% w/v pH 8.0, 18 hr, 4°C).

0.1 ml volumes of the concentrated material (concentrated 22 fold, 128 Hu/ml) were added to 0.9 ml volumes of 0.1 M phosphate, Tris - HCl or borate buffered salines (0.85% v/v NaCl) at pH 8.0. After incubation (24 hr, 4°C) the samples were tested for H-lysin activity. The results indicated that samples in phosphate buffer showed a 25% loss in activity whilst samples in Tris - HCl showed a 75% loss in activity. In borate buffer 63% of the initial activity was lost.

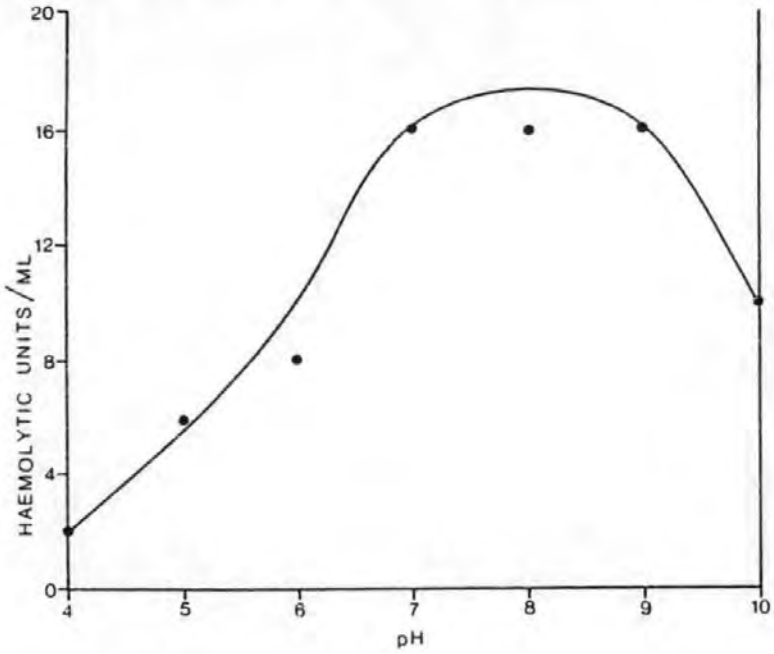
5.2.7 The effect of sodium chloride concentration on haemolysin stability

Sodium chloride is commonly used to raise the ionic strength in buffers used for protein purification techniques. It is also known that some proteins are unstable at low or high ionic strengths.

In view of these facts it was decided to investigate the effect of sodium chloride concentration on the H-lysin.

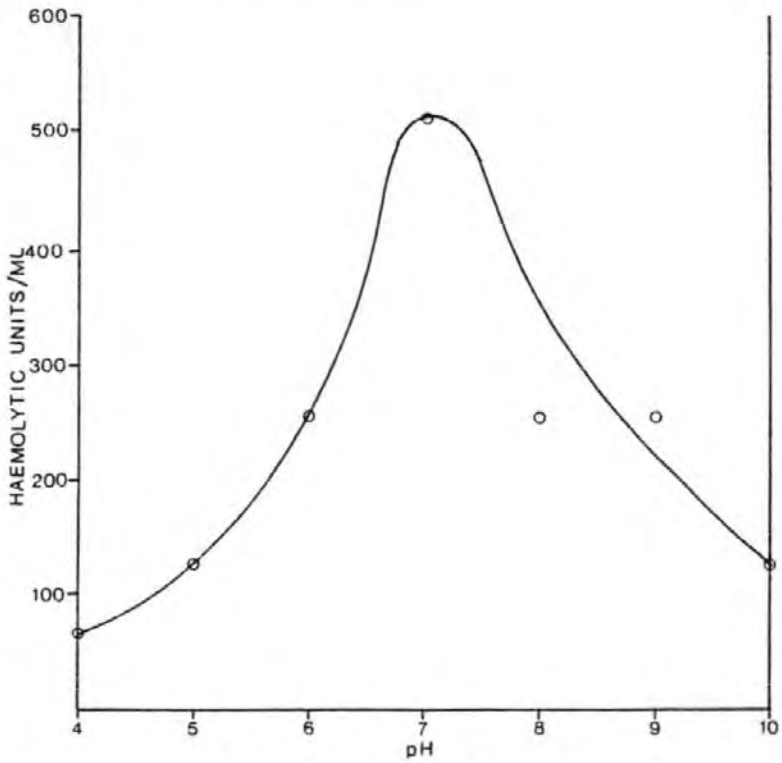
H-lysin precipitated using ammonium sulphate (6.2.3) was mixed (0.1 ml + 0.1 ml) with phosphate buffer (0.1 M, pH 7.2) containing NaCl (0.063 M, 0.125 M, 0.25 M, 0.5 M, 1.0 M and 2.0 M) and incubated for 1 hour at 4°C in a microtitre tray.

Figure 24 The effect of pH on H-lysin stability



● = H-lysin activity (Hu/ml)

Figure 25 The effect of pH on T-lysin stability.



o = T-lysin activity (Hu/ml)

The samples were assayed for H-lysin activity as described previously (2.4.2). The results indicated that all samples contained similar H-lysin activity (32 Hu/ml) to the control sample mixed with PBS only (0.1 ml + 0.1 ml). In view of the dilution of samples required to reach the 50% endpoint (x 32 dilution) the concentration of added NaCl in these wells was considered to have an insignificant effect on the total ionic strength in these wells.

5.2.8 Evaluation of suitable methods for the concentration of H-lysin activity

In view of the fact that concentration steps are often important in bacterial product purification schemes it was decided to evaluate the suitability of some concentration techniques for the H-lysin.

Samples of unshaken culture supernate (3.2.15) containing H-lysin (10 ml, 16 Hu/ml) were lyophilised, dialysed against polyethylene glycol (PEG 6,000, Fisons, 30% w/v, 4°C, +0.02% NaN_3) or mixed with lyphogel (Sigma, 1.82 g, 4°C). The volume of material after PEG dialysis was 0.9 ml and lyophilised material was resuspended to a similar volume in distilled water. Control (unconcentrated samples) were frozen (-80°C) until required.

The samples were tested for H-lysin activity (2.4.2) and the results indicated that whilst 72% of the control haemolytic activity was recovered after lyophilisation, only 9% of the control activity was detected in PEG concentrated samples. Samples concentrated using lyphogel were not tested due to difficulties experienced in separating the lyphogel from the concentrated fluid.

5.2.9 The effect of trout serum on haemolytic activity

It has been demonstrated that some bacterial haemolysins are inhibited by serum components. It was therefore decided to investigate the effect of normal trout serum on A. salmonicida haemolytic activity. Rainbow trout serum was heated to destroy complement activity (45°C, 20 min) and mixed with shaken (containing

48, 24 or 128 Hu/ml T-lysin) or unshaken (containing 24, 16 or 12 Hu/ml H-lysin) supernate (0.1 ml + 0.1 ml). The mixture was diluted out in two fold steps in 0.1 ml aliquots of supernate and PBS (50 ml supernate + 50 ml PBS).

Horse or trout erythrocyte suspensions were added to the wells and incubated for 1 hour at 37°C. The supernates were also assayed for T or H-lysin activity after an initial dilution of the supernate (1 : 1 with PBS) as a control.

The results indicated that the serum was capable of inhibiting T-lysin activity but that H-lysin activity was unaffected.

The experiment was repeated using sera from different rainbow trout and different culture supernate samples with similar results. 0.1 ml of trout serum was calculated to be capable of inhibiting 1,100 Hu of T-lysin (mean value of three experiments).

When tested in a double diffusion Ouchterlony plate (2% Oxoid purified agar in PBS) no precipitation arcs were seen between serum (50 µl undiluted and diluted x 1, x 2, x 4) and supernatant fluid containing T-lysin (128 Hu/ml, undiluted and diluted x 1, x 2 and x 4).

In a separate experiment, rat serum was tested for its ability to inhibit T-lysin activity. The serum was found to completely inhibit T-lysin activity (512 Hu/ml).

5.2.10 The effect of enzyme treatment on haemolytic activity

The investigation of the susceptibility of haemolytic activity to inactivation by a variety of enzymes with different substrate specificities may indicate the biochemical nature of the haemolytic activities produced by A. salmonicida.

To investigate this, the enzymes δ chymotrypsin (BDH, 15,000 ATEE units /mg), pronase (BDH, 45,000 PUK units/mg), RNAase (Sigma, 95 Kunitz units/mg), DNAase (Sigma, 1548 Kunitz units/mg), lysozyme (Sigma 44,400 units/mg) or lipase (Sigma, protease and amylase free) dissolved in PBS (1 mg/1 ml) were mixed (0.1 ml), with shaken or unshaken culture supernate (0.9 ml). After incubation (2 hr, 25°C), the mixtures were tested for H and T-lysin activity (2.4.2).

The results (Table 18) indicated that the proteases - pronase and δ chymotrypsin - were capable of abolishing H-lysin activity but had no apparent effect on the T-lysin. The other enzymes

tested had no detectable effect on either T or H-lysin activity.

Further addition of soyabean trypsin inhibitor (0.1 ml, 10 mg/ml in PBS) to reaction mixtures containing pronase or chymotrypsin with incubation (2 hr, 25°C) did not affect these results.

Control experiments indicated that horse erythrocytes treated with the proteases tested, under similar conditions, did not differ in their sensitivity to H-lysin.

5.2.11 Investigation of the effect of metal ions on haemolytic activity

Many enzymes have been shown to require a metal ion for the full expression of activity. Knowledge of such a requirement may be particularly important in purification schemes since the ion may be "lost", resulting in the apparent disappearance of activity.

In view of these facts it was decided to investigate the effects of deprivation of divalent cations, along with the addition of some commonly required cations, on haemolytic activity.

Divalent cations were effectively "removed" by the addition of ethylenediaminetetraacetic acid (EDTA, sodium salt, 16 mM, 31 mM, 63 mM, 125 mM final concentration) to an equivalent volume of culture supernatant fluid (0.2 ml). After incubation (4 hr, 4°C) the samples were tested for haemolytic activity. The results indicated that there was no loss of either T or H-lysin activity when compared with control supernate samples incubated with PBS only (0.2 ml + 0.2 ml, 4 hr, 4°C). EDTA (16 mM, 31 mM, 63 mM, 125 mM in PBS) had no lytic effect on horse or trout erythrocytes.

Calcium (calcium chloride), zinc (zinc sulphate), magnesium (magnesium chloride) and iron (ferrous sulphate) salts were dissolved in tris - HCl buffered saline (0.1 M, pH 7.2, 0.85% NaCl), 0.1 ml volumes of metal ion solutions (5 mM or 10 mM) were placed in their respective rows in a microtitre tray.

The first well in each row contained metal ion solution at twice the tested concentration. Material precipitated from unshaken broth culture supernate, using ammonium sulphate (6.2.2) was dialysed against tris - HCl buffer and assayed for haemolytic

Table 18 Effect of enzyme treatments on haemolytic activity

Enzyme tested	% remaining haemolytic activity	
	T-lysin	H-lysin
Chymotrypsin	100%	0%
Pronase	100%	0%
DNA ase	100%	100%
RNA ase	100%	100%
Lysozyme	100%	100%
Lipase	100%	100%

Samples were incubated for 2 hours at 25°C with enzyme (1 mg/ml final concentration in PBS).

Table 19 The ability of antiserum raised against crude H-lysin to inhibit H-lysin activity.

Broth culture supernate tested	Presence of the A layer	H-lysin activity detected (Hu/ml) when supernate was mixed with an equal volume of:		
		PBS	Antiserum	Control serum
Strain 25/77	-	24	0	24
Strain CM30	+	8	0	8
Strain 451	+	4	0	4
Strain 451/3	-	6	0	6
Strain 84/77	+	16	0	16
Strain 10/76	-	16	0	16

Supernate samples were mixed with serum or PBS, incubated (30 min, 25°C) and tested for H-lysin activity.

Table 20 Comparison of properties of crude H and T-lysins

	H-lysin	T-lysin
Dialysability	Not dialyzable	Not dialyzable
Optimum pH for stability	pH 7 - 8	pH 7
Effect of membrane filtration	Loss of activity	No effect
Thermal stability	Unstable 48°C 5 min.	Stable 48°C 5 min.
Effect of trout serum	No effect	Potent inhibitor
Effect of proteolytic enzymes	Loss of activity	No effect?
Effect of metal ions	Zinc (10 mM/5 mM) inhibits activity	No effect

activity (2.4.2) in the microtitre tray containing metal ion solutions.

The results indicated that H-lysin activity was unaffected by calcium iron or magnesium ions, however added zinc ions caused a reduction in activity (50% at 5 mM, 75% at 10 mM). No effect on T-lysin activity was observed.

Experiments performed using atomic absorption spectroscopy techniques indicated that glass distilled water used for buffer solutions contained zinc ions at 40 ppb (0.6 μM).

5.2.12 The immunogenicity of the H-lysin

The immunogenicity of a crude sample of H-lysin was investigated. Ammonium sulphate precipitated broth culture extracellular products (strain 25/77) were sterilized by membrane filtration and a sample (78 μg protein) injected into rats using a multiple site intradermal route (2.8.0). A second sample (300 μg protein) was administered by the same route 15 days later. After a further 14 days the serum was obtained and tested for the presence of antibody. Serum from uninoculated rats served as control serum. The sera were heated to destroy complement (2.8.0) and mixed with an equal volume of broth culture supernate. After incubation (30 min, 25°C) the mixtures were tested for H-lysin activity (2.4.2).

The results (Table 19) indicated that although the control serum had no effect on H-lysin activity, the antiserum completely inhibited H-lysin activity from strain 25/77. At a dilution of 1:32 the serum was capable of causing 50% inhibition of H-lysin activity (24 Hu/ml).

The H-lysin from a variety of other virulent and avirulent strains of A. salmonicida was also inhibited by the antiserum raised against strain 25/77 H-lysin.

5.3.0 DISCUSSION

The thermal lability, susceptibility to proteolytic degradation and immunogenicity of the H-lysin all indicate that the molecule is a protein. The H-lysin appears to have no divalent metal ion

requirement for full activity although zinc ions appear to suppress haemolytic activity. The inhibiting effect of zinc has been reported for a variety of other haemolysins (Avigad and Bernheimer 1976); the authors suggesting that zinc ions bind to the erythrocyte surface, altering the lipid bilayer and rendering the cell more resistant to haemolysis.

One of the most important observations concerning the H-lysin was the effect of membrane filtration on haemolytic activity. The finding that membrane filters bind the H-lysin is of importance when considering previous work on the toxicity of crude membrane filtered extracellular products (Ellis et al 1981); the H-lysin may have been excluded from these studies.

The biochemical properties of the caseinase component of the T-lysin have been reported by other workers (Shieh and Maclean 1975, Sheeran and Smith 1981, Ellis et al 1981). The properties of the T_1 component of the T-lysin are, however, unclear. The T-lysin was sensitive to thermal denaturation, however it was apparently unaffected by various enzyme treatments. The sensitivity of the activity to proteolytic degradation is difficult to evaluate since such proteases contribute to T-lysin activity (4.6.0).

The inhibitory effect of serum on T-lysin activity has been reported by Ellis et al (1981) and the results obtained in this project, indicating that the T-lysin activity was due to two components, mean that the nature of this inhibition can be partially explained. Serum anti-proteases may block the activity of the caseinase component of the T-lysin (Heimburger 1975). It is not clear, however, why the serum was capable of blocking the T_1 activity. The inhibition of S. aureus δ lysin by serum has been reported (Kreger et al 1971) where the inhibiting component is thought to be serum phospholipids (Kapral 1972). The inhibition of the caseinase and T_1 activities may therefore occur by different mechanisms. This interesting possibility merits further attention perhaps by testing individual serum components for their ability to inactivate the caseinase and T_1 activities. The inhibition of

T-lysin activity by trout serum may be of consequence in the pathogenesis of furunculosis since the effects of this extracellular product *in vivo* may be inhibited. The observation that H-lysin activity was not affected by trout serum strengthens the case for this extracellular product being important in the pathogenesis of furunculosis.

A number of comparisons have been made in this project between *A. salmonicida* T_1 activity and *S. aureus* δ-lysin. The serum inhibition, susceptibility of fish erythrocytes to lysis and apparent molecular weight heterogeneity are properties shared by both these haemolysins. *S. aureus* δ-lysin is thought to possess a detergent like action (Alouf 1976), however this mode of action seems unlikely to apply to the T_1 activity. Unlike δ-lysin (Thelestam et al 1973), the expression of T_1 activity appeared to be temperature dependant with very little activity detected at $4^{o}C$. This observation tends to confirm the previous suggestion that the T_1 activity possesses an enzymatic or pore forming mode of action (4.6.0), however further work is required to elucidate the mode of action of T_1 activity in detail.

CHAPTER 6 THE PURIFICATION AND PROPERTIES OF THE H-LYSIN

6.1.0 INTRODUCTION

Purification of potential bacterial toxins is a prerequisite for detailed studies concerning the mode of action and properties of these activities. The importance of bacterial product purification is exemplified by considering studies concerning the haemolysins of S. aureus. The results of many early studies, using ostensibly pure haemolysins, are now of dubious significance in view of recent work indicating that these preparations probably contained other S. aureus extracellular products. The presence of such 'contaminating' activities may have been responsible for many of the properties attributed to individual haemolysins in this early work.

This section of the project was devoted to the investigation of purification techniques applicable to A. salmonicida H-lysin. The properties of the H-lysin obtained after the use of these purification techniques were investigated. In addition, the possible mode of action of the H-lysin was investigated and its potential contribution to the pathogenesis of furunculosis evaluated.

6.2.0 RESULTS

6.2.1 Initial studies involving the precipitation of haemolytic activity

The large volumes of culture supernate required for bacterial haemolysin purification often result in the initial purification stage not only effecting an increase in the specific activity of the haemolysin but also resulting in a decrease in volume of the preparation. Agents capable of precipitating proteins are often used for this purpose.

The precipitants polyethylene glycol (Fisons, PEG 400), acetone and ammonium sulphate (BDH low in heavy metals) were tested for their

suitability as haemolysin precipitants. Culture supernate containing haemolysin was obtained by centrifugation (7,500 x g, 20 min, 4°C) of unshaken cultures in nutrient broth No. 2 (96 hr, 25°C).

One ml volumes of culture supernate were added dropwise to acetone (-20°C) to give various acetone - culture supernate ratios (5:1, 7:1, 8:1, 9:1, 10:1 and 12:1). After mixing and incubation (-20°C,18 hr), precipitated material was collected by centrifugation (12,000 x g, 20 min, -20°C). Residual acetone was removed from the pellet using an air line, the pellets resuspended in PBS (1 ml) and tested for H and T-lysin activity. The results indicated that whilst limited T-lysin activity was recovered (50%) no H-lysin was detected.

Precipitation using PEG was achieved by adding PEG 400 to culture supernate (25 ml) to give various final PEG concentrations (1%, 5%, 10% or 20% v/v). After mixing and incubation (1 hr, 0°C) precipitation material was collected as detailed above (0°C) and dialysed to remove residual PEG (18 hr, 4°C, 2 x 1 l PBS). No H or T-lysin activity was detected in the non-dialysable material.

Precipitation using ammonium sulphate was accomplished by the sequential addition of saturated ammonium sulphate solution (at 0°C, pH 8) to the culture supernate to give 40, 50 and 60% saturation. Precipitated material was collected by centrifugation. Solid ammonium sulphate was added to give 100% saturation (2.3.1). After dialysis of the precipitated material against PBS the non-dialysable material was assayed for H and T-lysin activity (2.4.2). The results (Table 21) indicated that both T-lysin and H-lysin were precipitated using this technique. Recovery of T-lysin was 75% and material precipitated at 40 - 60% saturation possessed a specific activity 9.5 times higher than the starting material. Recovery of H-lysin in these fractions was 18%.

6.2.2 Evaluation of precipitation techniques using ammonium sulphate

The results obtained suggested that ammonium sulphate was the haemolysin precipitant of choice. Recovery of H-lysin was, however,

Table 21 Ammonium sulphate precipitation of haemolytic activity

Fraction	Volume (ml)	Protein (mg/ml)	Haemolytic activity (Units/ml/60 min)		Specific activity (Units/mg/60 min)		Recovery (%)		Purification factor	
			H-lysin	T-lysin	H-lysin	T-lysin	H-lysin	T-lysin	H-lysin	T-lysin
Culture supernate	440	7.75	32	8	4.1	1.03	-	-	1	1
0-40% saturation	94	0.17	1	1	5.7	5.7	1	3	1.39	5.5
40-50% saturation	80	0.3	16	16	53.3	53.3	9	36	13	52
50-60% saturation	79	0.65	16	16	24.6	24.6	9	36	6	24
60-100% saturation	319	0.94	0	0	-	-	-	-	-	-
Remaining material	995	1.77	0	0	-	-	-	-	-	-

Ammonium sulphate was added sequentially to culture supernate as a saturated solution (to 60% saturation) or as solid ammonium sulphate (60-100% saturation). Precipitated material was dialysed against PBS (18 hr, $4^{o}C$) before determination of haemolytic activity.

low (6.2.1). A number of ammonium sulphate precipitation techniques were evaluated to attempt to increase the recovery of H-lysin.

The addition of solid, finely ground, ammonium sulphate to give 40-60% saturation resulted in H-lysin recovery of 19%. The use of saturated ammonium sulphate solution, in place of the solid material, resulted in an H-lysin recovery of 35% in this experiment.

In a separate experiment the use of saturated ammonium sulphate solution (to give 40-60% saturation) which had been adjusted to various pH values (pH 6, 7 or 8) indicated that maximal H-lysin recovery was achieved when the pH was 7 to 8. In a variety of later experiments the recovery of H-lysin precipitated at 40-60 saturation was found to be variable (5% to 46% in spite of similar precipitation conditions.

It was thought that the low recovery of the H-lysin may have reflected the fact that a factor necessary for full H-lysin expression was excluded from the material precipitated at 40-60% saturation ammonium sulphate. To test this possibility material precipitated at 40-60% saturation was mixed with other ammonium sulphate precipitated fractions (0-40%, 60-100% saturation and remaining material) and tested for H-lysin activity (2.4.2). The results indicated that there was no increase in activity under these conditions.

The poor recovery of the H-lysin may have reflected the fact that this material was present at too low a concentration in the culture supernate to be effectively precipitated. The prior addition of BSA to the culture supernate increased the recovery of the H-lysin. The addition of 0.5% (w/v) BSA resulted in a 3.3 fold increase in recovery in comparison with control (no BSA) samples precipitated at 40-60% saturation. The prior addition of 0.1% (w/v) BSA did not increase the recovery of H-lysin activity.

It was also decided to investigate the possibility that an inert 'carrier' material added to the culture supernate would increase the recovery of H-lysin on subsequent ammonium sulphate precipitation (40-60% saturation). Silica gel G (Merck) or Alumina (Sigma) were added to culture supernate at 1% (w/v). The recovery

of H-lysin from these supernates was no higher than that observed if these carriers were omitted from the culture supernate.

6.2.3 Precipitation of H-lysin activity from broth cultures for use in further purification studies

In view of the fact that recovery of H-lysin was low following ammonium sulphate precipitation, large volumes of culture supernate were required to obtain sufficient H-lysin for further studies. To reduce the volume of material for subsequent ammonium sulphate precipitation the culture supernate was concentrated using a freeze fractionation technique. Volumes of culture supernate (3-4l) were frozen (-80^{o}C) and allowed to thaw slowly (4^{o}C). The first 500-700 ml of liquid were collected and used for subsequent ammonium sulphate precipitation. Recovery of H-lysin after freeze fractionation was typically 70-100%.

In later experiments it was found that the addition of ethylenediaminetetracetic acid (EDTA, sodium salt, 40 mM) to the ammonium sulphate solution was effective in consistently increasing the recovery of H-lysin (43% to 56%).

6.2.4 Ion exchange chromatography of H-lysin

The material obtained after ammonium sulphate precipitation of culture supernate was known to represent a relatively impure preparation of H-lysin, containing a variety of other extracellular products (4.2.3).

To effect further H-lysin purification the ammonium sulphate precipitated material (6.2.3) was dialysed to lower the salt concentration (PBS, 1 l, 3 hr, 0^{o}C; tris-HCl , 10 mM, 2 x 1 l, 15 hr, 0^{o}C) and non-dialysable material centrifuged (36,000 x $\underline{g}$, 10 min, 4^{o}C). The refractive index of the supernate was checked to ensure that the salt concentration was sufficiently low ($<$ 50 mM).

Initial experiments at this stage indicated that haemolytic activity would bind to an ion exchange gel (DEAE Separose DCL 68) in a suitable buffer system (10 mM tris-HCl, pH 7, 8 or 9). Binding could be prevented if NaCl (0.3 M) was added to the buffer system used.

In an experiment to evaluate this system fully, ammonium sulphate precipitated material (20 ml, 32 Hu/ml H-lysin, 128 Hu/ml T-lysin) was applied to an ion exchange chromatography column (2.9 cm x 3.1 cm, DEAE Sepharose DCL 6B) at pH 7.1 (10 mM tris-HCl). The column was eluted using a linear gradient of NaCl (0 - 0.4 M in tris-HCl, pH 7.1). The fractination process was carried out at $4^{o}C$ and the column eluant optical density (280 nm) continuously monitored.

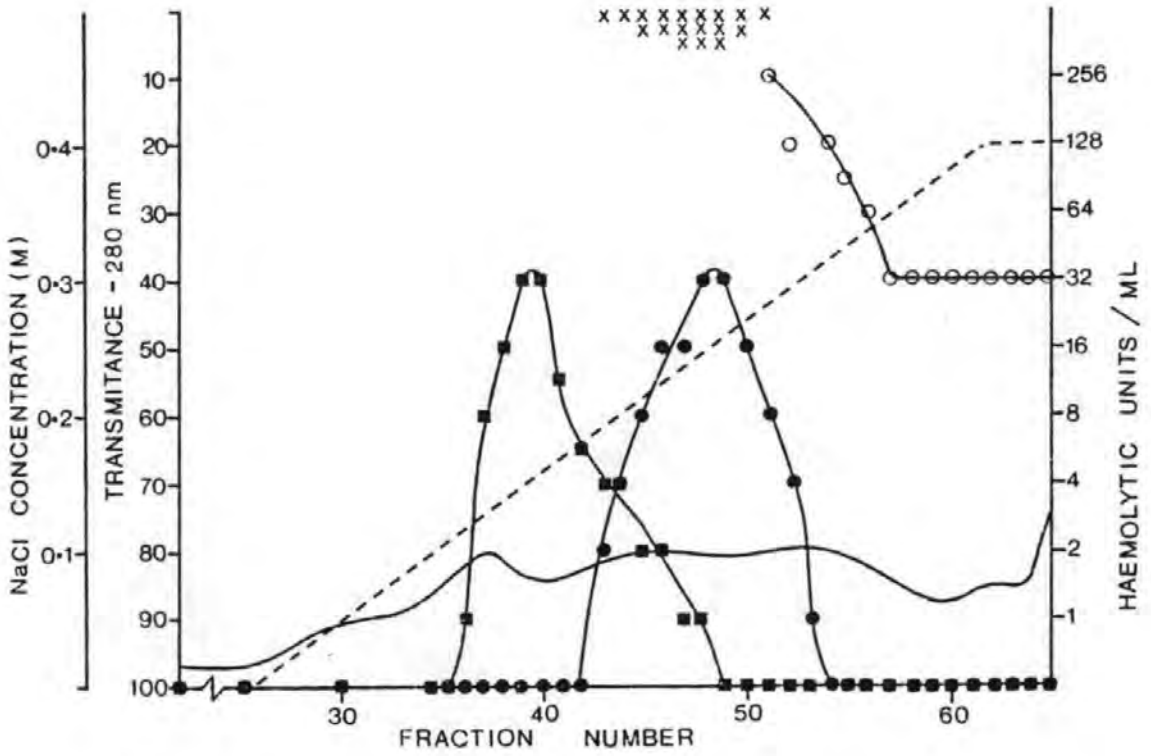
Fractions (3.7 ml) were tested for H-lysin, T-lysin, T_1 activity (2.4.2) and caseinase activity (2.4.5). The NaCl concentration was determined using refractometry.

The results (Fig. 26) indicated that the H-lysin could be separated from T-lysin, T_1 activity and caseinase activities using this technique. The H-lysin was eluted maximally at an ionic strength of 0.15 M (NaCl) with a recovery of 67%. T-lysin activity was eluted maximally at 0.25 M (NaCl) though recovery of this activity was low (20%). Fractions containing T-lysin also contained caseinase activity. The T_1 activity was not eluted as a single peak but appeared as a broad tailing peak of activity. T_1 activity could not be detected before fraction 50 because any activity was masked by T-lysin present in these fractions. By extrapolation of the T_1 activity elution curve, the T-lysin peak of activity would also appear to contain T_1 activity.

6.2.5 Purification of the H-lysin using gel filtration chromatography

Further purification of the H-lysin was effected using gel filtration chromatography. The H-lysin obtained after ion exchange chromatography (6.2.4) was dialysed to remove excess NaCl (2 x 1 l distilled water, $0^{o}C$, 3 hr), the non-dialysable material divided into two equal fractions, and lyophilised. One lyophilised fraction was resuspended in distilled water (0.5 ml), applied to a gel filtration

Figure 26 The purification of H-lysin using ion exchange chromatography



- - - = NaCl concentration (M)

■ = H-lysin activity (Haemolytic units/ml)

● = T-lysin activity (Haemolytic units/ml)

○ = T_1 activity (Haemolytic units/ml)

x x x = Visually estimated caseinase activity

—— = Optical density of column eluant (280 nm)

Material obtained after ammonium sulphate precipitation of unshaken culture supernate was applied to an ion exchange chromatography column (DEAE Sepharose DCL 6B, 2.9 x 3.1 cm) and eluted using a linear gradient of NaCl (0-0.4 M in tris-HCl buffer, 10 mM, pH 7.1).

column (1.5 cm x 28 cm, Ultrogel ACA 54) and the column eluted using PBS (pH 7.2). The fractionation was carried out at 4°C and the optical density of the column eluant (280 nm) continuously monitored. Fractions (2.5 ml) were tested for H-lysin activity (2.4.2) and stored at -80°C until required.

The results (Fig. 27) indicated that the H-lysin could be further purified using this technique though recovery of the H-lysin was low (12%).

A representation of the complete purification scheme for H-lysin is shown in Table 22. Using this scheme the H-lysin was purified by a factor of 1,770 with a recovery of 4.75%. The specific activity of the preparation was 3,980 Hu/mg/60 min.

6.2.6 Evaluation of the purity of the purified H-lysin

The H-lysin obtained after purification (6.2.5) was tested for purity using a number of techniques. The H-lysin preparation was not found to contain detectable T-lysin, T_1 activity (2.4.2), caseinase, gelatinase, egg yolk digesting activity or tributyrin digesting activity (2.4.1). Glycerophospholipid: cholesterol acyltransferase (GCAT) activity was detected using human erythrocyte membranes as a substrate for the enzyme (2.4.6). The reaction products were separated using thin layer chromatography (TLC) and visualised using iodine vapour. Cholesterol ester production, a characteristic reaction product of GCAT activity, was detected after incubation of the H-lysin preparation with erythrocyte membranes.

The H-lysin preparation was lyophilised, resuspended in distilled water (0.84 mg/ml) and 20 µl samples analysed by polyacrylamide gel electrophoresis (2.5.1) (T - 7.5%). The results (Fig. 28) indicated that there was one major band of protein (Rf = 0.27) in addition to six other minor bands of protein (Rf values = 0.42, 0.46, 0.52, 0.59, 0.61, 0.75). The main protein band appeared to represent 65% of the total stained protein in the sample. Overlaying a similar gel track with horse erythrocyte agar

Figure 27 The purification of H-lysin using gel filtration chromatography

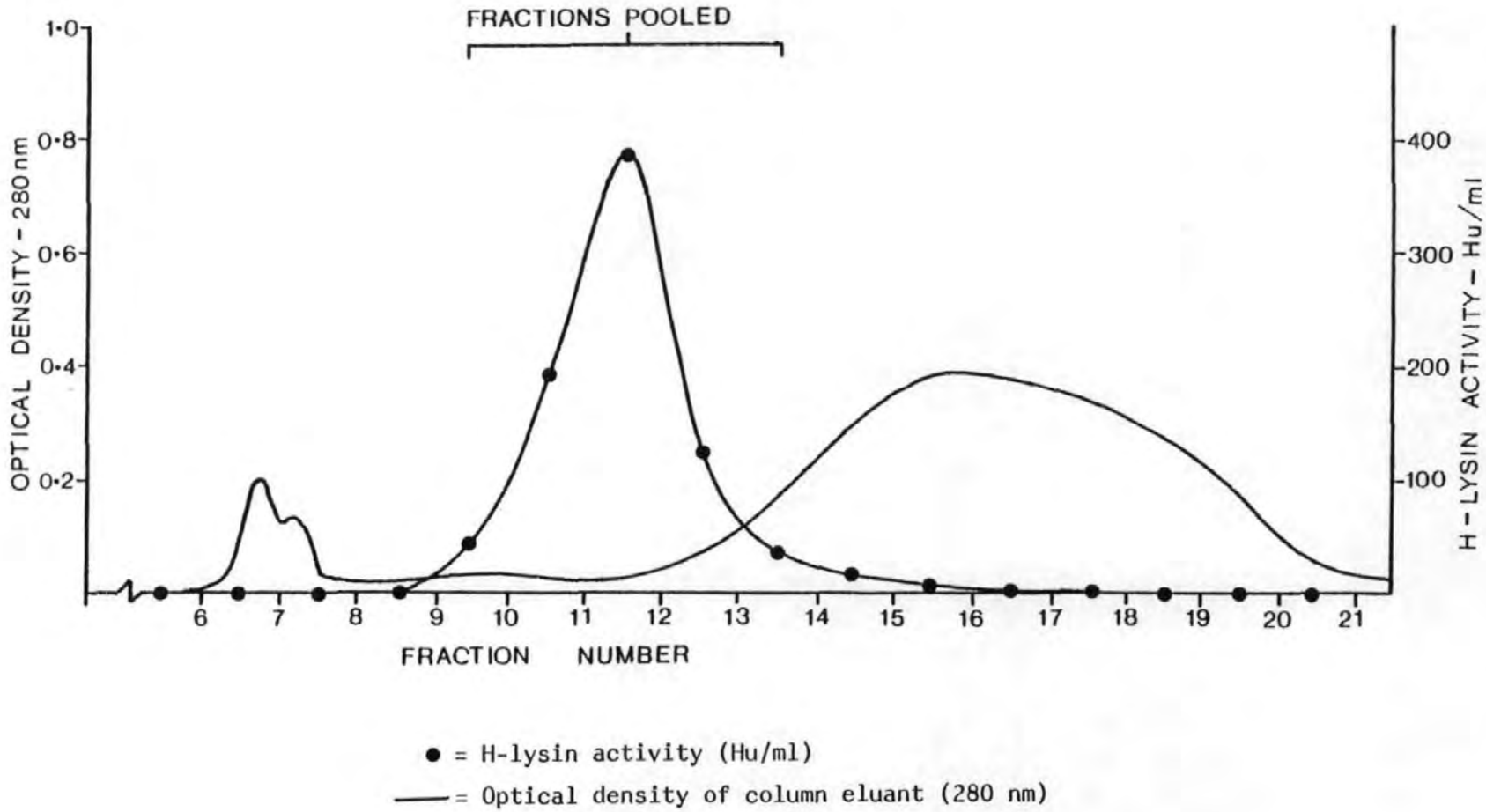
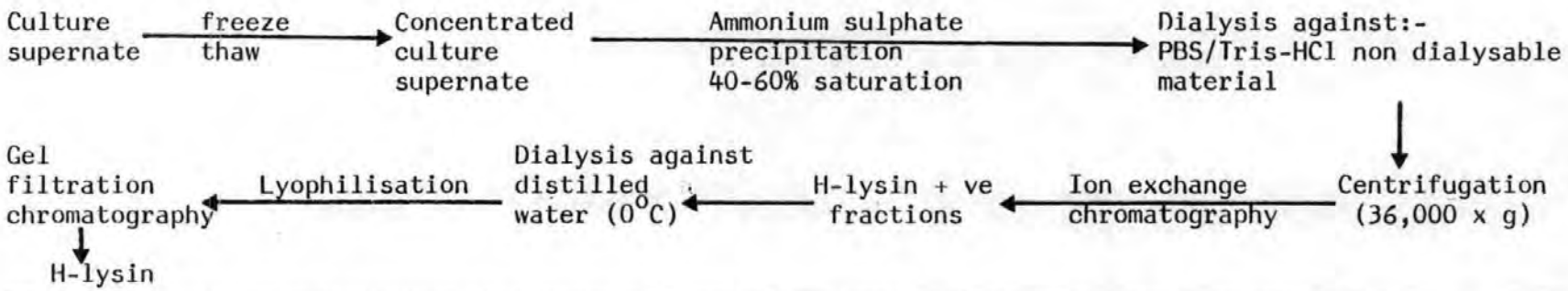


Table 22 The Purification of A. salmonicida H-lysin



Purification stage	Volume (ml)	H-lysin activity (Hu/ml)	Total H-lysin activity (Hu)	Total H-lysin used for next step (Hu)	Protein (mg/ml) Lowry method	Protein (mg/ml) O.D.260/280 nm method*	Specific activity (Hu/mg/ 60 min)	Purification factor	Recovery %
Culture supernate	2,720	32	87,040	87,040	14.2	N.D.	2.25	1	
Concentrated culture supernate	460	192	88,320	86,400	75.6	N.D.	2.53	1.12	101
Ammonium sulphate precipitation	118	320	37,760	37,120	1.32	N.D.	242	108	45
Ion exchange chromatography	52.5	650	34,125	33,800	0.383	0.471	1697	754	41
Gel filtration chromatography	25	160	3,920	-	N.D.	0.0394	3980	1770	4.75

* Calculated from the method of Warburg and Christian (1942)

(2.5.2) with subsequent incubation (up to 24 hr, 25°C) did not reveal any zones of cell lysis.

Using a double diffusion (Ouchterlony) technique (1% Oxoid purified agar in PBS + 0.02% NaN_3) 40 µl samples of H-lysin were tested against an antiserum raised against ammonium sulphate precipitated culture supernate (2.8.0). After 48 hours incubation (25°C) no precipitation arcs were observed.

6.2.7 The effect of membrane filtration on H-lysin and GCAT activities

In view of the finding that H-lysin preparations contained GCAT activity (6.2.6) it was decided to attempt to selectively remove one of these activities from the preparation.

The H-lysin was membrane filtered (Millipore 0.45 µm, 13 mm) to remove H-lysin activity (5.2.3) (24 Hu/ml). The eluant fluid contained no detectable H-lysin activity however GCAT activity was still detectable (2.4.6).

Visual examination of the TLC plates suggested that there was no decrease in cholesterol ester production in membrane filtered samples when compared with control unfiltered samples.

6.2.8 Evaluation of methods for the storage of the H-lysin

A number of potential methods for the storage of H-lysin with minimal loss of activity were evaluated. Samples of H-lysin were stored at -80°C (with or without 10% glycerol) or stored at 4°C after ammonium sulphate precipitation (80% saturation). After one month storage the ammonium sulphate precipitated samples were centrifuged (10,000 x g, 20 min, 4°C) and the pelleted material resuspended to its original volume in PBS. Lyophilised samples were also resuspended in PBS to their original volume. All samples were tested for H-lysin activity. Samples stored at -80°C possessed the greatest H-lysin activity when tested in the microtitre tray assay system (32 Hu/ml) (2.4.2). The addition of glycerol did not enhance the stability of frozen samples. Ammonium suphate precipitated samples possessed 25% of the H -lysin activity in frozen samples.

Figure 28 Electrophoretic analysis of purified H-lysin

Figure 29 The ultraviolet absorption spectrum of purified H-lysin

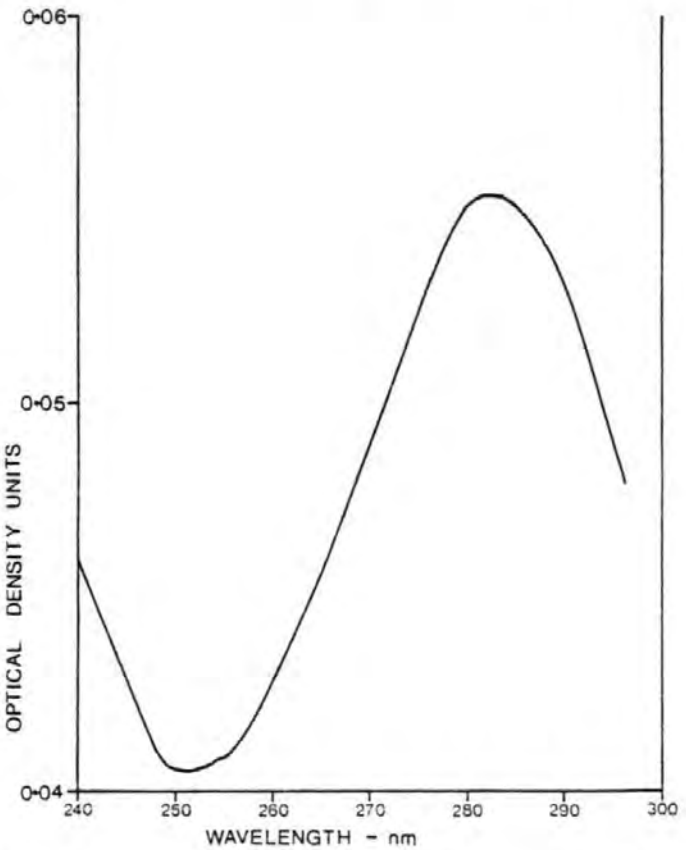


Fig. 28 legend A sample of H-lysin was electrophoresed (7.5% gel), the gel stained to locate proteins and a densitometer used to record the locations of stained material.

Samples of H-lysin stored at -80°C were not completely stable and showed losses of H-lysin activity of 60% over storage periods of one month.

6.2.9 The ultraviolet absorption spectrum of the H-lysin

The ultraviolet absorption spectrum of the H-lysin was investigated since the use of this technique may have indicated the biochemical nature of the preparation.

The absorption spectrum (240 - 296 nm) of a sample of H-lysin (0.049 mg/ml) was determined using a scanning spectrophotometer (Pye Unicam SP 1800). The results (Fig. 29) indicated that there was an absorption maximum at 282 nm (optical density = 0.0553 units) and a minimum at 252 nm (optical density = 0.0405 units). The ratio of optical densities at 282 and 252 nm was 1.23.

6.2.10 Determination of the apparent molecular weight of the H-lysin

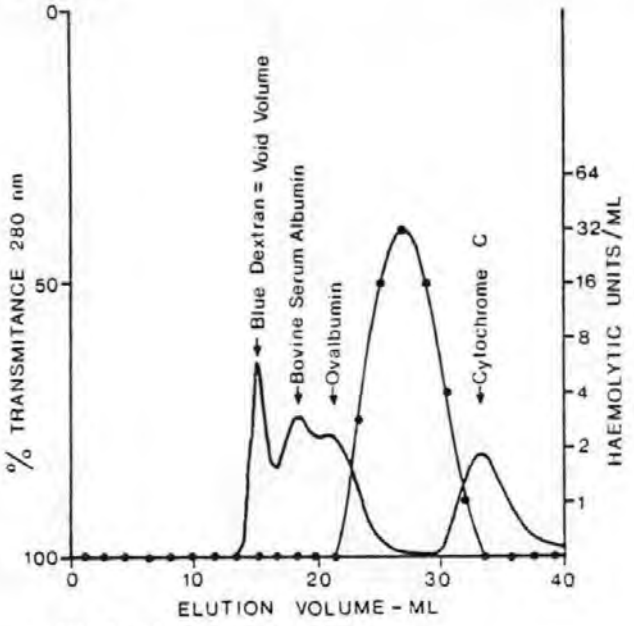
The apparent molecular weight of the H-lysin was determined using gel filtration chromatography. A variety of proteins of known molecular weight were dissolved in a sample of H-lysin (0.5 ml) and applied to a gel filtration column (Ultrogel ACA 54, 1.5 cm x 28 m). The column was eluted using PBS and the optical density of the column eluant monitored (280 nm). Fractions collected (1.8 ml) were tested for H-lysin activity (2.4.3). The standard proteins employed were bovine serum albumin (Mol. wt. 66,000, 3.1 mg), ovalbumin (mol. wt. 45,000, 2 mg) and cytochrome C (mol. wt. 12,500, 0.7 mg).

The results (Fig. 30) indicated that by comparison of the elution volumes of the protein standards and the H-lysin, the H-lysin possessed a molecular weight of 25,900 (mean of three determinations) (Fig. 31).

6.2.11 The thermal stability of H-lysin

The effect on stability of exposure of H-lysin to a variety of temperatures was determined. Samples of H-lysin (6.2.5) (1.1 ml,

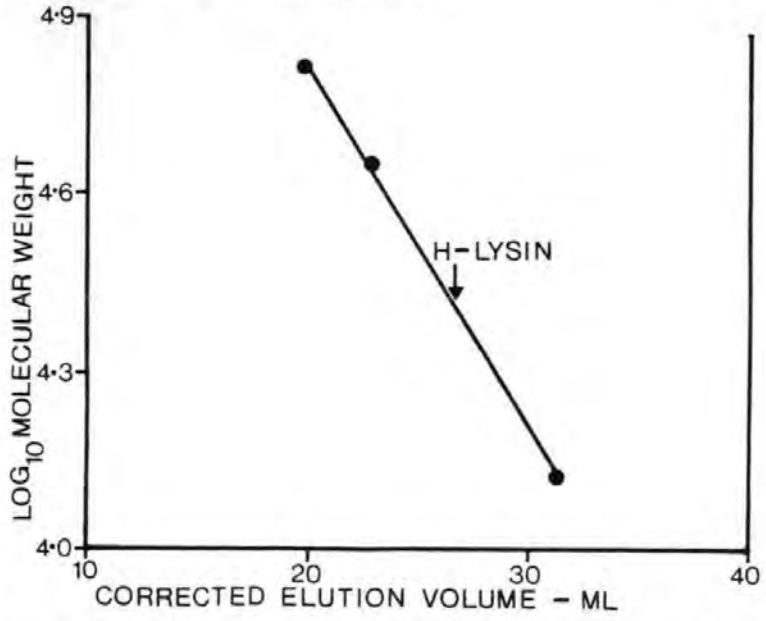
Figure 30 The elution pattern of H-lysin and a number of standard proteins from a gel filtration chromatography column



—— = Optical density (280 nm)
● = H-lysin activity

Elution points of the standard proteins are indicated by an arrow.

Figure 31 The estimation of H-lysin molecular weight using gel filtration chromatography



The corrected elution volumes of proteins of known molecular weight was plotted against the $\log_{10}$ of the molecular weight

20 Hu/ml) were maintained at a variety of temperatures (0°C, 2.5°C, 15.5°C, 23°C, 37°C, 40°C) and at various times (t_0, t+15 min, t+30 min, t+45 min, t+60 min) samples were taken and tested for H-lysin activity (2.4.3).

The results (Fig. 32) indicated that losses in H-lysin activity were observed at all the temperatures tested. At 0°C, 50% of the input haemolytic activity was lost after 60 minutes incubation whilst at 40°C no activity was detected after 15 minutes incubation . At 2.5°C, 15.5°C, 23°C and 32°C the H-lysin showed a similar pattern of activity loss.

6.2.12 The ability of a variety of compounds to enhance H-lysin thermal stability

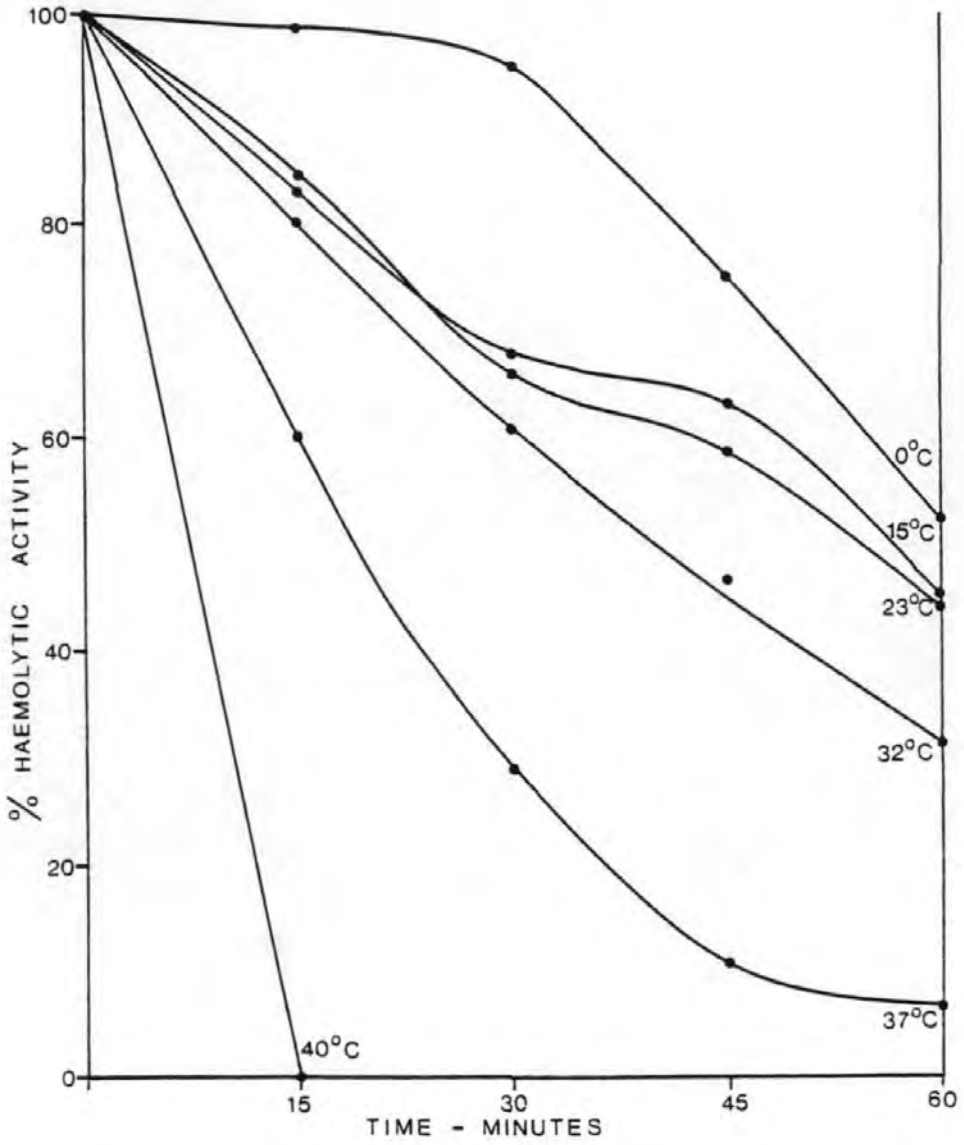
The extreme instability of the H-lysin in aqueous solution (6.2.11) may have reflected an unfavourable environment, and would present problems involving further work with the H-lysin. A variety of potential stabilising agents were tested for their ability to enhance the stability of the H-lysin.

Samples of H-lysin (6 Hu/ml) were incubated at 37°C in the presence of EDTA (2 mM or 20 mM), BSA (1 mg/ml), glycerol (10% v/v) or in the presence of tris-HCl buffered saline (0.1 M, pH 7.2, 0.85% NaCl).

The results indicated that glycerol had no effect on H-lysin stability whilst in tris-HCl buffer decreased stability was observed (70% of control activity at t+ 30 min). BSA had a marked effect on the stability of H-lysin (2.5 fold increase in stability at t+ 60 min) whilst EDTA had a less pronounced effect (20 mM - 2 fold increase in stability, 2 mM - 1.6 fold increase in stability).

In a later experiment H-lysin samples (5.3 Hu/ml) were incubated in the presence of PMSF (1 mM) or NaCl (0.25 M) at 37°C (60 min). Neither of these compounds were capable of enhancing the stability of the H-lysin when compared with control samples (H-lysin in PBS).

Figure 32 The stability of purified H-lysin at various temperatures in PBS.



Samples of H-lysin (20 Hu/ml) were maintained at the temperatures indicated and tested at intervals for H-lysin activity.

6.2.13 The kinetics of horse erythrocyte lysis by H-lysin

The kinetics of horse erythrocyte lysis by H-lysin were investigated using a recording spectrophotometer (Pye Unicam SP1800) at a wavelength which showed the maximum difference between lysed and unlysed cells (590 nm). The blank cuvette contained horse erythrocyte suspension (0.6 ml) + PBS (0.6 ml) whilst the sample cuvette contained H-lysin in place of the PBS. The time course for the haemolysis of erythrocytes was determined for a variety of H-lysin concentrations. The experiments were performed at 30°C because higher temperatures have been shown to rapidly inactivate the H-lysin. To further enhance H-lysin stability, BSA (0.5 mg/ml) was also added to the reaction mixture (6.3.12).

The results indicated that lysis of cells was preceded by a phase when the cells were apparently intact (pre-lytic or lag phase) (Fig. 33). The length of the pre-lytic phase showed an inverse relationship to the H-lysin concentration. The length of the pre-lytic phase showed a linear relationship to the $\log_{10}$ of the H-lysin concentration (Fig. 34).

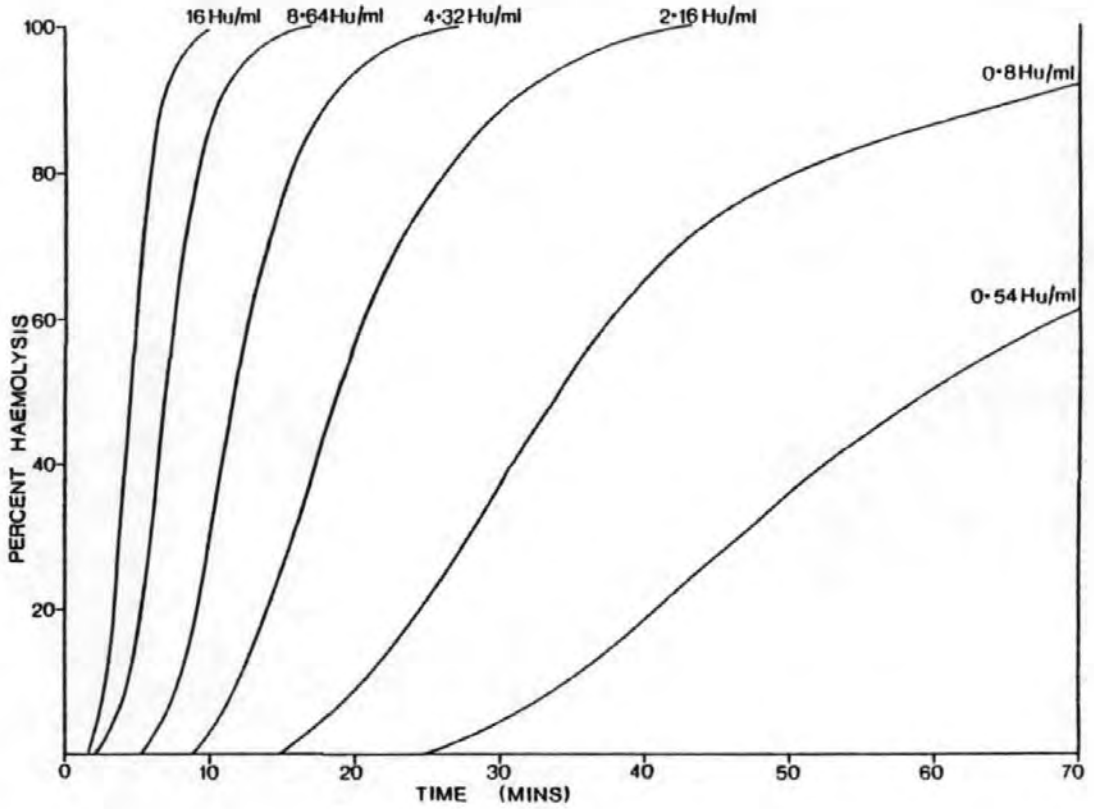
The maximum rate of haemolysis (% change/min) appeared to be related to the H-lysin concentration. A graphic representation of these variables indicated that the maximum rate was directly related to H-lysin concentrations up to 2.2 Hu/ml. H-lysin concentrations above 2.2 Hu ml did not result in a proportional increase in the maximum rate of cell lysis (Fig. 35).

6.2.14 The effect of temperature on the kinetics of horse erythrocyte lysis

Using a recording spectrophotometer (6.2.14) the effect of temperature on the kinetics of horse erythrocyte lysis was determined. The temperature of the reaction and blank cuvettes was maintained at a constant level and the lysis of horse erythrocytes followed after the addition of 9 Hu/ml of H-lysin. Temperatures in the range 9-37°C were investigated.

The results (Fig. 36) indicated that the incubation temperature was related to the log of the pre-lytic phase length (Fig. 37). The maximum rate of cell lysis (% change/min) increased with

Figure 33 The kinetics of horse erythrocyte lysis by increasing concentrations of H-lysin



The experiment was performed at 30°C in the presence of 0.5 mg/ml BSA.

Figure 34 The relationship between H-lysin concentration and the length of the pre-lytic phase of haemolysis

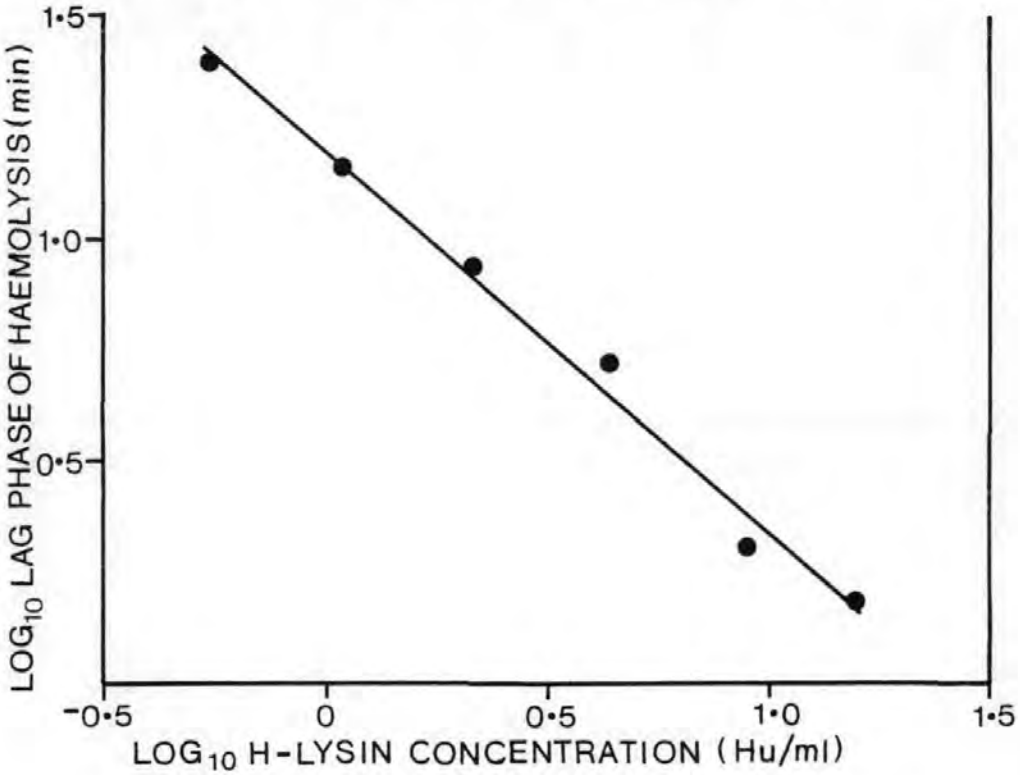
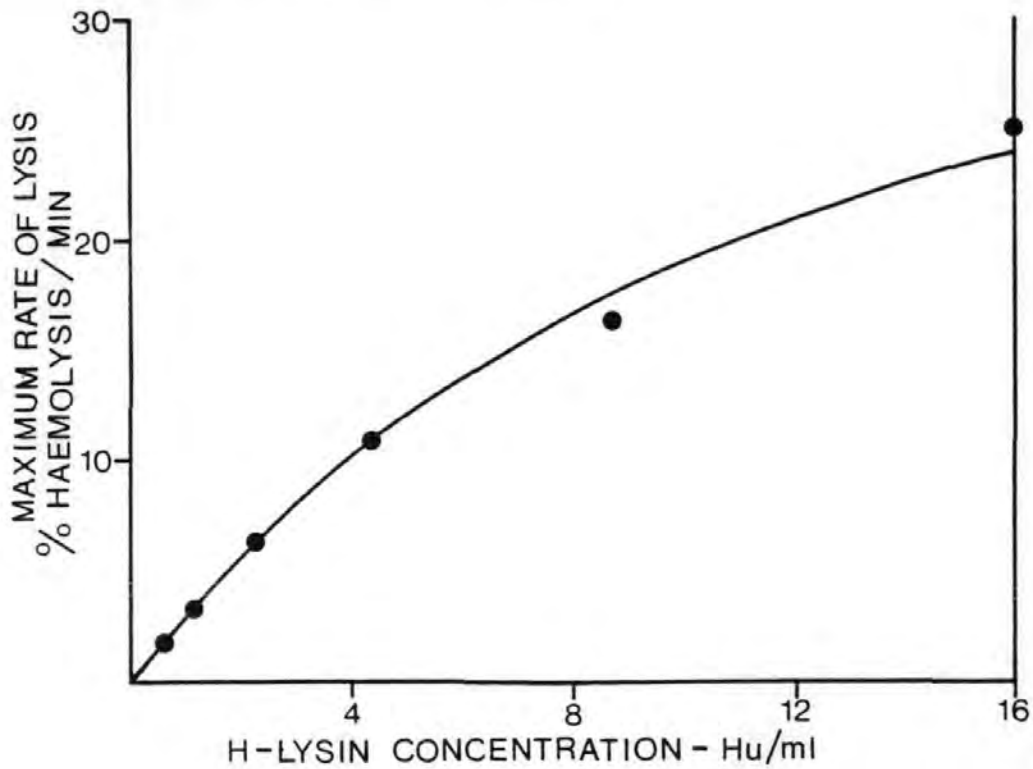


Figure 35 The relationship between H-lysin concentration and the maximum rate of cell lysis



increasing temperature in the range 9-33°C. At 37°C a decrease in the maximum rate of cell lysis was observed. The results suggested that maximum cell lysis was observed at between 25 and 33°C (Fig. 38).

The decrease in rate of cell lysis observed at temperatures above 33°C may have reflected the increased lability of the H-lysin at these temperatures. To investigate this possibility BSA (0.5% wv) was added to the incubation mixture to stabilise the H-lysin (6.3.12) (8.2 Hu/ml). The kinetics of cell lysis were determined over the temperature range 22.5 - 37°C.

The results table indicated that in the presence of BSA the maximum rate of cell lysis was observed at 33°C with a decline in maximum rate at 37°C. The difference in maximum rate between experiments at 33°C and 37°C was, however, not as marked as that observed when BSA was omitted from the reaction mixture.

6.2.15 Investigation of the pre-lytic phase of horse erythrocyte lysis by H-lysin

The effect of H-lysin on horse erythrocytes was characterised by a pre-lytic phase (lag phase) when no haemolysis was detected. It was of interest to know whether cells exposed to H-lysin during this period would subsequently lyse if the H-lysin was removed. To test this possibility 0.2 ml volumes of horse erythrocyte suspension (2.4.2) (+ 1.0 mg/ml BSA) were placed into microcentrifuge tubes (Eppendorf) and pre-incubated at 30°C (15 min). Samples of H-lysin (2.6 Hu/ml) were pre-incubated at 30°C (5 min) and 0.2 ml volumes of the lysin added to the erythrocytes. After incubation for various times, the mixtures were centrifuged (10,000 x g, 2 min, 4°C) and the supernate removed by aspiration. PBS (0.4 ml + 0.5 mg/ml BSA) was added to the pelleted cells and after resuspension of the cells 0.2 ml volumes of the mixture were placed into wells in a micro-titre tray. After further incubation (30°C, 1 hr) the percentage lysis of cells was determined using a microelisa reader (2.4.3). The pre-lytic phase of horse erythrocyte lysis by a similar sample of H-lysin (30°C + 0.5 mg/ml BSA) was found to be 9.8 minutes using a recording spectrophotometer (6.2.13). The supernatant fluid containing the H-lysin which was removed after centrifugation of

Figure 36 The kinetics of horse erythrocyte lysis at various temperatures

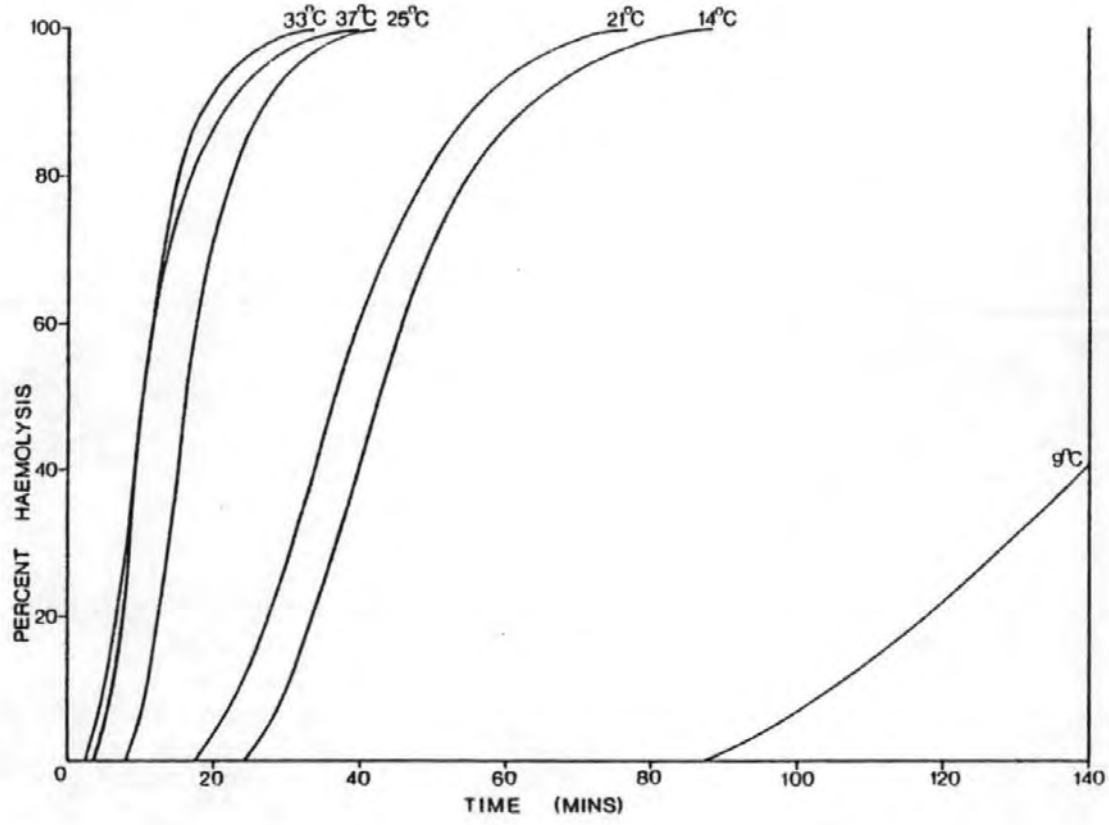


Figure 37 The effect of incubation temperature on the duration of the pre-lytic (lag) phase of haemolysis by H-lysin

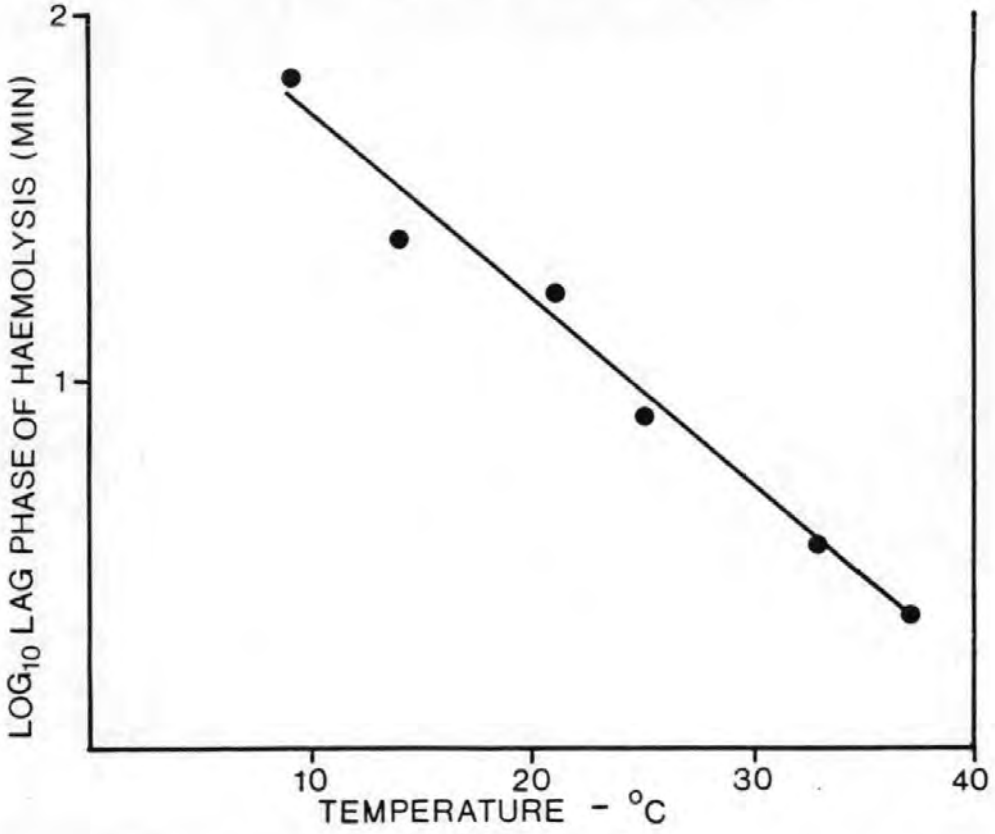


Figure 38 The effect of incubation temperature on the maximum rate of horse erythrocyte lysis by H-lysin

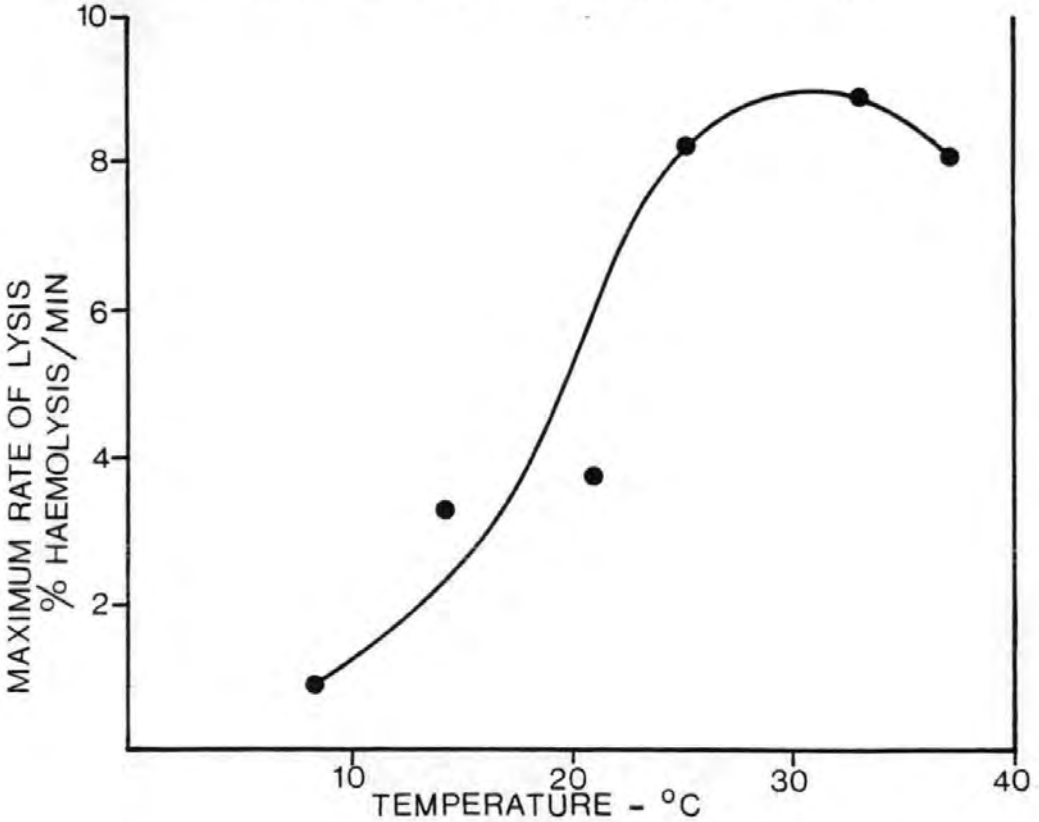


Table 23 The effect of incubation temperature on the maximum rate of horse erythrocyte lysis by H-lysin in the presence of BSA (0.5% w/v)

Incubation temperature (^{o}C)	Maximum rate of cell lysis (% change in cells lysed/min)
22.5	4.44
29.5	7.14
33.0	7.41
37.0	7.04

the cells, was also tested for H-lysin activity. The results indicated that cells exposed to H-lysin during the pre-lytic phase lysed on subsequent incubation, after removal of the H-lysin (Fig. 39). The degree of cell lysis appeared to reflect the length of time that the cells were exposed to the H-lysin. However, cells treated with H-lysin during the pre-lytic phase did not lyse completely, after incubation of cells with H-lysin throughout the whole of the pre-lytic phase (9.8 min) only 70% of the cells lysed on further incubation. Control samples indicated that horse erythrocytes treated with H-lysin (2.6 Hu/ml) for 1 hour at 30°C lysed completely.

No decrease in the H-lysin activity in the supernate removed from the cells exposed during the pre-lytic phase was observed. In another experiment using 0.47 Hu/ml of H-lysin,a limited decline in the H-lysin activity in the supernate (14% reduction) was observed over the course of the pre-lytic phase.

6.2.16 The effect of antiserum addition on the kinetics of horse erythrocyte lysis

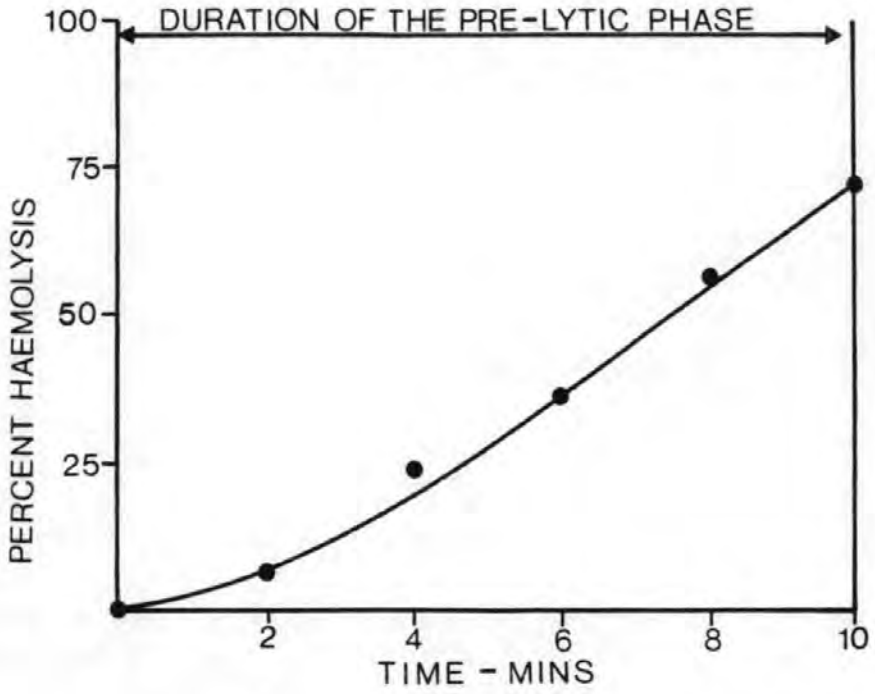
The results suggested that the H-lysin was capable of initiating the haemolytic process even if it was removed before haemolysis was detected. It was decided to investigate the effect of antiserum addition, during or after the pre-lytic phase, on the kinetics of erythrocyte lysis.

The kinetics of cell lysis were monitored using a recording spectophotometer. Horse erythrocyte suspension (0.6 ml) was mixed with H-lysin (0.6 ml, 2.3 Hu/ml) with the further addition of PBS (0.25 ml) and incubated at 30°C. The results of this control experiment indicated that there was a pre-lytic phase of 8 minutes (Fig. 40 a).

If antiserum against the H-lysin (5.2.12) was added in place of PBS no lysis of erythrocytes was observed on subsequent incubation (Fig. 40 b).

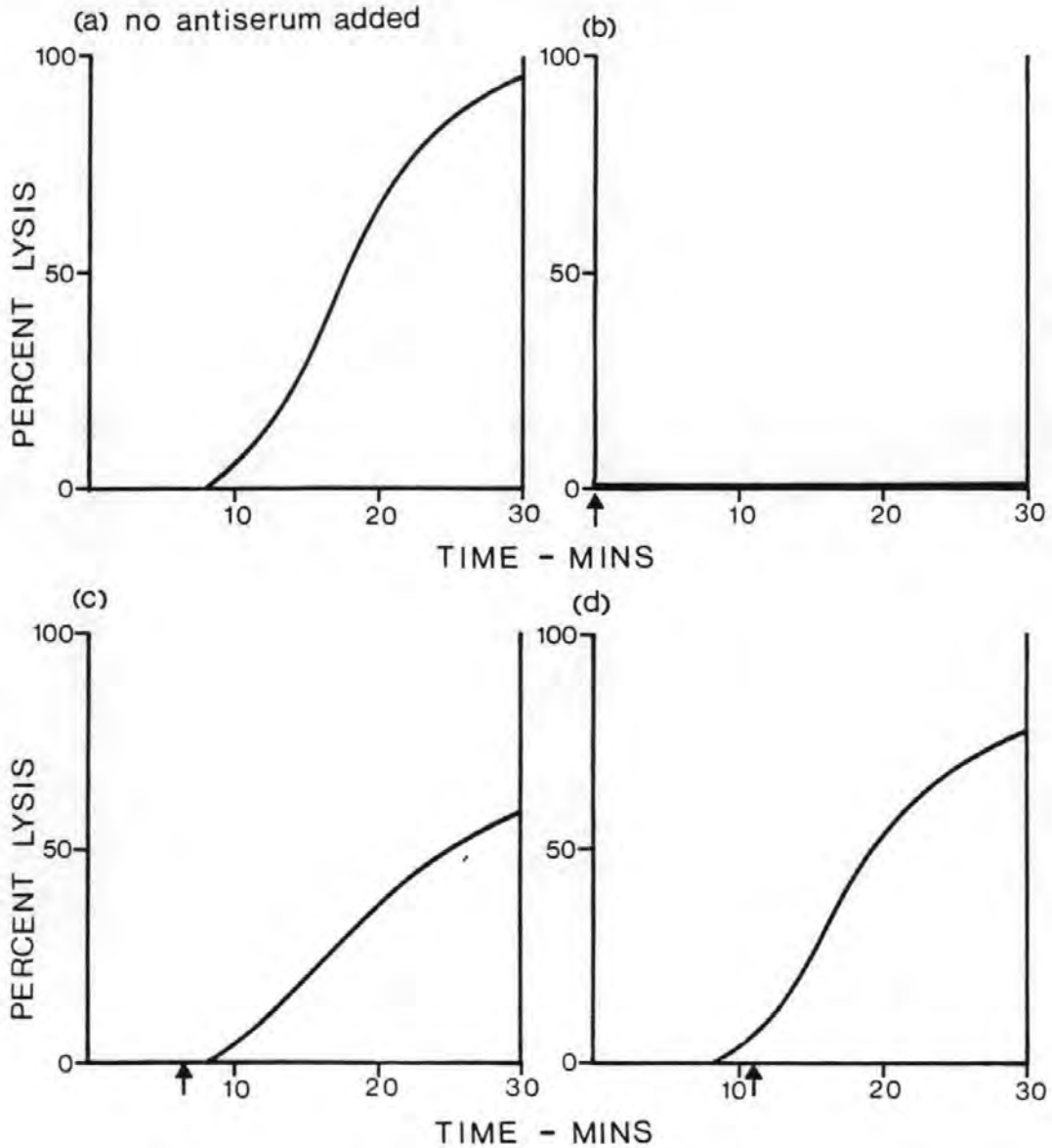
The addition of antiserum in place of the PBS, after 6 minutes resulted in subsequent lysis of the erythrocytes (Fig. 40 c). However,

Figure 39 The effect of treatment of horse erythrocytes with H-lysin during the pre-lytic phase of haemolysis, with the subsequent removal of the H-lysin and further incubation.



Horse erythrocytes were treated with H-lysin (2.6 Hu/ml) for the times indicated, the mixtures were then centrifuged briefly and the supernatant fluid removed. The pelleted cells were resuspended to their original volume in PBS, incubated for a further 60 minutes (30°C) and the percentage cell lysis determined.

Figure 40 The effect of antiserum against H-lysin on the kinetics of horse erythrocyte lysis by H-lysin



H-lysin (2.3 Hu/ml) was incubated with horse erythrocytes at 30°C (+PBS) and the kinetics of cell lysis monitored. In some cases antiserum was added to the mixture, in place of the PBS and the kinetics of cell lysis monitored.

→ = point of addition of antiserum.

the rate of cell lysis was reduced in comparison with control samples (Fig. 40 a). The addition of antiserum, in place of PBS, after the pre-lytic phase (11 min.) also resulted in subsequent erythrocyte lysis (Fig. 40 d). The maximum rate of cell lysis was higher than that observed when antiserum was added after 6 minutes, but lower than that observed with control samples (Fig. 40 a).

6.2.17 The effect of horse erythrocyte concentration on percentage haemolysis by H-lysin

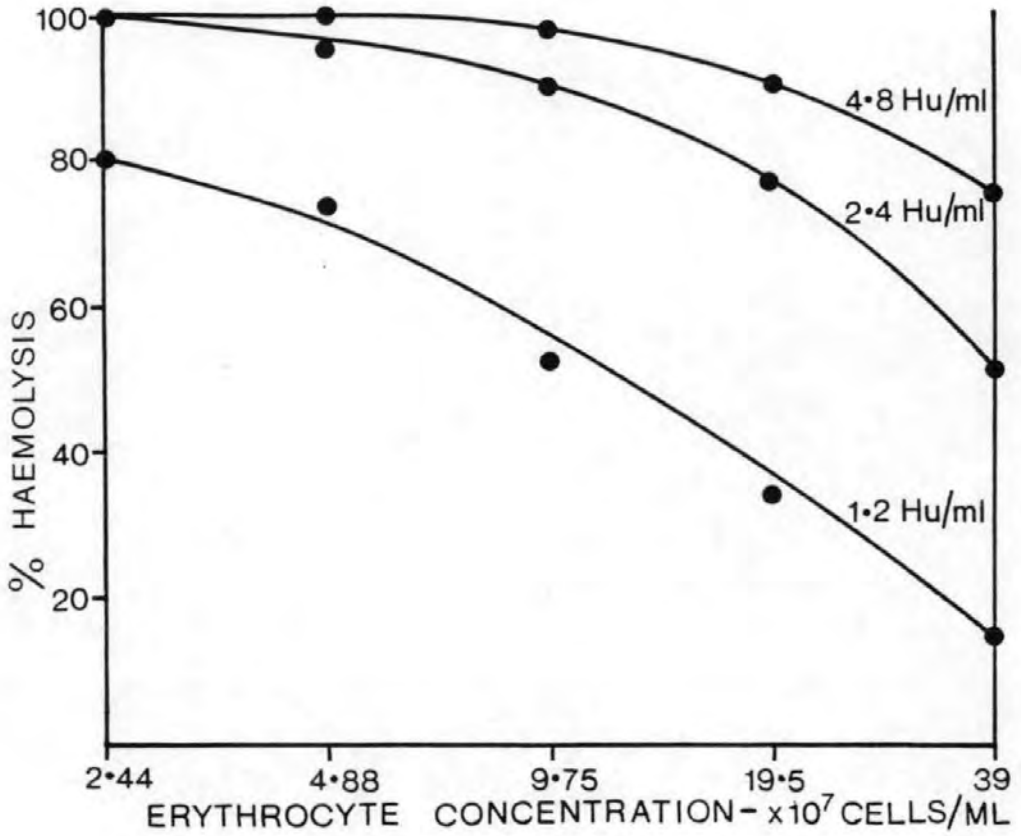
Bernheimer (1970) reported that the investigation of the degree of haemolysis of increasing concentrations of erythrocytes, by a fixed quantity of haemolysin, may indicate the likely mechanism of haemolysis. This experimental system was utilised to study the H-lysin.

Standardised horse erythrocyte suspension (2.4.2) (9.75×10^7 cells/ml) was either diluted (in PBS) or concentrated by centrifugation (3,500 x g, 10 min, 20°C) and resuspension (in PBS) to give cell concentrations in the range 2.4×10^7 to 3.9×10^8 cells/ml. 9.75×10^7 cells/ml corresponded, approximately, to 1.4% v/v erythrocytes. Other cell concentrations were chosen to lie within the range of concentrations tested by Bernheimer (1970).

The degree of haemolysis of erythrocytes by a sample of H-lysin was determined using the microtitre tray assay system. In addition to testing the H-lysin against standardised horse erythrocyte suspensions, samples of the H-lysin were also tested against horse erythrocyte suspensions of other concentrations. The percentage haemolysis of the suspensions was determined using a microelisa reader (Dynatech) with reference to standard curves (2.4.3).

The results (Fig. 41) indicated that a concave downward relationship between percentage lysis and erythrocyte concentration was observed with 2.4 Hu/ml of H-lysin. Raising or lowering the H-lysin concentration (1.2 Hu/ml or 4.8 Hu/ml) resulted in the appearance of a family of curves with similar forms.

Figure 41 The effect of horse erythrocyte concentration on percentage haemolysis observed with a fixed quantity of H-lysin



6.2.18 The ability of horse erythrocyte membranes to inhibit haemolytic activity

It was decided to investigate the possibility that horse erythrocyte membranes were capable of binding H-lysin. Horse erythrocyte membranes obtained by hypotonic lysis of cells in a standardised suspension (2.9.1) were resuspended to the original cell suspension volume (designated 1 erythrocyte equivalent). The membranes were centrifuged (10,000 x g, 20 min 4°C) and resuspended in various volumes of PBS to give other erythrocyte equivalent suspensions.

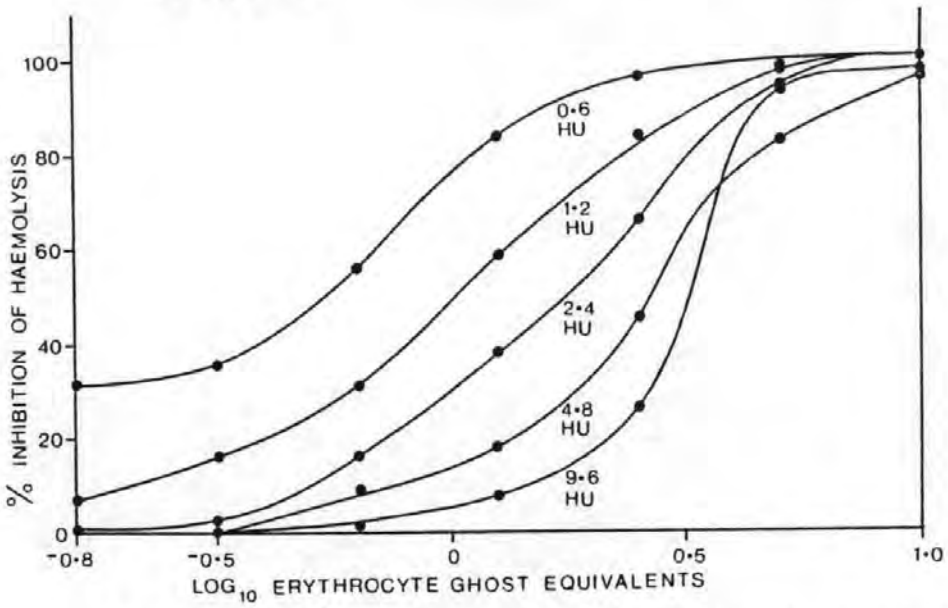
Samples of H-lysin (0.6 to 9.6 Hu/ml final concentration) were incubated with membranes (0.156 to 10 erythrocyte equivalents) for 30 minutes (30°C) then tested for haemolytic activity (2.4.3) in the presence of membranes at the requisite concentration. Control samples were included to enable changes in the optical density of lysed and unlysed cells in the presence of membranes to be compensated for in calculations of the H-lysin titre (2.4.3).

The results (Fig. 42) indicated that H-lysin activity was reduced after membrane treatment. The concentration of membranes required for inhibition of H-lysin activity increased as the quantity of H-lysin increased. Inhibition of H-lysin with respect to the concentration of membranes was sigmoidal in form when the data were plotted as a graph. The concentration of membranes required for 50% inhibition of haemolysis was determined from Figure 42 and these data plotted against the $\log_{10}$ of the H-lysin concentration. The results (Fig. 43) indicated a linear relationship between these variables. From Figure 42 it was estimated that 1 erythrocyte ghost equivalent caused 50% inhibition of 1 Hu/ml of H-lysin. However, only 3.3 erythrocyte ghost equivalents were required for 50% inhibition of 10 Hu/ml of H-lysin.

6.2.19 The effect of sugars on haemolytic activity

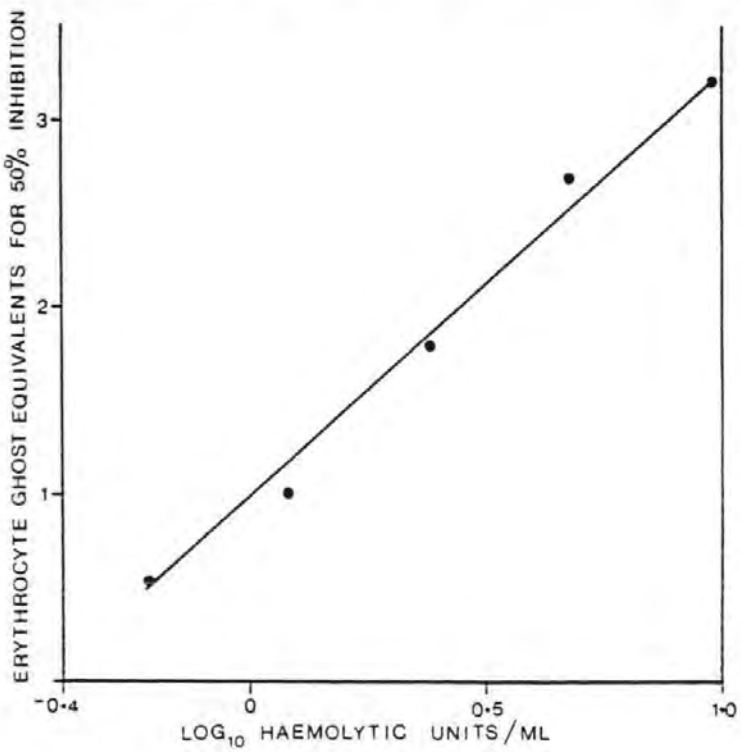
In view of the fact that many erythrocyte membrane proteins are glycosylated it was decided to investigate the possibility that sugar residues were H-lysin receptors.

Figure 42 H-lysin activity detected in the presence of increasing concentrations of horse erythrocyte membranes



● = H-lysin activity detected after incubation of membranes with the stated initial concentration of H-lysin

Figure 43 The relationship between erythrocyte membrane concentration required for 50% inhibition of H-lysin activity and $\log_{10}$ H-lysin concentration



Samples of H-lysin (22 Hu/ml) were tested for haemolytic activity (2.4.3) in the presence of lactose, glucose, melibiose or glucose (2.5 mM final concentration in PBS). The results (Table 23) indicated that the presence of these sugars had little effect on the H-lysin activity (79-93% of control activity).

6.2.20 The ability of enzyme-treated erythrocyte membranes to inhibit H-lysin

Horse erythrocyte membranes had been shown to inhibit H-lysin activity. It was decided to investigate the effect of enzyme treatment of the membranes on their capacity to inhibit the H-lysin.

Horse erythrocyte membranes (20 fold concentrated) were incubated (0.3 ml) with an equal volume of chymotrypsin, papain phospholipase C, lipase or neuraminidase (2.9.2). The treated membranes were centrifuged (10,000 x g, 20 min, 4°C) washed (3 x 0.8 ml PBS) and resuspended in PBS to their original volume. The membranes were mixed with an equal volume of H-lysin (16.6 Hu/ml), and after incubation (30 min, 30°C) and centrifugation (10,000 x g, 20 min, 4°C) the supernatant fluid tested for H-lysin activity (2.4.3). Control samples contained H-lysin mixed with untreated membranes.

The results (Table 24) indicated that H-lysin activity was reduced (55%) after incubation with erythrocyte membranes. Pre-treatment of the membranes with the enzymes tested did not reduce their ability to bind the H-lysin. Pretreatment of the membranes with chymotrypsin or neuraminidase appeared to increase the ability of the membranes to bind the H-lysin (reduction in H-lysin activity of 33% and 36% respectively).

6.2.21 The sensitivity of erythrocytes to H-lysin activity after treatment with proteases

The possibility that proteins represented receptor sites for the H-lysin was also investigated by treating horse erythrocytes with proteases.

Chymotrypsin (250 µg/ml final concentration, 15,000 ATEE units/ml) or papain (250 µg/ml, 21.5 units/ml) was added to a standardised

Table 24 The effect of sugars on H-lysin activity

Sugar tested	H-lysin activity %
Control (no sugar)	100
Lactose	93
Glucose	79
Galactose	93
Melibiose	82

Sugars were tested at 2.5 mM final concentration

Table 25 The ability of enzyme treated erythrocyte membranes to inhibit H-lysin activity

Enzyme treatment	H-lysin activity %
Control (no membranes)	100
Control (+ untreated membranes)	55
Neuraminidase	36
Chymotrypsin	33
Papain	59
Lipase	58
Phospholipase C	49

Membranes were incubated with appropriate enzyme, washed, and incubated with H-lysin (16 Hu/ml). The supernate obtained after centrifugation was tested for H-lysin activity.

suspension of horse erythrocytes (2.4.2) and the mixture incubated for 30 minutes at 25°C. After centrifugation (3,000 x g, 10 min, 25°C) and washing (2 x 20 ml PBS) the erythrocytes were resuspended to their original volume in PBS. H-lysin samples (28 Hu/ml) were tested for haemolytic activity against untreated and protease-treated erythrocytes.

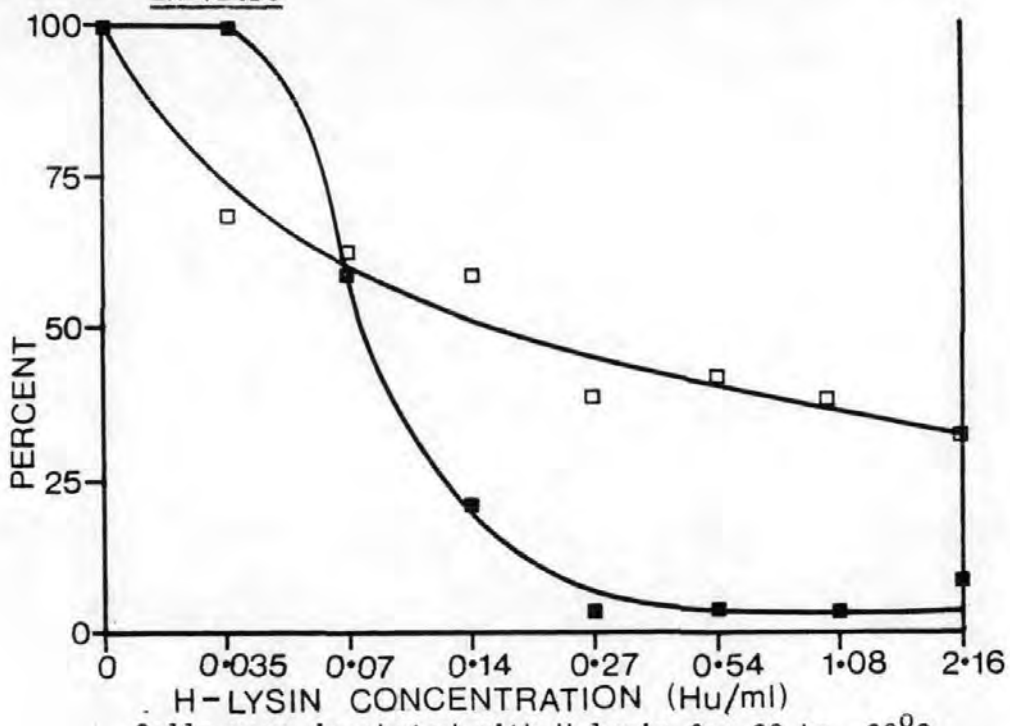
The results indicated that the H-lysin activity was higher against chymotrypsin (108%) or papain (128%) treated erythrocytes than against untreated cells (100%).

6.2.22 The effect of H-lysin on trout leucocytes

It was of interest to know whether the H-lysin was active against cells other than erythrocytes. Initial studies involved the evaluation of the effect of H-lysin on trout leucocytes. Pooled trout blood was mixed with sterile PBS (equal volume) and the leucocytes obtained after centrifugation (2.9.3) over a cushion of Lymphoprep (Nyegaard). The leucocytes were washed twice with tissue culture medium (2.9.4) and resuspended in tissue culture medium to 2 x 10^6 cells/ml. 0.1 ml aliquots of this suspension were aseptically pipetted into the wells of a sterile microtitre tray (Sterilin) and the plate incubated at room temperature (22°C) for 4 hours. H-lysin (2.16 Hu/ml) was sterilised by membrane filtration (2.9.5) and twofold dilutions of the preparation made in sterile PBS (Flow). 0.1 ml volumes of the H-lysin dilutions were added to the wells containing trout leucocytes and the plate incubated for 20 hours in a humid chamber at 20°C. Control wells contained PBS only added to the leucocytes; this PBS was also membrane filtered before testing (2.9.5). After incubation, the well contents were gently aspirated and the tissue culture medium removed, the number of cells in this fluid was determined using a haemocytometer. The number of cells attached to the microtitre tray bottom was determined with an invert microscope.

The results (Fig. 44) indicated that the numbers of adherent and non-adherent leucocytes decreased in the presence of H-lysin.

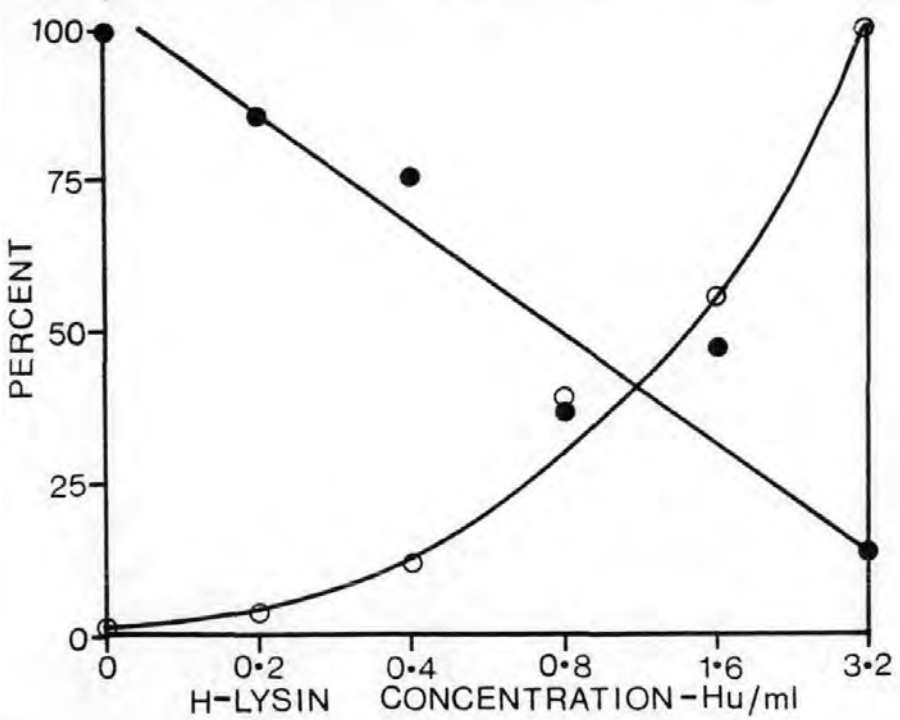
Figure 44 The effect of H-lysin on the number of trout leucocytes in vitro



Cells were incubated with H-lysin for 20 hr, 20°C.

■ = adherent leucocytes (control cells = 100%)
□ = non-adherent leucocytes (control cells = 100%)

Figure 45 The effect of H-lysin on the number and viability of rainbow trout gonad tissue culture cells.



Cells were incubated with H-lysin for 20 hr at 20°C.

● = Total number of cells (control cells = 100%)
○ = Percentage of cells non-viable

The effect of changes in H-lysin concentration on the number of adherent leucocytes was most pronounced at between 0.035 and 0.27 Hu/ml.

The number of non-adherent leucocytes appeared to decrease steadily with increasing concentrations of H-lysin, however, the decline in cell numbers was not as marked as that observed with adherent cells.

6.2.23 The effect of H-lysin on rainbow trout gonad tissue culture cells

The results obtained suggested that the H-lysin was active against trout leucocytes. It was therefore decided to investigate whether other trout cells were also susceptible to H-lysin activity. Rainbow trout gonad tissue culture cells (RTG-2) were grown in a microtitre tray (2.9.4) and the tissue culture medium replaced with 0.1 ml of fresh medium. H-lysin (3.2 Hu/ml) was prepared as described previous (2.9.5) and added to the microtitre tray wells containing RTG-2 cells. After incubation (20 hr, 20°C) the tissue culture medium was removed, the cells stained for 3 minutes with trypan blue (0.4% in PBS) and the number of cells stained using this procedure determined using an invert microscope. Following trypan blue staining the cells were washed (PBS), fixed (formol-PBS), stained (carbol fuchsin) and the total number of cells determined.

The results (Fig. 45) indicated that an increase in H-lysin concentration was accompanied by a decrease in the total number of cells. The proportion of these cells that were stained by trypan blue (non-viable cells) increased with increasing H-lysin concentration. Incubation of cells with 3.2 Hu/ml of H-lysin resulted in all remaining cells taking up the trypan blue stain. The cells appeared to be rounded up with the loss of the characteristic spindle-like cell processes.

6.2.24 The effect of H-lysin *in vivo* in the rainbow trout

In view of the fact that toxic factors are thought to be important in the pathogenesis of furunculosis, it was decided to

Table 26 The effect of H-lysin *in vivo* on some blood parameters in the rainbow trout

Fish weight (g)	H-lysin injected (Hu)	Time to death	Blood leucocyte count at 24 hr (cells/ml)
180	160	5 hr *	
150	160	**	2.1×10^6
200	160	**	
190	80	**	
215	80	4 hr *	2.5×10^6
180	80	**	
190	40	**	
145	40	**	1.8×10^6
180	40	**	
190	20	**	2.2×10^6
160	20	**	
205	10	**	
180	10	**	2.7×10^6
195	10	4 hr *	
250	CONTROL	**	
195	CONTROL	**	
220	CONTROL	**	2.75×10^6
175	CONTROL	**	
200	CONTROL	**	

** fish sacrificed at 48 hours
* leucocyte counts made at the time of death

investigate the effect of H-lysin *in vivo* after injection into rainbow trout.

Groups of three rainbow trout (145-250 g , mean weight 190 g) were anaesthetised (MS 222, Sandoz, 1:10,000) and injected intravenously (caudal sinus) with 0.25 ml or 0.5 ml volumes of H-lysin (160 Hu, 80 Hu, 40 Hu, 20 Hu or 10 Hu). Control fish received (five fish) 0.25 ml or 0.5 ml volumes of PBS only. The H-lysin was sterilised, prior to injection, by membrane filtration (Millipore, 0.45 μm, 13 mm) after pre-eluting the filter with sterile BSA solution (2 ml, 10 mg/ml in PBS) and PBS (10 ml) (2.9.5). The fish were maintained at 10°C (2.10.0).

At 4 hour post injection,one fish in the 10 Hu group and one in the 80 Hu group died. After 5 hours one fish in the 160 Hu group died. All remaining fish appeared normal with no apparent changes in behaviour or colour. Post mortem examination of the dead fish revealed no visible changes in internal organs.

At 24 hour post-injection blood samples were taken from remaining fish and examined for haemolysis and changes in leucocyte counts. The results (Table 25) indicated that there was no decline in leucocyte counts. No haemolysis was detected.

At 48 hour post-injection remaining fish all appeared normal and were sacrificed and examined for gross histopathological changes. No changes in the heart, kidney, liver, spleen or gut were observed.

6.3.0 DISCUSSION

Although the H-lysin has been purified 1,760 fold, the resultant preparation does not appear to be a single protein. Further purification of the H-lysin may, however, prove to be problematical in view of the extreme lability of the molecule and the low recovery of the H-lysin after the use of the purification scheme outlined here. A number of results suggest that the low protein concentration in the H-lysin preparation was the main factor leading to instability. Further purification of the H-lysin may require much larger volumes of starting material to be used since the H-lysin appears to be a minor component of the crude extracellular product preparation. An alternative approach may be to alter the culture medium to selectively increase production of the

H-lysin by the bacterium. Affinity chromatography or iso-electric focusing techniques applied to the purification scheme may also be useful because of the inherent protein concentrating abilities of these techniques. Problems involving the low recoveries of bacterial extracellular products have been reported for a variety of other bacteria and as with A. salmonicida H-lysin, these problems can often be attributed to the increased lability of the extracellular product as purification progresses. The lability of the H-lysin was not thought to be attributable to the effects of proteolytic degradation since no proteolytic activity was detected in the preparations and PMSF had no stabilising effect on the H-lysin. EDTA was found to have a limited effect on increasing H-lysin stability, which may indicate the sensitivity of the H-lysin to a divalent action. The finding that BSA had a marked effect on increasing H-lysin stability tends to confirm that low protein concentration is the main reason for instability.

The preparations obtained appeared to contain GCAT activity. However the relationship of the H-lysin to GCAT activity is not clear. The GCAT activity is apparently not haemolytic (Trust, T.J., personal communication) although it has been reported to act on erythrocyte membranes. Several observations suggest that H-lysin and GCAT activities are not due to the same molecule. In particular, the loss of H-lysin activity was not accompanied by the loss of GCAT activity. The GCAT has also been reported to associate with membrane vesicles and is apparently stable in shaken cultures (MacIntyre et al 1979), neither of these features were apparent with the H-lysin. The investigation of the properties of H-lysin or GCAT-negative mutants may indicate the relationship of these two activities, however, numerous attempts to isolate H-lysin-negative mutants in this project were unsuccessful. The separation of H-lysin and GCAT activities may prove problematical since these activities appear to have similar molecular weights and are eluted from ion exchange columns at only slightly different ionic strengths, suggesting they possess similar PI values (Buckley et al 1982).

Several other bacterial species have also been reported to produce GCAT (MacIntyre et al 1979), and Thelestam and Ljungh (1981) have

suggested that the activity of A. hydrophila haemolysin I may reside in the GCAT produced by the bacterium. The possibility that a similar situation occurs with A. salmonicida H-lysin and GCAT cannot be excluded at this time; if the two activities are due to different molecules then it is probable that the GCAT would tend to enhance the haemolytic potential of the H-lysin.

The lysis of erythrocytes by the H-lysin was temperature dependant with the maximum rate observed at 33°C, suggesting that the H-lysin possesses an enzymatic mode of action. This possibility was also indicated by the observation that the relationship of percentage cell lysis to increasing erythrocyte concentration was typical of that observed for other haemolysins with enzymatic modes of action (Bernheimer 1970). The maximum rate of cell lysis of a fixed concentration of erythrocytes appeared to be proportional only to low concentrations of H-lysin. This result would also be expected for an enzyme mediated reaction with a fixed substrate concentration.

Horse erythrocyte membranes were capable of inhibiting H-lysin activity though the concentration of membranes required to inhibit increasing concentrations of H-lysin showed a logarithmic, rather than directly proportional, relationship. This result may be expected if the H-lysin possesses an enzymatic mode of action, the experiment being essentially similar to that involving increasing erythrocyte concentrations in place of the membranes.

The kinetics of erythrocyte lysis showed a sigmoidal form which was preceded by a phase of no apparent lysis. Similar results have been reported for a variety of other bacterial haemolysins (Bernhemier 1947, Oberley and Duncan 1971, Duncan and Mason 1976) with the notable exception of the detergent like haemolsins such as S. aureus δ lysin, when no pre-lytic phase is observed (Wadström and Möllby 1972). The pre-lytic phase observed with the H-lysin may represent a period of increasing membrane damage, allowing the leakage of molecules of increasing molecular weights until haemoglobin release occurs. Such a situation has

been observed with streptolysin S (Duncan and Mason 1976) and aerolysin (Howard and Buckley 1982) where the results have been interpreted as indicative of a colloid-osmotic mode of action of cell lysis. By contrast, the concimitant release of molecules of various molecular weights has been reported to occur with a haemolysin from the group B streptococci which may indicate an enzymatic mode of action (Marchlewicz and Duncan 1981). Future work using intracellular marker molecules may help to indicate the mode of action of the H-lysin.

The finding that there was little or no decrease in the amount of H-lysin present in the supernate of cells treated with H-lysin may be indicative of an enzymatic rather than colloid-osmotic mechanism of cell lysis.

Erythrocytes treated with H-lysin during the pre-lytic phase only lysed on subsequent further incubation; this observation is more consistent with a colloid-osmotic lytic process than an enzymatic one. It is, however, interesting to note that cells treated with H-lysin during the pre-lytic phase only, with the addition of antiserum after this time did not lyse to the same extent as control cells. This result may be indicative of an enzymatic mode of action or alternatively may reflect the heterogeneity of the erythrocyte population, varying in their susceptibility to colloid-osmotic cell lysis. The evidence obtained therefore suggests that lysis occurs by an enzymatic mode of action, although a colloid-osmotic mechanism cannot be excluded at this time.

The nature of the red cell substrate for the H-lysin is unclear. One approach to elucidating the nature of the substrate is to attempt to correlate the sensitivity of erythrocytes from various species to the erythrocyte membrane composition (Nelson 1967). Such an approach applied to the H-lysin did not reveal a single potential substrate in the horse erythrocyte membrane. Horse erythrocyte membranes are rich in gangliosides, however pig erythrocytes membranes contain little ganglioside (Nelson 1967). If the H-lysin and GCAT activities are due to the same molecule then the sensitivity of erythrocytes should be correlated with the membrane composition. Because the reaction catalysed by

GCAT involves both cholesterol and phosphatidyl choline as substrates, the presence of both of these components in the membrane may be required for lysis. The proportions of cholesterol and phosphatidyl choline do not, however, correlate with the sensitivity to lysis (Table 25). A number of factors may result in the inability of membrane composition to be correlated with sensitivity to lysis; the H-lysin preparation tested was of a crude nature and this may have influenced the results. Also the exact erythrocyte membrane composition may show considerable intra-species variation (Degier and Van Deenen 1961, 1964).

Erythrocyte membranes or erythrocytes treated with a variety of enzymes failed to indicate likely substrates for the H-lysin. Treatment of intact erythrocytes with proteases appeared to increase their sensitivity to the H-lysin which may have indicated that the membranes were less stable after protease treatment. The finding that protease-treated membranes showed a decreased ability to inhibit the H-lysin may, however, indicate that membrane proteins tend to mask some of the H-lysin substrate.

The nature of the erythrocyte membrane substrate is not clear; further work involving the use of individual membrane components may indicate possible substrates.

The H-lysin appeared to be cytotoxic to both rainbow trout leucocytes and tissue culture cells. The time taken for this effect to be observed was much longer than that required for A. salmonicida leucocidin to elicit cytotoxic effects (Fuller et al 1977). The leucocidin was found to have a molecular weight of between 100,000 and 300,000 daltons which is considerably higher than the value obtained for the H-lysin. These observations suggest that the H-lysin and GCAT activities are not due to the same molecule.

The lack of H-lysin toxicity observed in vivo suggests that this molecule is not a key factor in the pathogenesis of furunculosis. Alternatively, this result may reflect the fact that insufficient material was injected into the fish which would have been rapidly diluted in the blood.

Recent work has suggested that A^+ strains of A. salmonicida are preferentially ingested by macrophages (Emes et al 1983) suggesting that the bacterium can survive intracellular killing mechanisms - perhaps by the combined effects of the A layer and melanin production (Ellis 1982). If this situation occurs then extracellular products may accumulate within the phagocyte until lethal levels are reached, allowing the release of the bacterium at a site distant from the initial focus of infection at a later stage. The possibility that phagocytes provide containment vessels for the accumulation of toxins has been suggested by Arbuthnott to occur in other bacterial infections (Arbuthnott 1981). If this occurs in A. salmonicida infections,then the H-lysin concentration may reach a lethal level within the phagocytic cell. In view of the array of cytotoxic or potentially cytotoxic factors produced by A. salmonicida, the H-lysin may not however be the only factor involved in this process; it may be useful in future work to not only establish the properties of individual factors but also to elucidate the combined effects of a variety of known extracellular products.

Table 2.6 The cholesterol and phosphatidyl choline content of erythrocytes from various species

Animal species	Cholesterol[1] ($\times 10^{-10}$ mg/cell)	Phosphatidyl[2] choline ($\times 10^{-10}$ mg/cell)	H-lysin[3] activity (Hu/ml)
Horse	0.632	0.57	32
Pig	0.675	0.35	24
Rabbit	1.2	0.925	16
Rat	0.778	1.00	8
Cow	0.71	0	4
Guinea pig	1.19	1.01	1
Sheep	0.429	0	0

[1] Calculated from the data of Nelson (1967)

[2] Calculated from the data of Nelson (1967)

[3] (3.2.3)

GENERAL DISCUSSION AND CONCLUSIONS

Though previous workers have reported that A. salmonicida produces a haemolysin, this is the first report that two haemolytic activities are produced by the bacterium in vitro. This is also the first detailed report concerning the production of various extracellular products in vitro. Cultural conditions have been described which promote production of the broad spectrum H-lysin activity or the narrow spectrum T-lysin. In the course of this project the observation by Sheeran and Smith (1981) that the bacterium produces two proteolytic enzymes in vitro has also been confirmed.

The mode of action of the T and H-lysins in vitro has not been fully elucidated. The interesting possibility that the T_1 activity is a modified bacterial cell membrane porin merits further attention. The results obtained suggest that the H-lysin possesses a pore forming, or more probably, an enzymatic mode of action. Only further investigation can indicate the mechanism of haemolysis of this lysin and its potential relationship to the GCAT activity produced by the bacterium. The results obtained suggest that these activities are due to two different molecules; however, it is possible that the GCAT activity enhances the haemolytic potential of the H-lysin.

Although a variety of workers have indicated that the bacterium produces a toxic factor in vitro (Munro et al 1980, Pol et al 1980) the identity of this factor has yet to be conclusively demonstrated. The leucocytolytic factor described by Fuller et al (1977) was apparently non-toxic to fish, whilst there have been conflicting reports concerning the toxicity of the caseinase in vivo (Sakai 1977, Sakai 1978, Sheeran and Smith 1981). Although tissue digesting effects have been ascribed to the gelatinase produced by the bacterium this factor would also appear to be non-toxic in vivo (Sheeran and Smith 1981).

Observations by Pol et al suggested that the H-lysin may have been an important toxic factor. The toxic factor in culture supernate was reported to be difficult to precipitate using ammonium sulphate unless BSA was previously added to the supernate. This observation along with the reported proteinaceous nature of the

toxic factor were typical of properties ascribed to the H-lysin in this project. Such properties of the toxic factor would not appear to be typical of the T-lysin. However, although the H-lysin has been found to be cytotoxic in vitro, no effect has been observed in vivo in this project. It may be important to note that Pol et al worked with carp whilst only the rainbow trout has been used in this project, and the possibility arises that the relative importance of various extracellular products in the pathogenesis of disease varies according to the host fish species.

Munro et al (1980) reported that the toxicity of a crude extracellular product preparation was diminished in the presence of trout serum. This observation may implicate either the caseinase or T-lysin activity as toxic factors, however these workers found no evidence of erythrocyte lysis after administration of extracellular products to rainbow trout. It is difficult to interpret the results of Munro et al with relation to the H-lysin because it is likely that the use of the AOT to prepare extracellular products, and the practise of membrane filtration of the material before inoculation, resulted in the exclusion of this factor from their preparations.

The results obtained in this project have failed to indicate a correlation between possession of the A-layer and levels of production of H-lysin, T-lysin or caseinase in vitro, suggesting that these extracellular products are not virulence factors. These extracellular products may, however, have important roles as aggressins. The possibility that A^+ strains of A. salmonicida are preferentially phagocytosed by fish phagocytes leads to the suggestion that cytotoxic activity may be directed against these cells from within rather than outside the cell. In this case the serum inhibition of the T-lysin or T_1 activity would not be important and the injection of cytolytic activities directly into the fish would fail to indicate such a mode of action of the haemolysins. This possibility can only be resolved by further investigation and establishment of the fact that these extracellular products are produced, in vivo, during the course of the infection.

This project provides an important basis for further studies concerning haemolytic activities. It is also of considerable

importance to other workers in this field since this is the first report of the complex interactions that occur between individual extracellular products. In particular, recent work using caseinase negative mutants to elucidate the role of this enzyme in the pathogenesis of disease may require re-interpretation. Only further work in relation to detailed investigation of the pathogenesis of the disease can resolve these possibilities.

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Evidence for two haemolytic activities from *Aeromonas salmonicida*

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1. INTRODUCTION

Aeromonas salmonicida is the aetiological agent of furunculosis, an economically important disease of fish, particularly salmonids reared under intensive conditions [1]. There has been much interest recently in the investigation of toxins and other pathogenic mechanisms with a view to producing effective vaccines. A factor having leukocytolytic activity has been partially characterized [2]; proteolytic activity may be responsible for the necrosis of tissue seen in infected fish [3,4]. However, the anaemic response of infected fish [5] and the presence of extensive haemosiderin deposits in haemopoietic tissue [6] have resulted in the suggestion that haemolysins may play a role in pathogenesis.

Extracellular products obtained by the agar overlay technique have been shown to be toxic and contain haemolytic activity against trout erythrocytes [7]. However, the complex nature of such material is likely to make it difficult to purify the factors responsible. We report here the first detailed study of haemolysin production by *A. salmonicida* and present evidence for the existence of two distinct haemolytic activities.

2. MATERIALS AND METHODS

2.1. Bacterial strains and cultivation

Of 16 strains of *A. salmonicida* examined for haemolysin production on tryptone soya agar (TSA, Oxoid) +5% v/v horse blood, all were haemolytic; strain 25/77, a particularly haemolytic strain isolated from rainbow trout, was chosen for further study. It was identified as *A. salmonicida* using biochemical tests in comparison with the neotype strain NCMB833 [8,9]; the strain was preserved under liquid nitrogen refrigeration and cultured weekly onto TSA (25°C).

Haemolysin production in liquid media was investigated by inoculating 100-ml volumes of different broth media in 250-ml erlenmeyer flasks with 1.0 ml of an 18-h culture in tryptone soya broth (TSB, Oxoid). Viable counts were determined by the method of Miles and Misra [10] or using a spiral plater (Spiral Systems, Bethesda, USA). Peptone broth contained Peptone (Oxoid, 10 g/l), NaCl (5 g/l) and K_2HPO_4 (5 g/l). Lab Lemco broth contained Lab Lemco powder (Oxoid, 10 g/l), yeast extract (5 g/l), L-tyrosine (BDH, 1 g/l) and NaCl (5 g/l). Nutrient Broth No. 2 and Brain Heart Infusion were supplied by Oxoid. Nutrient Broth No. 2 was supplemented with 1% (w/v) yeast ribonucleate, 5% (v/v) glycerol, 1% (w/v) glucose (all BDH) 0.3% (w/v) yeast extract, 2% (w/v) haemoglobin (Oxoid) or 0.5% (w/v) bovine serum albumin (BSA, Sigma) as required. The pH of all media was adjusted to 7.5 before autoclaving (121°C, 15 min).

Agar overlay material was obtained by inoculating 5.0 ml of an 18-h culture onto TSA (250 ml) overlaid with cellophane in large assay plates.

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After incubation (72 h) material was removed using a glass slide resuspended in 9.0 ml phosphate-buffered saline (PSB) and shaken with glass beads for 15 min. The supernate obtained after centrifugation (7500×*g*, 20 min, 4°C) and dialysis against PBS (18 h, 4°C) was designated agar overlay material.

2.2. *Haemolysin assays*

Freshly collected erythrocytes were washed three times in PBS (0.85% NaCl). Due to instability of eel and dogfish cells, appropriate isotonic PBSs were used (eel 1.05% NaCl; dogfish 2.2% NaCl, 2.9% urea). Cells were resuspended in appropriate diluent to give $9.7 \cdot 10^7$ cells/ml.

Culture supernates were collected by centrifugation (10000×*g*, 10 min, 4°C) and haemolytic activity determined in microtitre plates. Samples (0.1 ml) were diluted in twofold steps in appropriate saline and 0.1 ml of erythrocyte suspension added. After incubation (37°C, 1 h) the haemolytic titre (expressed as haemolytic units, HU) was obtained from the reciprocal of the dilution that gave an end product of 50% haemolysis.

2.3. *Haemolysin properties*

Culture supernates were made cell-free by membrane filtrates using 25 mm, 0.45 μm cellulose acetate filters (Millipore). Chymotrypsin (BDH, 15000 U/mg) was used at 0.1 mg/ml and 1.0 mg/ml in PBS. In experiments to study haemolysin degradation by shaking, bacterial growth was inhibited by the addition of 0.02% sodium azide to 25 ml aliquots of 96 h supernate from unshaken cultures in 100-ml erlenmeyer flasks. Inhibition of haemolytic activity by rainbow trout serum (heated 45°C, 20 min) was tested by diluting serum in culture supernates in the microtitre plate assay system.

3. RESULTS AND DISCUSSION

Initially unshaken and shaken liquid cultures were examined for the presence of haemolysin. The results (Table 1) suggest that two haemolytic activities are produced in vitro. Supernatant fluid from unshaken cultures contained a broad-spectrum haemolytic activity against erythrocytes from a wide variety of species with maximum activity against horse erythrocytes (H activity). Supernate from shaken cultures, however, contained an activity against trout erythrocytes only (T activity). Thus agitation selectively favoured the production of T activity, whilst static conditions favoured the production of H activity. This pattern of production was observed with a variety of media. Results (Table 2) indicate that maximal T and H activities were obtained in nutrient broth No. 2. Additions known to increase the production of other bacterial toxins did not increase the production of either haemolytic activity. Previously, Foster and Hanna [11] found that unshaken cultural conditions resulted in maximal haemolytic activity in broth cultures of *A. proteolytica.*

TABLE 1

Sensitivity of erythrocytes to lysis by 72 h culture supernates

Erythrocyte Suspension tested	Haemolytic units/ml	
	Stationary culture	Shaken culture
Horse	32	0
Pig	24	0
Rabbit	16	0
Eel	16	0
Rat	8	0
Mouse	8	0
Trout	7	128
Cow	4	0
Guinea pig	1	0
Dogfish	1	0
Sheep	0	0
Frog	0	0

High yields of T haemolytic activity (up to 1024 HU/ml) were obtained in agar overlay material whereas H activity was low (2 HU/ml). Variation in titres of T activity in separate experiments was observed, which may reflect variation in the sensitivity of erythrocytes used. Environmental, seasonal and nutritional variations are known to result in changes in haematological parameters in fish [12] and control of such varia-

TABLE 2

Effect of growth medium on production of haemolytic activity

Medium tested	Maximum haemolytic activity (HU) against	
	Horse erythrocytes (shaken culture)	Trout erythrocytes (shaken culture)
Tryptone soya broth	4	16
Furunculosis broth	4	32
Peptone broth	3	48
Peptone + yeast extract broth	4	64
Lab lemco broth	4	32
Lab lemco + yeast extract broth	4	32
Brain heart infusion	3	64
Nutrient broth	16	16
Nutrient broth No. 2 (NB 2)	64	128
NB 2 + BSA	64	32
NB 2 + Glycerol	64	3
NB 2 + Yeast ribonucleate	8	32
NB 2 + Yeast extract	32	32
NB 2 + Glucose	1	1
NB 2 + Haemoglobin	8	32

tions may be achieved by the long-term storage of cells [13].

The production of T and H activity throughout the growth cycle is shown in Fig. 1. Like many extracellular products both activities accumulated at the end of the logarithmic phase of growth; this was particularly evident in static cultures. This suggests that the haemolysins are produced during the logarithmic phase but are activated or released during the stationary phase. The results confirm that T activity is produced maximally in shaken cultures whilst unshaken conditions favour H activity production and/or stability. The peak in activity in shaken cultures at 33 h (H activity) and 50 h (T activity) is not apparent in unshaken cultures when both activities seem to be produced throughout the stationary phase. The decrease in H activity apparent after 33 h in shaken cultures

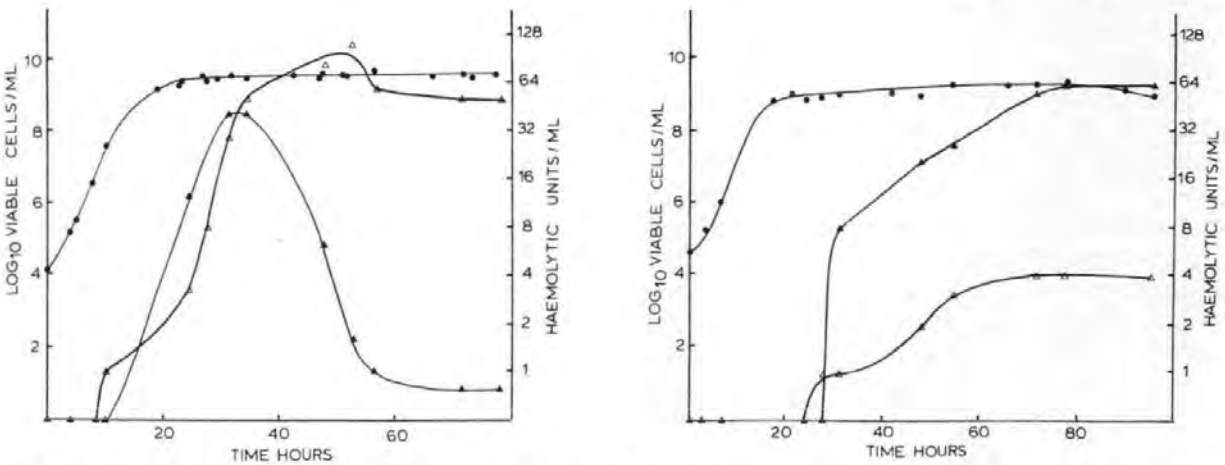

Fig. 1. Production of lytic activity in nutrient broth No. 2. (a) Shaken culture; (b) static culture. ●, Viable count (cells/ml); △, T haemolytic activity; ▲, H haemolytic activity.

TABLE 2

Effect of growth medium on production of haemolytic activity

Medium tested	Maximum haemolytic activity (HU) against	
	Horse erythrocytes (shaken culture)	Trout erythrocytes (shaken culture)
Tryptone soya broth	4	16
Furunculosis broth	4	32
Peptone broth	3	48
Peptone + yeast extract broth	4	64
Lab lemco broth	4	32
Lab lemco + yeast extract broth	4	32
Brain heart infusion	3	64
Nutrient broth	16	16
Nutrient broth No. 2 (NB 2)	64	128
NB 2 + BSA	64	32
NB 2 + Glycerol	64	3
NB 2 + Yeast ribonucleate	8	32
NB 2 + Yeast extract	32	32
NB 2 + Glucose	1	1
NB 2 + Haemoglobin	8	32

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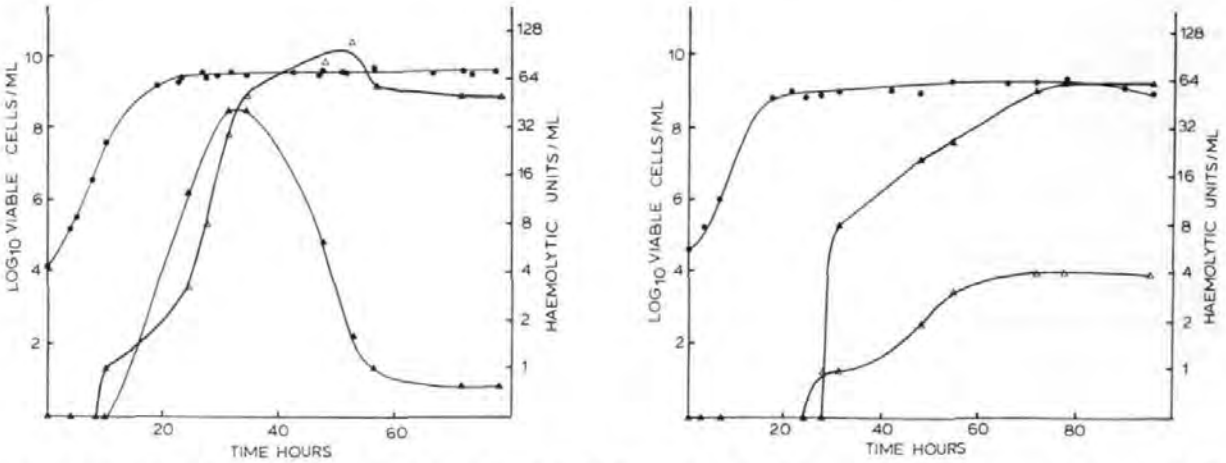

Fig. 1. Production of lytic activity in nutrient broth No. 2. (a) Shaken culture; (b) static culture. ●, Viable count (cells/ml); △, T haemolytic activity; ▲, H haemolytic activity.

ERRATA:-

2.3 Haemolysin properties:-

Line two to read:- membrane filtration using 25 mm 0.45 um cellulose

TABLE 2. Effect of growth medium on production of haemolytic activity.

Second column heading to read:- Horse erythrocytes (unshaken culture)

3. RESULTS AND DISCUSSION.

Third paragraph, sixth line to read:- suggests that the haemolysins are either produced during

Third paragraph, eighth line to read:- during the stationary phase, or are secondary metabolites. The results confirm