CHARACTERISTICS OF *VIBRIO CHOLERAE* PROTEINASES

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DECLARATION

This thesis is the original work of the author

To my family

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Throughout this thesis the word proteinase, although biochemically correct, has been substituted by protease to allow ease of quoting the literature which mostly uses the term protease.

Summary

V. cholerae extracellular proteases were isolated, characterized and their role in adherence and as vaccine additives considered.

Gel filtration of the crude protease produced one large peak of protease activity quite distinct from the major protein band. Isoelectric focussing of the crude protease or the pooled G100 protease peak (G100/P) yielded three peaks of protease activity. These were termed IEF-1 which was a broad peak with a pI of 4.5 - 5.5 $\pm$ 0.1, IEF-2 with a pI of 6.5 $\pm$ 0.1 and IEF-3 with a pI of 9.0 $\pm$ 0.1.

The three IEF proteases were shown by peptide digest analysis to have distinct catalytic activities.

Gelatin-PAGE of the crude protease, G100/P, IEF-1, IEF-2 and IEF-3 showed that each preparation contained at least three areas of protease activity, suggesting either the presence of several proteases in each preparation or autocatalytic degradation of a single protease which had produced multiple activities on the PAGE gel.

Characterisation of the three IEF proteases by inhibitors revealed that IEF-1 was inhibited by metalloprotease and serine protease inhibitors, IEF-2 was inhibited by metallo, serine and thiol protease inhibitors and IEF-3 was inhibited by only metalloprotease inhibitors. This suggested that more than one protease was present in IEF-1 and IEF-2 as it is unusual for a single protease to be inhibited by more than one class of protease inhibitor. IEF-3 was only inhibited by metalloprotease inhibitors but showed multiple protease activities in gelatin-PAGE suggesting either several metalloproteases or autodegradation of one metalloprotease which has then displayed electrophoretic heterogeneity.

The crude protease, G100/P and IEF-3 all showed mucinase and haemagglutinating activity.

Fibronectin and lactoferrin, two host substances that may be involved at the level of mucosal defence were tested for their susceptibility to V. cholerae proteases. Fibronectin was found to be digested after 1 h and further digestion occurred after 24 h. No digestion of lactoferrin was seen even after a 24 h incubation with V. cholerae proteases.

IgA is the main immunoglobulin present in mucosal secretions and so is the major antibody involved in mucosal defence. V. cholerae proteases were shown to digest IgA specifically with the loss of three peptide bands. Immunoelectrophoresis showed that the V. cholerae protease(s) was not a typical IgA protease as no distinct Fc and Fab fragments were produced. The antigenicity of the IgA remained but the single precipitin arc was shortened following protease treatment.

The binding of V. cholerae enterotoxin to ileal segments was found not to be affected by enzyme treatment of the tissue. However it is interesting that treatment of the cholera enterotoxin with the protease-containing mixture G100/P caused an increase in toxin activity. peptide fragments with similar molecular weights to A_1 and A_2 following protease treatment.

The effect of V. cholerae proteases on the binding of the vibrios to ileal segments in vitro was assessed by a radioactive adherence assay. Three different types of tissue preparation were used. Experiments which involved the use of tissue segments where both the mucosal and serosal surface of the ileum were present during the assay procedure showed no effect on the number of vibrios binding following enzyme treatment. V. cholerae was found to adhere to the serosal surface of

the ileum and the numbers adhering to the serosal surface increased following protease treatment of the tissue segment. Adherence of V. cholerae to the mucosal surface was found to decrease following protease treatment of the tissue segments and neuraminidase had no effect on the adherence of vibrios to either the mucosal or serosal surfaces.

The use of the protease-containing mixture G100/P as a vaccine to produce active protection against V. cholerae was tested in guinea-pigs. Initial experiments were done by giving guinea-pigs one parenteral vaccination. These guinea-pigs were found only to be significantly protected if given a vaccine containing both protease plus neuraminidase. Guinea-pigs given one parenteral vaccination and one parenteral booster dose with various concentrations of protease and neuraminidase were highly protected when the vaccine contained 80 µg of protease and 0.02 I.U. of neuraminidase.

A parenteral vaccination followed by an oral booster dose of 80 µg of protease and 0.02 I.U. of neuraminidase significantly protected guinea-pigs and for the first time protease alone (80 µg) was a protective vaccine.

In conclusion, it is apparent that V. cholerae produces several proteases which play important roles in the pathogenesis of cholera.

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Abbreviations

$Al(OH)_3$	aluminium hydroxide gel
B.D.	blueing dose
BSA	bovine serum albumin
^{o}C	degrees centigrade
CFA	colonization factor
cpm	counts per minute
E	extinction
ELISA	enzyme linked immunosorbent assay
F.C.A.	freunds complete adjuvant
F.I.A.	freunds incomplete adjuvant
g	gram
h	hour
IEF	isoelectric focussing
IgA	immunoglobulin A
IgG	immunoglobulin G
i.m.	intramuscular
i.p.	intraperitoneal
KDO	ketodeoxyoctonate
k.Dal.	kilodaltons
L	litre
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
M.Wt.	molecular weight
N	normal

nm	nanometre
NS	not significant
P	probability value
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMN	polymorphonuclear leukocytes
psi	pounds per square inch
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Tris	tris-(hydroxymethyl)-methylamine
v/v	volume per volume
w/v	weight per volume
WHO	world health organisation
%	per cent
$\geqslant$	greater than or equal to
$\leqslant$	less than or equal to

INTRODUCTION

1. GENERAL INTRODUCTION

The existence of cholera, or a similar disease, in the ancient civilizations of India and possibly China was suggested by Pollitzer (1959) in his definitive text on cholera.

During the London outbreak of cholera in 1854 an anaesthetist, John Snow "with a notebook, a map and his five senses", proved that many persons acquired the disease from "spoiled" water from the Broad Street pump. Boehm (1838) made the first claim to have seen the causative organism of cholera, a claim which was subsequently repeated by other workers including Pacini and Hassal (1854), Leyden (1866) and finally Kleb (1867) described intestinal organisms identical to Vibrio cholerae. Through the studies of Koch (1884) on cholera victims in Egypt and India it became generally accepted that V. cholerae was the causative organism. He reported the comma-shaped organism increased in number in the stools during infection and gradually disappeared on recovery. The bacteria were invariably present in the intestine of cholera victims and absent in patients suffering any other morbid disease (cited from Pollitzer, 1959).

Until the end of the 18th Century cholera as far as is known, was contained largely within India and neighbouring regions. From this time however, there have been a succession of cholera pandemics, the first six of which were described by Pollitzer (1959). The seventh pandemic of cholera began in 1961, after an absence of the disease from most of the world since the nineteen twenties. After 1961 the disease gradually spread over a large area of the world in several distinct phases:-

(i) 1961-1962: the disease was recognized in many countries of south-east Asia, eg. Phillipines.

(ii) 1963-1969: the disease reached the countries of mainland Asia, eg. Thailand.

(iii) 1970-1979: there was a significant rise in the incidence of cholera in the Middle East and parts of Africa and Europe.

In this seventh pandemic, the el tor biovar of the cholera vibrio was more prevalent than the classical V. cholerae identified by Koch in 1884. The el tor biovar was first isolated in 1906 at the el tor quarantine station on the Gulf of Suez. Unlike the classical cholera vibrios, the el tor vibrio forms a soluble haemolysin (Gotschlich, 1906; Moor, 1936; cited in Buchanan and Gibbons, 1974), and is unusual in its phage susceptibility (Mukerjee, 1963). The el tor vibrios have a longer survival time in the environment and are more resistant to antibiotics than the classical vibrio strain (Gan and Tjia, 1963). It also causes a higher incidence of asymptomatic infection than the classical V. cholerae (Bart et al, 1970). Consequently there are more undetected and untreated cholera cases which preserve a reservoir of infection in el tor outbreaks.

The W.H.O. estimates that half of the world's population lives in or near the cholera endemic areas of Asia, so the disease obviously poses an immense public health problem. Because of the enormity of the problem, means of prevention and treatment of cholera have been sought. Treatment of the disease is now so well developed that infection with V. cholerae need not be fatal.

Unfortunately, cholera is endemic in areas of high population density, and where restricted medical facilities hinder large-scale treatment. Until clean drinking water and proper sewage disposal systems become available, there is little hope of eradication of the disease. Emphasis should therefore be placed on the production of an effective vaccine which is capable of eliciting long-term protection against the disease.

2. CHOLERA, THE DISEASE

2.1 Definition of cholera infection

Finkelstein (1975) defined cholera as an acute (frequently severe) dehydrating, diarrhoeal disease, usually occurring with epidemic incidence, caused by infection with cholera vibrios.

2.2 The causative organism

Cholera is caused by a Gram-negative uniflagellate, facultatively anaerobic curved rod belonging to the family Vibrionaceae (Vernon, 1965: see Buchanan and Gibbons 1974). It is contained in the genus Vibrio (Pacini, 1854: see Buchanan and Gibbons, 1974). The species Vibrio cholerae is subdivided into four biovars:

(i) V. cholerae biovar cholerae

(ii) V. cholerae biovar el tor (Pribram, 1919)

(iii) V. cholerae biovar proteus (Buchner, 1885)

(iv) V. cholerae biovar albensis (Lehmann and Neumann, 1896)

Only biovars (i) and (ii) are associated with human cholera. Biovar proteus causes gastroenteritis in human beings and biovar albensis is pathogenic only to certain animals, eg. guinea-pigs and pigeons. All four biovars are intestinal pathogens (Buchanan and Gibbons, 1974).

2.2.1 Antigenic constitution

V. cholerae possesses both 'H' and 'O' antigens. The 'H' antigens are non-specific, and are shared by all cholera vibrios. The diagnosis of cholera is, therefore, dependent upon the use of O-specific antisera (Gardner and Venkatram, 1935). The majority of strains are classified into six O antigenic groups. Serogroup "O", type 1 (O:1)

contains the biovar cholerae and el tor, whereas proteus and albensis biovars produce cell wall antigens other than the O:1 antigen (Sakazaki et al, 1970).

2.2.2 Serovars

Three antigenic factors A, B and C are used to sub-divide the O:1 group into the serovars, Ogawa, Inaba and Hikojima (Kauffmann, 1950). Table 1 depicts the combination of antigens found in serogroup O:1.

Antigenic conversion among the serovars Ogawa, Inaba and Hikojima can occur in both experimental animals and in natural infection (Kauffman, 1950; Sack and Miller, 1969). Serological conversion appears to be related to the appearance of agglutinating antibody in the serum. Rough to smooth reversion was found with Inaba and Ogawa serotypes (Miller et al, 1972).

2.2.3 Somatic Antigens

The exact chemical structure of the O-somatic antigens of V. cholerae are still unknown, despite the efforts of numerous research workers (Watanabe and Verwey, 1965; Verwey et al, 1965; Jackson and Redmond, 1971; Sur, Maiti and Chaterjee, 1974). Raziuddin and Kawasak (1976) presented detailed analyses of V. cholerae cell envelope lipopolysaccharide (LPS). The absence of 2-keto-3 deoxyoctonate (KDO), the typical linking sugar between the polysaccharide and lipid moieties in enterobacterial LPS was outstanding. In addition galactose, a typical core sugar in other Gram-negative bacteria was missing (Jackson and Redmond, 1971). Redmond (1975) discovered a highly-labile sugar, 4-amino-4,6 dideoxy-D-mannose; this amounted to at least 6% in the LPS of V. cholerae 569B (Inaba).

TABLE 1. Antigenic Determinants of Serogroup 0:1

SEROVAR	'O' FACTORS
Ogawa	AB
Inaba	AC
Hikojima	ABC

Recently Kabir (1982) characterized the LPS from V. cholerae 395 (Ogawa) and compared it with the LPS of the Inaba serovar and with the LPS of other species of the Enterobacteriaceae. The LPS of the Ogawa 395 serovar also lacked KDO but possessed an acid-labile amino sugar, 4-amino arabinose. This observation confirmed the earlier findings of Redmond (1975) who also detected 4-amino arabinose in the Ogawa serovar. Although this amino sugar was present in the LPS from several Gram-negative bacteria (Volk, Galanos and Lüderitz, 1970), the Inaba serovar of V. cholerae does not possess this sugar. Thus, the LPS from V. cholerae 395 (Ogawa) possesses both common and distinct features when compared with the LPS from enterobacterial species and the Ogawa serovar.

The role of the somatic antigen of V. cholerae in the adherence of the vibrios to the intestinal epithelium was considered by Chitinis and his colleagues (Chitinis, Sharma and Kamat, 1982a,b). Lipopolysaccharide preparations from V. cholerae 395 (Inaba) inhibited adherence of two different Inaba strains to the intestinal mucous membrane. It was concluded that the somatic antigen may play a role in the adherence of V. cholerae to the intestinal mucosa.

2.3 Production of Clinical Disease

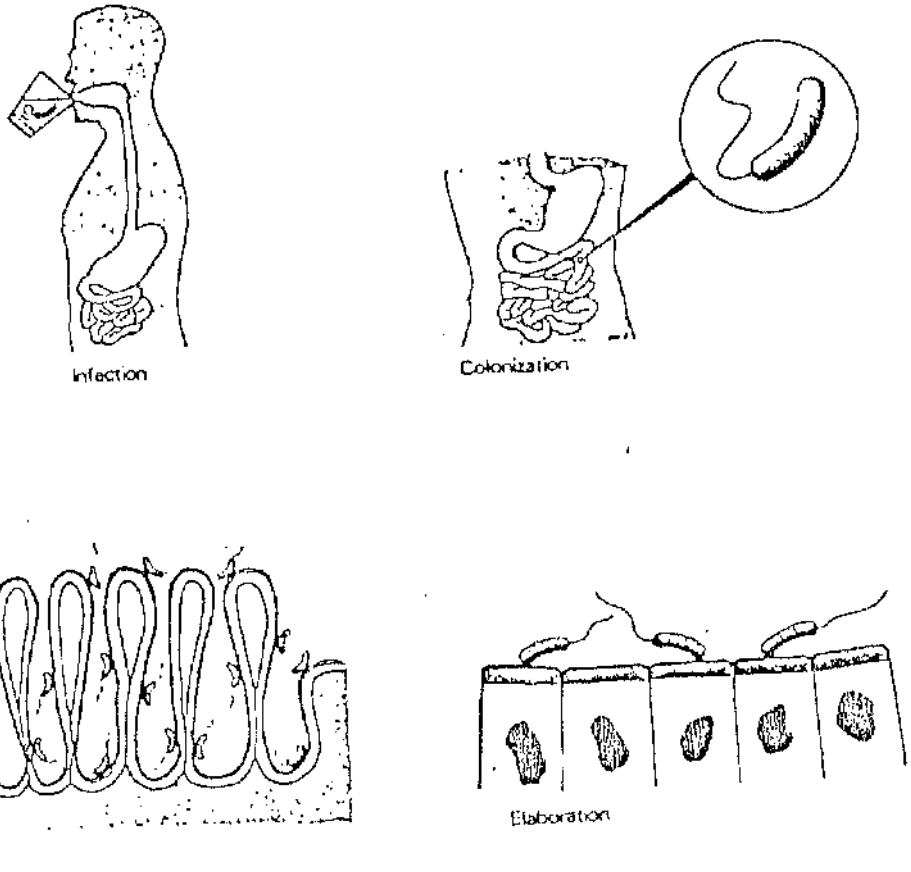
The events preceding the development of clinical cholera are: ingestion of viable V. cholerae, colonization of the small intestine and production of enterotoxin (Figure 1).

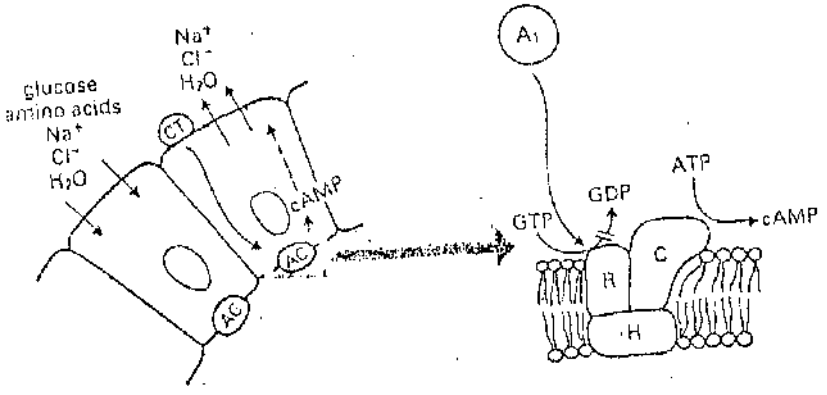
2.3.1 Ingestion of viable V. cholerae

Cholera infection is established via the orogastric route, the disease being dependent on the ingestion of live vibrios in food or water.

FIGURE 1 CHOLERA PATHOPHYSIOLOGY

(adapted from Nalin, 1975; Stephen and Pietrowski, 1981)





AC : adenylate cyclase H : specific hormone receptor
R : component responsible for GTP-dependent regulation of enzyme activity.
C : component responsible for enzymatic conversion of ATP to cAMP

V. cholerae organisms are very acid-sensitive, so that protection from gastric acidity, eg. within a bolus of food or ingestion of extremely large numbers of organisms is required for their safe passage into the small intestine. The organism grows well at the slightly alkaline pH (7.0-8.0) of the small intestine and is resistant to high bile salt concentrations, so that rapid multiplication may occur.

2.3.2 Colonization of the small intestine

V. cholerae remains localized within the intestine during infection, and although no penetration of the tissue occurs, Freter (1969) demonstrated that 50% of the infecting vibrios in rabbit ileal loops adhered strongly to the intestinal lining. Cholera adherence seems to be necessary for colonization of the small intestine and appears to be a complex process involving several factors (see sections 4 and 5).

More than two decades ago Lankford (1960) suggested that cholera vibrios colonized the mucosa of the small intestine. This possibility was supported by the observations of Freter, Smith and Sweeney (1961) who concluded that because of the short transit time of fluid through the intestine of cholera patients and the inability of the contents of the lumen to support the growth of vibrios, the vibrios most probably colonized and grew on the intestinal mucosa.

Nelson, Clements and Finkelstein (1976) studied the colonization of the small intestine of infant and adult rabbits by V. cholerae, by scanning and transmission electron microscopy. They demonstrated a lag period of one hour before significant numbers of bacteria attached to the intestinal epithelium of adult animals. This lag was absent in the infant rabbit where colonization began immediately on infection, and where greater concentrations of vibrios accumulated than in the adult animal. It was

suggested that natural differences in the susceptibility of the intestinal surface to bacterial attachment could be important in the different morbidity rates of infant and adult human beings for enteric diseases such as cholera.

Recent work has shown that a classical V. cholerae strain and mutants derived from it show significant differences in their abilities to colonize the infant mouse upper bowel and to induce diarrhoea (Baselski and Parker, 1978; Baselski, Upchurch and Parker, 1980; Baselski, Medina and Parker, 1978 ; Sigel, Finkelstein and Parker, 1981). Differences in virulence were correlated with differences in the ability to multiply in the upper bowel and to evade normal host clearance mechanisms.

Sigel et al (1980) found that the ability to multiply in the upper intestine appeared to be a preliminary determinant of virulence for V. cholerae strains and a good predictor for the ability to cause human disease. Srivastava, Shina and Srivastava (1980) found that if the intestine was exposed to an optimum number of vibrios, multiplication would not in itself have a role in the pathogenesis of cholera. However, multiplication of vibrios probably has a very important role in natural infection in man, in whom the infecting dose is likely to be small in relation to the number of vibrios needed to cause disease.

2.3.3 Production of enterotoxin

Growth of the cholera vibrios in the small intestine is accompanied by the production of an exo-enterotoxin. The enterotoxin of V. cholerae was first isolated and purified by Finkelstein and LoSpalluttoin 1969. This was 85 years after Koch first suggested that the symptoms of cholera were caused by a potent exotoxin secreted by the

organism in the gut. Koch's ideas were not accepted till the late 1950s when De and his colleagues showed that injection of V. cholerae or sterile culture filtrates into ligated segments of rabbit ileal loops in vivo led to rapid fluid accumulation in the lumen and consequent distention of the loop (De, 1959; De, Ghose and Sen, 1960).

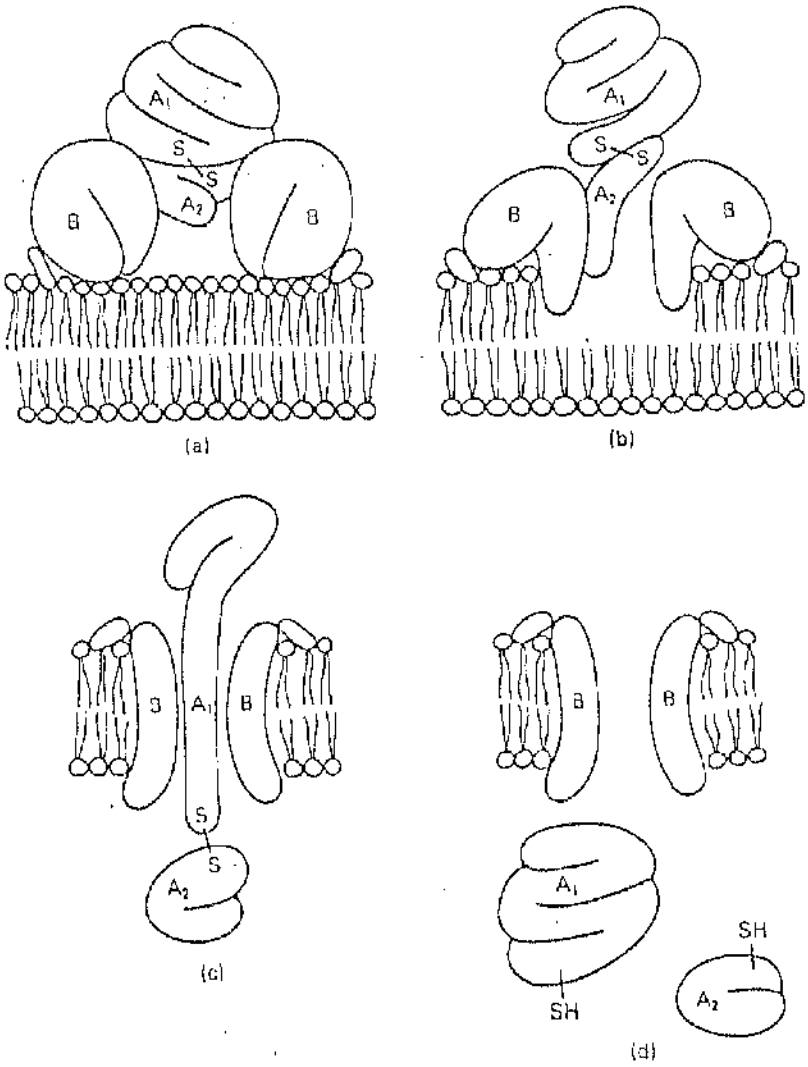
Cuatrecasas, Parikh and Hollenberg (1973) stated that enterotoxin from V. cholerae was composed of two oligomeric subunits which were non-covalently linked, one of about 60 k.Dal. and another of approximately 30 k.Dal. The 60 k.Dal. toxin unit, the B subunit, was found to be an aggregate of five 8-10 k.Dal. peptides. The 30 k.Dal. component, the A subunit, contained two disulphide linked peptides of unequal size, A_1 and A_2 with molecular weights of 28 and 5 k.Dal. respectively (Finkelstein et al, 1974). It was proposed that the B subunit was responsible for the initial interaction with the intestinal cell surface, while the A subunit represents the biologically active portion of the molecule (Figure 2: Van Heyningen, 1974; Holmgren and Lönnroth, 1975; Finkelstein, 1975).

During purification of the enterotoxin Finkelstein and his colleagues also purified a protein that was immunologically identical to enterotoxin but was biologically inactive. This protein was named enterotoxoid or choleragenoid (Finkelstein and LoSpalluto, 1969). The B subunit was common to both enterotoxin and enterotoxoid but the A subunit was absent in the latter (Finkelstein et al, 1974). This was in agreement with earlier work by Finkelstein, Fujita and LoSpalluto, 1971).

The functioning toxin binds specifically to GM_1 ganglioside receptors on the intestinal cell surface by means of its B subunit (Holmgren, Lönnroth and Svennerholm, 1973; Cuatrecacas, 1973; Van Heyningen, 1974). The GM_1 ganglioside is an acidic glycolipid containing

FIGURE 2 PROPOSED MECHANISM OF ENTRY OF CHOLERA TOXIN FRAGMENT A1

(adapted from Stephen and Pietrowski, 1981)



Binding of B subunits to specific receptors (a) induces a conformational change in these sub-units and their insertion into the membrane (b) to create a hydrophilic channel through which the A sub-unit can diffuse into the cell (c). Here, thiol agents reduce the disulphide bond, allowing fragment A_1 to diffuse into the cytoplasm and activate adenylate cyclase.

sialic acid and a terminal galactose (Figure 3; Fishman and Brady, 1976). After B subunit attachment the A subunit is inserted into the cell membrane resulting in activation of adenyl cyclase, which leads to increased levels of cyclic AMP and a net secretion of chloride ions into the bowel lumen and a net reduction in the adsorption of sodium (Figure 4; Field, 1971; Strewler and Orloff, 1977).

The net fluid deficit which this produces in the body is responsible for the entire range of clinical manifestations of cholera (Carpenter, 1970; Carpenter, Greenough and Gordon, 1974), such as vomiting, diarrhoea and abdominal cramps and finally complete dehydration of the body.

2.4 Clinical Symptoms and Treatment

Pollitzer (1959), Pierce and Mondal (1974) and Mahalanabis, Watten and Wallace (1974) have comprehensively reviewed the clinical aspects of cholera.

Cholera has an incubation period varying from a few hours to five days with a mean of two to three days (Oseasohn et al, 1966; Hornick et al, 1971). The onset is abrupt, vomiting occurring in most patients shortly after the onset of severe diarrhoea. In severe cases the voluminous liquid ("rice-water") stools contain mucus, epithelial cells and large numbers of vibrios ($10^6 ml^{-1}$, or more). As the infection progresses fluid loss in the stool exceeds the ratio of readsorption by the colon, resulting in an overall deficit of isotonic fluid in the body. Fluid loss in severe cases approaches 15 to 20 litres per day. In untreated cases the fatality rate was over 60% and higher rates were seen in children.

FIGURE 3.

The structure of monosialoganglioside GM_1

(van Heyningen, 1977)

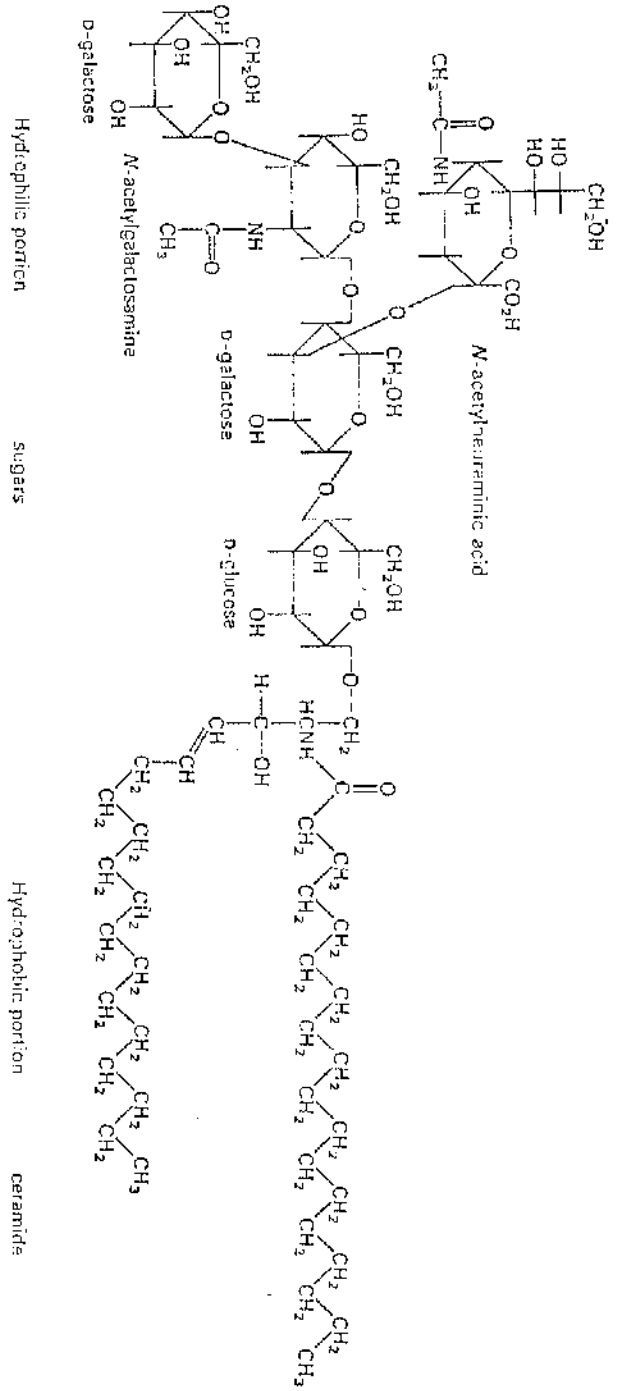
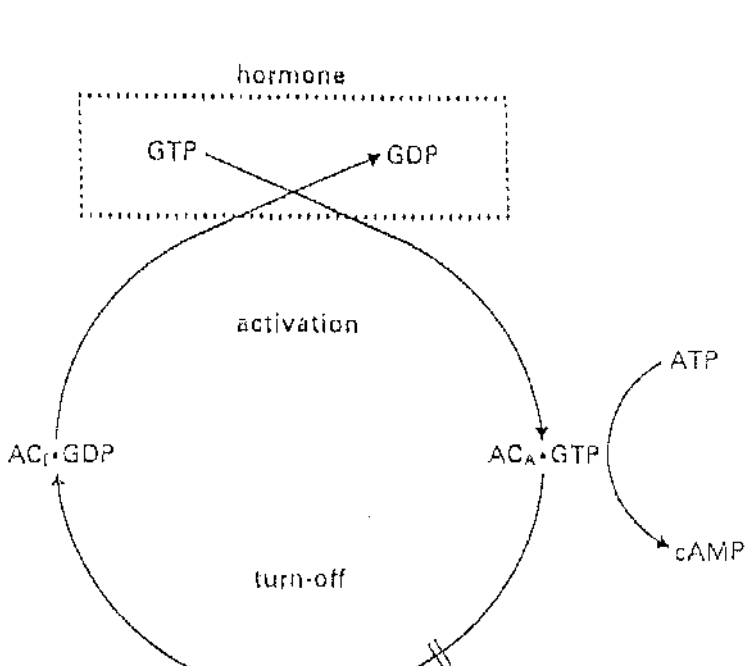


FIGURE 4.

Proposed mechanism for the regulation of adenylate cyclase activity and site of action of cholera toxin

(adapted from Stephen and Pietrowski, 1981)



AC_A : activated adenylate cyclase

AC_I : inactive adenylate cyclase

Since the symptoms of cholera are entirely the result of water and electrolyte loss from the soft tissues and the blood into the gut, replacement of this fluid can remedy the ill-effects of the toxin action. Fluid replacement therapy reduces the mortality rate from 60% to less than 1%. Methods of treatment of cholera were reviewed by Hirschhorn et al (1974) and outlined by Frost (1976). A suitable solution for oral rehydration of cholera patients was glucose 20.0 g, NaCl, 4.2g, $NaHCO_3$, 4.0g, KCl, 1.8g, dissolved in one litre of drinking water. Glucose is included in this solution as inclusion of 2% (w/v) glucose in oral fluids increases the absorptive capacity of the small intestine (Taylor et al, 1968). The fact that oral therapy can be administered by paramedical personnel is a significant advantage in rural areas of developing countries.

Tetracycline, chloramphenicol or furazolidene therapy significantly lowers the numbers of infecting organisms in the gut, and thereby reduces fluid loss (Carpenter, 1971; Gordon et al, 1966). Antimicrobial therapy is, however, not a substitute for adequate fluid replacement. Antibiotic treatment does assist in the prevention of the carrier state, where the person harbours the cholera vibrios in the gall bladder and sheds them intermittently.

3. DEVELOPMENT OF EFFICACIOUS V. cholerae VACCINE

3.1 Standard Vaccine

The standard cholera vaccine now in use is a saline suspension containing 8×10^9 killed vibrios ml^{-1}. The vaccine is bivalent, containing equal numbers of Ogawa and Inaba serotypes.

Two doses of 0.5 ml and 1.0 ml are given to adults (0.1-0.3 ml for children under the age of 10 years) by the subcutaneous or intramuscular route. In epidemics, however, single 1.0 ml doses are used for mass immunization, and may be almost as effective as the two-dose regime in an endemic area.

The immunity induced by this vaccine is of short duration, persisting for no longer than 6 months. The dosage cannot be increased since concentrations greater than 8×10^9 cells ml^{-1} tend to produce local tenderness, and less frequently lead to a slightly more severe reaction lasting for several days (Feeley and Gangerosa, 1980).

3.2 Prospective Cholera Vaccines

3.2.1 Cell Fractions

The possibility of cell fractions being effective immunogens against cholera infection was explored in several studies. Watanabe and Verwey (1965) isolated a lipopolysaccharide antigen from Ogawa cell supernates which was protective against V.cholerae challenge in mice. Verwey et al (1965) also found Inaba lipopolysaccharide antigens to be protective in mice. From these studies an effective cholera vaccine could possibly be prepared from the outer envelope layers of V. cholerae.

3.2.2 Enterotoxin preparations

Whole cell cholera vaccine contains no enterotoxin (Feeley and Roberts, 1969); however due to its central role in the production of disease, this toxin cannot be ignored as a potentially important immunogen (Finkelstein, 1969). Immunizing agents intended to prevent cholera by means of stimulating anti-toxic immunity include (i) formaldehyde-treated cholera toxin (Feeley and Roberts, 1969; Thomson, Walker and Knight, 1972), (ii) glutaraldehyde-treated cholera toxin (Rappaport et al, 1976; Levine et al, 1979), (iii) purified B-subunits (Svennerholm et al, 1982) and (iv) procholeragenoid (Finkelstein, Fujita and LoSpallutto, 1971). These immunizing agents are not capable of eliciting good protection against V. cholerae but may be useful in a combined vaccine with whole cells.

3.2.3 Attenuated V. cholerae vaccines

An ideal cholera vaccine would be one which stimulated prolonged antibody production at the mucosal surface of the small intestine. Following the observation that clinical cholera infections in North American volunteers stimulated a high degree of protective immunity for at least 3 years (Levine et al, 1981a,b) an immunological approach was taken to try to control cholera by means of attenuated non-enterotoxigenic V. cholerae strains used as oral vaccines.

Naturally occurring non-toxigenic strains failed to provide protection in experimental challenge (Levine et al, 1982).

Honda and Finkelstein (1979) isolated a V. cholerae mutant by mutagenesis with nitrosoguanadine which produced the immunogenic B-subunit but failed to produce detectable A-subunit. This mutant was termed Texas-Star SR. Texas-Star SR was extensively evaluated in volunteers

(Levine et al, 1981b; Levine et al, 1983) and was found to confer significant protection against V. cholerae challenge. However the Texas Star strain suffers from certain drawbacks:- (a) the method of attenuation, mutagenesis with nitrosoguanadine, induces multiple mutations, not all of which are necessarily recognized and (b) the precise genetic lesion presumed to be responsible for the attenuation of Texas Star is not known. Therefore, until this is clarified there always remains the theoretical possibility of reversion to virulence.

To overcome these problems Kapar et al (1984) developed an attenuated V. cholerae strain by techniques of recombinant DNA. Mekalanos and his co-workers have also reported attenuated V. cholerae strains constructed by similar methods (Mekalanos et al, 1983). Attenuated V. cholerae vaccines constructed by this method eliminate some of the disadvantages of the previously tested attenuated strain, Texas Star. A very specific method of mutagenesis was used so that no unexpected secondary mutations were present and the precise genetic lesion was known and in addition no possibility of reversion to virulence existed. Volunteer studies are under way to evaluate the safety and efficiency of candidate vaccines produced by this method.

4. BACTERIAL ADHERENCE TO MUCOSAL SURFACES

To induce disease, many pathogens must establish some degree of association with the mucosal surface. It seems likely that the extent to which a pathogen can attach to a surface influences colonization, and thus it may be related to virulence. Many pathogens have been observed to adhere to mucosal epithelium and a direct correlation between this ability and the initiation of infection exists.

The efficiency of the cleansing mechanisms operating on various mucosal surfaces to prevent colonization with bacteria was reviewed by Ofek and Beachy (1980) and Gibbons (1977). Briefly, the mucosal and endothelial surfaces are constantly bathed by mucous gels, blood flow or urine which together with mechanical anatomical mechanisms (sneezing, coughing, ciliary action and peristalsis) sweep such surfaces free of pathogenic bacteria. Therefore bacteria must adhere to host cells to counteract the normal cleansing mechanism. The importance of the initial colonization of mucosal surfaces is realized for a varied selection of pathogenic microbes, as shown in Table 2 (adapted from Mims , 1982).

Neisseria gonorrhoeae adherence to urethral mucosa cells has been studied by many workers (Ward and Watt, 1972; Swanson et al, 1975; Pearce and Buchanan, 1978) and there is agreement that fimbriation facilitates gonococcal attachment to eukaryotic cells. By human fallopian tube organ culture (Ward, Watt and Robertson, 1974) which is highly relevant to the study of gonococcal adhesion, a four-fold increase in the adhesion of fimbriated gonococci to the mucosal surface occurred than found with a non-fimbriate variant of the same strain (Ward and Watt, 1980). Fimbriate gonococci not only bind in greater numbers to human cells than

TABLE 2 Microorganisms whose attachment has a role in pathogenicity

(adapted from Mimms, 1982)

Microorganism	Disease	Attachment site	Mechanism
Influenza virus	Influenza	Respiratory epithelial cells	H.A. reacts with neuraminic acid receptor on cell
Polio virus	Poliomyelitis	Susceptible tissue cell (neurone)	Viral capsid protein reacts with specific receptor on cell
Chlamydia	Conjunctivitis	Conjunctival epithelium	Unknown-sialic acid receptor on epithelial cell
Mycoplasma pneumoniae	Atypical pneumonia	Respiratory epithelial cells	'Foot' attaches to neuraminic acid receptor on cell
Neisseria gonorrhoeae	Gonorrhoea	Urethral epithelium	Bacterial fimbriae involved and perhaps outer membrane proteins
Vibrio cholerae	Cholera	Intestinal epithelium	Receptor may be fucose or mannose
Escherichia coli	Diarrhoea	Intestinal epithelium	Requires specific bacterial surface component eg K88
Streptococcus mutans	Dental caries	Tooth	Dextran 'glue' synthesised by bacteria
Bordetella pertussis	Whooping cough	Respiratory epithelium	Evidence suggests involvement of bacterial fimbriae
Plasmodium vivax	Malaria	Erythrocyte of susceptible host sp.	Malarial merozoite attaches to antigen on erythrocyte surface

do non-fimbriate cells but also they show an increased rate of attachment. This may be critical in the transmission of the natural infection where flows of mucus or urine would tend to flush the bacterium from the mucosal surface. Buchanan, Pearce and Chen (1978) presented evidence for a carbohydrate containing receptor on Neisseria gonorrhoeae, somewhat resembling the carbohydrate moiety found on gangliosides.

However, as well as fimbriae, outer membrane proteins may play an important role in neisserial adhesion. Virji and Everson (1981) using variants of N. gonorrhoeae strain P9 which differs in its outer membrane proteins (Swanson, 1978; Lambden and Heckels, 1979), showed that one variant (P9-16) with no fimbriae but protein IIb in the outer membrane, had high avidity of attachment, for a Chang conjunctiva cell line. They also found that protein II increased the final level of adhesion compared to the prototroph P9-1 which is non-fimbriate and lacks protein II in the outer membrane. The rate of attachment of these non-fimbriate variants however was slow compared to a variant such as P9-20 which was fimbriate and possessed protein II (Virji and Everson, 1981). Therefore, both fimbriae and outer membrane proteins play a role in adherence of N. gonorrhoeae.

Fimbriae have been shown to be important in E. coli adherence to the intestinal epithelium from as early as 1957 (Duguid and Gilles, 1957). The fibrillar K antigen of E. coli, K88, was shown to be produced in vivo and to be responsible for the colonization of pathogenic E. coli to the intestinal mucosa of neonatal piglets (Jones and Rutter, 1972). Bacteria without the antigen failed to adhere to the mucosa and hence failed to colonize and cause disease (Jones and Rutter, 1972). Also, there is clear evidence that a similar mannose-resistant adhesin, K99, promoted intestinal E. coli infection in calves and lambs (Orskov

Sellwood and Gibbons, et al, 1975; Burrows, 1976). Ninety-eight percent of enterotoxigenic strains of E. coli from human adults also have a mannose-resistant haemagglutinin, either Colonization Factor (CFA) I or II. As the name suggests, these are also involved in colonization of the small intestine (Evans and Evans, 1978; Evans et al, 1975; Evans et al, 1978). The role of CFA/I in the pathogenesis of diarrhoeal disease was demonstrated in human volunteers given a virulent strain of E. coli which was either CFA^+ or CFA^-. Only those given CFA^+ strains contracted diarrhoea and showed prolonged excretion of bacteria.

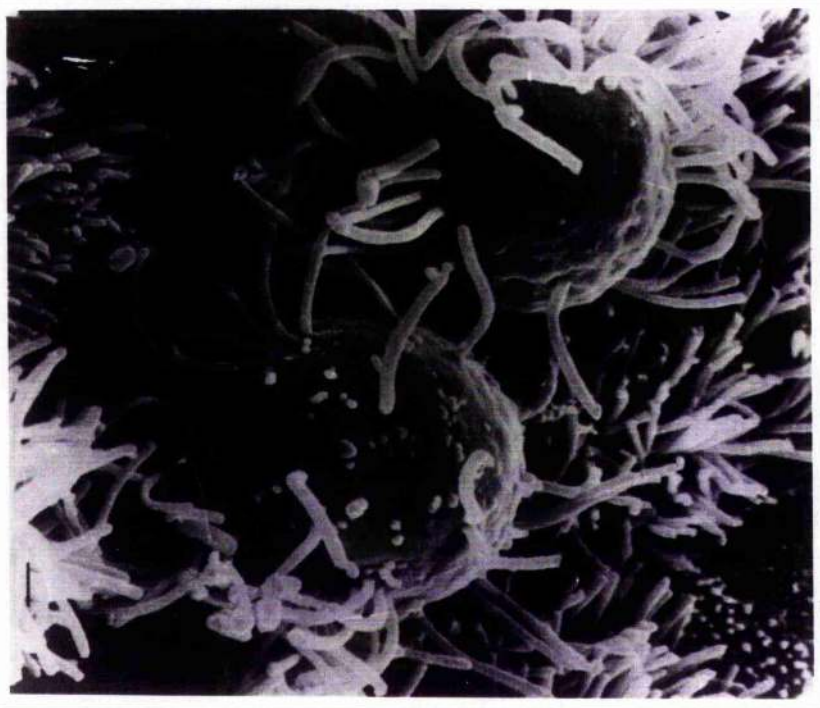
Streptococcus pyogenes is thought to adhere to the pharyngeal mucosa via the specific M protein surface layer (Ellen and Gibbons, 1974). Beachy and Ofek (1976) also suggested the involvement of lipoteichoic acid in the adherence of Strep. pyogenes to eukaryotic cells. Ofek et al (1977) found that the new born animal had a low capacity to bind streptococci and lipoteichoic acid to oral mucosal cells; this ability increased a few days after birth. They suggested that a lipoteichoic acid specific site develops or is unmasked during this period.

Mycoplasma pneumoniae is the causative organism of atypical pneumonia, although much smaller than bacteria, are also dependent upon attachment to the respiratory epithelium for production of the clinical disease. Radestock and Bradt (1977) demonstrated that virulent M. pneumoniae cells possessed a gliding motility and that an avirulent mutant was non-motile and showed reduced adherence. An adhesin present on the membrane of M. pneumoniae was identified as a high molecular weight protein (Gorski and Bredt, 1977). One receptor on eukaryotic cells for M. pneumoniae was demonstrated to be a sialic acid moiety (Razin, 1978).

Bordetella pertussis infects the lower respiratory tract and is found mainly among the bronchial cilia (Mallory and Horner, 1912). The specificity of the organisms for the ciliated cells is illustrated in scanning electron microscope pictures of organisms attached to the ciliated cells in tracheal organ cultures (Figure 5) (Muse, Collier and Baseman, 1977). Whether the sites of adhesion are on the cilia themselves (Muse, Collier and Baseman, 1977) or on the microvilli (Hopewell, Desombre and Holt, 1972) of the ciliated cells is unclear. The identity of filamentous haemagglutinins with the filamentous appendages was shown by the uniform attachment of specific anti-haemagglutinins antibody molecules to the bacterial fimbriae (Sato et al, 1979). Avirulent phase III organisms lacked fimbriae and did not attach to HeLa cells (Sato et al, 1979) nor to rabbit tracheal organ cultures (Matsuyama, 1977). Antibody to phase III organisms did not prevent phase I bacteria adhering to HeLa cells whereas homologous phase I antibody or antibody to isolated haemagglutinin largely prevented attachment (Sato et al, 1979). The weight of evidence suggests that fimbriae play a part in the pathogenesis of B. pertussis by an involvement in the attachment of the organism to the mucosal surface although the possibility of other outer membrane proteins being involved in adhesion cannot be ruled out.

FIGURE 5

Scanning electron micrograph of Bordetella pertussis infected tissue



From,

Muse, Collier and Baseman (1977)

5. ADHESIVE PROPERTIES OF V. cholerae

5.1 Association with and adhesion to the intestinal mucosa

V. cholerae interacts intimately with mucosal surfaces of the small intestine and this phenomenon is of prime importance in the pathogenesis of cholera (Freter, 1969). By definition, mucosae are epithelial layers moistened by mucus (Warwick and Williams, 1973). This term is collectively applied to two major categories:-

(i) the inner habitat of the brush-border surface of the epithelial cells. This consists of glycoproteins and glycolipids which appear to be synthesized by the epithelial cell (Bennet, 1963). The carbohydrate-rich surface layer formed in this manner is the glycocalyx (Ito, 1969) which consists of a fringe of polysaccharide fibres produced by the branching sugar chains on the glycoprotein molecules of the cell membrane (Costerton, Creesey and Cheng, 1978). The oligosaccharide components of the glycoproteins tend to differ in composition not only at different positions along the intestine but also from the tips of the villi to the crypts of Lieberkühn (Etzler and Branstrator, 1974);

(ii) the outer habitat of the mucous gel. Specialized cells, goblet cells, secrete glycoproteins which differ in various characteristics from those of the glycocalyx. The mucous gel can be of varying thickness over the epithelium, the classical work of Florey (1933) observed a rather patchy mucous blanket with areas of free villi. In contrast, Shrank and Verwey (1976) suggested a continuous mucous blanket. The thickness of the mucous probably determines the degree and rate of penetration of the vibrios to the epithelium.

As reviewed by Jones (1977), there is evidence that heterosaccharides of the glycocalyx act as receptors for bacterial adhesins.

The interaction of the vibrios with this mucosal surface is of a complex nature and appears to involve more than one mechanism. These mechanisms include bacterial motility, chemotactic attraction, penetration of the mucous gel on the intestinal villi, adhesion to receptors in the mucous gel, chemotaxis into deeper intervillous spaces, adherence of the organism to the epithelial surfaces and finally elaboration of an injurious toxin (Freter, 1978).

5.1.1 Motility

V. cholerae was shown by several electron microscopy studies (Follet and Gordon, 1963; Barua and Chatterjee, 1964; Das and Chatterjee, 1966) to possess a polar monotrichous sheathed flagellum. This is also true of other vibrio species (Glauert, Kerridge and Horne, 1963).

Numerous reports suggested a correlation between the possession of a flagellum and adhesion to mucosal surfaces by cholera vibrios. Guentzel and Berry (1975) demonstrated a relationship between motility and virulence of V. cholerae. Non-motile V. cholerae mutants showed a decreased ability to kill suckling mice than wild type parents despite the fact that they produced enterotoxin. The lack of a flagellum and/or motility in these mutant strains greatly decreased their ability to bind to the mouse epithelium. Non-motile mutants were also less virulent for adult mice infected intra-peritoneally with the organisms suspended in hog gastric mucin than parental strains (Eubanks, Guentzel and Berry, 1976).

Guentzal et al (1977) found that motile organisms penetrated in larger numbers into intervillous spaces and crypts of Lieberkühn than did non-motile strains.

Jones and Freter (1976) and Freter and Jones (1976) found that non-flagellate mutant strains lacked the ability to adhere to brush-border membranes of rabbit intestine and that adherence of these motile strains could be inhibited by L-fucose and to a lesser extent by D-mannose.

By electron microscopy Nelson et al (1977) demonstrated vibrios bound to the epithelial surface in a horizontal position with their flagella extending into the lumen of the intestine. It was suggested that since the cholera vibrio was propelled by a polar flagellum, its first contact with the intestinal surface would be via the "front-end". After the initial binding the vibrio was thought to align in a position parallel to the surface to allow greater contact. During this sequence of events it is difficult to imagine how a binding site on the flagellum, which was not in contact with the epithelial cells, could be effective, as proposed by Jones and Freter (1976).

Freter et al (1977) and Freter, O'Brien and Macsai (1978b) showed that not only were fewer non-motile bacteria present in the mucus, but that motile vibrios were more widely distributed and penetrated more deeply between the villi. Studies by Yancey and Berry (1978) and Yancey, Willis and Berry (1978) strengthened the earlier evidence that motility played a critical role in the pathogenicity of V. cholerae. These studies demonstrated that motility, or, as was suggested by the studies of Jones and Freter (1976), a motility related factor was required for the association with the ileal mucosa.

Yancey, Willis and Berry (1979) extended the original work of Eubanks, Guentzal and Berry (1976) on the use of a crude flagellar vaccine. The protection conferred by the crude flagellar immunization was superior to that of a commercial bivalent vaccine and also to that

of glutaraldehyde-treated cholera toxoid. There is no definite explanation for the high degree of active protection afforded by immunity to the crude flagellar preparation. Bacterial motility is probably inhibited or the crude flagellar vaccine may contain vibrio surface components as contaminants. Neither assumption can, however, be confirmed until the specific antigenic components of the crude flagellar preparation are determined.

It is not clear at present whether the correlation between loss of motility and loss of virulence in cholera vibrios is actually a consequence of the loss of locomotion as such or whether this is simply related to the activity of some other virulence/adhesive factor which is phenotypically linked to the expression of motility. Recent work by Attridge and Rowley (1983a) suggested that motility was not the primary role of the flagellum, but rather that this structure might act as a carrier of the adhesins responsible for attachment. Using a variety of assay systems they were unable to detect any adherence capacity associated with non-motile strains and this capacity was only obtained by the simultaneous acquisition of a functional flagellum.

5.1.2 Chemotaxis

Chemotaxis, which adds a directional component to microbial motility, was suspected for many decades of being important in the localization of mammalian pathogens *in vivo*. As reviewed by Weibull (1960) little was known about the chemotactic reactions of *V. cholerae*. Guentzel *et al* (1977) speculated on various possible virulence mechanisms of *V. cholerae* and listed chemotaxis among these, although no experimental data were given. Allweiss *et al* (1977) suggested that chemotaxis might play a role in the initial attraction of cholera vibrios

to the intestinal mucosa. A pepsin digest of rabbit intestinal mucosa neutralized a positive chemotactic response of V. cholerae, E. coli and S. typhimurium to the villous surface of pieces of rabbit intestine. Non-chemotactic motile mutants of the three bacterial strains had a significantly lower association with the mucosal tissue than the parent chemotactic strains. Mucosal extracts contained chemotactically active substances, or taxins, which apparently blocked the chemotactic attraction of cholera vibrios to the surface of the intestinal mucous gel (Freter et al, 1977). Additional work by Freter and others (Freter, O'Brien and Halstead, 1978; Freter, O'Brien and Macsai, 1979) using in vivo studies showed an increase in the mucosal association of chemotactic vibrios, as compared to non-chemotactic strains.

The taxin gradients not only increased the efficiency of the initial contact between the vibrios and the mucosal surface but, in addition, appeared to extend some distance towards the crypts where the relative numbers of chemotactic vibrios far exceeded the number of non-chemotactic vibrios (Freter, O'Brien and Macsai, 1978a,b).

V. cholerae is attracted to twenty amino acids and several carbohydrates (Freter and O'Brien, 1981a). L-fucose inhibited the adhesion of V. cholerae to the brush border membrane but lacked taxin activity (Freter and O'Brien, 1981a,b). Recently, in vivo (Freter, O'Brien and Macsai, 1981) and in vitro studies (Freter et al, 1981) provided conclusive evidence that the taxin gradient which the vibrios follow extends deeply into the intervillous spaces of the intestinal mucosa.

From the various chemotactic studies with V. cholerae it would appear that chemotaxis aids in the initial association of the vibrios

with the mucosa and also assists in their penetration through the mucous gel towards the base of the villi. Conceivably, the taxins which initially attract bacteria to the mucosal surface may differ from the taxins that attract the vibrios towards the base of the villi.

5.1.3 Association with the mucus

The mucous gel does not present an impenetrable barrier to vibrios (Freter et al, 1977) and indeed may provide a suitable habitat (Freter and Jones, 1976; Freter et al, 1977). The vibrios penetrate into and through the mucous gel for considerable distances in short periods of time (Freter, O'Brien and Macsai, 1978a,b). How this is achieved is not really known and is the subject of controversy.

The mucinase activity of the vibrios is likely to be of assistance (see Section 7.1) although it was suggested that vibrios may move along tracts created within the gel as it flows from the epithelial surface to the lumen (Jones, Abrams and Freter, 1976). The migration of vibrios towards the tissue surface was suggested to be analogous to that proposed for migration of spermatazoa within cervical mucus (Gibbons and Sellwood, 1973). Schrank and Verwey (1976) suggested that vibrio interaction with the mucus was caused by entrapment within the mucus itself. Association of the vibrios to mucous receptors was suggested by Freter and Jones (1976). In both mouse and rabbit models V. cholerae populations are found within the mucous gel (Williams et al, 1973; Schrank and Verwey, 1976; Freter et al, 1977). The apparent ease with which vibrios migrate through the mucus (Freter, O'Brien and Macasi, 1978a,b) suggests that little permanent attachment occurs in the mucous zone.

5.1.4 Adhesion to the brush-border membrane of the epithelial cells

Cholera vibrios adhered to the brush-border surfaces of intestinal epithelium cells of the rabbit and accumulated there to form layers several cells deep (Nelson, Clements and Finkelstein, 1976). Work by Jones and his colleagues (Jones, Abrams and Freter, 1976; Jones and Freter, 1976) provided evidence that vibrios attached to brush-border membranes of both rabbit and mouse intestinal epithelial cells.

V. cholerae showed an apparent degree of selectivity in its attachment to the epithelial cells of the guinea-pig (LeBrec et al, 1965) and rabbit intestines (Hirschberger, Thalar and Mirelman, 1978). The unequal distribution of vibrios on adjacent villi, reported by Nelson, Clements and Finkelstein (1976), was probably due to an unequal distribution of mucus over the epithelium providing the vibrios with easier access to some areas compared to others. The absence of vibrios from the tips of the villi (Nelson, Clements and Finkelstein, 1976), although this site is readily accessible to the vibrios (Freter et al, 1977), was probably due to lack of suitable receptors. It appears that V. cholerae cannot attach to the surfaces of all epithelial cells and that the attachment to eukaryotic cells depends upon the presence of appropriate receptors.

The extent of adhesion of one vibrio strain to brush-border membranes in vitro was directly related to the vibrio concentration and inversely related to the number of brush-border membranes present (Jones, Abrams and Freter, 1976). The adhesion was also both temperature and time dependent. At 4°C little or no adhesion occurred whereas marked adhesion occurred at 25°C and 37°C. The adhesion was not permanent at 37°C (Jones, Abrams and Freter, 1976) and the bacterium eventually dissociated. The membranes, after dissociation of the vibrios, remained

receptive to the attachment of fresh vibrio suspensions. Therefore, marked enzymic destruction of the brush-border receptors was an unlikely, but not excluded, reason for dissociation. Dissociation from brush borders *in situ* was accompanied by morphological changes in the microvilli (rough and patchy appearance) and perhaps increased mucous secretion (Nelson, Clements and Finkelstein, 1976). It may be that this movement of vibrios is the result of a reversed chemotactic gradient or enzymatic dissolution of the mucous gel.

The adherence of vibrios to the intact intestinal mucosal surface is a complicated process and the differences between the adhesive properties of the intact mucosa and the brush border membranes is quite distinct when inhibition studies are considered (see section 5.3).

5.2 *V. cholerae* adhesins

The forms taken by bacterial adhesins have been reviewed by Jones (1977).

5.2.1 Fimbriae

Unlike other bacteria such as *E. coli* and *N. gonorrhoeae* fimbriae have not been shown to play an important part in the adhesion of *V. cholerae* to the small intestinal mucosa.

Fimbriae have been observed on some vibrio species and unlike the straight fimbriae of *E. coli*, those of *V. cholerae* are curved and up to 2.5 μm in length. The diameters of the fimbriae ranged from 6-8nm (Barua and Chatterjee, 1964) or approximately 10nm (Tweedy, Park and Hodgkiss, 1968). Only some 10% of the classical vibrios examined had fimbriae and there were 10 or less fimbriae per cell. In contrast almost half of the *el tor* vibrios examined produced up to 50 fimbriae

per cell (Tweedy, Park and Hodgkiss, 1968). It seemed that el tor types produced fimbriae more readily than the classical cholera vibrios and this may account for the greater adhesiveness of el tor vibrios. Several investigators have failed to find fimbriae on vibrio cells (Finkelstein and Mukherjee, 1963; Lankford and Legsomburana, 1965) and fimbriae have not been observed on vibrios attached to brush borders (Nelson, Clements and Finkelstein, 1976; Nelson et al, 1977).

Lateral strands have been observed on the surface of vibrios attached to mucosal surfaces (Nelson, Clements and Finkelstein, 1976). These are not unlike the lateral fimbriae of some vibrios (Tweedy, Park and Hodgkiss, 1968). Whether these lateral strands are involved in V. cholerae adhesion is not known.

5.2.2 Slime envelopes

Substances often called slime envelopes or slime agglutinin have been shown to be produced by V. cholerae (Bales and Lankford, 1961; Barua and Chatterjee, 1964; Lankford and Legsomburana, 1965). Under the electron microscope the material appeared as a dense network of strands quite unlike fimbriae (Tweedy, Park and Hodgkiss, 1968). Extracts believed to contain the slime material produced by cholera vibrios were proteinaceous (Lankford and Legsomburana, 1965). The slime envelope or agglutinin was not present in organisms harvested from agar plates. It was more stable on the surface of el tor vibrios (Lankford and Legsomburana, 1965). Ghulasamaya and Lankford (1965) found no evidence that the slime envelope possessed adhesive properties. However, Attridge and Rowley (1983b) have correlated the production of this slime agglutinin with the superior adherence of el tor strains to inert particles, erythrocytes and the serosal surface of murine intestine.

Similar levels of attachment were observed with vibrios of the classical biovar if these were cultured and assayed in conditions which support the development of slime agglutinin.

The slime agglutinin does not promote attachment to an intact mucosal surface, *in vitro*, and there is no evidence at present to suggest that the slime agglutinin plays a role in the pathogenesis of cholera.

5.2.3 *Haemagglutinins*

The haemagglutinating activity of *V. cholerae* was reviewed by Finkelstein (1973). Earlier studies on haemagglutinins were concerned with the haemagglutination test as a means of differentiating *el tor* and classical types for classification purposes. Haemagglutinating activity has been associated with the ability to adhere to the surface of epithelial cells (Jones and Freter, 1976). D-mannose may inhibit the haemagglutinins of *V. cholerae* completely (Barua and Mukherjee, 1965); partially (Barua and Mukherjee, 1965; Tweedy, Park and Hodgkiss, 1968) or not at all (Jones and Freter, 1976; Bhatterjee and Srivastava, 1978). The haemagglutinins of some *V. cholerae* strains were also inhibited by L-fucose (Jones and Freter, 1976) but some strains were not inhibited by either D-mannose or L-fucose. From these carbohydrate inhibition studies there was good reason to believe that *V. cholerae* produces several haemagglutinins.

Finkelstein *et al* (1978) isolated a haemagglutinin from *V. cholerae* which they termed "cholera lectin". This isolated haemagglutinin inhibited the attachment of heterologous and homologous vibrios to tissue *in vivo* and *in vitro*.

Four distinct *V. cholerae* haemagglutinins were described by Hanne and Finkelstein (1982).

(i) a cell-associated mannose-sensitive haemagglutinin (MSHA) was produced only by the el tor biovar. This haemagglutinin appeared to be responsible for the differentiation of el tor from the classical biovar V. cholerae. The MSHA was inhibited by D-mannose and L-fucose.

(ii) a cell-associated haemagglutinin inhibited by L-fucose (FSHA) which was present during the late logarithmic phase of growth.

(iii) a cell-associated haemagglutinin produced in late logarithmic to stationary phase that was not inhibited by either D-mannose or L-fucose.

(iv) a "soluble" haemagglutinin was detected in late log phase cultures of all strains tested. This soluble haemagglutinin is the "cholera lectin" reported earlier by Finkelstein et al (1978). This particular haemagglutinin is discussed further in section 7.3.

The exact role, if any, of these haemagglutinins in colonization and adherence of V. cholerae to the intestinal mucosa is still uncertain.

5.3 Inhibition of V. cholerae adhesion and association

The interactions of vibrios with rabbit brush border membranes and with intact slices of rabbit intestinal mucosa were compared (Jones and Freter, 1976; Freter and Jones, 1976).

L-fucose inhibited the attachment of V. cholerae to the brush-border membranes of the rabbit. The L-isomer was inhibitory and the inhibition increased with increasing molecular size of the fucoside (Jones and Freter, 1976). D-mannose also inhibited the attachment of some strains but to a lesser extent than L-fucose. The association of the vibrios with the intact intestinal mucosa appears to be quite different than to the isolated brush-border membranes as neither L-fucose nor D-mannose inhibited the attachment of vibrios to the intact

mucosa (Freter and Jones, 1976). Bhattarjee and Srivastava (1978) in contrast to Freter and Jones (1976) found that D-mannose inhibited the association of el tor vibrio strains to intact slices of rabbit intestinal tissue.

The results obtained from these inhibition studies with D-mannose must be considered carefully as D-mannose is a chemo-attractant for V. cholerae. It is possible that the inhibition observed in these studies was a consequence of inhibition of adherence of the vibrios to the brush-border membrane, but it is equally possible that the reduced association was a result of D-mannose interfering with normal chemotaxis. Unlike D-mannose, L-fucose was not a chemo-attractant for cholera vibrios.

Pepsinised mucosal scrapings (Freter and Jones, 1976) inhibited both adherence to brush borders and association with mucosal slices. Fractionation of this crude mixture of substances revealed the presence of two activities one of which inhibited association with intact mucosa, possibly acting as a chemoattractant while the other inhibited adherence and possibly contained L-fucose (Freter et al, 1977).

Although there is no direct evidence that specific receptors exist, the evidence of selectivity with which vibrios attach to animal cells and the inhibition of adhesion by some monosaccharides suggests that this may be so.

Evidence against the concept of host receptor structures being required for attachment has recently been presented by Attridge and Rowley (1983a,b). They found that organisms selected for their capacity to adhere to inert substances simultaneously acquired the capacity to bind to intestinal tissues in vivo. Inhibition studies with monosaccharides, such as L-fucose, showed no decrease in the capacity

of *V. cholerae* to attach to the intestinal mucosal surface. They also found that *V. cholerae* showed undiminished binding to boiled tissue segments.

6. PROTEASES

6.1 General Microbial Proteases

Extracellular protease activity has been noted in many eukaryotic microorganisms such as fungi, protozoa and slime moulds (North, 1982) and in many bacterial species (discussed below). There have been correlations between protease activity and pathogenicity of eukaryotic and bacterial microorganisms. At the beginning of this study, since very little was known about V. cholerae protease(s), it was necessary to rely on studies with other bacterial proteases.

Pseudomonas aeruginosa, an important opportunistic pathogen, is known to produce several proteases (Jensen et al, 1980; Kessler, Kennah and Brown, 1977; Morihara and Tsuzuki, 1977), two of these proteases were isolated, purified and characterized. Both were metalloproteases but they were immunologically distinct and differed in their molecular weights, substrate specificities and pH optima. One was designated alkaline protease because of its activity at alkaline pH, the other as elastase because it degraded elastin.

Another opportunistic pathogen, Serratia marcescens, was shown by Aiyappa and Harris (1976) to produce an extracellular metalloprotease. Unlike Ps. aeruginosa the Serratia protease was a neutral protease with maximum activity at pH 5.5-7.5. Lyerly and Kreger (1979) found that the purified Serratia protease although homogeneous by Ouchterlony double immunodiffusion showed three to four closely migrating bands in SDS-polyacrylamide gel electrophoresis. However, zymogram analysis of the

patterns showed that protease activity was associated with each component, they therefore termed the protease 'microheterogeneous'. This characteristic of Serratia protease was also reported by McQuade and Crewther (1969), although their protease preparation was not rigorously examined for purity. Purified Ps. aeruginosa elastase was also reported to exhibit microheterogeneity (Kreger and Gray, 1978), so this observation was not unique to a single protease. In addition, preparations of crystalline bovine trypsin were found to contain variable quantities of at least five molecular weight species, which apparently resulted from partial autodigestion.

Pseudomonas proteases were implicated in the pathogenesis of several infections including keratitis and corneal ulcers (Kawaharajo et al, 1974; Hirao and Homma, 1978; Kreger and Gray, 1978) and in burned skin infections (Carney, Dyster and Jones, 1973; Cicmanec and Holder, 1979; Pavlovskis and Wretlind, 1979; Holder and Haidaris, 1979).

Ps. aeruginosa is the major pathogen associated with persistent pulmonary infections in children and young adults with cystic fibrosis. Data presented by Jagger, Bahner and Warren (1983) suggested that protease production may be important in the initial colonization of the respiratory tract of cystic fibrosis patients by Ps. aeruginosa.

Protease activity was associated with the production of Serratia pneumonia by Lyerly and Kreger (1983). They showed that (i) a homogeneous Serratia protease preparation elicited gross lung damage in guinea-pigs and mice which was similar to that observed during experimental pneumonia, (ii) vaccination against this protease stimulated active immunity against experimental lethal pneumonia, and (iii) the

protease was produced *in situ* during this type of pneumonia. These characteristics support the experimental evidence that this protease is involved in the pathogenesis of experimental *Serratia* pneumonia.

The viridans streptococci form the single most prevalent group of bacteria causing subacute bacterial endocarditis. Strains of *Streptococcus sanguis* produced more than one type of proteolytic enzyme (Straus, Mattingly and Milligan, 1980). Recently, *Strep. sanguis* isolated from a patient with subacute bacterial endocarditis (Straus, 1982) yielded four distinct proteases which were isolated and purified from the supernatant fluids of stationary phase cultures. The four proteases had different molecular weights and substrate specificities but all four had pH optima between 8.0 and 9.0. The finding that *Strep. sanguis* excreted potentially destructive enzymes when it was not actively multiplying may explain some of the damage it causes in heart tissue during subacute bacterial endocarditis.

The natural environment of *Aeromonas hydrophila* is surface fresh-water, including chlorinated drinking supplies (LeChevallier, Siedler and Evans, 1980). *A. hydrophila* has been associated with wound infections, septicaemia (particularly in immunologically compromised patients) and with diarrhoea in human beings (Ljungh, Popoff and Wadström, 1977; Echeverria *et al*, 1981; Goodwin *et al*, 1983). *A. hydrophila* produces a number of surface lectin-like adhesins (Adams, Atkinson and Wood, 1983) and extracellular proteases (Riddle,

Graham and Ambaorski, 1981). The role, if any, of the proteases, or their interaction with the surface lectins/haemagglutinins in the pathogenicity of A. hydrophila is unknown.

The ability to hydrolyse casein or liquefy gelatin is widely distributed among members of the family Micrococcaceae. According to to Baird-Parker (1963), the majority of the staphylococcal strains produce extracellular proteases. S. aureus produces at least three proteases, one which is EDTA-sensitive and two which are EDTA-insensitive; of the latter one is active at a neutral pH and the other is an alkaline protease (Arvidson, 1973; Arvidson, Holme and Lindholm, 1973). The function of the proteases from staphylococci and micrococci is unknown but it has been suggested that they ensure a supply of amino acids for growth and synthesis (McDonald and Chambers, 1966).

Vibrio species other than cholera have been shown to produce extracellular protease activity. Vibrio parahaemolyticus (Tanaka and Iuchi, 1983) produced four proteases separated by analytical polyacrylamide gel electrophoresis, two of which were inhibited by serine protease inhibitors and two which were inhibited by metalloprotease inhibitors. Vibrio alginolyticus produced alkaline proteases (Hare, Scott-Burden and Woods, 1983) which yielded five bands of protease activity in gelatin-PAGE. All five proteases were inhibited by both serine and metalloprotease inhibitors.

Extracellular protease activity was found in many other bacterial species including Bacillus subtilis (McGonn, Tsuru & Yasunobu, 1964), Bacillus megaterium (Keay et al, 1971), Bacillus cereus (Feder, 1971) and Proteus mirabilis (Hampson, Mills and Spencer, 1963) although no relationship of the enzymes to pathogenesis was considered.

Therefore, protease activity appears to be widely distributed among many microorganisms. The reason for this enzyme production is, in some cases, unknown, in some organisms it is used to obtain amino acids as a nutritional source and in many instances there is a correlation between protease production and the pathogenicity of the organism.

6.2 Microbial IgA Proteases

In all mammals studied, secretions bathing mucous membranes contain antibodies and the predominant isotype of antibody is IgA (Tomasi and Grey, 1972). Mucosal IgA has binding specificity for surface and secreted antigens of bacteria, viruses, microbial toxins and food antigens (Tomasi, 1976). The precise mechanism by which antigen clearance at mucosal sites takes place is unclear because secretory IgA apparently does not utilize complement for disposal of antigen and is not opsonic (Wilson, 1972; Van Epps and Williams, 1976). There is however evidence that secretory IgA functions by preventing adherence of bacteria to relevant tissues. Williams and Gibbons (1972) showed that preparations of secretory IgA specifically inhibited the adherence of Streptococci to epithelial cells. Because such adherence is a prerequisite for colonization and disease, inhibition of this event would apparently be an adequate means by which secretory IgA could function in mucosal defence.

IgA is present in both serum and external secretions of the alimentary, respiratory and reproductive tracts. Serum IgA is primarily monomeric with a molecular weight of about 160 k.Dal, and composed of two light and two heavy chains characteristic of most immunoglobulins. In contrast IgA in secretions is mainly dimeric, composed of two covalently bound monomeric subunits of IgA, and is secreted in this form by the mucosal plasma cells. Each secreted IgA molecule also contains one J-chain, a polypeptide of molecular weight 16 k.Dal. During transport of the IgA dimers through the mucosal epithelium to the lumen they bind to a glycoprotein, the secretory component, that is contributed by the epithelial cells. The molecular weight of the fully assembled secretory IgA (sIgA) is about 380 k.Dal.

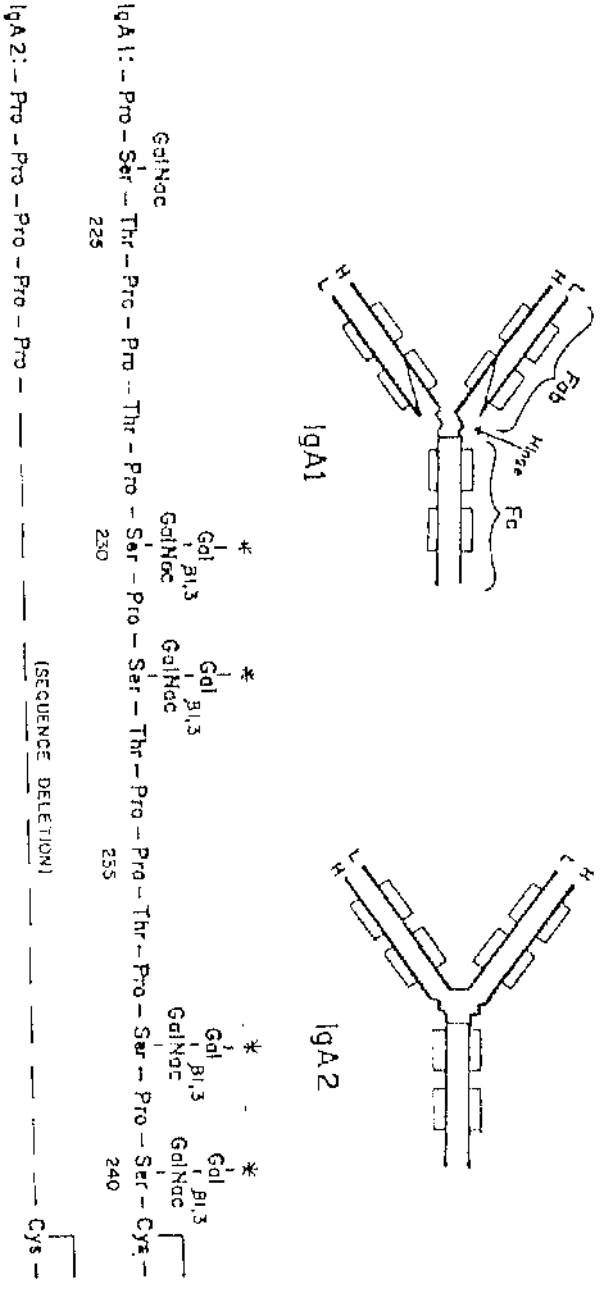
Serum and sIgA contain two isotypes (subclasses) of IgA designated as IgA1 and IgA2. The two isotypes are distributed unequally in human serum and secretions. Grey et al (1968) in a careful analysis of the two isotypes in human colostrum found that 35-50% was of the IgA2 type but less than 10% of serum proteins were IgA2. The primary structure of IgA1 and IgA2 are similar (Putman, Liu and Low, 1979), the only major difference in their amino acid sequence is in the hinge region peptides between Fab and Fc regions where IgA2 has a deletion of 13 amino acid residues (Figure 6).

IgA proteases are specific to human IgA and attack only proteins of the IgA1 isotype, cleaving one of the peptide bonds within the hinge region of the heavy chain. Human IgA2 proteins are resistant to this enzyme because the hinge region of their heavy chains has a primary sequence deletion that involves most of the protease susceptible bonds found in IgA1.

FIGURE 6

Structure of human immunoglobulin A

(adapted from Kornfield and Plaut, 1981)



The IgA1 protease-positive bacteria so far identified include species that cause such human infections as meningitis, sinusitis, otitis media, bronchitis, pneumonitis, sexually transmitted diseases and bacterial endocarditis. IgA1 protease was first clearly identified in S. sanguis, a species implicated in the early stages of dental caries (Gibbons and van Houte, 1975) and in causing endocarditis in persons with structural heart abnormalities (Genco, Plaut and Mellering, 1975).

IgA1 protease production by Neisseria gonorrhoeae is a constant feature of nearly all isolates of these organisms. Isolates harmlessly carried in the nasopharynx of normal persons and those obtained from cerebrospinal fluid of patients acutely ill with meningitis are all IgA protease positive (Plaut et al, 1975; Mulks and Plaut, 1978).

Haemophilus influenzae, a major respiratory, middle ear and meningeal pathogen was IgA protease positive in 95% of all strains (Mulks, Kornfield and Plaut, 1980; Kilian et al, 1980). All clinical isolates of Streptococcus pneumoniae, which causes meningitis and pneumonia in man, were IgA protease positive (Male, 1979; Kilian, Mestecky and Schrohenloher, 1979). Kilian and Holmgren (1981) reported that several pathogens of the periodontal tissues have extracelluar proteolytic enzymes similar to IgA1 proteases. IgA1 specific enzymes were identified in Bacteroides melaninogenicus and Streptococcus mitior.

The extent to which IgA proteases become involved in the pathogenesis of infectious disease is not known because the mechanism by which limited proteolysis of IgA into Fab and Fc fragments may affect antibody function at secretory sites has not been determined. It is likely that the formation of single Fab fragments reduces the functional affinity of the antibody for its antigen by decreasing its multivalency

and binding efficiency (Hornick and Karush, 1972). Moreover, the separation of the antigen-binding Fab portion of the molecule from the Fc "effector" segment would be expected to interfere greatly with the disposal of antigen by IgA antibody.

6.3 Protease Inhibitors

Proteases can be placed in four well-characterized biochemical groups (Cohen, 1980; Laskowski and Kato, 1980.)

(i) proteases with a serine residue at the active site - serine proteases.

(ii) proteases with a nucleophilic cysteine residue at the active site - sulphydryl(thiol) proteases.

(iii) metal-containing enzymes - metalloproteases.

(iv) proteases with an acid pH optimum - acidic (carboxyl) proteases.

By their reaction with enzyme inhibitors the proteases can be classified into one of these four groups (Table 3).

Alpha-2-macroglobulin (α2m) is a plasma protease inhibitor with a molecular weight of about 800 k.Dal. (Barrat and Starkey, 1973). α2m is quite unusual in being able to inhibit all four classes of proteases (Hartley, 1960). The mechanism by which α2m causes inhibition is known as the "trap" mechanism (Barrat and Starkey, 1973). The protease hydrolyzes one or more particularly susceptible peptide bonds in α2m, and triggers a conformational change in α2m which traps the enzyme molecule. Proteases which are bound to α2m retain their ability to attack low molecular weight substances but are prevented from attacking high molecular weight substances due to steric hindrance.

TABLE 3

CLASSIFICATION OF PROTEASES INTO FOUR BIOCHEMICAL GROUPS

GROUP	IDENTIFICATION	
	FUNCTIONALITY	INHIBITORS
Serine	Active serine	Soyabean trypsin inhibitor Eggwhite trypsin Anti-trypsin Phenylmethylsulphonyl fluoride
Metallo-	Metal ion	8-hydroxyquinoline Phosphoramidon Ethylenediaminetetraacetic acid Chelating agents Heavy metals
Sulphydryl	CySH	Iodoacetate N'Ethylmaleimide Dithiothreitol
Carboxyl	Acidic pH optimum	Pepstatin Epoxy(p-nitrophenoxy) propane

7. *V. cholerae* EXTRACELLULAR ENZYMES

7.1 Mucinase

Burnet and Stone (1947a) described an activity present in cultures of *V. cholerae* that caused desquamation of guinea-pig intestinal mucosa. They regarded this activity as enzymatic in nature and thought it might play a role in the pathogenesis of cholera; this new enzyme was referred to as mucinase. Burnet (1948, 1949) again drew attention to the existence of an enzyme or group of enzymes produced by *V. cholerae* that rapidly destroyed the viscosity and hence the mechanical protective properties of intestinal mucus. It is significant that in these early papers Burnet had realised the possibility that there might be a mucinase complex containing a number of enzymes, including possibly mucinases, neuraminidases and proteases.

The immunological studies of Singer, Wei and Hoa (1948) showed that the mucinase activity could be neutralized by antiserum raised against products of *V. cholerae* in the culture filtrates, but in addition, they found that the activity was neutralized by antisera to the "O"-antigen of *V. cholerae*. On the other hand, Jensen (1953) showed that antisera to culture filtrates had a high neutralizing power but that "O"-antisera were ineffective, this confirmed the earlier work of Burnet (1947a). Narayanou, Devi and Menon (1953) and Singh and Ahuta (1953) also found mucolytic activity in *V. cholerae* cultures, and that some *el tor* and non-agglutinating vibrio strains isolated from water also produced enzymes capable of desquamating intestinal mucosa. The production of a mucinase enzyme by some non-pathogenic vibrios was also noted by Singh and Ahuja (1953). Lam and Mandle (1954) demonstrated

that vibrio mucinase increased the permeability of isolated mouse intestinal loops and they were able to neutralize this effect by active and passive immunization against the homologous mucinase.

Freter (1955) reported the presence of two serological types of mucinase occurring in varying combinations in V. cholerae strains. The data he presented indicated that the titre and serological type of mucinase, produced in vitro, had no direct relation to virulence.

After a period of 35 years since Burnet's first paper on mucinase, Schneider and Parker (1982) reexamined the purification and characterization of V. cholerae mucinase. The mucinase possessed protease activity (discussed in section 7.3) but no neuraminidase activity was detected in this particular preparation. The lack of neuraminidase may have been due to the growth conditions and purification procedure, neither of which were optimal for neuraminidase production (Ada, French and Lind, 1961).

The mucinase activity produced in vitro appears to have only protease activity but whether this is the case in vivo is not known. It would seem more likely that the in vivo mucinase is a complex of enzymes rather than one single enzyme.

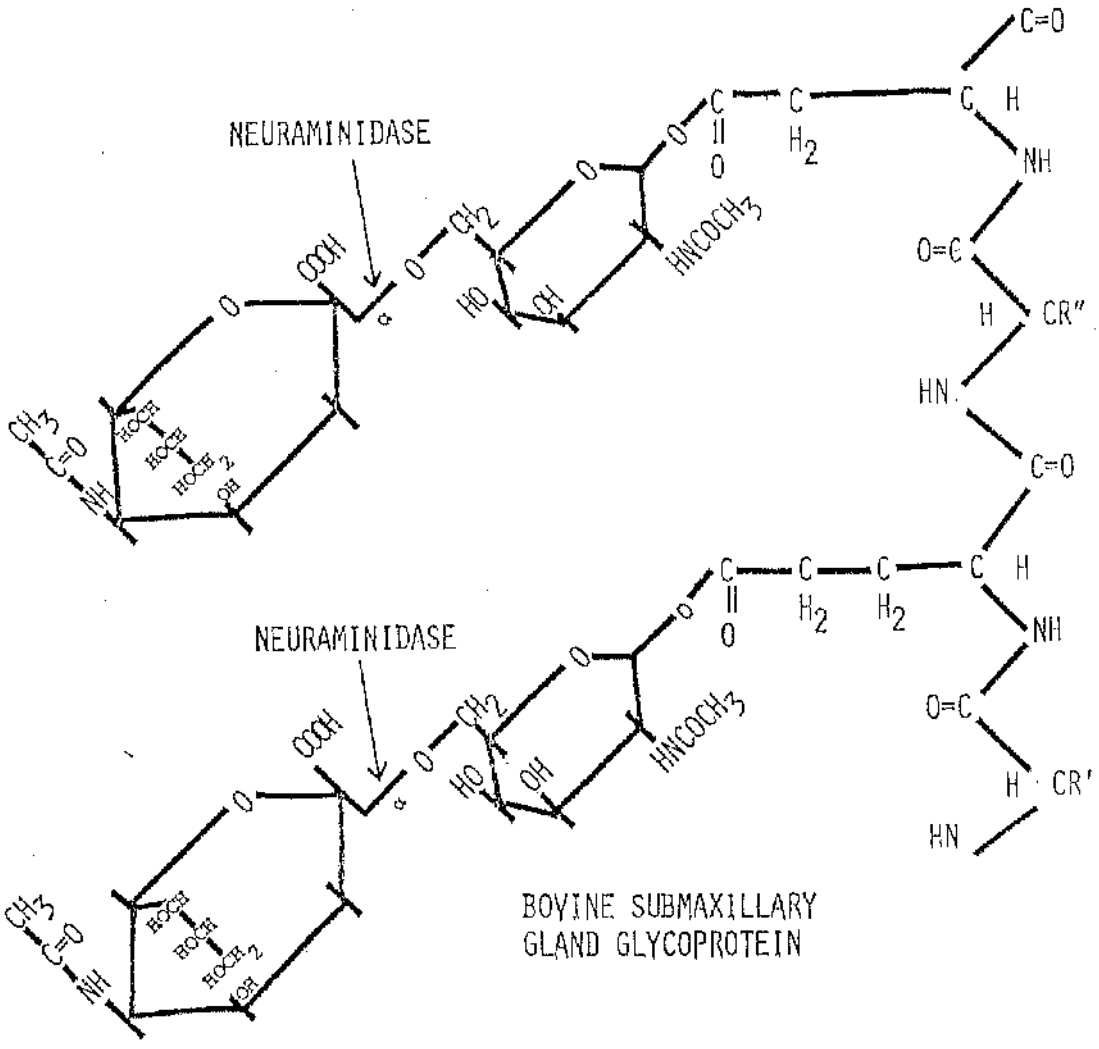
7.2 Neuraminidase

Neuraminidases are enzymes with the common ability to hydrolyse the α-O-ketosidic bond between sialic acid residues and neighbouring carbohydrates (Figure 7). The existence of these enzymes was shown in viruses, bacteria, protozoa and vertebrates (Drzenaik, 1972; Gottschalk and Drzeneik, 1972).

The ability of culture fluids of V. cholerae to render human

FIGURE 7

Action of neuraminidase on bovine submaxillary gland glycoprotein



human red cells inagglutinable by influenza virus was first noticed by Burnet, McCrea and Stone (1946). They described the active principle as the receptor-destroying enzyme (RDE) of V. cholerae (Burnet and Stone, 1947). Gottschalk and Lind (1949) provided early evidence about the chemical activity of RDE but the enzyme was not characterized as a glycosidase until 1956 (Gottschalk, 1956; Heimer and Meyer, 1956). The enzyme was originally named sialidase (Heimer and Meyer, 1956) but it is now more commonly known as neuraminidase (Gottschalk, 1957).

Early purification of RDE (Ada and French, 1950) was difficult and complex but some of these difficulties were overcome when it was found that the addition of N-acetyl neuramin-lactose to a simple medium greatly stimulated enzyme production (Ada and French, 1957; Ada and French, 1959a,b). Purification procedures and chemical and physical properties of the RDE were reported by various groups (Ada and French, 1959b; Ada, French and Lind, 1961; Pye and Curtain, 1961).

Burnet (1949) reported that the mucinase and the RDE were different enzymes. Studies by Kusama and Craig (1970) supported this statement since the time course for the production of the two enzymes was quite different.

Comparatively little is known about the function of bacterial neuraminidases although it is believed that one function may be to prepare the cell surface for attachment of pathogens particularly as more neuraminidase is excreted by pathogenic than non-pathogenic bacteria. Indeed only a few instances of non-pathogenic organisms which produce neuraminidase have been reported (Kunimoto et al, 1974). Therefore bacterial neuraminidase may "process" complex glycoproteins by removing

removing those sialic acid residues joined to the backbone of the glycoprotein by susceptible chemical bonds (Hutchison and Kabyo, 1977).

Durihin, Popova and Kobrinskii (1976) found a high degree of correlation between synthesis of both enterotoxin and neuraminidase and it was suggested that these products acted cooperatively during the development of cholera. Neuraminidase was shown to increase the number of receptor sites on the epithelial membrane available for the binding of enterotoxin. This property of the enzyme, however, was rejected by Holmgren *et al* (1975). Holmgren (1981) found that neuraminidase treatment of intestinal epithelium tissue did not create new receptor sites for cholera enterotoxin.

Neuraminidase was implicated as a virulence factor not only in *V. cholerae* but also in streptococci (Milligan, Straus and Mattingly, 1977; Milligan *et al*, 1978; Davis, Mansoorbaig and Ayoub, 1979), *Pasteurella* strains (Frank and Tabataki, 1981) and in other bacterial species that secrete or produce cell bound neuraminidase (Ray, 1977; Smith, 1977; Pardoe, 1974)

7.3 Proteases

Proteases are regarded as degradative enzymes which are capable of cleaving proteins into small peptides and amino acids. This proteolytic activity is generally detected by digestion of proteins such as casein or gelatin.

Until 1981, very little work had been done on *V. cholerae* protease activity. Since then a number of workers have taken an interest in this area expanding the knowledge of extracellular protease activity in *V. cholerae*.

A protease-type activity in the culture filtrates of V. cholerae was first noted by Burnet and Stone (1947a). Kusama and Craig (1970) suggested that the protease and lytic activity factor, also found in V. cholerae supernatant culture fluids, were the same enzyme. However, these were quite distinct from the neuraminidase.

Hsieh and Liu (1970) used a serological approach to study the protease activities of the so-called non-agglutinable (NAG) vibrios and those of V. cholerae. Proteases from the NAG vibrios were serologically indistinguishable from the enzymes produced by classical strains of V. cholerae, but were distinct from those produced by Vibrio metchnikovii and Aeromonas liquefaciens. Dahle and Sandvik (1971) also examined the serological characteristics of V. cholerae proteases. In contrast to Hsieh and Liu (1970), they found serological cross reactions between protease fractions of Aeromonas liquefaciens and V. cholerae.

Dahle and Sandvik (1971) used a zymogram technique to analyse the protease activity of V. cholerae. This technique involved the electrophoretic separation of proteases in polyacrylamide gels which were subsequently covered with a sodium caseinate gel allowing localization of the different proteases. Most of the strains examined produced more than one extracellular protease fraction. Sandvik and Dahle (1971) showed that some strains of V. cholerae produced two or more serologically different proteases. These results suggested the idea of a complex protease system in V. cholerae.

Schneider, Sigel and Parker (1981) used a simple method, peptide digest analysis, to characterize the proteolytic activities of various V. cholerae isolates. This technique utilized the catalytic specificities of proteases as a basis for typing strains of V. cholerae.

A standard protein was used as the substrate for proteases found in culture supernatant fluids. The unique peptides generated by cleavage were separated on the basis of relative molecular weights by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The unique banding patterns generated in the gels were used as the basis for catalytic typing. V. cholerae supernatant culture fluids from most isolates revealed the presence of similar proteases as monitored by their peptide digest patterns and immunological reactivities. These observations agree with the earlier work of Hsieh and Liu (1970).

A genetical approach was used by Schneider and Parker (1978) to consider the role which extracellular enzymes may play in experimental infections with V. cholerae. Evidence was presented that most mutants deficient in proteolytic activity had reduced virulence for experimental animals. These authors suggested that a specific protease, rather than the total proteolytic activity, was involved in the virulence of V. cholerae as protease-deficient mutants showed no correlation between absolute protease levels and the LD_{50} values. The protease(s) was thought to be involved in either maintenance or growth of the organisms in the gut. This work was done with protease-deficient mutants but 90% of these mutants were also neuraminidase-deficient, so this particular set of results should be interpreted with care as the neuraminidase deficiency may also be aiding in reducing the virulence of these mutant strains.

Increased interest in V. cholerae proteases has been apparent during the course of my studies. Three major papers which support the experimental work to be described in this thesis have appeared in the

literature during the past two years. Consequently, these will be reviewed in some detail. Table 3 summarizes the protease nomenclature used in these papers.

During studies on the role of the mucinase of V. cholerae CA401 in virulence, Schneider and Parker (1982) found the enzyme was an alkaline protease. Under the conditions used the mucinase was the only extracellular protease produced. The protease was purified by precipitation with 70% (w/v) ammonium sulphate and gel filtration on a column of either BioGel P-100 or Sephadex G100. The elution profiles from these columns showed two peaks of protease activity. These were termed fraction 3 and fraction 4. The apparent molecular weights of fraction 3 and fraction 4 on Sephadex G100 columns were 38 k.Dal. and 22 k.Dal. respectively. The comparative peaks off BioGel P100 columns were 18 k.Dal and 10 k.Dal. SDS-PAGE and non-denaturing PAGE gels of fraction 3 showed a single band with a molecular weight of approximately 36 k.Dal. Schneider and Parker suggested that the elution of the fractions from the BioGel P100 was retarded, possibly due to an interaction with the gel matrix. Both molecular weight forms, fraction 3 and fraction 4, exhibited similar properties, both were inhibited by (i) heavy metals but not by naturally occurring protease inhibitors and (ii) antiserum to purified mucinase. Antiserum to mucinase passively protected infant mice from diarrhoea caused by V. cholerae challenge.

A high protease-producing strain, V. cholerae el tor 1621 hip, was obtained by N-methyl-N'-nitro-N-nitrosoguanadine mutagenesis of V. cholerae el tor 1621 (Young and Broadbent, 1982). Isoelectric focussing of the culture filtrates showed the presence of at least three types of V. cholerae extracellular proteases. The wild type strain contained predominantly a protease with a pI of 4.2 which was sensitive

TABLE 4

Summary of *V. cholerae* protease nomenclature

Reference	Strain	Protease
Scheider and Parker (1982)	*V. cholerae* CA401	Fraction 3 Fraction 4
Young and Broadbent (1982)	*V. cholerae* eltor 1621	I II
	V. cholerae eltor 1621 hip	IIA IIB IIIA IIIB
Finkelstein and Hanne (1982)	*V. cholerae* CA401	pI 6.3 pI 5.3 pI 4.7

to serine protease inhibitors. This was termed type I protease. Activation of cholera toxin by limited proteolysis of the A subunit was also sensitive to serine protease inhibitors (Gill and Rapport, 1979; Makalanes, Collier and Romig, 1979) therefore Young and Broadbent suggested this may involve type I protease. No results have been shown to confirm this suggestion. The minor protease in the wild-type strain and the major protease in the mutant strain was termed protease II. Protease II resembled the proteases described by Schneider and Parker (1982) as it was resistant to metalloprotease and serine protease inhibitors and possessed mucinase activity. Two peaks of type II protease were found and differed by 1.2 pI units in isoelectric focussing point and by 1.5 k.Dal. in molecular weight; these were termed IIA and IIB. The mutant strain also contained a minor protease, proteaseIII, which was a typical metalloenzyme. Two peaks of type III protease were found and differed by 0.3 pI units; these were termed IIIA and IIIB. Protease III was not detected in the wild-type strain.

The presence of four types of haemagglutinins in V. cholerae CA401 was described by Hanne and Finkelstein (1982). One of these haemagglutinins, the soluble haemagglutinin was purified to apparent homogeneity by a sequence of ammonium sulphate fractionations, gel filtration and preparative isoelectric focussing (Finkelstein and Hanne, 1982). The tremendous loss of activity during purification led to the observation that the soluble haemagglutinin was also a protease. Analysis of protease activity after preparative isoelectric focussing revealed major regions of activity which focussed at pH 6.3 and in a broad area from 5.3 to 4.7. These corresponded almost exactly with

the pIs of the haemagglutinin activity. Antiserum raised against the purified haemagglutinin inhibited the protease activity and also inhibited attachment of V. cholerae to intestinal epithelium. However, further studies indicated that the two activities had different temperature requirements. The haemagglutinin titres were relatively constant over the temperature range 4° to $37^{\circ}C$, whereas 100-fold differences in protease activity were observed between 4° and $25^{\circ}C$. These observations suggest that the haemagglutinin and protease activities may be independent functions of the same molecule.

This soluble haemagglutinin/protease was also found in a cell-associated form in vivo (Finkelstein, Boesman-Finkelstein and Holt, 1983). Finkelstein and his colleagues suggested that the purified haemagglutinin/protease was functionally identical to the mucinase described earlier by Burnet and Stone (1947a) and Burnet (1949). They came to this conclusion because (a) the haemagglutinin/protease degraded ovomucin and (b) antiserum against the purified haemagglutinin inhibited the mucinase activity. This correlation between mucinase and protease activity was noted previously by Schneider and Parker (1982) and Young and Broadbent (1982).

The haemagglutinin/protease hydrolysed fibronectin and lactoferrin (Finkelstein, Boesman-Finkelstein and Holt, 1983) two proteins that may participate in host defence against cholera. Fibronectin is an adhesive high molecular weight glycoprotein that is present on the surface of mammalian cells (Yamada and Olden, 1978) and has been shown to be synthesized by the crypt cells of the intestinal epithelium (Quaroni, Isselbacher and Ruoslahti, 1978). Fibronectin was reported to interfere with the adherence of Pseudomonas aeruginosa to epithelial

cells (Woods et al, 1981). Lactoferrin has been assumed to play a major role in host defence by withholding essential iron and thus reducing its availability to microbes in mucosal secretions (Bullen, 1981).

Finkelstein, Boesman-Finkelstein and Holt (1983) found that the A subunit of the choleragen-related heat-labile enterotoxin of E. coli was nicked at a specific site by the haemagglutinin/protease. It was suggested that this was the mechanism by which cholera enterotoxin is activated, although no work has been done as yet with cholera enterotoxin.

Recently the soluble haemagglutinin/protease was shown to be a zinc metalloenzyme by Booth, Boesman-Finkelstein and Finkelstein (1983). Both its haemagglutinating and protease functions were inhibited by chelating agents including a specially designed inhibitor of zinc metalloenzymes, Zincov (Nishino and Powers, 1979).

As mentioned above, the soluble haemagglutinin/protease was shown (Finkelstein, Boesman-Finkelstein and Holt, 1983) to nick the A subunit of the heat-labile enterotoxin of E. coli. Young and Broadbent (1982) suggest that this proteolytic activity would be sensitive to serine protease inhibitors, as was apparent with protease I isolated from culture supernatant fluids of V. cholerae el tor 1621. Both, Boesman-Finkelstein and Finkelstein (1983) have shown the haemagglutinin/protease to be resistant to serine protease inhibitors but still capable of nicking the A-subunit of the heat-labile enterotoxin of E. coli.

By considering this recently published work it would appear that V. cholerae has numerous extracellular proteases. They differ in their molecular weights, their pI values and susceptibility to inhibitors.

There also appears to be a close relationship between protease, mucinase and the soluble haemagglutinin.

The work of Din and his colleagues (Din et al, 1981) showed that proteolytic enzymes altered the biochemical and biophysical nature of cervical mucus and so enhanced sperm migration through the mucus. Since V. cholerae must move through the mucus layer covering the mucosa the cholera proteases may be involved in this movement in a similar manner to the enhanced sperm migration through mucus gel treated with proteolytic enzymes. Protease activity was also shown to enhance the adherence of bacilli to human buccal epithelial cells by altering the epithelial cell surface (Woods et al, 1981). Cholera proteases may be involved in the adherence of vibrios to the epithelial cell surface by altering this surface in a manner that makes it more susceptible to vibrio attachment.

Object of Research

The main aim of this project was to determine the role of Vibrio cholerae extracellular protease(s) in the pathogenesis of cholera. Initially, the plan was to purify and characterize the proteases and to consider their role in adherence. Since existing vaccines for V. cholerae were shown to be ineffective in the duration of immunity induced the main objective was to examine the possible beneficial effects of adding the characterized protease(s) to experimental V. cholerae vaccines.

This thesis describes a variety of procedures used to prepare and purify the protease enzymes of V. cholerae, characterize the specific activities of these enzymes in an attempt to explain their role in colonization, adherence and virulence of the organism. Finally laboratory animals were immunized with vaccines containing the protease enzymes either alone or in conjunction with neuraminidase or enterotoxoid.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Bacterial Strain

The organism used throughout these studies was Vibrio cholerae NCTC 10732, this is a classical biovar with Inaba serovar obtained from the National Collection of Type Cultures, London.

2. Growth Media

Trypticase soy broth (TSB), Appendix 1A from BBL (Becton, Dickson & Co., Cockeysville, M.D.) was used to grow V. cholerae for the production of proteases both in batch and fermenter-cultures.

A modified minimal medium syncase (Appendix 2, Finkelstein and Lankford, 1955) was used to grow V. cholerae for the radioactive adherence assays. This medium was modified by changing the carbon source from sucrose to glucose (Appendix 2B).

V. cholerae for use in ileal loop challenges was grown in 2% (w/v) protease peptone (Gibco Biocult Diagnostics).

Starter cultures of V. cholerae used to inoculate these media were grown on BBL trypticase soy agar (TSA) plates (Appendix 1C).

3. Production of V. cholerae proteases

3.1 Batch Cultivation

V. cholerae was grown overnight on TSA plates at 37°C, the growth was harvested by flooding the agar plate with sterile TSB. This cell suspension, 10 ml, was added to 500 ml of sterile TSB in 2 litre dimpled flasks. Six 2 litre flasks were inoculated for each batch and these were shaken on a rotary shaker at 150 rpm for 24 h at 37°C. The cultures were centrifuged at 9,000 rpm (7,000xg) for 20 min at 4°C in an

MSE 21 High-spin centrifuge. The supernatant culture fluids were decanted and ammonium sulphate (BDH Chemicals, Poole, England, specially low in heavy metals) added slowly with stirring at 4°C to a final concentration of 85% saturation. The ammonium sulphate precipitates were stored at 4°C until required. The precipitate was removed by centrifugation at 9,000 rpm (7,000xg) for 15 min at 4°C in an MSE 21 High-spin centrifuge. The supernatant fluid was decanted and the precipitate redissolved in a minimum volume of distilled water, this was extensively dialysed against distilled water for 48 h at 4°C to remove the ammonium salts.

3.2 Fermenter cultivation

V. cholerae was grown overnight in 500 ml TSB and this culture was added to 14.5 litres of sterile TSB in the fermenter vessel (L.H. Engineering Co. Ltd.). The stirrer was started at 500 rpm and the air flow at 10 litre min^{-1}, after 2 h the stirrer and air flow were turned down to 300 rpm and 2 litre min^{-1} respectively. The antifoam used was silcolapse 5000 (Sigma Chemical Co. Ltd.). After 24 h, 1 g of merthiolate (Sigma) in 100 ml sterile distilled water was added and left for 1 h. The suspension of killed V. cholerae was removed from the fermenter vessel under positive air pressure. An attempt was made to precipitate the cells by adding 75 g of calcium chloride but this was not very effective. Therefore, the culture was centrifuged at 9,000 rpm (7,000xg) for 20 min at 4°C in an MSE 21 High-spin centrifuge , the supernatant decanted and ammonium sulphate added slowly with stirring at 4°C to 85% saturation. The precipitate was removed by centrifugation at 9,000 rpm for 15 min at 4°C in an MSE 21 High-spin centrifuge. The

precipitate was redissolved in a minimum volume of distilled water and dialysed for 48 h against distilled water at 4°C. Any precipitate not required immediately was stored at 4°C.

4. Enzyme Assays

4.1 Protease Assays

4.1.1 Dimethylcasein assay

Proteolytic activity was measured by the method of Lin, Means and Feeny (1969) with N,N-dimethylcasein (DMC) as the substrate. This assay was used for all protease measurements unless otherwise stated.

Preparation of substrate

The substrate for the DMC assay was prepared by dissolving 1.5 g of casein (Sigma) in 150 ml of 0.1M borate buffer, pH 9.0 by warming at 37°C and subsequently cooling to 0°C. The slightly cloudy solution was rapidly stirred and 300 mg of sodium borohydride were added. A few drops of 2-octanol (B.D.H. Chemicals Ltd.) were added to prevent any subsequent tendency to foam. Formaldehyde, 3 ml, was added in 100 µl increments over a period of 30 min. Stirring was continued for a further 2-3 min and the solution acidified to pH 6.0 by the addition of 50% acetic acid. This solution was dialysed against distilled water for 24 h at 4°C and the desalted protein was lyophilized and stored at -20°C as a fluffy white powder.

Assay procedure

The *V. cholerae* protease sample 0.1 ml was added to 1.0 ml of a solution of 0.1% (w/v) DMC at pH 9.0 and incubated for 30 min at 38°C. The proteolytic reaction was stopped by heating the mixture briefly (1 min)

in a boiling-water bath. After cooling, 1.0 ml of a solution of 0.1% (w/v) trinitrobenzene sulphonic acid (Sigma) and 1.0 ml of a 4% (w/v) sodium bicarbonate solution, pH 8.5, were added to each mixture. The mixture was incubated in the dark for 30 min at 50°C. Subsequently, 1.0 ml of 10% (w/v) sodium dodecylsulphate solution and 0.5 ml of 1N HCl solution were added. The absorbance of the resultant yellow solution was read at 340 nm in a Unicam SP500 spectrophotometer. To measure cell-associated protease activity, an overnight V. cholerae culture was washed twice in PBS and resuspended in PBS to give an absorption value of 0.4 at 600 nm. This V. cholerae suspension, 0.1 ml, was added to 1.0 ml of 0.1% (w/v) DMC and incubated for 30 min at 37°C. Following incubation the samples were centrifuged to remove the vibrios, the supernatant removed, and the assay continued as explained previously.

Calculation of enzyme activity

The molar extinction coefficient is the absorbance of a 1M solution of the material of interest at the chosen wavelength and has the dimensions litre $mol^{-1}cm^{-1}$. The molar extinction coefficient values of various trinitrophenol α-amino acids have been determined by many workers (Okuyama and Satake, 1960; Satake et al, 1960; Ozols and Strittmatter, 1966). These molar extinction coefficient values vary slightly with different α-amino acids from 1.1×10^4 to 1.5×10^4, but have an average value of 1.3×10^4. By using the value 1.3×10^4, the results from the DMC assay can be expressed in terms of the number of primary amino groups present irrespective of the peptide bond from which they were derived (Lin, Means and Feeny, 1969). This allows the direct expression of proteolytic activity in terms of the number of bonds split.

The molar concentration of cleaved bonds can therefore be calculated from the relationship,

$$NH_2\text{-terminal} = \frac{E_{340\ nm}}{1.3 \times 10^4}$$

The enzyme activity is then calculated as

One enzyme unit (EU) = 1 μmol of free amino groups generated $min^{-1}ml^{-1}$ of protease sample.

4.1.2 Azoalbumin Assay

The azoalbumin protease assay was modified from Tomarelli, Charney and Harding (1949) as used by Finkelstein and Hanne (1982). Azoalbumin (Sigma Chemical Co.) 5 $mgml^{-1}$ in distilled water was the enzyme substrate. This substrate, 100 μl (0.5 mg) was mixed with 50 μl of the cholera sample being tested, and incubated at 37°C. After 1 h, 3 ml of 3% (w/v) trichloroacetic acid was added to precipitate undigested substrate. The mixtures were left at room temperature for 20 min and centrifuged at 900xg for 10 min in an MSE minor bench centrifuge. The supernatant fluid, 2 ml, was decanted and added to 2 ml of 0.5M NaOH. The neutralized samples were agitated briefly and the optical density at 440 nm was determined in a Unicam SP500 spectrophotometer. A standard curve of digested substrate was generated by incubating different concentrations (0.05 mg - 0.5 mg) of the azoalbumin substrate with 1 mg of pronase (Sigma) per ml for 1 h to effect complete digestion. One unit of protease activity by this assay was defined as that which will digest 20% of the substrate (ie. 0.1 mg) in 1 h.

4.1.3 Digestion of casein

Casein plates were prepared by the method of Schneider and Parker (1978). Trisodium citrate (4.41 g) was dissolved in 1 litre of distilled water. Nutrient broth powder (8.0 g, Gibco), 5.0 g tryptone (Difco), 2.5 g yeast extract (Oxoid) and 1.0 g glucose (B.D.H.) were dissolved in 500 ml of the citrate solution. Sodium caseinate, 10.0 g (Difco) was dissolved in the remaining 500 ml. The two solutions were combined, the pH was adjusted to 7.5 with 1N NaOH and 20 g of purified agar (Oxoid) was added. After autoclaving for 15 min at 121 psi the medium was cooled to 50°C, merthiolate was added to a final concentration of 0.001% (w/v) and 1.0 ml of 1M calcium chloride solution was added before the plates were poured. Casein agar (10 ml) was added to each petri-dish and when the medium was solid, walls were cut with a no.2 cork borer. Samples, 15 µl, were added to each well and the plates incubated at 37°C for 18 h. The diameter of the zone of precipitation was measured and this was compared with the zones of precipitation produced by trypsin (Sigma, type III) standards.

4.2 Neuraminidase Assay

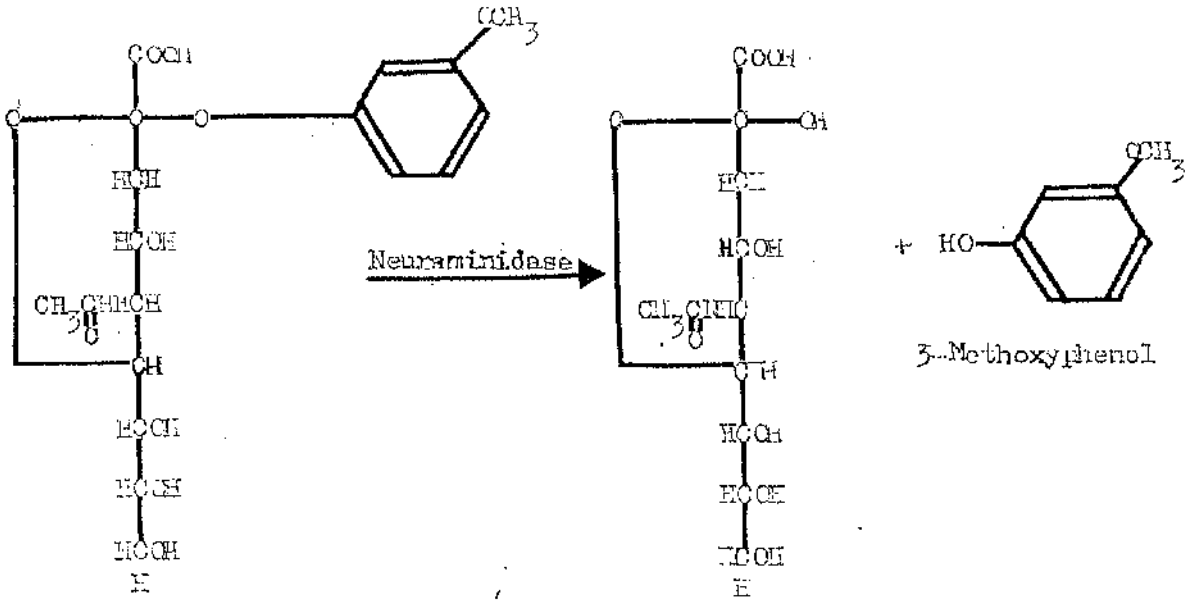
Neuraminidase was measured by the method of Sedmack and Grossberg (1973). Neuraminidase was measured by its hydrolysis of 2-(3methoxyphenol)-N-acetyl-α neuraminic acid (MPN) which liberated 3-methoxyphenol (MP) (Figure 8). MP can combine with the diazonium salt of 4-amino 2, 5-dimethoxy-4-nitroazobenzene (DS.ADNB) to give a water soluble chromogen.

Assay procedure

The test sample (0.1 ml) was warmed to 37°C for 2-3 min and

FIGURE 8

Enzymatic hydrolysis of 2-(3'-methoxyphenyl)-N-acetyl-alpha-neuraminic acid (MPN). The enzyme hydrolyses the ketosidic linkage at C-2 of N-acetyl Neuraminic acid



2-(3'-Methoxyphenyl)-N-acetyl-alpha neuraminic acid

N-acetylneuraminic acid

the reaction started by the addition of 0.1 ml of MPN (10^{-2}M in 0.1M phosphate buffer, pH 5.9). The mixture was incubated at 37°C and after 30 min the reaction was stopped by the addition of 25 µl of 1M tetra-sodium EDTA in 0.1M phosphate buffer, pH 5.9. The samples were immersed in ice and 0.2 ml of 6 $mgml^{-1}$ DS-ADNB (Koch Light, Fast black K salt) in 0.4M phosphate buffer, pH 7.0 was added. The precipitate which formed after 30 min at 0°C was solubilized by the addition of 4 ml of 0.5N NaOH. The absorbance at 580 nm of the resultant blue solutions were read in a Pye-Unicam SP500 spectrophotometer.

Standard curve

A standard curve was obtained to determine the absorption of the solubilized chromogen formed from MP and the DS-ADNB, as a function of the concentration of MP.

Standard solutions of MP from 20-100 n.moles were prepared in 0.1M phosphate buffer, pH 5.9 containing 2×10^{-3}M $CaCl_2$. The standard solutions were added to 0.1 ml of 0.1M phosphate buffer, pH 5.9 and incubated at 37°C. After 30 min the reaction was stopped by the addition of 25 µl of 1M tetra-sodium EDTA. The mixtures were immersed in ice and 0.2 ml of DS-ADNB added. After 30 min at 0°C the precipitates that formed were solubilized in 4 ml of 0.5N NaOH. The absorbance was measured at 580 nm in the SP500 spectrophotometer.

Calculation of specific activity

One enzyme unit (EU) was defined as 1 µmol of methoxyphenyl generated $min^{-1}ml^{-1}$ of sample. This was read from the standard curve.

4.3 Mucinase Assay

Ovomucin was prepared by the method of Kusama and Craig (1970). The whites of 12 eggs were freed of chalazea and poured into 4 litres of distilled water at 4°C. The precipitate formed after 30 min of stirring was centrifuged off (7,000xg, 20 min) and dissolved in 10% NaCl. This was diluted with distilled water to a salt concentration of 0.85%. To the final preparation thiomersal was added to a final concentration of 0.001% (w/v). Serial two-fold dilutions of mucin in PBS in 1 ml amounts, were made and one drop of 1% (w/v) cetyltrimethylammonium bromide was added. The highest dilution which gave a characteristic fibrous clot was considered one clot forming unit.

Two clot forming doses in 0.5 ml were added to equal volumes of serial two-fold dilutions of the protease samples being tested. These mixtures were kept at 37°C for 1 h. One drop of 1% (w/v) cetyltrimethylammonium bromide (Sigma) was added to each sample to detect undigested mucin. The reciprocal of the highest dilution of the protease sample which prevented clot formation was taken as the number of units in the sample. All samples tested were standardized to 15 EU ml^{-1} of protease activity before use in the assay.

5. Other Assays

5.1 Haemagglutination Assay

The samples being tested were diluted in serial two-fold dilutions in round-bottomed microtitre plates in 25 µl of PBS. Red blood cells (rbc), either sheep or chicken, (1.5%) were added in 25µl, the plates were tapped to mix interactants, and rbcs were allowed to

settle at 25°C for 30 min. The titre was defined as the reciprocal of the highest dilution in which haemagglutination was visible to the naked eye. For determination of cell-associated haemagglutination, vibrios were washed twice and suspended in PBS to give an absorption value of 0.4 at 600 nm and the assay continued as described.

5.2 Limulus Amoebocyte Lysate Assay

The Limulus amoebocyte lysate (LAL) was used to rapidly detect Gram-negative bacterial endotoxin. Single test vials were supplied by M.A. Bioproducts, Maryland.

These single test vials contain a lysate prepared from the circulating amoebocytes of the horse shoe crab Limulus polyphemus, standardized to detect 0.25 $ngml^{-1}$ of reference endotoxin.

All glassware was treated with Toxa-clean (Sigma) to remove any pyrogen by soaking overnight in a 1% (w/v) solution and drying in an oven. Pyrogen-free water (Sigma) was used for sample dilutions and strict aseptic techniques were employed throughout the assay.

The test sample (200 µl) was added to a vial of lysate, mixed gently until the contents were in solution and incubated at 37°C for 1 h. 200 µl of pyrogen-free water was added to another vial of lysate as a negative control. Positive controls were set up with E. coli endotoxin, 10 $ngml^{-1}$ and 1 $mgml^{-1}$ (Sigma). Reading of the test after exactly 1 h incubation was done by gently everting the vials on a flat surface. A positive test is characterized by the formation of a solid gel, a negative test shows no gel or clot but may show an increase in turbidity. Inhibition control vials were supplied. These contain the lysate as in the test vials but also 1 $ngml^{-1}$ of E. coli endotoxin. At the end of the incubation period a positive reaction should be observed with all

samples in the inhibition control. If the reaction is negative the sample is inhibitory and any associated LAL test with this sample is invalid. Table 5 shows the possible combination of test results.

5.3 Toxin Assay - Intradermal Blueing Test in Rabbits

The animal injections in this assay were done by Dr. D.E.S. Stewart-Tull.

New Zealand white rabbits were injected with 0.1 ml of test sample intradermally, into an area of skin shaven with Ostler Animal Grooming Clippers (Model A-5). After 18-24 h, 3 ml of a 5% (w/v) solution of Pontamine Sky Blue 6XB (Difco) were injected intravenously into an ear vein. One hour later the diameter of the zones of blueing on the skin were measured. The toxin dose which produced a blueing zone of 8 mm in diameter was equivalent to one blueing dose (1 BD).

5.4 Protein Estimations

(i) The method of Warburg and Christian (1941) was used to estimate the protein concentration in samples. The extinction of the samples at 260 nm and 280 nm was measured on a Pye-Unicam SP500 spectrophotometer. The ratio of E_{280}/E_{260} was calculated and from this a factor is obtained for the calculation of protein concentration.

The protein concentration is given by:

$$\text{Protein concentration } (\text{mgml}^{-1}) = E_{280} \times \text{Factor} \times {}^{1}/_{d},$$

where d = length of the light path in cm.

(ii) The method as described by Lowry *et al* (1951) was used for protein estimations with bovine serum albumin (Sigma) as the standard protein.

TABLE 5

POSSIBLE COMBINATIONS OF TEST RESULTS FROM

LIMULUS AMOEBOCYTE LYSATE ASSAY

Test Sample		Test Results (Gel Formation)				
Endotoxin	Inhibitor	Test Vial	Neg. Control	Pos. Control	Inhib. Control	Acceptable Test
+	-	+	-	+	+	yes
-	-	-	-	+	+	yes
-	+	-	-	+	-	no (inhibitor present)
+	+	-	-	+	-	no (inhibitor present)
+	+	+	-	+	-*	no (inhibitor present)

\+ = gram negative endotoxin present

\- = gram negative endotoxin absent

*With high concentrations of endotoxin and an inhibitor present in test sample, this combination may occur.

6. Purification of Proteases

6.1 Ammonium sulphate precipitation

The precipitation was carried out as described in section 3. The ammonium sulphate precipitates were centrifuged at 9,000 rpm (7,000xg) for 15 min at 4°C in an MSE 21 High Spin centrifuge. The precipitate was dissolved in a minimal amount of distilled water and dialysed for 48 h at 4°C with several changes of distilled water. The dialysed sample was concentrated to about a quarter of its original volume using Aquacide IIA (Calbiochem, La Jolla, C.A.). This was termed "crude protease", and stored at -20°C until required.

6.2 Gel Filtration

The column (2.5 cm x 55 cm) was filled with a slurry of Sephadex G100 (Sigma) and was equilibrated with borate buffer (0.05M, pH 9.0) by running several litres of this buffer through the gel. The crude protease (approximately 10 mg in 5 ml borate buffer) was applied to the column and was eluted with borate buffer (500 ml, 0.05M, pH 9.0) at a rate of 1 $mlmin^{-1}$. Fractions (3.0 ml) were collected on an LKB Ultrorac 7000 fraction collector and their absorbance at 280 nm was measured in a Unicam SP500 spectrophotometer.

Molecular Weight estimation from gel filtration

The Sephadex G100 column was equilibrated with borate buffer and a sample containing 10 mg of each of the following standards in 5 ml borate buffer were added to the column:

Cytochrome c, 12.4 k.Dal. (Sigma)

Chymotrypsinogen A, 25 k.Dal. (Sigma)

Ovalbumin 45 k.Dal. (Sigma)

Bovine serum albumin 67 k.Dal. (Sigma).

The column was eluted with borate buffer (500 ml) at a rate of 1 $mlmin^{-1}$. Fractions (3.0 ml) were collected and their absorbance at 280 nm measured as before.

A graph was drawn of eluate volume against $E_{280\ nm}$ readings and from this a graph of eluate volume (V_e) against log molecular weight of the standard proteins was drawn. From this second graph the molecular weight of the protease sample was determined.

6.3 Isoelectric Focussing

Crude protease was dialysed against 1% (w/v) glycine for 24 h before electrofocussing. Isoelectric focussing was carried out in a step gradient of 0 to 50% (w/v) sucrose containing Ampholine buffers at pH 3.5-10.0 (LKB Instruments Inc., Rockville) in a 110 ml LKB electrofocussing column (Vesterberg, 1971). The voltage across the column (anode at the bottom) was gradually increased to 800V (maximum power, 2.0W), and the column was kept at 0 to 4°C by circulating ice-cold water through the cooling jacket. After 24 h the power supply was switched off, and fractions (2.0 ml) were collected from the bottom of the column using a Hiloflow peristaltic pump (F.A. Hughest and Co., Surrey).

The carrier ampholines were separated from the protein samples in each fraction by prolonged dialysis (7 days) against borate buffer (0.05M, pH 9.0). The fractions were assayed for protein, protease, neuraminidase, mucinase and haemagglutinating activity. The reagents used for isoelectric focussing are shown in Appendix 4.

7. Polyacrylamide gel electrophoresis (PAGE)

7.1 Sodium dodecyl sulphate (SDS)-PAGE

The method used was based on that of Laemmli (1970) as modified by Ames (1974). Electrophoresis was done in the presence of SDS. Separating and stacking gels contained respectively 11% (w/v) and 5% (w/v) acrylamide (Appendix 5).

The test sample was prepared by mixing an equal volume with one volume of solubilizing buffer (Appendix 5) and heating to 100°C for 5 min. Samples of 75 µl were added to each track in the gel. Gels were run in 1/10 dilution of running buffer at a constant current of 15 mA for approximately 3 h in a Shandon analytical PAGE outfit (Shandon Southern Instruments Ltd., Surrey, England). When the tracking dye front had reached the bottom of the resolving gel the gels were stained for 1 h and destained. The buffers, gels, stain and destain are described in Appendix 5. Molecular weight markers, BSA, ovalbumin, chymotrypsinogen, trypsin and cytochrome c (Sigma) were used. The distance of migration of these standards against log molecular weight was plotted to produce a standard curve for the molecular weight estimation of other peptides.

7.2 Gelatin-PAGE

The method used was a modified method of Heussen and Dowdle (1980). The separating gel contained 1% (w/v) gelatin. The separating and stacking gels contained 11% (w/v) and 3% (w/v) acrylamide respectively. Electrophoresis was done in the presence of SDS. The buffers and gels are described in Appendix 6.

The samples were added to the sample wells in a final volume of 75 µl of solution containing 2.5% (w/v) SDS, 1% (w/v) sucrose and 4 $\mu g ml^{-1}$ phenol red. Electrophoresis was performed at a constant current of 8mA for approximately 3.5 h in a Shandon analytical PAGE outfit (Shandon Southern Instruments Ltd., Surrey, England). When the tracking dye front had reached the bottom of the resolving gel the gel was removed and shaken gently at room temperature for 1 h in 2.5% (v/v) Triton X-100 to remove SDS. The gel slabs were transferred to a bath containing 0.1M glycine-NaOH, pH 8.9 and incubated at 37°C for 5 h. The gels were fixed and stained for 1 h and destained (Appendix 6).

Since microbial contamination of electrophoretic buffers and other solutions may have added some contaminatory bacterial proteases all solutions were autoclaved or sterilized by filtration.

7.3 PAGE gel with gelatin overlay

A PAGE gel was run with no SDS present and a gelatin overlay or Zymogram technique was used to detect the protease bands (Foissey, 1974).

The gels and buffers were prepared as in Appendix 7, the gels were run in a horizontal system using a 20 cm x 20 cm gel. The samples were all standardized to have approximately 10 $EUml^{-1}$ of protease activity. Each sample (50 µl) was added to the wells and the gels were run at 150V in an electrophoresis tank till the tracking dye had reached 1 cm from the end of the gel.

The gel was sliced through the horizontal plane with a wire suture; one half was stained in 0.1% (w/v) Amido black for 1 h and destained in 7% acetic acid. The other half was placed on top of a

gelatin agar plate and left at 37°C for 18 h. The PAGE gel was removed and the gelatin plate flooded with a mercuric chloride solution (Appendix 7). This caused precipitation of the gelatin and clear bands showed where the gelatin was hydrolyzed by the proteases.

8. Inhibition Studies

The inhibitors used are shown in Table 6.

The inhibitor (50 µl) was incubated with 50 µl of protease (approximately 5 $EUml^{-1}$) at 37°C for 30 min. To this mixture 1.0 ml of DMC was added and the DMC protease assay carried out as described previously (see page 66). For each inhibitor a blank was set up consisting of 50 µl of inhibitor plus 50 µl borate buffer (0.05M, pH 9.0).

In a few cases the inhibitor interfered with the DMC protease assay and so the casein plate method (see page 69) for measuring protease activity was used. In these cases equal volumes of inhibitor and protease were incubated as before but it was necessary to use approximately 10 $EUml^{-1}$ of protease to detect a clear zone of precipitation.

The samples (15 µl) were added to the wells in the casein plates and incubated at 37°C for 18 h. The diameter of the zone of precipitation was measured as mentioned previously.

9. Peptide Digest Analysis

Peptide digest analysis was carried out by the method of Schneider, Sigel and Parker (1981).

The protein digestion substrate solution consisted of 4 mg of

TABLE 6

PROTEASE INHIBITORS

INHIBITOR	SOURCE
Soyabean Trypsin Inhibitor	Sigma
Eggwhite Trypsin Inhibitor	"
Anti-Trypsin	"
Pepstatin	"
2-nitro,4-carboxyphenyl NN-phenylcarbonate (NCDC)	"
Ethylenediaminetetraacetic acid (EDTA)	BDH Chemicals Ltd.
Mercuric Chloride ($HgCl_2$)	"
Sodium Fluoride (NaF)	"
Phenylmethylsulphonyl fluoride (PMSF)	Sigma
N'ethylmaleimide	"
Sodium Iodoacetate	BDH Chemicals Ltd.
8-Hydroxyquinoline *	Sigma
α-2 Macroglobulin *	"

*Plate assay used as these inhibitors interfered with DMC assay.

bovine serum albumin (Sigma) per ml dissolved in a solution of 20% (w/v) glycerol, 0.5% (w/v) SDS, 0.1% (w/v) bromophenol blue (Sigma) in 0.05M borate buffer, pH 9.0. This digestion solution was heated for 5 min at 100°C and stored at -20°C. The solution was warmed to 37°C before the addition of the enzyme preparation.

The enzyme preparation was mixed with equal volumes of the digestion solution and incubated at 37°C for either 30 min or 2 h. After incubation, the enzyme was inactivated by boiling for 5 min after the addition of 2-mercaptoethanol and SDS to a final concentration of 10 and 1%, respectively. These enzyme-treated stocks were stored at -20°C until use.

SDS-PAGE of these digests was carried out as described previously.

10. IgA Digestion

Human IgA (Sigma) 5 $mgml^{-1}$ was incubated with an equal volume of approximately 10 $EUml^{-1}$ of V. cholerae G100/P. These mixtures were incubated at 37°C for 1, 9 and 24 h at 37°C. The digestion was stopped by freezing the mixtures in liquid nitrogen and the samples stored at -20°C. The digests were examined by SDS-PAGE (see page 78) and immunoelectrophoresis (see page 84).

11. Fibronectin Digestion

Fibronectin (Sigma) 1 $mgml^{-1}$ was incubated with an equal volume of V. cholerae, G100/P (approximately 10 $EUml^{-1}$) for 1, 9 and 24 h at 37°C. The digestion was stopped by freezing in liquid nitrogen. SDS-PAGE (see page 78) was used to examine these digests.

12. Lactoferrin Digestion

V. cholerae G100/P (approximately 10 $EUml^{-1}$) was incubated with an equal volume of lactoferrin (Sigma), 1 $mgml^{-1}$ for 1, 9 and 24 h at 37°C. The digestion was stopped and the digests examined by SDS-PAGE, (see page 78).

13. Digestion of cholera enterotoxin A-subunit

The A-subunit (1 $mgml^{-1}$) of cholera enterotoxin (Sigma) was incubated with an equal volume of G100/P (10 $EUml^{-1}$) for 1, 9 and 24 h at 37°C. The digestion was stopped and SDS-PAGE (see page 78) used to examine the mixtures.

14. Immunological Techniques

14.1 Ouchterlony double diffusion

Ouchterlony plates were prepared by dissolving 15 g purified agar (Oxoid) and 16 g NaCl in 1 litre of distilled water by steaming for 45 min. Methyl orange (0.12 g) and 50 ml of 10% phenol saline solution were added. The molten agar (20 ml) was poured into scratch-free glass plates, allowed to set and dried at 37°C. A template was used to cut wells in the agar by the method of King (1957). A drop of molten medium was added to each well to prevent leakage of reagents under the agar layer. Antigen and antiserum were added to the wells and the plate incubated in a moist atmosphere at 37°C for 18 h. The appearance of a precipitin line between the wells showed the presence of antibody in the serum specific to the antigen being tested.

14.2 Immunoelectrophoresis

Barbitone agar (100 ml; Appendix 8) was poured over a glass plate (20 x 10 cm) and allowed to set. Troughs and wells were cut with a No. 2 cork borer, according to the template shown in Appendix 9. The agar wells were removed and filled with the samples. Electrophoresis was carried out in a Shandon electrophoresis tank at 150V for approximately 2 h using 0.05M barbitone buffer, pH 8.4. Following electrophoresis the agar in the troughs was removed and the troughs were filled with antiserum. The plate was left in a damp chamber for 24-48 h to allow precipitin lines to develop. The plate was washed in saline for 48 h, stained in 1% (w/v) Amido Black for 1 h and destained in 7% (v/v) acetic acid.

14.3 Antiserum production

Antisera were produced in collaboration with Dr. D.E.S. Stewart-Tull who injected and bled the animals. New Zealand white rabbits were vaccinated intramuscularly (i/m) with 2 ml of Freunds complete adjuvant containing 1.0 ml Bayol F, 0.4 ml Arlacel A, 1.0 ml antigen (containing 100 µg protein) and 250 µg freeze-dried Mycobacterium tuberculosis (Appendix 10). Two weeks later the rabbits were given an intraperitoneal (i/p) booster injection of Freunds incomplete adjuvant (2 ml) containing 1 ml (100 µg) of the same antigen, 1.0 ml Bayol F and 0.4 ml Arlacel A (Appendix 10). Ten days later blood samples were obtained from the rabbit's ear. The blood was allowed to clot and left at 4°C overnight. The serum was removed and tested for antibody by the Ouchterlony technique. If antibody was present the animal was exsanguinated by cardiac puncture. The clotted blood was left at 4°C overnight before the serum was removed. Any residual erythrocytes were

removed from the serum by centrifugation at 900xg for 10 min on an MSE bench centrifuge. The sera were stored at -20°C until required.

15. Radio-active adherence assay

15.1 Radio-labelling of V. cholerae

V. cholerae was grown overnight at 37°C with shaking in the modified Syncase medium. The cells were centrifuged at 900xg for 20 min in an MSE minor bench centrifuge and the supernatant fluid was discarded. The cells were resuspended in the Syncase medium but without the carbon source. The cells were resuspended to give an optical density of 0.3 at 600 nm and 2 $\mu Ci ml^{-1}$ of ^{14}C-glucose (Amersham International, Buckinghamshire, England) was added. The culture was shaken for a further 5 h at 37°C. Subsequently, the cells were washed three times in 0.1% (w/v) peptone - 0.85% NaCl and finally resuspended in the peptone-NaCl to give an optical density of approximately 0.3 at 600 nm. By a Miles and Misra viable count technique, a culture with an optical density of 0.35 was found to have 1.2×10^8 organisms ml^{-1}.

15.2 Preparation of tissue segments

The tissue used throughout these experiments was the ileum from an adult Wistar rat. The rats were starved of solid food overnight, killed under CO_2 gas and the ileum removed and placed in 10 ml of physiological saline. Tissue segments were prepared by one of the following methods (Figure 9).

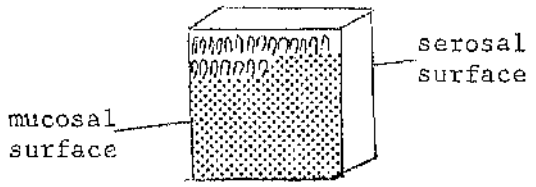
Type A - One square centimetre of tissue

The ileum was placed on filter paper and opened longitudinally.

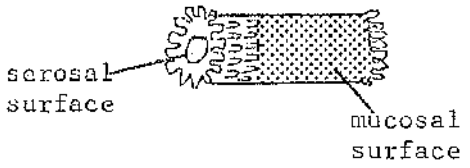
FIGURE 9

Types of tissue preparation

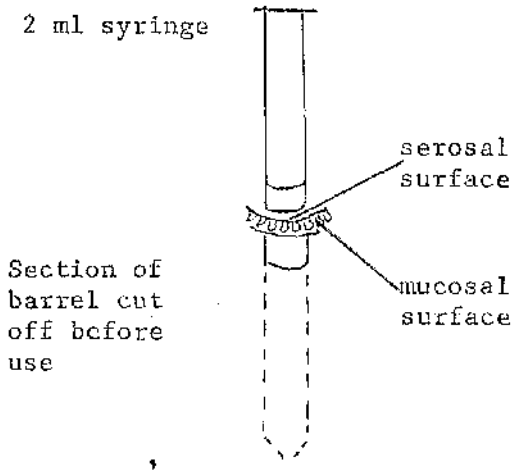
TYPE A. One square centimetre



TYPE B. One centimetre cylindrical lengths



TYPE C. Tissue segment mounted on specially prepared syringe



Segments (1 cm^2) or discs (20-113 cm^2) were cut using a scalpel or different sized cork borers respectively.

Type B - One centimetre cylinders of tissue

1 cm lengths of intact ileum were cut and everted to ensure that the brush border surface was exposed to the V. cholerae organisms.

Type C - Mucosal disc of tissue

The ileum was opened longitudinally and 1.5 cm lengths cut. A specially prepared 2 ml syringe was used (Figure 9) to mount the tissue segment which ensured that only either the mucosal or serosal surface of the ileum was in contact with the V. cholerae organisms.

15.3 Assay Procedure

The tissue segments were pretreated for 30 min at 37°C with either saline, as a control, or the enzyme preparation being tested. The tissue segments were added to 2 ml of labelled and standardized organisms. After 30 min at 37°C the tissue segments were removed, washed three times in individual 2 ml aliquots of 0.1% (w/v) peptone - 0.85% (w/v) NaCl and placed in a scintillation vial for digestion (Mahin and Lofberg, 1966). The digestion was carried out by the addition of 0.25 ml of 70% (v/v) perchloric acid and 0.5 ml of 30% (v/v) hydrogen peroxide. The vials were sealed and placed in an 80°C water bath for 1-2 h depending on the size of the tissue segment. After cooling 4.5 ml of scintillation fluid (Appendix 11) was added and the samples were counted in a Packard Tri Carb 300C scintillation counter.

The radioactivity present in the three 2 ml washes for each tissue segment and in the residual, 2 ml of the suspension of organisms originally added to the tissue segment were also measured. This was

done by adding 0.5 ml of each of these samples to 4.5 ml of scintillation fluid and counting the radioactivity as previously described. To obtain the total counts in the 2 ml washes and the 2 ml of organisms the counts obtained for the 0.5 ml that were counted were multiplied by four to give the radioactivity present in the 2.0 ml samples.

15.4 Calculation of the number of organisms attached to the tissue segment

An example of a worked calculation is shown in Figure 10. The counts of A, B, C, D and E were added together to give the total count which is equivalent to the number of organisms in the original 2 ml sample of *V. cholerae* added to the tissue segment. In the example shown in Figure 10, 30×10^{10} organisms were present in the 2 ml sample, which gave a total count of 299,659 c.p.m. The c.p.m. in the tissue segment were 13,187 which was equivalent to the number of organisms bound to the tissue segment. Therefore the number of organisms associated with the tissue segment can be calculated by

No. of *V. cholerae* associated with tissue segment =

$$\frac{13{,}187 \times 30}{299{,}659} \times 10^{10} = \frac{1.32 \times 10^{10}}{}.$$

This method was used for the calculation of all adherence assay results throughout this study.

15.5 Paraformaldehyde treatment of tissue segments

Paraformaldehyde vapour fixation (Tock and Pearce, 1965) was used to retain the mucus on the surface of the tissue segments prior to the assay procedure.

FIGURE 10

TYPICAL EXAMPLE OF RESULTS OBTAINED FROM

RADIOACTIVE ADHERENCE ASSAY

A.	Remaining activity of 2 ml *V. cholerae* culture after incubation with tissue segment	273656 c.p.m.
B.	Activity in wash 1	7488 cp.m.
C.	Activity in wash 2	3400 c.p.m.
D.	Activity in wash 3	1928 c.p.m.
E.	Activity associated with tissue segment (Number of *V. cholerae* bound to tissue segment)	13187 c.p.m.
	By adding A, B, C, D and E obtain total count in the original 2 ml *V. cholerae* sample added to tissue segment.	299,659
	Total activity in a 2 ml suspension of organisms alone - control suspension.	298,867

Paraformaldehyde, 3 g (BDH Chemicals Ltd., Poole) was placed in an evaporating vessel in a desiccator. This was heated to 50°C for 1 h and the desiccator opened carefully in a fume cupboard and the tissue segments added. The desiccator with the tissue segments was left at 50°C for 1 h, the tissue segments were used immediately.

16. Toxin Binding to Ileal Segments in vitro

16.1 Toxin

Cholera toxin, 2 mg (N.I.A.I.D. Cholera Advisory Committee, Wyeth Laboratories) was added to 2 ml of sodium acetate buffer. This solution contained 300 $BDml^{-1}$ as 2 mg of toxin is equivalent to 600 BD. Dilutions were prepared to give 600, 400, 200, 100 and 20 B.D. 2.0 ml^{-1} in sodium acetate buffer, pH 5.5.

16.2 Tissue preparation

The method of tissue preparation was identical to type C preparation used in the radioactive adherence assay which allowed the use of the mucosal surface alone, (see page 86).

16.3 Assay procedure

The tissue segments were incubated for 30 min with 2 ml PBS or the enzyme preparation at 37°C. The tissue segments were added to 2 ml of the toxin solutions for 1 h at 37°C. The tissue segments were removed from the toxin solution and the tissue was discarded as bound toxin cannot be detected. The toxin remaining was measured by the intradermal blueing test in rabbits as described previously (see page 74). Since the rabbit was injected with 0.1 ml of the toxin solutions the

expected BDs in the rabbit were 30, 20, 10 5 and 1.0 BD if no toxin had bound to the tissue segment. The values of BD obtained from the rabbits' back were multiplied by 20 to measure the remaining toxin in the 2 ml sample. Therefore the amount of toxin bound to the tissue segment was calculated by subtracting the BD of cholera toxin in the remaining solution from the expected BD, ie. 600, 400, 200, 100 or 20.

17. Toxin activation

Cholera toxin (N.I.A.I.D., Cholera Advisory Committee, Wyeth Laboratories) 2 $mgml^{-1}$ was used, this solution contained 600 $BDml^{-1}$ as 2 mg of toxin is equivalent to 600 BD.

An equal volume of the toxin solution was incubated with protease (G100/P), with an activity of 10.5 $EUml^{-1}$ for either 5 or 24 h at 37°C. An equal volume of distilled water and toxin was also incubated for 24 h at 37°C, as a control sample.

Following incubation the toxin solutions were diluted in distilled water to give 30, 20, 10, 5.0 and 1.0 $BDml^{-1}$. The intradermal blueing test (see page 74) was used to measure the toxin activity. Since the rabbit was injected with 0.1 ml of the toxin solutions the expected BDs in the rabbit were 3.0, 2.0, 1.0, 0.5 and 0.1 BD. Any increase in toxin activity would be seen by an increase in the blueing dose of the 0.5 and 0.1 BD samples.

18. Protection Studies

18.1 Animals

Dunkin Hartley guinea-pigs were used for all protection experiments.

18.2. Vaccine components

(i) Protease - G100/P prepared during this study.

(ii) Neuraminidase - Calbiochem, La Jolla, California.

(iii) Enterotoxoid - gluteraldehyde treatment of toxin from N.I.A.I.D. Cholera Advisory Committe, Wyeth Laboratories.

18.3 Vaccinations

All vaccinations were given in Freunds complete adjuvant (F.C.A.), Freunds incomplete adjuvant (F.I.A.) (Freund and Bonanto, 1944) or aluminium hydroxide gel ($Al(OH)_3$). The composition of these adjuvants is shown in Appendix 10. This work was done in collaboration with Dr. D.E.S. Stewart-Tull who carried out all experiments on living animals.

For each vaccine preparation 12 guinea-pigs were used; 3 were vaccinated with F.C.A., 3 with F.I.A., 3 with $Al(OH)_3$. Three guinea-pigs were unvaccinated controls.

(i) Originally experiments were done by giving a group of guinea-pigs (D.S-T 1287-1345) one 0.5 ml i/m vaccination followed three weeks later by ileal loop challenge using approximately 1×10^5 virulent V. cholerae.

(ii) Further experiments involved giving guinea-pigs (D.S-T 1346-1454) a 0.5 ml i/m vaccination followed three weeks later with a 0.5 ml i/m booster dose. Two weeks later the guinea-pigs were challenged.

(iii) Subsequently, guinea-pigs (D.S-T 1455-1477) were given a 0.5 ml i/m vaccination followed three weeks later by an oral boost. The 0.3 ml oral dose of each vaccine was administered to each guinea-pig using a 1.0 ml syringe, without a needle, as an applicator and releasing the

mixture at the back of the mouth. In this instance the guinea-pigs vaccinated i/m with F.C.A. adjuvant were given an oral booster with F.I.A. and not F.C.A. The guinea-pigs vaccinated with F.I.A. and $Al(OH)_3$ were boosted with F.I.A. and $Al(OH)_3$ respectively.

18.4 Ileal loop test

The ileal loop operations were done by Dr. D.E.S. Stewart-Tull.

18.4.1 Preparation of *V. cholerae* for challenge

V. cholerae was grown overnight in 2% (w/v) protease peptone at 37°C without shaking. 5 ml of this culture was added to 20 ml of sterile 2% (w/v) protease peptone and left for 5 h at 37°C without shaking. This culture was used for the ileal loop challenge. The control injection was done with 2% (w/v) protease peptone.

18.4.2 Operation procedure

Guinea-pigs were starved of solid food for 24 h as a preoperative measure. The animals were anaesthetised by an injection of 0.15 ml Valium 20 (Rothe Products Ltd., England) and 0.3 ml of small animal Immobilon (Reckitt & Colman, Pharmaceutical Divn., Hull, England). A length of small intestine above the caecum was lifted out of the peritoneal cavity. The intestine was ligated with cotton approximately 2 cm and 9 cm above the caecum. A third ligature, 7.0 cm above the caecum, was tied loosely, resulting in a 5.0 cm long test loop and a 2 cm interloop. To prevent leakage from the test loop the sample was injected by inserting the hypodermic needle into the 2.0 cm interloop and through the loose ligature into the test loop. The ligature was tightened onto the needle before injection of the sample, and finally tied off after removal of the needle.

Two 5 cm loops, separated by 2.0 cm interloops, were prepared in each animal. One loop was injected with 0.1 ml of the cholera culture, the other with 0.1 ml of sterile 2% (w/v) proteose peptone.

The intestine was replaced in the peritoneal cavity and the wound was stitched with monofilament nylon (metric 2; Ref. No. V4022; Arnolds Veterinary Products Ltd., Reading) and the animal allowed to recover from the anaesthetic. After 16-18 h the animal was killed and the ileal loops were removed. Blood was taken from each animal by cardiac puncture and antiserum collected as described previously.

18.4.3 Measurement of ileal loops

Measurement of the length of the loops (cm) were made using calipers (Gamlab, Cambridge, type 6921). The accumulated fluid in each loop was measured by lowering the loop into a measuring cylinder containing dilute Hibitane and noting the volume of fluid displaced (ml). The fluid accumulation in the loop was expressed as

$$\text{ml of fluid displaced (cm length of loop)}^{-1}.$$

18.4.4 Analysis of ileal loop test results

In each guinea-pig the mlcm^{-1} of the protease peptone loop was subtracted from the mlcm^{-1} of the *V. cholerae* loop to give a value of fluid accumulation produced solely from the *V. cholerae* organisms. These values for the vaccinated and unvaccinated guinea-pigs were compared. A 1-tail t-test was used since it was unreasonable to expect a *V. cholerae* culture to produce less fluid accumulation than the culture medium alone.

An Olivetti model 101 computer was programmed for t-test analysis and the t-value for each group of 3 guinea-pigs compared to the

3 unvaccinated control guinea-pigs was obtained. The P-values were obtained from statistical tables. The results of these values were expressed as

+++ significant difference at P = 0.1% level

++ " " " P = 1% level

\+ " " " P = 5% level

+--- no significant difference detected.

RESULTS

RESULTS

1. Production of protease

Initial experiments were done to find the best growth conditions for the extracellular protease production by *V. cholerae*.

1.1 Variation in protease production in different media

V. cholerae was grown at 37°C for 24 h in various growth media. After 24 h, 1.0 ml samples of the cultures were removed, the cells centrifuged and the protease activity in the supernatant fluid measured by the DMC assay. Samples, 1.0 ml, were evaporated to dryness in an 80°C drying oven and the dry weight of the cells was measured. The extracellular protease activity in each of the different media was expressed as $EUml^{-1}mg^{-1}$ dry weight of *V. cholerae* cells (Table 7). Trypticase soy broth (BBL) yielded the best protease production, 0.73 $EUmg^{-1}$ compared with the poorest yield of 0.22 $EUmg^{-1}$ with the tryptic soy broth (Gibco). Consequently the trypticase soy broth (BBL) was used as the growth medium for the production of *V. cholerae* protease.

1.2 Variation in protease production during the growth of *V. cholerae*

The *V. cholerae* culture was grown aerobically at 37°C and 3.0 ml samples taken at various time intervals. The growth of *V. cholerae* was measured by the optical density of the culture at 600 nm. The samples were centrifuged and the protease activity in the supernatant fluids was measured by the DMC assay. Maximum protease activity was detected in samples taken between 23-25 h (Figure 11). Consequently, *V. cholerae* cultures were grown for 24 h for the production of cholera proteases.

TABLE 7

COMPARISON OF GROWTH MEDIA FOR EXTRACELLULAR PROTEASE PRODUCTION BY *V. cholerae*

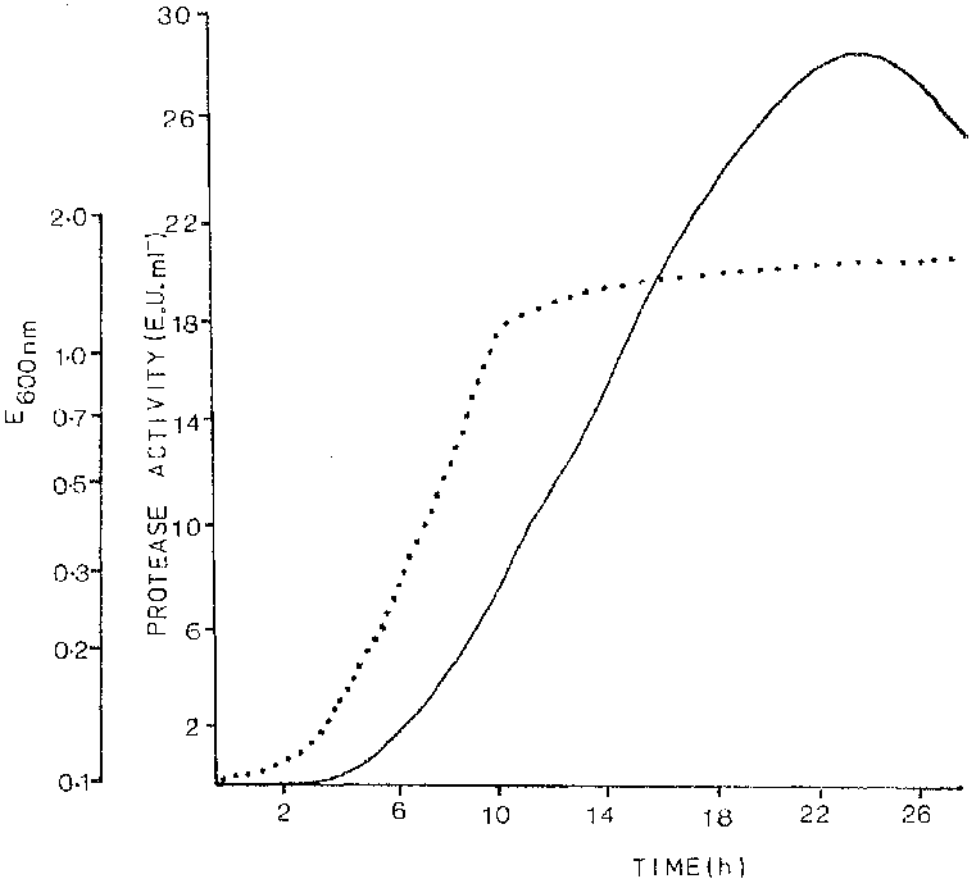
MEDIUM	PROTEASE ACTIVITY ($E.U.ml^{-1}$) per mg DRY WEIGHT OF *V. cholerae* CELLS
Trypticase Soy Broth (BBL)	0.73 $\pm$ 0.15
Tryptic Soy Broth (Gibco)	0.22 $\pm$ 0.06
2% (w/v) protease peptone (Difco)	0.56 $\pm$ 0.08
Syncase (Appendix 2)	0.52 $\pm$ 0.05

The above results are the mean values from three experiments.

$\pm$ = standard deviation.

FIGURE 11

Extracellular protease activity detected in the culture fluid during growth of V. cholerae



2. Stability and Storage

The culture filtrate of a 24 h culture of V. cholerae was precipitated with ammonium sulphate to 85% (w/v) saturation. The precipitate was stored at 4°C and in this form retained at least 95% of its protease activity after three months. This precipitate once redissolved and dialysed was termed "crude protease". The stability of this "crude protease" at 37, 26, 4, -20 and -70°C was tested over a four week period (Table 8). The crude protease was unstable at 37°C and 26°C as after one day only 56% and 59% of the original protease activity remained. Storage at 37°C for 7 days resulted in only 2% of the original protease activity remaining and at 26°C only 10% of the protease activity remained. When the crude protease was stored at 4°C it lost nearly 50% of its activity after 28 days, although shorter storage time (1 day) did not result in loss of much activity. However, following storage for 28 days at -20°C only 6% of the protease activity was lost and storage at -70°C for 28 days resulted in the loss of only 5% of the protease activity. Subsequently as storage at -70°C had no greater effect than storage at -20°C, for convenience all protease samples were stored at -20°C.

3. pH optimum for protease activity

The protease activity of the crude protease was measured over a pH range of 1-12. The activity of the protease was measured by the DMC assay. Maximum activity was found at pH 8.9-9.0 (Figure 12). Unfortunately dimethyl casein (DMC) was not soluble in the pH range of 3.5-5.0 so the protease activity could not be measured at this pH. To try to overcome this problem the azoalbumin assay was used but this

TABLE 8

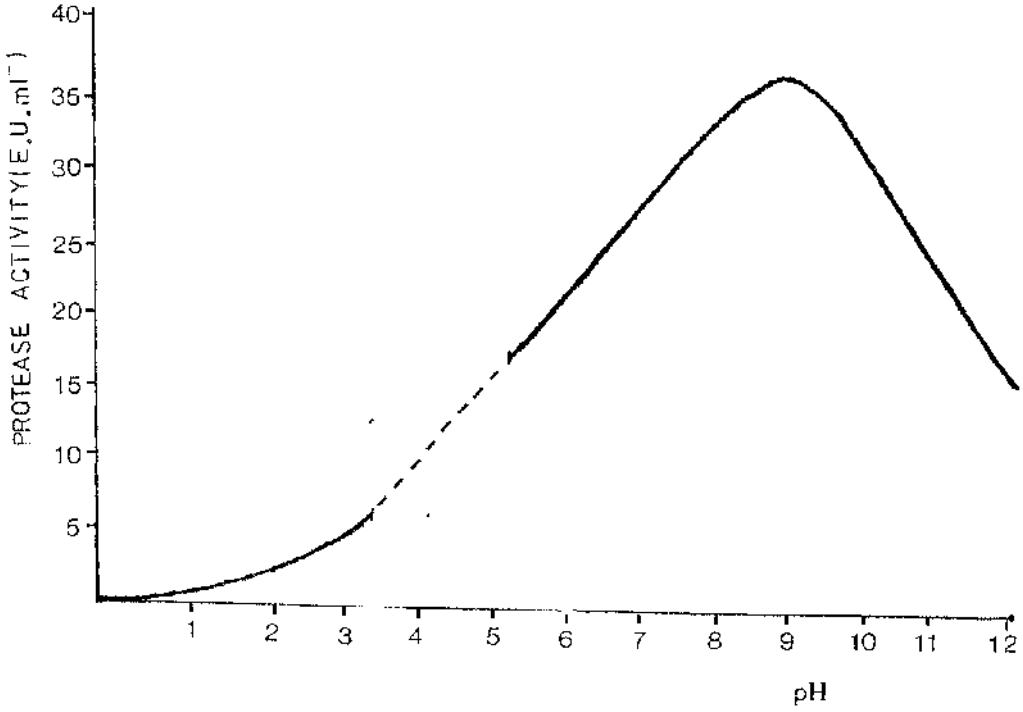
EFFECT OF TEMPERATURE AND TIME OF STORAGE ON PROTEASE STABILITY

TEMPERATURE (°C)	TIME (Days)	PROTEASE ACTIVITY ($EUml^{-1}$) BEFORE STORAGE	AFTER STORAGE	PERCENTAGE PROTEASE ACTIVITY REMAINING
37	1	9.5	5.3	56
	7	9.8	0.2	2
	28	10.1	0	0
26	1	9.5	5.6	59
	7	9.8	0.9	10
	28	10.1	0	0
4	1	9.5	9.3	98
	7	9.8	8.3	85
	28	10.1	5.5	55
-20	1	9.5	9.4	99
	7	9.8	9.3	95
	28	10.1	9.5	94
-70	1	9.5	9.4	99
	7	9.8	9.3	95
	28	10.1	9.6	95

These results are the average of one experiment done in duplicate. This experiment has been repeated another twice and similar results obtained.

FIGURE 12

pH activity profile of *V. cholerae* crude protease



– – – – – DMC not soluble

assay was also found to be inactive at pH 3.5-5.0. However, the results from the azoalbumin assay paralleled those of the DMC assay showing maximum protease activity at pH 9.0 $\pm$ 0.1.

4. Partial Purification of the Protease(s)

4.1 Gel filtration of crude protease

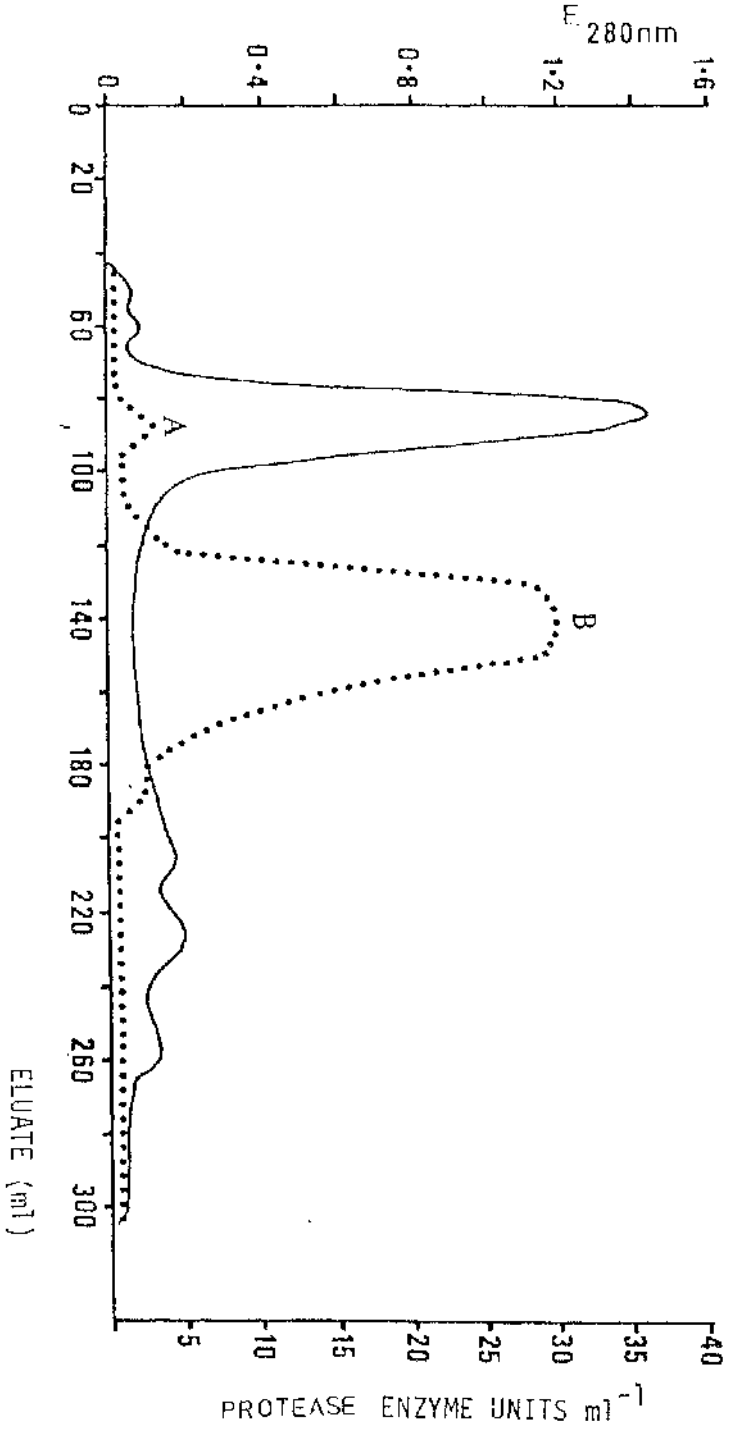
Gel filtration of the crude material on Sephadex G100 eluated with borate buffer (0.05M, pH 9.0) produced the E_{280nm} elution profile shown in Figure 13. However, only one major peak of protease activity, B, measured by the DMC assay, was detected. This peak of protease activity did not correspond to the major protein peak suggesting that a lot of contaminating material had been lost during gel filtration. No neuraminidase activity was detected in any of the fractions using the methoxyphenyl neuraminic acid assay. The fractions in peak B were pooled and termed G100 protease (G100/P). By the use of appropriate markers the molecular weight of G100/P was found to be 21.5-31 k.Dal. (Figure 14).

4.2 Isoelectric focussing of crude protease

Proteases were also separated from non-enzymatic protein in the crude material by isoelectric focussing (IEF) in a pH gradient of 3.5-10.0. The E_{280nm} elution profile is shown with the pH gradient and the protease activity, measured by the DMC assay, in Figure 15. Neuraminidase activity of the fractions was determined using the MPN assay but no neuraminidase activity was found in any of the fractions. The elution profile shows three major peaks of protease activity; peak 1 (protease(s) IEF-1) was focussed as a broad peak with pI values of

FIGURE 13

ELUTION PROFILE OF CRUDE PROTEASE ON SEPHADEX G100



..... Protease activity

——— $E_{280}{}^{nm}$

FIGURE 14

Standard curve for molecular weight determination by gel filtration

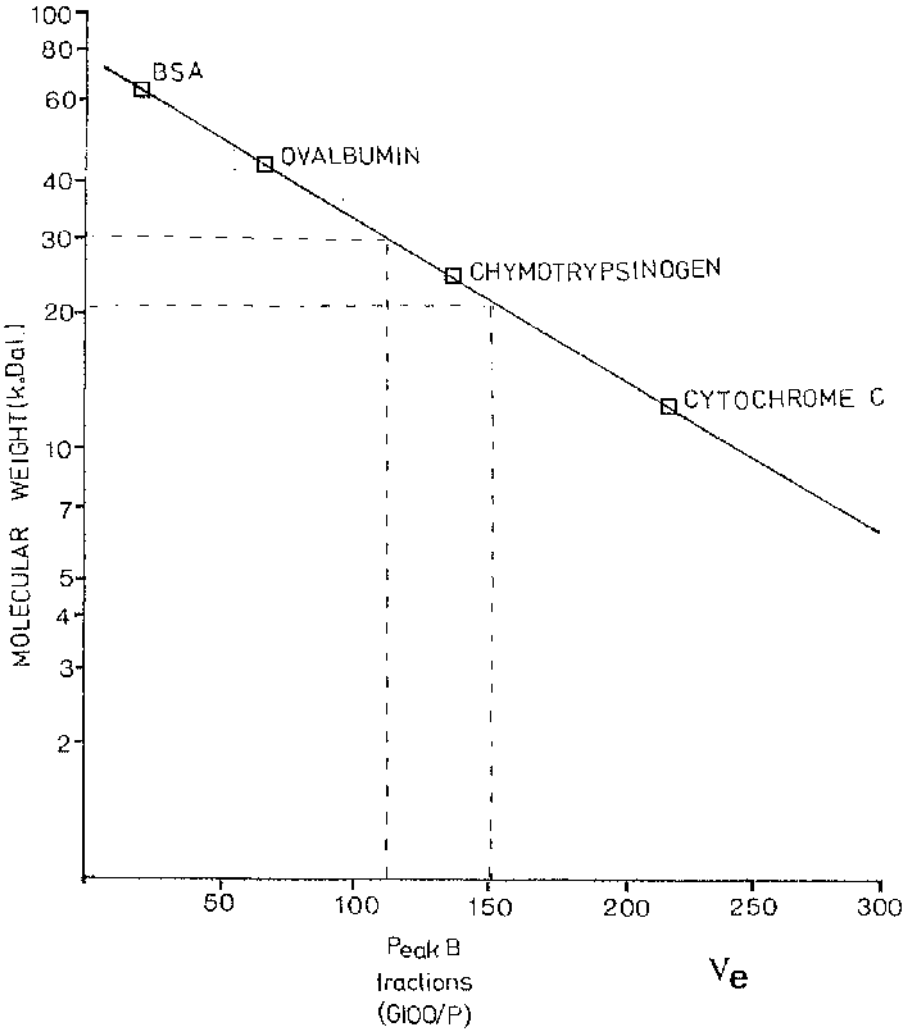
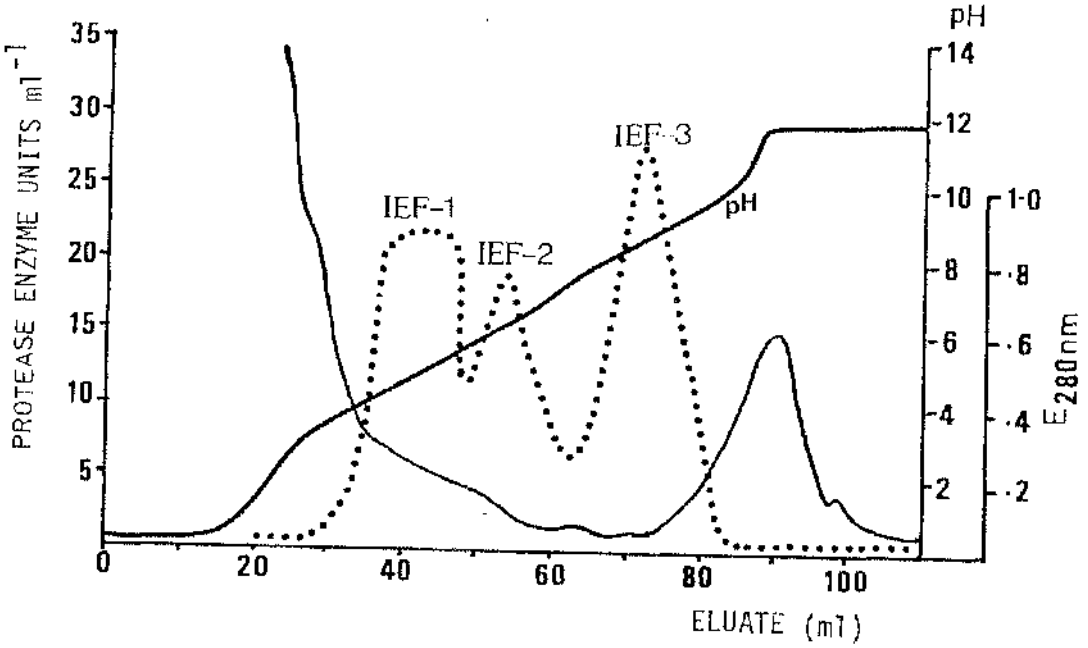


FIGURE 15

Elution profile of crude protease from batch grown cultures following isoelectric focussing



——— $E_{280}{}^{nm}$

..... Protease activity

4.5-5.5, peak 2 (protease(s) IEF-2) had a pI value of 6.5 and peak 3 (protease(s) IEF-3) had a pI value of 9.0. The crude protease used was from batch-grown cultures of V. cholerae. When crude protease from fermenter-grown cultures was subjected to IEF a similar elution profile was obtained (Figure 16). Three peaks of protease activity were detected with similar pI values to the IEF of the batch-grown production of crude protease. The proportions of IEF-1 and IEF-2 varied from those obtained in the batch cultures. In the batch-grown cultures IEF-1 was present in greater activity than IEF-2 but with the fermenter-grown culture more IEF-2 was present than IEF-1. In both types of culture IEF-3 had the maximum protease activity. IEF of G100/P also produced three peaks of protease activity with a similar elution profile to Figure 15. Unless otherwise stated IEF-1, 2 and 3 used throughout this study were prepared from crude protease and not G100/P.

4.3 Calculation of specific enzyme acrivity of V. cholerae proteases during purification

The enzyme activity was calculated $(\text{mg protein})^{-1}$ for the supernatant fluid, the crude protease, G100/P, IEF-1, IEF-2 and IEF-3 (Table 9). The specific activity increased some 50 times from the supernatant fluid to the semi-purified G100/P preparation. Isoelectric focussing increased the specific activity as compared to gel filtration of the crude protease quite markedly.

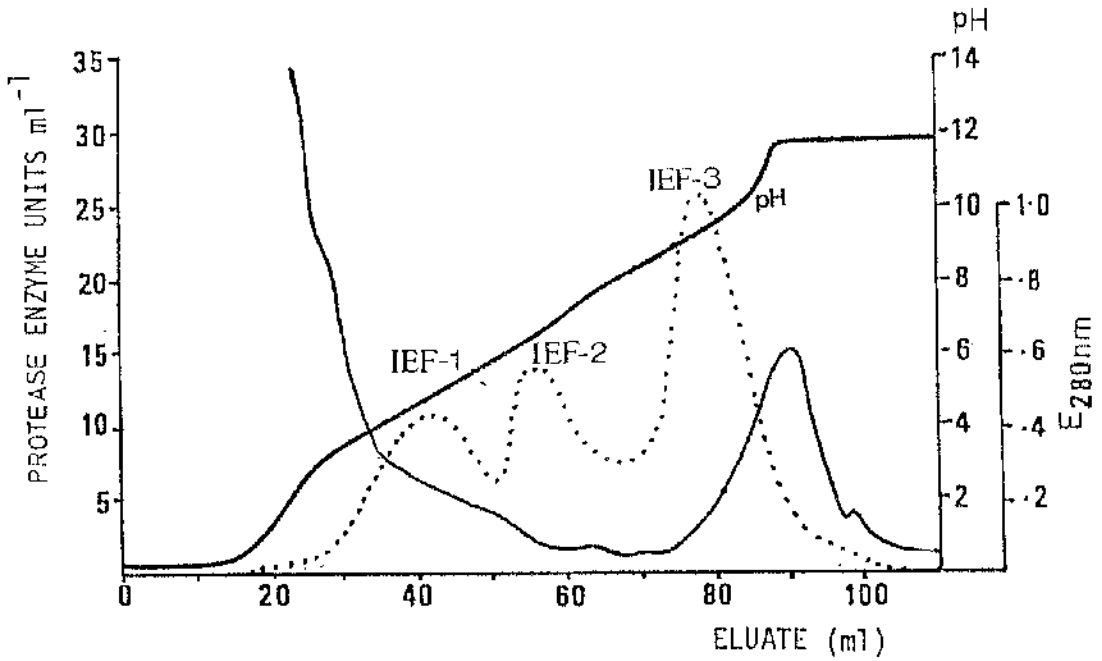
5. Characterization of V. cholerae proteases

5.1 Polyacrylamide gel electrophoresis (PAGE)

5.1.1 SDS-PAGE

FIGURE 16

Elution profile of crude protease from fermenter grown cultures following isoelectric focussing



——— E_{280}^{nm}

····· Protease activity

TABLE 9

SPECIFIC ENZYME ACTIVITY OF *V. cholerae* PROTEASES DURING PURIFICATION PROCEDURES

PROTEASE PREPARATION	SPECIFIC ENZYME ACTIVITY ($EU.ml^{-1}$ mg protein)*
Supernatant Culture Fluid	2.9
Crude Protease**	17.2
G100 Protease	120.0
IEF-1 Protease***	178.6
IEF-2 Protease***	195.0
IEF-3 Protease***	192.2

* Protease activity measured by DMC assay, protein determinations by Lowry method

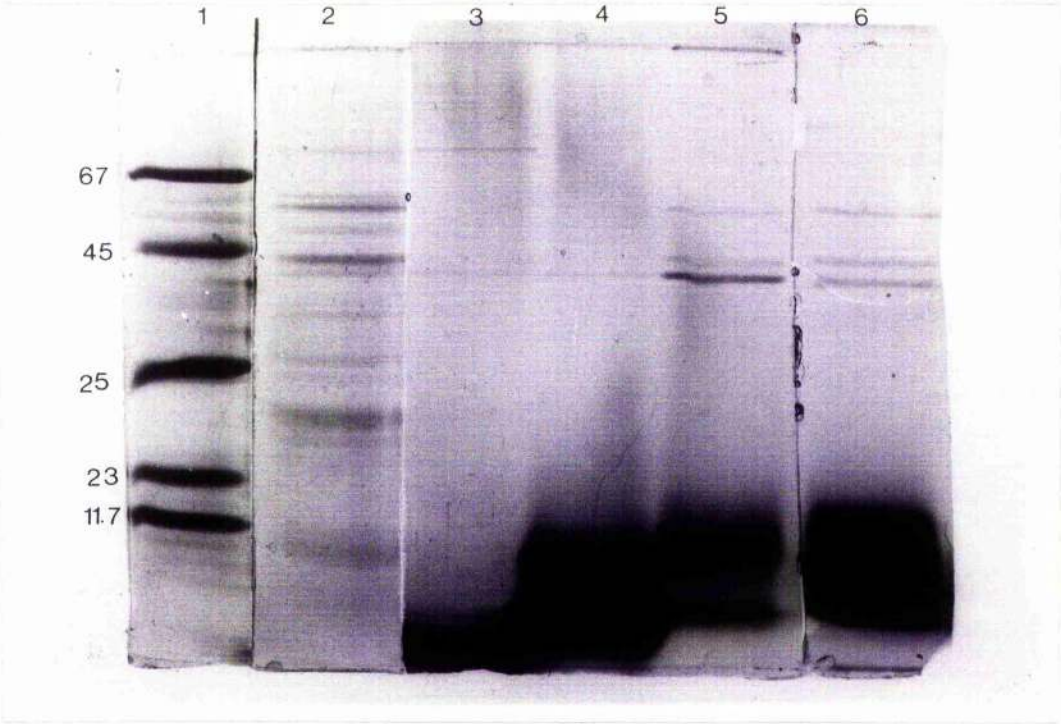
** Ammonium sulphate precipitate of culture fluid

*** IEF-1, 2 and 3 all prepared from crude protease.

A 12% polyacrylamide gel was run in the presence of sodium dodecyl sulphate (SDS) in a discontinuous buffer system. The samples used for the gel were crude protease, G100/P, IEF-1, IEF-2, IEF-3 and culture medium (TSB) alone (Figure 17). The culture medium alone showed no detectable bands when the gel was stained with bromophenol blue. The molecular weight markers in lane 1 (Bovine serum albumin. 67 k.Dal; ovalbumin, 45 k.Dal; chymotrypsinogen, 25 k.Dal; trypsin, 23 k.Dal. and cytochrome c, 11.7 k.Dal.) were used to produce a standard curve of log molecular weight against migration distance in the PAGE gel (Figure 18). From this standard curve the approximate molecular weights of other peptide bands was calculated. The G100/P, IEF-1, IEF-2 and IEF-3 all showed the presence of one common band with a molecular weight of approximately 42 k.Dal. (Figure 17). IEF-1 (lane 5) also showed two other bands with molecular weights of about 45 k.Dal. and 57 k.Dal., these two bands were not present in IEF-2 or IEF-3 but they were present in G100/P (lane 6). IEF-3 (lane 3) had one other band with a molecular weight of about 75 k.Dal. This was not present in IEF-1 or IEF-2 but was present in G100/P. The latter also showed one other very weak band with a molecular weight of about 82 k.Dal. not present in any of the IEF proteases. On comparing G100/P and the IEF proteases with the crude protease (lane 2) it can be seen that numerous contaminating bands have been removed during gel filtration and isoelectric focussing. On comparing the crude protease (lane 2) with any of the partially purified proteases (lanes 3-6) during purification there was an increase in the staining of low molecular weight material. This may have been due to digestion of contaminating proteins by the cholera proteases.

FIGURE 17

SDS-PAGE of crude protease G100/P, IEF-1, IEF-2 and IEF-3



Lane 1 - Molecular weight markers (R.Dal.)

" 2 - Crude protease

" 3 - IEF-3

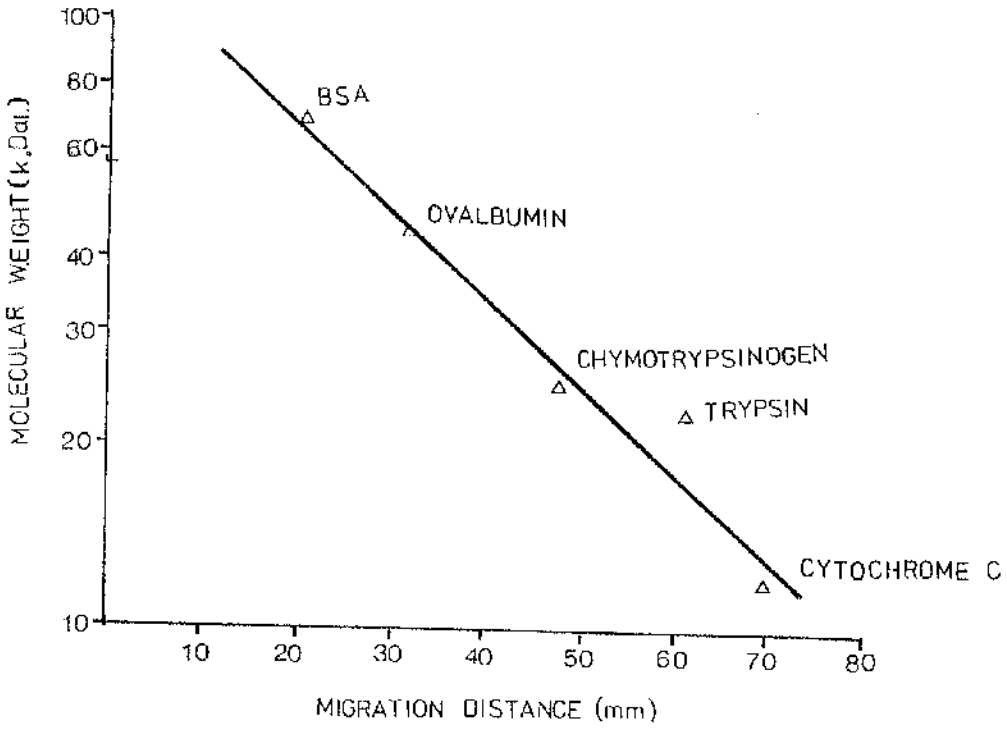
" 4 - IEF-2

" 5 - IEF-1

" 6 - G100/P

FIGURE 18

Standard for molecular weight determination by SDS-PAGE



5.1.2 PAGE with gelatin overlay

A polyacrylamide gel containing no SDS was run with samples of the crude protease, G100/P and the IEF proteases. Following electrophoresis a gelatin overlay was placed on the gel and after 18 h the gelatin overlay was stained with an acid sublimate solution. Bands of clearing were expected where the protease had digested the gelatin. However, no distinct bands of clearing were found; instead whole areas of clearing were seen from the sample wells of the gel to about 4 cm from these wells (Figure 19) This resulted in about one third of the gel being clear. The only distinct observation from this gel was that the crude protease gave a larger area of clearing than any of the other proteases although each protease preparation was standardized to 10 $EUml^{-1}$. As this method was not very informative the following technique was used.

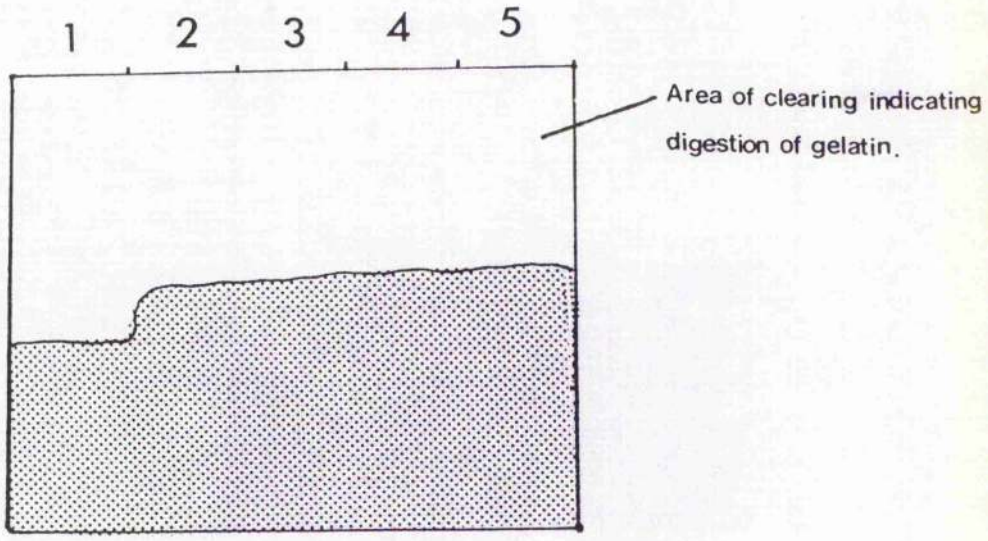
5.1.3 SDS-PAGE incorporated with gelatin

A polyacrylamide gel containing 1% (w/v) gelatin was run in the presence of SDS in a discontinuous buffer system. Following electrophoresis the gels were treated with 2.5% (v/v) Triton X-100, to remove the SDS, and then incubated for 5 h in buffer. The gelatin protein was stained black with 1% (w/v) Amido Black, the areas of clearing indicated where the protease had digested the gelatin (Figure 20).

All protease preparations showed areas of protease activity at A and B (Figure 20) and also a more broad area of activity at E. However, the intensity of these areas of protease activity varied between the different preparations, ie. IEF-2 (lane 2) and IEF-3 (lane 1) showed a stronger activity at A than B, whereas IEF-1 (lane 3) showed a stronger

FIGURE 19

Diagrammatic representation of gelatin agar overlay following incubation with PAGE gel of crude protease, G100/P, IEF-1, IEF-2 and IEF-3

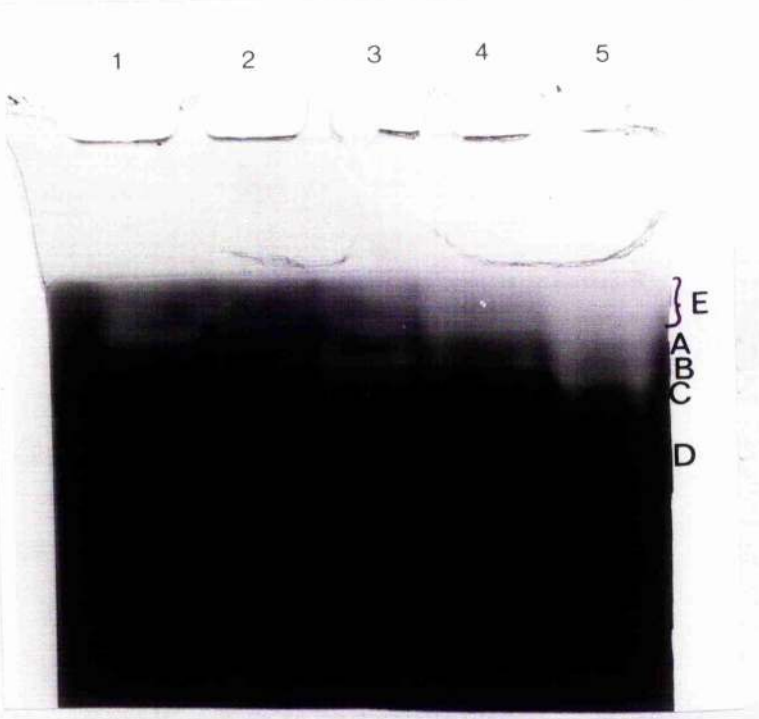


Lane 1 - Crude protease
" 2 - G100/P
" 3 - IEF-1
" 4 - IEF-2
" 5 - IEF-3

All protease samples contained approximately 10 $EUml^{-1}$

FIGURE 20

SDS-PAGE incorporated with gelatin of crude protease G100/P, IEF-1, IEF-2 and IEF-3



Lane 1 - IEF-3
" 2 - IEF-2
" 3 - IEF-1
" 4 - G100/P
" 5 - Crude protease

All samples contained approximately 10 $EUml^{-1}$ of protease activity.

activity at B. IEF-1 also showed protease activity at C which was absent from IEF-2, IEF-3 and G100/P but was present in the crude protease. The crude protease (lane 5) also had one band of protease activity (area D) not present in any of the other preparations.

5.2 Peptide Digest Analysis of BSA digestion by cholera proteases

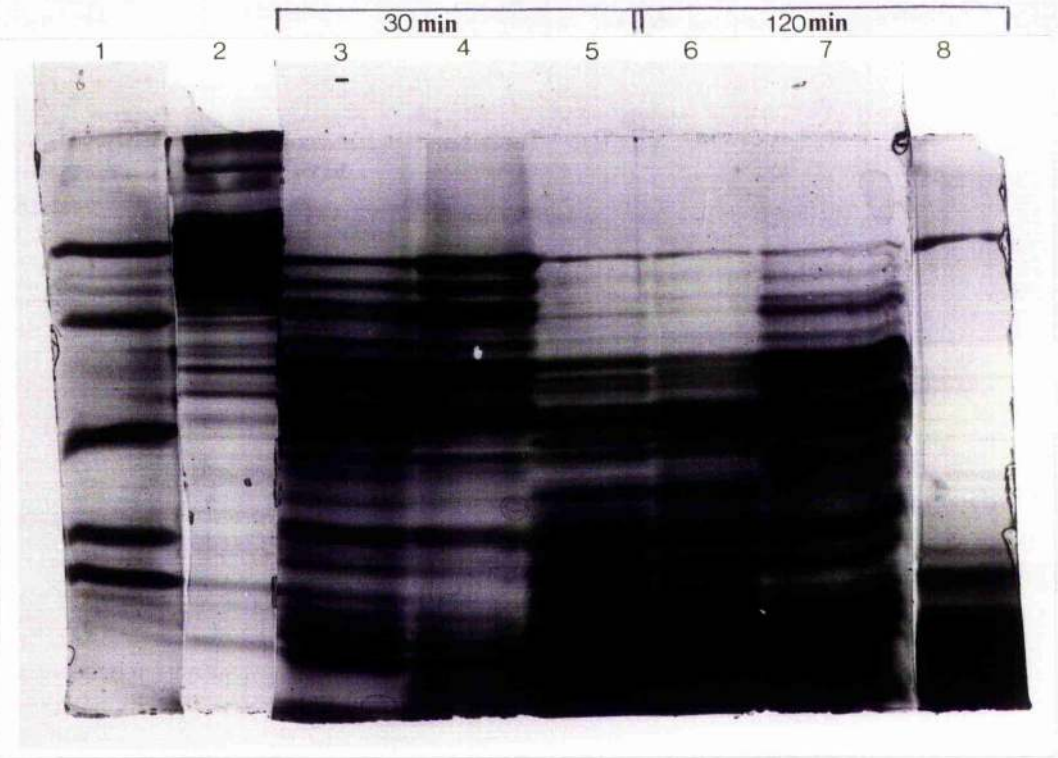
BSA was used as the standard protein to observe the peptide patterns obtained after digestion with IEF-1, IEF-2 and IEF-3. The three IEF proteases (15 $EUml^{-1}$) were incubated with BSA for 30 min and 2 h and the unique peptides were separated by SDS-PAGE; the patterns obtained after the digestion are shown in Figure 21. Lanes 3, 4 and 5 show the 30 min digestion patterns by IEF-1, 2 and 3 respectively. IEF-1 and IEF-2 gave similar patterns but IEF-3 was quite different. The 2 h digestion of BSA by IEF-1, 2 and 3 (lanes 6, 7 and 8 respectively) showed distinct peptide patterns for each of the IEF proteases, with IEF-3 showing most of the BSA in low molecular weight peptides. IEF-1 and IEF-2 did not show such active digestion of BSA as IEF-3, but their peptide patterns were distinct from each other and from the IEF-3 peptide pattern. In addition, it was obvious that there was continued protease activity, as shown by comparing lanes 3 and 6, with 30 min and 2 h digests of BSA by IEF-1.

5.3 Inhibition of protease activity by enzyme inhibitors

Characterization of the IEF proteases was attempted using enzyme inhibitors. The effect of the inhibitors on the crude protease was tested using three different concentrations of each inhibitor. The initial concentration of inhibitors tested (Table 10, concentration A) was taken from the papers of Young and Broadbent (1982) and Schneider and

FIGURE 21

Peptide Digest Analysis of Bovine Serum Albumin by IEF-1, IEF-2 and IEF-3 by SDS-PAGE



Lane 1 - Molecular weight markers
" 2 - BSA
" 3 - IEF-1 digestion of BSA at 30 min
" 4 - IEF-2 " " " " "
" 5 - IEF-3 " " " " "
" 6 - IEF-1 " " " " 120 min
" 7 - IEF-2 " " " " "
" 8 - IEF-3 " " " " "

IEF-1, 2 and 3 had a protease activity of approximately 10 $EUml^{-1}$.

TABLE 10 EFFECT OF ENZYME INHIBITORS AT DIFFERENT CONCENTRATIONS ON CRUDE PROTEASE

Inhibitor	Concn.A	Percentage Inhibition *	Concn.B	Percentage Inhibition *	Concn.C	Percentage Inhibition *
Soyabean Trypsin	0.1 $mgml^{-1}$	3	1.0 $mgml^{-1}$	16	10 $mgml^{-1}$	26
anti- Eggwhite Trypsin	0.1 "	2.5	1.0 "	6	10 "	26
Anti-Trypsin	0.1 "	3.0	1.0 "	17	10 "	44
Pepstatin	0.1 "	0	1.0 "	2	10 "	0
2-nitro-4carboxyphenyl N'-N'phenylcarbamate	0.1 "	9.5	4 mM	10	40 mM	10
Ethylenediamine tetraacetic acid	5 mM	0	25 "	14	250 "	28
Mercuric chloride	0.1 "	8.5	25 "	19	250 "	22
Sodium fluoride	100 "	4	250 "	18	250 "	18
Phenylmethyl sulphonyl fluoride	1.0 "	5	40 "	11	80 "	20
N'ethylmalemide	5 "	11.5	25 "	43	250 "	20
Sodium Iodoacetate	5 "	6	25 "	3	250 "	38

* Average figures from four separate assays

Parker (1982). These concentrations were not very effective so two higher concentrations of each inhibitor were tested (Table 10, concentrations B and C). Concentration B of the inhibitors was used against proteases IEF-1, IEF-2 and IEF-3 to obtain the optimum effect and to avoid some of the solubility problems obtained with concentration C of the inhibitors. The IEF protease preparations were all standardized to approximately 5 $EUml^{-1}$ except in the cases where the casein plate assay was used in which instance the protease was standardized to approximately 10 $EUml^{-1}$. The protease activity was measured before and after incubation with the inhibitor and the inhibition classed on a scale from - to ++ for 0 to 100% inhibition.

Table 11 shows the effect of the inhibitors on IEF-1. This IEF protease was inhibited by mercuric chloride, the metalloprotease inhibitors, EDTA and 8-hydroxyquinoline, the serine protease inhibitors, egg white trypsin and anti-trypsin, and by α-2 macroglobulin.

IEF-2 (Table 12) was inhibited by mercuric chloride, the metalloprotease inhibitors, EDTA and 8-hydroxyquinoline, the serine protease inhibitor anti-trypsin, the thiol protease inhibitor N-ethylmaleimide and by α-2 macroglobulin.

IEF-3 (Table 13) was inhibited by mercuric chloride, the metalloprotease inhibitors, EDTA and 8-hydroxyquinoline, and by α-2 macroglobulin.

The percentage inhibition by each inhibitor on the protease activity of IEF-1, IEF-2 and IEF-3 was calculated and is shown in Table 14. The figures in boxes represent where the protease was inhibited by more than 30%. From this table it is quite clear that all three IEF proteases were inhibited by α-2 macroglobulin, mercuric chloride, EDTA and 8-hydroxyquinoline.

TABLE 11 EFFECT OF ENZYME INHIBITORS ON IEF-1

Inhibitor	Concentration	PROTEASE ACTIVITY, E.U.ml^{-1} * Prior to incubation with inhibitor	After incubation with inhibitor **	Inhibition
Soyabean Trypsin	1.0 mgml^{-1}	5.1	5.1	-
Eggwhite anti-Trypsin	"	5.1	2.9	+
Anti-Trypsin	"	5.1	3.0	+
Pepstatin	"	5.1	5.1	-
2-nitro-4carboxyphenyl N'N-phenylcarbamate	4 mM	5.1	4.7	-
Ethylenediaminetetra acetic acid (EDTA)	25 "	5.1	0	++
Mercuric chloride	25 "	5.1	0	++
Sodium fluoride	250 "	5.1	5.2	-
Phenylmethyl sulphonyl fluoride (PMSF)	40 "	5.1	4.1	$\pm$
N'ethylmaleimide	25 "	5.1	4.7	-
Sodium iodoacetate	25 "	5.1	3.9	$\pm$
8-hydroxyquinoline ***	1 mM	10.5	5.1	+
α-2 macroglobulin ***	1 mgml^{-1}	10.5	3.2	++

* Protease activity measured by DMC assay.
**Mean values from three experiments
***Casein plate assay was used to measure protease as inhibitors interfered with DMC assay. The loss of enzyme activity was measured by the decrease in the diameter of the zone of precipitation.

TABLE 12

EFFECT OF ENZYME INHIBITORS ON IEF-2

Inhibitor	Concentration	PROTEASE ACTIVITY, E.U.ml^{-1}* Prior to incubation with inhibitor	After incubation with inhibitor **	Inhibition
Soyabean Trypsin	1.0 mgml^{-1}	4.6	4.3	-
Eggwhite anta-Trypsin	1.0 "	4.6	4.4	-
Anti-trypsin	1.0 "	4.6	3.1	+
Pepstatin	1.0 "	4.6	4.45	-
2-nitro-4carboxyphenyl N'N-phenylcarbamate	4 mM	4.6	4.7	-
Ethylenediamine-tetra acetic acid (EDTA)	25 "	4.6	2.8	+
Mercuric chloride	25 "	4.6	1.9	++
Sodium fluoride	250 "	4.6	4.6	-
Phenylmethyl sulphonyl fluoride (PMSF)	40 "	4.6	3.8	$\pm$
N'ethylmaleimide	25 "	4.6	2.9	+
Sodium Iodoacetate	25 "	4.6	4.2	-
8-Hydroxyquinoline***	1 mM	12.1	4.2	++
α-2 macroglobulin***	1 mgml^{-1}	12.1	5.1	+

* Protease activity measured by DMC assay

** Mean values from three experiments

*** Casein plate assay was used to measure protease activity as inhibitors interfered with DMC assay. The loss of enzyme activity was measured by the decrease in the diameter of the zone of precipitation.

TABLE 13

EFFECT OF ENZYME INHIBITORS ON IEF-3

Inhibitor	Concentration	PROTEASE ACTIVITY, E.U.ml^{-1} * Prior to incubation with inhibitor	After incubation with inhibitor**	Inhibition
Soyabean Trypsin	1.0 mgml^{-1}	5.7	4.9	-
Eggwhite Trypsin	1.0 "	5.7	5.7	-
Anti-Trypsin	1.0 "	5.7	4.8	-
Pepstatin	1.0 "	5.7	5.8	-
2-nitro-4carboxyphenyl N'N'-phenylcarbamate	4 mM	5.7	4.7	-
Ethylenediaminetetra acetic acid (EDTA)	25 "	5.7	0.6	++
Mercuric chloride	25 "	5.7	0.9	++
Sodium fluoride	250 "	5.7	5.5	-
Phenylmethyl sulphonyl fluoride (PMSF)	40 "	5.7	4.75	-
N'ethylmaleimide	25 "	5.7	5.4	-
Sodium iodoacetate	25 "	5.7	5.5	-
8-Hydroxyquinoline ***	1 mM	11.3	3.5	++
α-2 macroglobulin ***	1 mgml^{-1}	11.3	4.8	+

* Protease activity measured by DMC assay

** Mean values from three experiments

*** Casein plate assay was used to measure protease activity as inhibitors interfered with DMC assay. The loss of enzyme activity was measured by the decrease in the diameter of the zone of precipitation.

TABLE 14 SUMMARY OF INHIBITION OF IEF-1, IEF-2 and IEF-3 BY ENZYME INHIBITORS

INHIBITOR	Concentration	Percentage inhibition of IEF-1	IEF-2	IEF-3
Soyabean Trypsin	1.0 $mgml^{-1}$	0	7	13
Eggwhite anti-Trypsin	1.0 "	43	4	0
Anti-Trypsin	1.0 "	48	33	15
Pepstatin	1.0 "	0	3	0
α-2 macroglobulin	1 $mgml^{-1}$	69	58	57
NCDC	4 mM	8	0	17
EDTA	25 "	100	39	90
8-hydroxyquinoline	1.0 "	51	65	69
$HgCl_2$	25 "	100	59	84
NaF	250 "	0	0	3
PMSF	40 "	20	17	16
N'ethylmaleimide	25 "	8	37	4
Sodium iodoacetate	25 "	24	9	3

Also noticeable was the inhibition of IEF-1 by egg white trypsin and anti-trypsin inhibitors but not by soyabean trypsin inhibitor although all three inhibitors belong to the same class of serine protease inhibitors. This was also true of IEF-2 where only anti-trypsin had any effect on the protease activity. Interestingly, IEF-1 and IEF-2 were both inhibited by more than one class of protease inhibitor.

6. Activities of the proteases associated with the environment in the small intestine

6.1 IgA digestion by cholera proteases

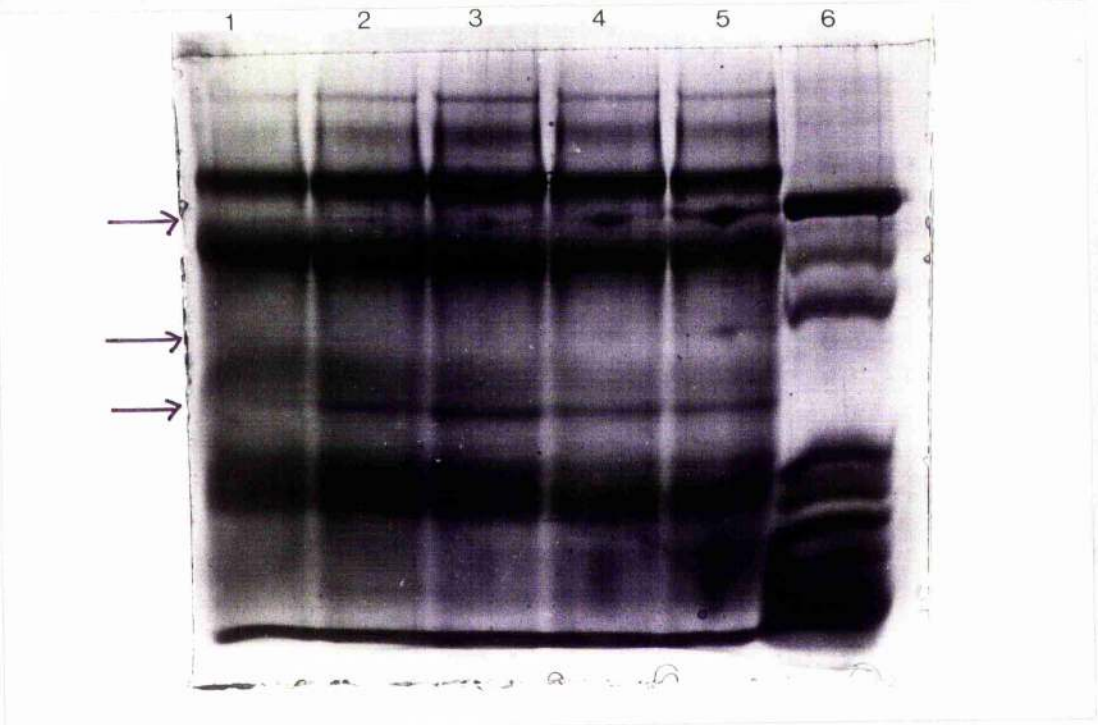
Human IgA were incubated with G100/P (10 $EUml^{-1}$) for 1, 9 and 24 h. These digests were examined by SDS-PAGE and immunoelectrophoresis. The SDS-PAGE of the digests (Figure 22) showed that after 24 h (lane 1) only three specific bands were digested when compared to untreated IgA (lanes 4 and 5). The immunoelectrophoresis plate was set up with these digests and untreated IgA against goat anti-human IgA serum (Figure 23). The antigenicity of the IgA was not destroyed but it was noticed in repetitive tests that the single precipitin arc was shortened.

6.2 Digestion of Fibronectin by cholera proteases

Cholera proteases G100/P (9.5 $EUml^{-1}$) were incubated with fibronectin for 1, 9 and 24 h. Fibronectin was digested by cholera proteases, as shown by SDS-PAGE of the digests (Figure 24). After only 1 h (lane 3) fibronectin digestion was noticeable. Further digestion occurred as seen in the pattern of the 24 h digest (lane 5).

FIGURE 22

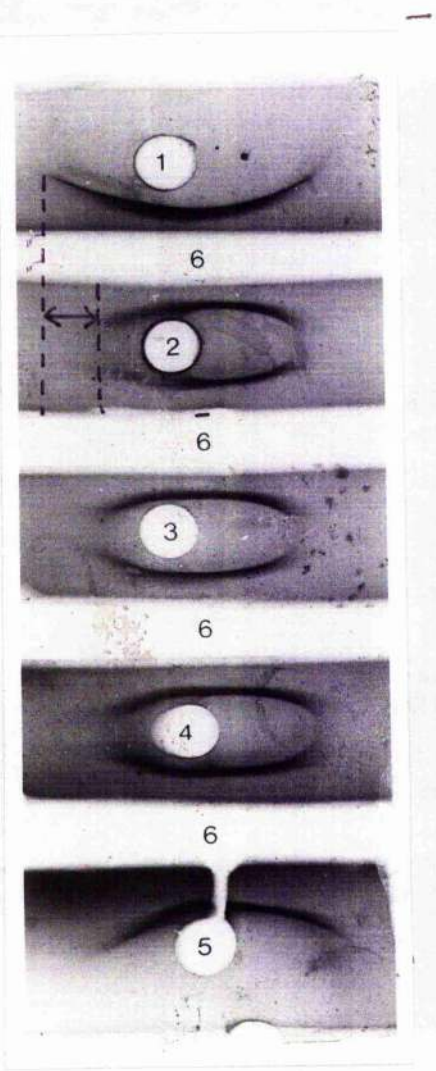
SDS-PAGE OF IgA DIGESTION BY G100/P



Lane 1 - 24 h digestion of IgA with G100/P
2 - 9 h " " " " "
3 - 1 h " " " " "
4 - IgA
5 - IgA
6 - Marker proteins

digestion of these bands has occurred following protease treatment.

FIGURE 23 IMMUNOELECTROPHORESIS OF IgA DIGESTION BY G1OO/P



WELL 1 - IgA alone

2 - 24 h digestion of IgA by G1OO/P

3 - 9 h " " " " "

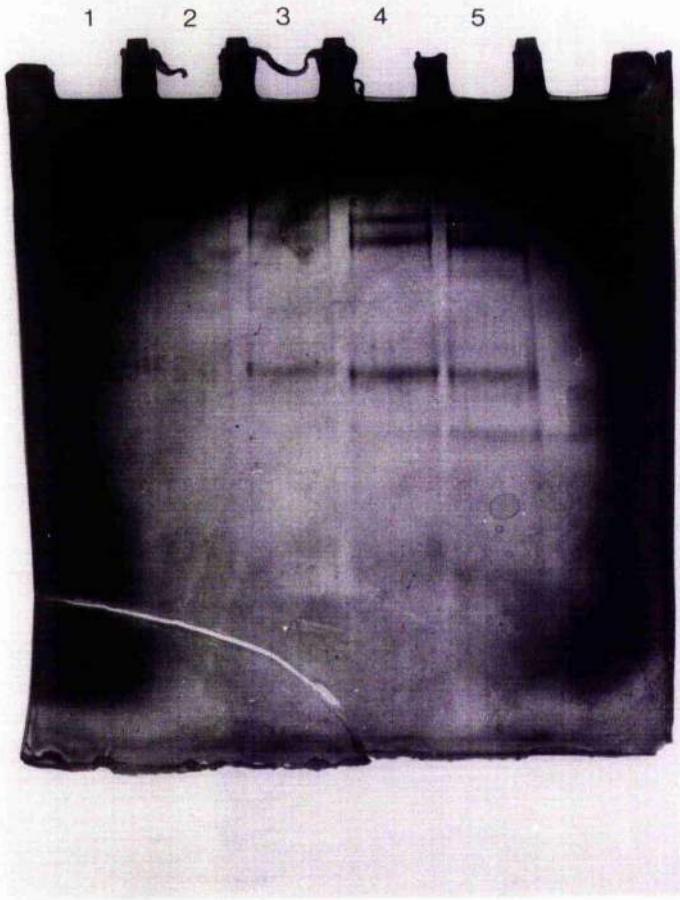
4 - 1 h " " " " "

5 - IgA alone

TROUGHS 6 - Anti-IgA serum

↔ area digested by protease.

FIGURE 24 SDS-PAGE OF FIBRONECTIN DIGESTION BY G1OO/P



Lane 1 - Fibronectin

2 - Fibronectin

3 - 1 h digestion of fibronectin by G1OO/P

4 - 9 h " " " " "

5 - 24 h " " " " "

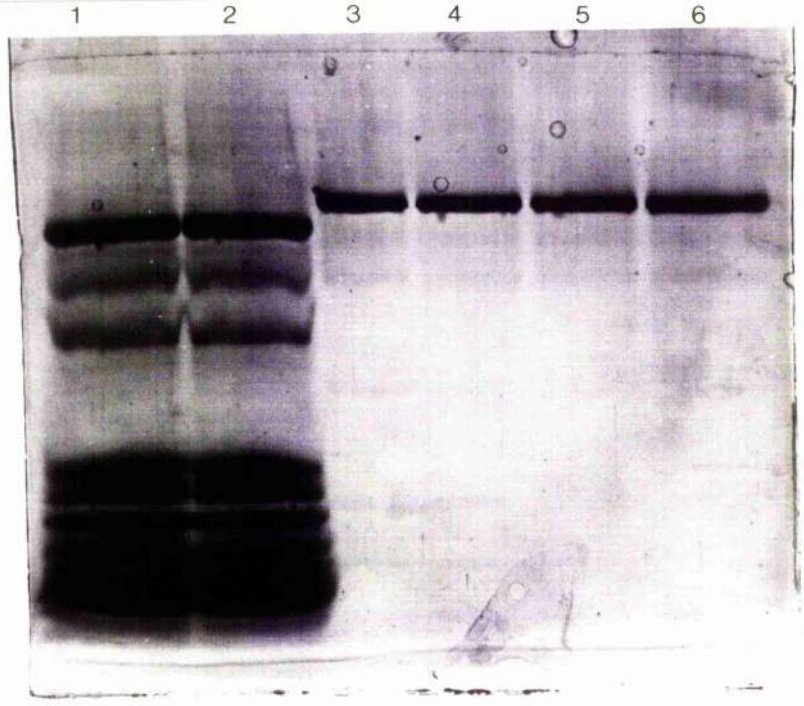
6.3 Lack of cholera protease activity on Lactoferrin

Lactoferrin was incubated with G100/P (9.5 $EUml^{-1}$) for 1, 9 and 24 h. SDS-PAGE of these digests (Figure 25) showed no digestion of the lactoferrin even after 24 h (lane 6).

6.4 Effect of cholera proteases and neuraminidase on toxin binding

Tissue segments were prepared as for type C in the adherence assay (see page 86). The mucosal surface of the rat ileal segment was suspended in PBS, protease, neuraminidase or protease plus neuraminidase for 30 min. Subsequently, the tissue segment was added to 2 ml of a solution containing 600, 400, 200, 100, 20 BD, in 2.0 ml, of cholera toxin for 1 h. The toxin bound to the tissue segment is inactivated and cannot be measured directly, therefore the residual toxin was measured by the rabbit intradermal blueing dose test. The toxin bound to the tissue segment was calculated by subtracting the blueing doses of the residual toxin from that originally added to the tissue segment. Table 15 shows the results obtained. When tissue segments were added to the toxin solution containing 600 $BDml^{-1}$, the buffer treated segments bound 576.4 BD, protease treated 572.4 BD, neuraminidase treated 573.2 BD and protease plus neuraminidase treated 573.6 BD of toxin. At the other end of the scale, when tissue segments were added to the toxin solution containing 100 BD, 2.0 ml^{-1}, 82.8, 88.0, 79.2 and 77.0 BD of toxin bound when treated with buffer, protease, neuraminidase and protease plus neuraminidase respectively. It is quite clear from these results that the enzyme treatment of the tissue segments had no effect on the binding of toxin to ileal segments *in vitro*. The enzyme preparations used for prior treatment of the tissue segments were tested for the presence of toxin and found to be negative.

FIGURE 25 SDS-PAGE OF LACTOFERRIN DIGESTION BY G100/P



Lane 1 and 2 - marker proteins

3 - lactoferrin

4 - 1 h digestion of lactoferrin by G100/P

5 - 9 h " " " " "

6 - 24 h " " " " "

TABLE 15 EFFECT OF PROTEASE AND NEURAMINIDASE ON THE BINDING OF CHOLERA TOXIN TO RAT ILEAL SEGMENTS in vitro

Tissue Treatment	Blueing Doses of Cholera Toxin ** Added to tissue segment	Remaining after removal of tissue*	Bound to tissue segment
BUFFER (PBS)	600	23.6	576.4
	400	20.8	379.2
	200	22.0	178.0
	100	17.2	82.8
	20	0.0	
PROTEASE (12.5 $EUml^{-1}$ (Gloo/P)	600	27.6	572.4
	400	26.0	374.0
	200	21.4	178.6
	100	12.0	88.0
	20	0.0	20.0
NEURAMINIDASE (0.05 IU)	600	26.8	573.2
	400	23.8	376.2
	200	23.6	176.4
	100	20.8	79.2
	20	0.0	20.0
PROTEASE (12.5 $EUml^{-1}$) (G100/P) + NEURAMINIDASE (0.05 IU)	600	26.4	573.6
	400	26.4	373.6
	200	25.2	174.8
	100	23.0	77.0
	20	0.0	20.0

* Average of triplicate results

** 1 blueing dose (1 BD) ≡ 8 mm diameter zone of blueing on rabbit back

(see p. 74)

6.5 Effect of cholera proteases on toxin activity

Toxin (2 mg ml^{-1}) was incubated with protease (G100/P, 10.5 EUml) for 5 and 24 h and with distilled water, as a control, for 24 h at 37°C. Following incubation the three toxin samples were diluted to give expected BD of 3.0, 2.0, 1.0, 0.5 and 0.1. The results of the intradermal blueing test of the 15 samples are shown in Figure 26A

It is quite clear from these results that the samples containing 0.1 and 0.5 BD produced BDs in excess of 1.0 BD, suggesting activation of the cholera toxin. However, the control toxin sample which was incubated with distilled water for 24 h also showed this increased activity which was unexpected. It was therefore decided to assay the N.I.A.I.D. toxin for protease activity. The toxin was found to have a high protease content of 15 EU of protease per mg of toxin. This meant that in the control samples 30 EU of protease were present, as the toxin sample was a 2 $mgml^{-1}$ solution. The test toxin samples also contained this 30 EU of protease plus the 10.5 EU of G100/P that were added to the toxin. These results suggest that proteases may be involved in toxin activation. The G100/P was assayed for toxin activity and found to be negative. Figure 26B shows the intradermal blueing dose results of toxin samples not incubated for 24 h before use in the assay system. The protease preparations alone showed no BD activity.

6.6 Digestion of the A-subunit of V. cholerae enterotoxin

Cholera toxin, A-subunit (1 $mgml^{-1}$) was incubated with G100/P (10 $EUml^{-1}$) for 1, 9 and 24 h. SDS-PAGE of these digests is shown in Figure 27. This gel has not run very well but the A-subunit appears to have been split to form two peptides, one with a very low molecular weight, indicated by the arrow in Figure 27.

A. Effect of V. cholerae proteases on toxin activity.

1. Control; 24h incubation of toxin with distilled water
2. 24h incubation of toxin with proteases (G100/P, 10.5 EU ml^{-1})
3. 5h incubation of toxin with proteases (6100/P, 10.5 EU ml^{-1})

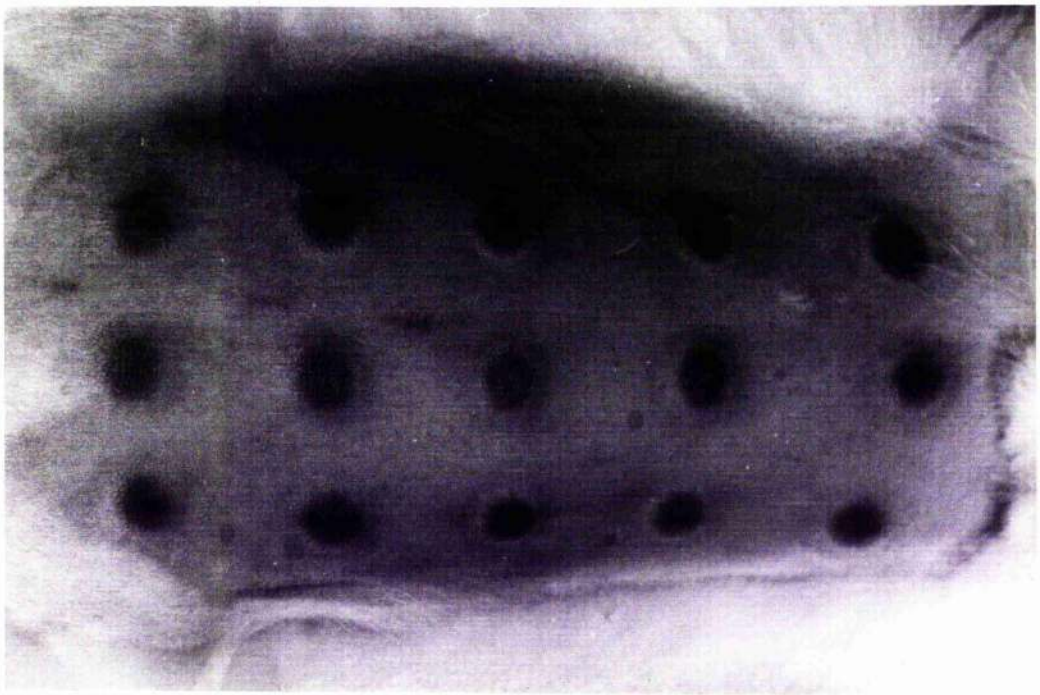
B. Titration to find the amount of NIAID cholera toxin equivalent to 1 OBD.

1. 0.1 BD
2. 1.0 BD
3. 1.0 BD
4. 2.0 BD
5. 4.0 BD
6. 8.0 BD

FIGURE 26

Intradermal blueing test of protease treated toxin samples

A



Expected BD 3·0 2·0 1·0 0·5 0·1

B

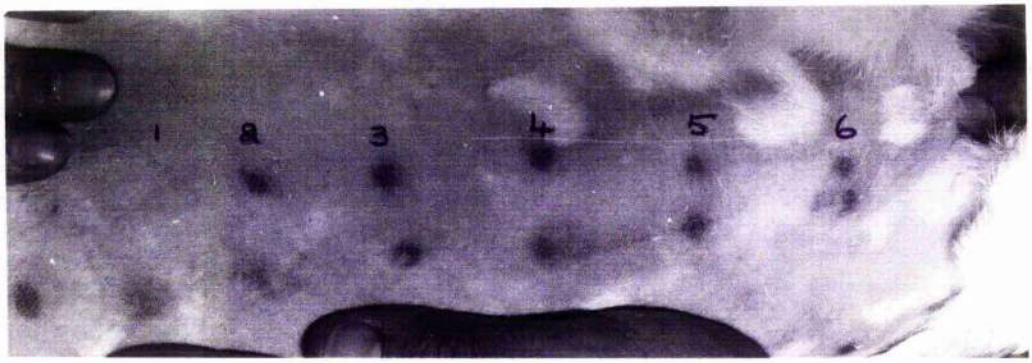
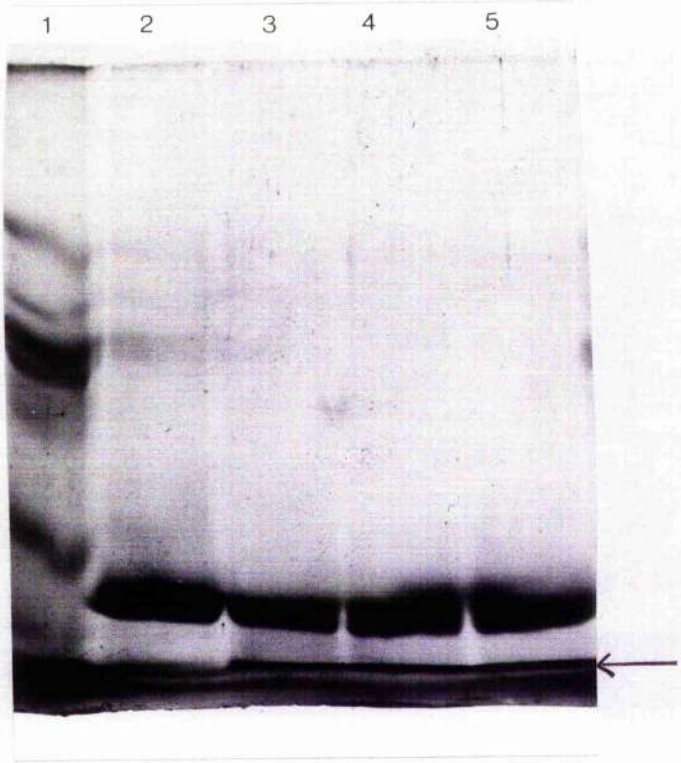


FIGURE 27 SDS-PAGE of A-subunit digestion by cholera proteases (G100/P)



Lane 1 - molecular weight markers
2 - A-subunit
3 - 1 h digestion of A-subunit by G100/P (10 $EUml^{-1}$)
4 - 9 h " " " " " "
5 - 24 h " " " " " "

6.7 Mucinase activity in protease preparations

The presence of mucinase activity in crude protease, G100/P, IEF-1, IEF-2 and IEF-3 was measured (Table 16). The crude protease, G100/P and IEF-3 all showed mucinase activity with values of 16.0, 16.0 and 32.0 respectively. IEF-1 and IEF-2 showed no detectable mucinase activity.

6.8 Haemagglutinating activity in protease preparations

Crude protease, G100/P, IEF-1, IEF-2 and IEF-3 were tested for haemagglutinating activity against sheep and chicken red blood cells (Table 17). Haemagglutinating activity was found in crude protease, G100/P and IEF-3 although the titres were low. No major difference in activity was seen between the two types of red blood cells and no difference in activity was obtained in the presence of calcium ions. IEF-1 and IEF-2 showed no detectable haemagglutinating activity.

6.9 Cell-associated haemagglutinin and protease activity

Haemagglutinin activity with a titre of 8.0 was found by assaying whole cells. Protease activity was also found associated with whole cells grown overnight in syncase medium, the specific activity was 14.7 $EUml^{-1}$. However, some of this protease activity measured may have been secreted by the vibrios during the 30 min incubation with the dimethylcasein substrate.

TABLE 16 MEASUREMENT OF MUCINASE ACTIVITY IN PROTEASE PREPARATIONS

PROTEASE SAMPLE (15 $EUml^{-1}$)	MUCINASE ACTIVITY *
Crude Protease	16
G100/P	16
IEF-1	0
IEF-2	0
IEF-3	32

* The reciprocal of the highest dilution which prevented clot formation (see p. 72)

TABLE 17

MEASUREMENT OF HAEMAGGLUTINATING ACTIVITY IN PROTEASE PREPARATIONS

PROTEASE SAMPLE ($10\ EUml^{-1}$)	HAEMAGGLUTININ TITRE *	
	SHEEP rbc	CHICKEN rbc
Crude protease	16.0	8.0
G100/P	8.0	8.0
IEF-1	0	0
IEF-2	0	0
IEF-3	8.0	8.0

* Reciprocal of highest dilution showing visible haemagglutination.

rbc : red blood cells.

7. Adherence Assay

Initial experiments were done to find the most suitable size of tissue segment and the most suitable number of organisms to be used in the assay.

The size of the ileal segment was increased from 20 mm^2 to 113 mm^2 by using different sized cork borers to cut the tissue segments. By varying the size of the tissue segment it was found that there was an increase in the number of organisms associated with the tissue segment with an increase in tissue area (Table 18). Some 6.4×10^8 V. cholerae organisms were associated with a 20 mm^2 tissue segment and 52.8×10^8 with a 113 mm^2 segment. When these results were shown diagramatically (Figure 28) this increase was shown to be exponential.

7.1 One square centimetre of tissue preparation type A

From the initial experiments it was decided to use 1 cm^2 tissue segments (area of 100 mm^2) as these were easy to prepare and a reasonable number of V. cholerae would adhere to a piece of ileal tissue of this size. With the 1 cm^2 tissue segments the number of binding organisms increased as the number of organisms in the suspension increased over the range $0.12 - 120 \times 10^9$ (Table 19). When 0.12×10^9 organisms were incubated with the tissue segment 0.2×10^8 organisms adhered to the tissue, but when 12×10^{10} organisms were incubated with the tissue segment 15×10^9 organisms adhered to the tissue segment. These results showed an increase in the number of organisms associated with the tissue segment as more organisms were used in the assay. However, when these results were shown diagrammatically (Figure 29) this increase was not exponential.

TABLE 18 COMPARISON OF *V. cholerae* ASSOCIATED WITH TISSUE SEGMENTS OF VARYING SIZE

Area of tissue Segment (mm^2)	*V. cholerae* organisms associated with the tissue segment, x 10^8 *
20	6.4 ± 2.3
38	5.2 ± 0.2
50	12.5 ± 2.3
78	25.7 ± 1.6
95	31.1 ± 0.8
100	46.8 ± 3.6
113	52.8 ± 0.8

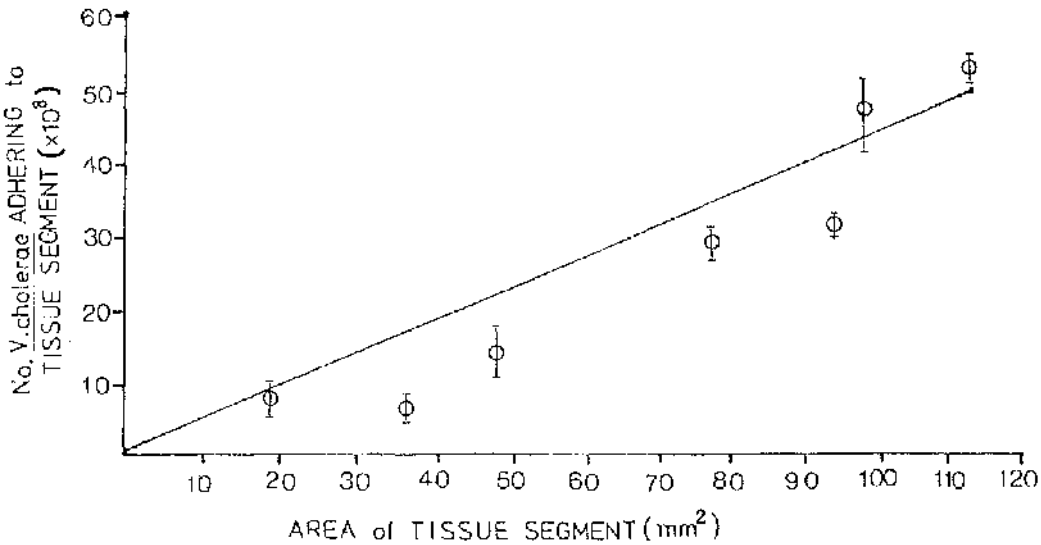
* Calculated from radioactive counts.

These values are the mean of three experiments.

± : the standard deviation.

FIGURE 28

Effect of increasing the area of ileal tissue on the number of adherent V. cholerae



The values shown are from three experiments.

Mean ○ Range |

TABLE 19 Effect of increasing the number of organisms in the assay but keeping the tissue size constant (1 cm^2) on the number of vibrios adhering to the tissue segment

Number of *V. cholerae* added to tissue segment, x 10^9 *	Number of *V. cholerae* associated with tissue segment, x 10^7 +
0.12	0.2 ± 0.03
1.2	1.5 ± 0.01
12	5.6 ± 0.05
15	6.7 ± 0.1
30	9.0 ± 0.7
60	10.6 ± 2.3
120	15.1 ± 0.4

* Calculated from absorbance at E_{600} nm

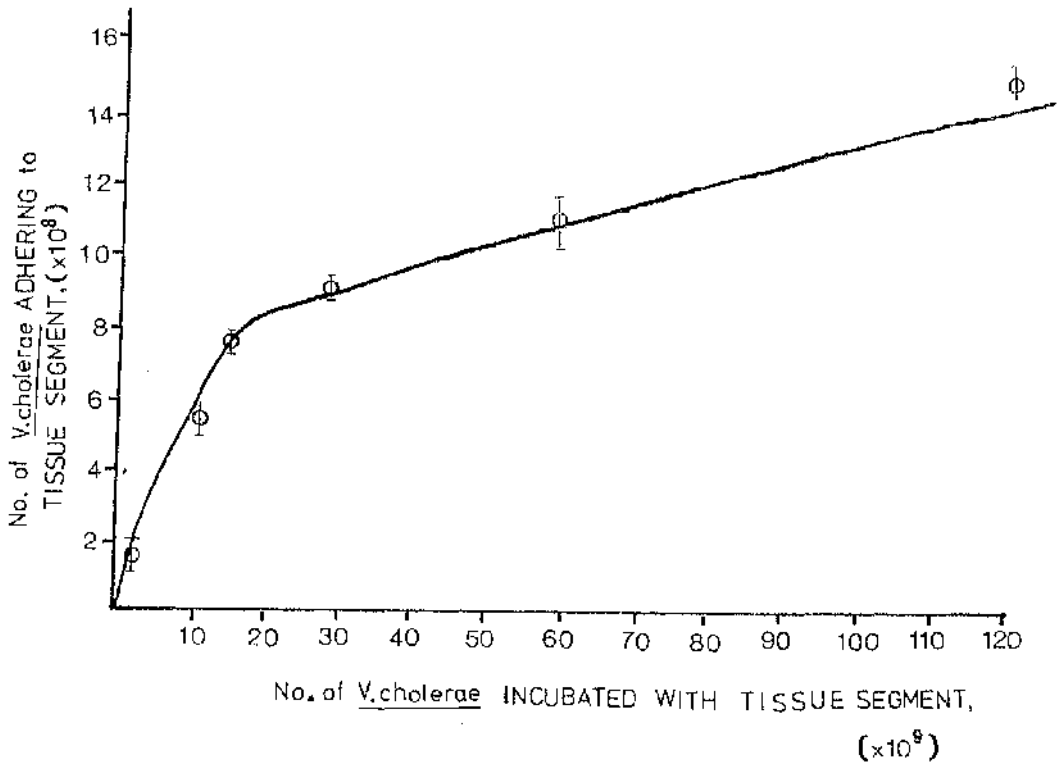
\+ Calculated from radioactive counts

These values are the mean of three experiments

± : standard deviation.

FIGURE 29

Effect of increasing the number of vibrios used in the assay system on the number of vibrios that adhered to a 1 cm^2 tissue segment



* Calculated from absorption at 600 nm.

** Calculated from radioactive counts.

The values are the mean from three experiments.

Mean o Range. I

Instead, there was an initial rapid increase followed by a gradual increase in the number of vibrios adhering to the tissue segments as more vibrios were added to the assay system.

It was decided to use approximately 1×10^{11} organisms in each individual assay as this value is on the slope of the graph and so would allow either an increase or decrease in the number of organisms adhering to a tissue segment to be seen.

Tissue segments (1 cm^2) were treated with Gl00/P at concentrations of 1.25 - 20 EU prior to the addition of the radiolabelled organisms (Table 20). This enzyme treatment had no significant effect on the number of vibrios binding to the tissue segment (11.8 - 15.7 $\times 10^7$) when compared to the untreated tissue (12.2 $\times 10^7$).

The 1 cm^2 tissue segments tended to curl into themselves and so to overcome this problem and make the assay system more consistent it was decided to use a different presentation of the mucosal surface of the ileum.

7.2 One centimetre cylindrical lengths of tissue preparation type B

Lengths of ileum (1 cm) were cut and inverted so that the brush-border surface of the ileum was the outer surface.

The cylindrical tissue segments were treated with protease, protease plus neuraminidase and neuraminidase alone prior to incubation with radioactive V. cholerae (Table 21). No effect on the binding of V. cholerae organisms to the tissue segments was seen following any of the enzyme treatments.

The time of incubation of the tissue segment with the radiolabelled vibrios was increased from 30 to 60 min. This was done to see if a longer incubation time would show differences in the number of

TABLE 20 Effect of protease (G100/P) on *V. cholerae* adherence to 1 cm^2 tissue segments

Expt.	*V. cholerae* associated with 1 cm^2 tissue segment, x 10^7					
	Borate Buffer (0.05M, pH9.0)	Protease (G100/P) $EUml^{-1}$ *				
		1.25	2.5	5.0	10.0	20.0
1	10.9	12.5	14.5	16.1	13.1	11.5
2	12.5	15.3	16.8	12.5	9.8	12.5
3	11.2	13.8	15.7	15.3	12.2	11.2
4	14.2	14.8	14.6	18.2	12.1	14.2
Mean	12.2	14.1	15.4	15.7	11.8	12.6

* Treatment with enzyme or buffer for 30 min before proceeding with assay.

TABLE 21 Effect of protease and neuraminidase on adherence of *V. cholerae* to 1 cm cylindrical tissue segments

Expt.	*V. cholerae* associated with 1 cm cylindrical tissue segments x 10^8			
	Borate Buffer (0.05M, pH9.0)	Protease 10EUml^{-1}*	Neuraminidase 0.05I.U.ml^{-1}	Protease, 10 EUml^{-1} + Neuraminidase 0.05 IUml^{-1}
1	4.1	3.1	5.0	3.6
2	3.8	4.3	5.2	4.5
3	3.9	2.9	4.6	3.6
4	4.3	4.4	4.3	5.1
Mean	4.0	3.65	4.8	4.2

* Tissue segments were pretreated with the enzyme or buffer preparations for 30 min before assay procedure was continued.

vibrios adhering to the enzyme treated compared to the untreated tissue segments (Table 22). This increase in incubation time caused a general 20-fold increase in the number of vibrios binding to the tissue segments. This was seen by comparing the buffer-treated segments in Table 21 and 22, after 30 min incubation 4.0×10^8 organisms adhered to the tissue segment but after 60 min the numbers adhering to the tissue segment had increased to 79.3×10^8 organisms. However, after a 60 min incubation there was still no difference between the number of vibrios binding to buffer-treated and any of the enzyme-treated tissue segments.

7.3 Presentation of mucosal disc to *V. cholerae* organisms preparation type C

To allow only the mucosal or serosal surface of the ileal tissue segment to be present during the assay procedure, the tissue segment was presented to the enzyme preparations and/or the radiolabelled organisms at the end of a specially prepared syringe.

Initially experiments were devised to see if the vibrios would actually bind to the serosal surface of the ileum. Table 23 shows buffer-treated segments of the mucosal and serosal surface with 1.85×10^8 vibrios binding to the mucosal surface and 0.45×10^8 vibrios binding to the serosal surface. Therefore, of the number of vibrios that will bind to the mucosal surface an equivalent 24% will also bind to the serosal surface of the ileal segment. Protease treatment (G100/P, 6 $EUml^{-1}$) of the mucosal surface (Table 23) caused the number of vibrios binding to fall from 1.85×10^8 to 0.82×10^8, a 56% decrease. Protease treatment of the serosal surface, however, caused a 60% increase in the number of vibrios binding, increasing the numbers from 0.45×10^8 to 1.12×10^8.

TABLE 22 EFFECT OF INCREASING THE INCUBATION TIME OF THE ASSAY TO 60 min ON THE EFFECT OF PROTEASE (G100/P) AND NEURAMINIDASE ON THE ADHERENCE OF *V. cholerae* TO 1 cm CYLINDRICAL TISSUE SEGMENT

Expt.	*V. cholerae* associated with tissue segment 1 cm tube x 10^8			
	BORATE BUFFER* (0.05M, pH9.0)	PROTEASE* 6 EUml^{-1}	NEURAMINIDASE* 0.05 I.U.ml^{-1}	PROTEASE, 6 EUml^{-1}* + NEURAMINIDASE 0.05IUml^{-1}
1	96	98	61	78
2	76	101	82	86
3	71	107	81	108
4	74	84	77	80
Mean	79.3	97.5	75.3	88.0

* Tissue segments were pretreated with enzyme or buffer for 30 min at 37°C before being used in the assay.

TABLE 23 EFFECT OF PROTEASE (G100/P) ON V. cholerae ADHERENCE TO THE MUCOSAL OR SEROSAL SURFACE OF THE TISSUE SEGMENT SEPARATELY USING MUCOSAL DISC TISSUE PREPARATION

	Vibrio cholerae organisms associated with the tissue segment ($x10^8$)			
	Mucosal surface		Serosal surface	
Expt.	Borate Buffer	Protease G100/P 6EUml^{-1}	Borate Buffer	Protease G100/P 6EUml^{-1}
1	2.51	0.77	0.43	0.69
2	1.72	0.86	0.56	1.00
3	1.49	1.08	0.46	1.67
4	1.71	0.59	0.35	1.14
Mean	1.85	0.82	0.45	1.12

Tissue segments were pretreated with protease or buffer for 30 min before the assay procedure continued.

Further experiments using 8 and 4 EU of protease (Table 24) showed that with 8 EU the number of vibrios binding to the tissue segment decreased from 11.2×10^8 to 4.5×10^8 and with 4 EU from 11.2×10^8 to 8.15×10^8. Therefore, with 8 EU there was a 60% decrease in the number of vibrios binding but with 4 EU there was only a 27% decrease. Neuraminidase treatment of the mucosal surface had no effect on the binding of the vibrios as after neuraminidase treatment 10.2×10^8 vibrios were associated with the tissue comparable with 11.2×10^8 vibrios binding to the tissue following buffer treatment (Table 24). Protease (8 EU) plus neuraminidase and protease (4 EU) plus neuraminidase caused a decrease in the number of vibrios binding to 4.9 and 8.4×10^8 respectively. These figures are comparable to the protease alone at 8 and 4 EU which gave figures of 4.5 and 8.15×10^8, so protease plus neuraminidase did not enhance the effect of protease alone.

TABLE 24 EFFECT OF PROTEASE AND NEURAMINIDASE TREATMENT ON *V. cholerae* ADHERENCE TO THE MUCOSAL SURFACE OF ILEAL SEGMENTS *in vitro*

Expt.	*Vibrio cholerae* associated with the mucosal surface of the tissue segment ($\times 10^8$)					
	Borate Buffer 0.05M, pH9.0	Protease 8 $EUml^{-1}$	Protease 4 $EUml^{-1}$	Neuraminidase 0.05 $IUml^{-1}$	Protease 8 $EUml^{-1}$ + Neuraminidase 0.05 $IUml^{-1}$	Protease 4 $EUml^{-1}$ + Neuraminidase 0.05 $IUml^{-1}$
1	13.0	3.1	10.5	13.4	5.3	6.8
2	8.1	4.0	7.2	6.7	4.1	7.9
3	14.5	4.0	8.4	10.7	6.3	13.2
4	9.2	7.0	6.5	10.0	3.9	5.6
Mean	11.2	4.5	8.15	10.2	4.9	8.4

8. Protection Studies

8.1 Parenteral immunization

Initial experiments were done by giving guinea-pigs one i/m injection followed later by an ileal loop challenge using virulent V. cholerae. The fluid accumulation in the vaccinated and control animals was measured in $mlcm^{-1}$. The mean $mlcm^{-1}$ for each group of three guinea-pigs is shown in Table 25. The unvaccinated control guinea-pigs had a mean fluid accumulation value of 0.72 $mlcm^{-1}$. The fluid accumulation in the guinea-pigs vaccinated with protease, protease plus neuraminidase, enterotoxoid plus neuraminidase, protease plus enterotoxoid and enterotoxoid alone all showed lower values than the controls. However, the neuraminidase alone vaccinated guinea-pigs showed no decrease in fluid accumulation.

These results were expressed as histograms (Figure 30) to compare the mean fluid accumulation obtained with each vaccine preparation containing one of three adjuvants with the mean fluid accumulation in the unvaccinated control guinea-pigs. Decreased fluid accumulation was noticeable in Figures 30, A, B, C, D and F but not in Figure 30E. This suggested that no protection was afforded by the neuraminidase vaccination.

To consider whether any of the other vaccine preparations afforded significant protection in the guinea-pigs a t-test analysis was used. The fluid accumulation in each group of 3 vaccinated guinea-pigs was compared to the fluid accumulation in the control guinea-pigs to evaluate whether the decrease in fluid accumulation seen with the vaccinated guinea-pigs was statistically significant (Table 26). This

TABLE 25 FLUID ACCUMULATION IN LIGATED ILEAL LOOPS MEASURED AS $ml cm^{-1}$ FOLLOWING PARENTERAL IMMUNIZATION OF GUINEA-PIGS

Guinea-pig Numbers	Vaccine**	Adjuvant	Mean $ml cm^{-1}$*
1287-1295	Protease (G100/P)	F.C.A.	0.513
	80 µg	F.I.A.	0.453
		$Al.(OH)_3$	0.183
1296-1304	Protease (G100/P 80 µg	F.C.A.	0.186
	+ Neuraminidase 60 µg	F.I.A.	0.250
		$Al.(OH)_3$	0.316
1305-1313	Protease 80 µg +	F.C.A.	0.513
	Enterotoxoid 80 µg	F.I.A.	0.333
		$Al.(OH)_3$	0.456
1314-1322	Enterotoxoid, 80 µg	F.C.A.	0.270
		F.I.A.	0.393
		$Al.(OH)_3$	0.430
1323-1331	Neuraminidase, 60 µg	F.C.A.	0.623
		F.I.A.	0.736
		$Al.(OH)_3$	0.670
1332-1340	Enterotoxoid 80 µg +	F.C.A.	0.463
	Neuraminidase, 60 µg	F.I.A.	0.493
		$Al.(OH)_3$	0.563
1341-1345	Control Animals - no vaccination		0.720

** Concentration that each animal obtained

* Mean of three values, ie. three guinea-pigs in each group.

F.C.A. - Freunds complete adjuvant

F.I.A. - Freunds incomplete adjuvant

$Al.(OH)_3$ - Aluminium hydroxide gel.

FIGURE 30

HISTOGRAMS OF MEAN FLUID ACCUMULATION IN CONTROL GUINEA-PIGS AND GUINEA-PIGS VACCINATED PARENTERALLY

A. Vaccine component - Protease (80 μg)
B. " " - Protease (80 μg) + Neuraminidase (60 μg)
C. " " - Protease (80 μg) + Enterotoxoid (80 μg)
D. " " - Enterotoxoid (80 μg)
E. " " - Neuraminidase (60 μg)
F. " " - Neuraminidase (60 μg) + Enterotoxoid (80 μg).

C - control guinea-pigs (unvaccinated)

F.C.A. - Freunds complete adjuvant

F.I.A. - Freunds incomplete adjuvant

$Al(OH)_3$ - Aluminium hydroxide gel.

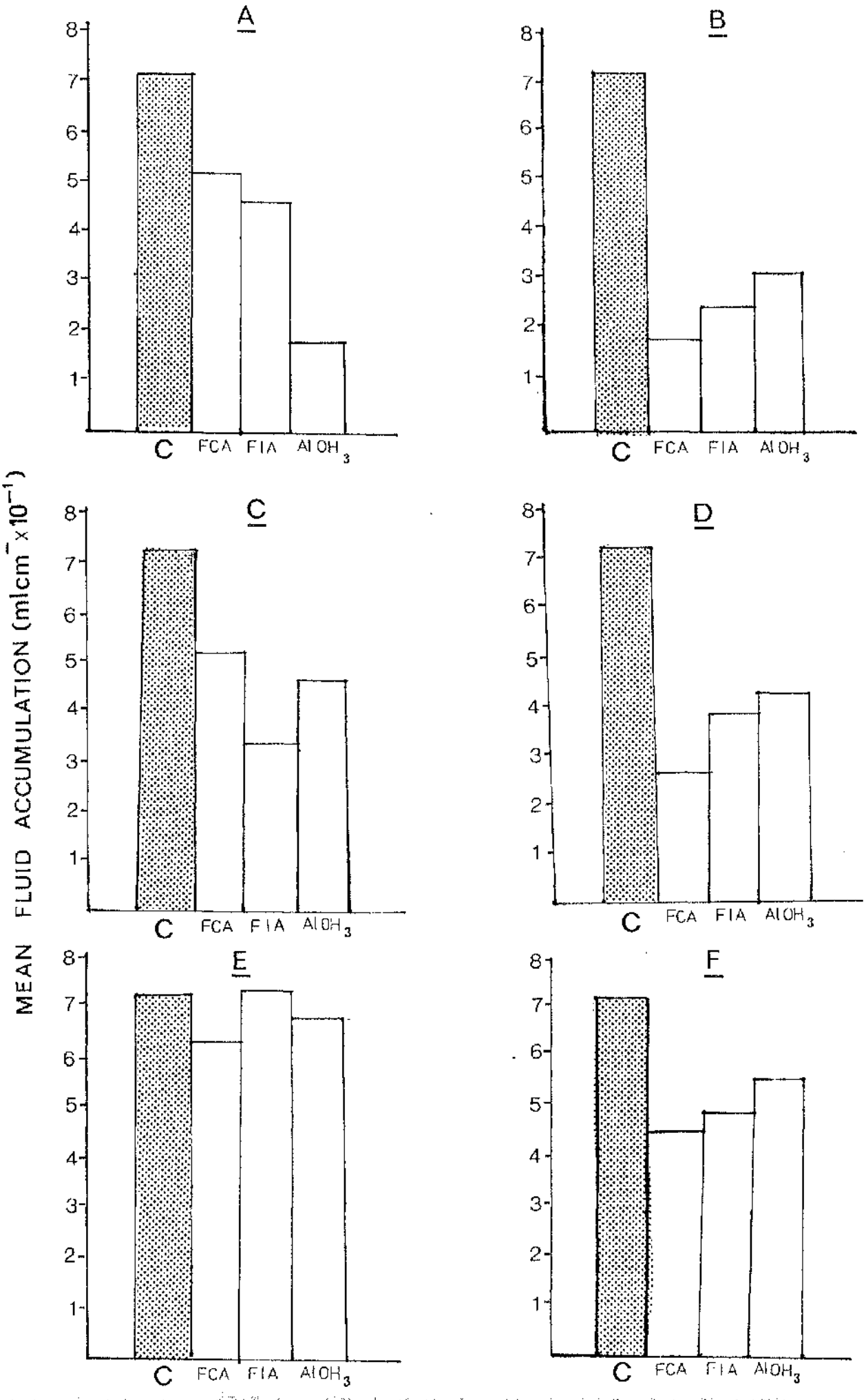

A
B
C
D
E
F
8
7
6
5
4
3
2
1
C
FCA
FIA
AlOH3
MEAN FLUID ACCUMULATION (mlcm⁻ x10⁻¹)

TABLE 26 STATISTICAL ANALYSIS OF PARENTERAL IMMUNIZATION OF GUINEA PIGS

Guinea-pig Numbers	Vaccine component	Adjuvant	P value	Significant Protection
1287-1295	Proteinase 80 µg	FCA	0.4	∓ -
		FIA	0.6	∓ -
		$Al(OH)_3$	0.005	+ +
1296-1304	Proteinase 80 µg + Neuraminidase 60 µg	FCA	0.001	+ + +
		FIA	0.001	+ + +
		$Al(OH)_3$	0.001	+ + +
1305-1313	Proteinase 80 µg + Enterotoxoid 80 µg	FCA	0.35	∓ -
		FIA	0.01	+ +
		$Al(OH)_3$	0.07	∓ -
1314-1322	Enterotoxoid 80 µg	FCA	0.01	+ +
		FIA	0.05	+
		$Al(OH)_3$	0.7	∓ -
1323-1331	Neuraminidase 60 µg	FCA	0.38	∓ -
		FIA	0.72	∓ -
		$Al(OH)_3$	0.75	∓ -
1332-1340	Neuraminidase 60 µg + Enterotoxoid 80 µg	FCA	0.06	+
		FIA	0.05	+
		$Al(OH)_3$	0.15	∓ -

∓ - not found to be protective

\+ - protective at $P \leqslant 0.05$ but $\geqslant 0.01$

++ - protective at $P \leqslant 0.01$ but $\geqslant 0.001$

+++ - protective at $P \leqslant 0.001$.

table shows clearly that only protease plus neuraminidase with all three adjuvants tested was found to be significantly protective, even although decreased fluid accumulation was seen in many other vaccine preparations they were not statistically significant.

8.2 Parenteral immunization followed by parenteral booster dose

Subsequently, animals were given an i/m injection of protease plus neuraminidase, neuraminidase alone or protease alone, followed three weeks later by an i/m booster dose. Two weeks later they were challenged with V. cholerae using the ileal loop method. Three concentrations of each vaccine preparation were used to try to find the optimal concentration for protective activity. The mean values for fluid accumulation in the guinea-pigs vaccinated with 20, 40 and 80 μg of protease are shown in Table 27. On comparing these values to the control value of 0.470 $mlcm^{-1}$ it was noticed that there was a slight decrease in fluid accumulation. Guinea-pigs vaccinated with 0.01, 0.02 and 0.04 I.U. of neuraminidase showed no decrease in fluid accumulation compared to the control guinea-pigs (Table 27). In some instances the mean fluid accumulation was higher than the 0.536 $mlcm^{-1}$ for the control guinea-pigs. A combination of both protease and neuraminidase (Table 27) showed a decrease in fluid accumulation with all concentrations tested, although the decrease was less with the lower concentration of protease (ie. 20 μg protease plus 0.02 I.U.neuraminidase).

These mean values for fluid accumulation in ligated ileal loops of guinea-pigs vaccinated with protease, neuraminidase and protease plus neuraminidase were displayed as histograms (Figure 31). Protease alone (Figure 31A) vaccination and booster dose showed decreased fluid accumulation in the guinea-pig ileal loop challenges for all three

TABLE 27 FLUID ACCUMULATION IN LIGATED ILEAL LOOPS, MEASURED IN $ml\,cm^{-1}$ FOLLOWING PARENTERAL IMMUNIZATION AND PARENTERAL BOOSTER DOSE OF PROTEASE (G100/P) AND NEURAMINIDASE IN GUINEA-PIGS

Guinea-pig Numbers	Vaccine *	Adjuvant	Mean $ml\,cm^{-1}$
1346-1354	Protease, 80 µg	F.C.A.	0.347
		F.I.A.	0.210
		$Al(OH)_3$	0.250
1355-1363	Protease, 40 µg	F.C.A.	0.457
		F.I.A.	0.317
		$Al(OH)_3$	0.247
1364-1372	Protease, 20 µg	F.C.A.	0.280
		F.I.A.	0.413
		$Al(OH)_3$	0.290
1373-1382	Controls - no vaccination		0.470
1383-1391	Protease, 80 µg plus Neuraminidase 0.02 I.U.	F.C.A.	0.213
		F.I.A.	0.170
		$Al(OH)_3$	0.205
1392-1400	Protease 40 µg plus Neuraminidase 0.02 I.U.	F.C.A.	0.515
		F.I.A.	0.170
		$Al(OH)_3$	0.350
1301-1309	Protease 20 µg plus Neuraminidase 0.02 I.U.	F.C.A.	0.400
		F.I.A.	0.160
		$Al(OH)_3$	0.580
1410-1418	Controls - no vaccination		0.622
1419-1427	Neuraminidase 0.04 I.U.	F.C.A.	0.580
		F.I.A.	0.730
		$Al(OH)_3$	0.670
1428-1436	Neuraminidase 0.02 I.U.	F.C.A.	0.410
		F.I.A.	0.603
		$Al(OH)_3$	0.656
1437-1445	Neuraminidase 0.01 I.U.	F.C.A.	0.625
		F.I.A.	0.530
		$Al(OH)_3$	0.653
1446-1454	Controls - no vaccination		0.536

* Concentration that each animal obtained in initial 0.5 ml injection and 0.5 ml booster dose.

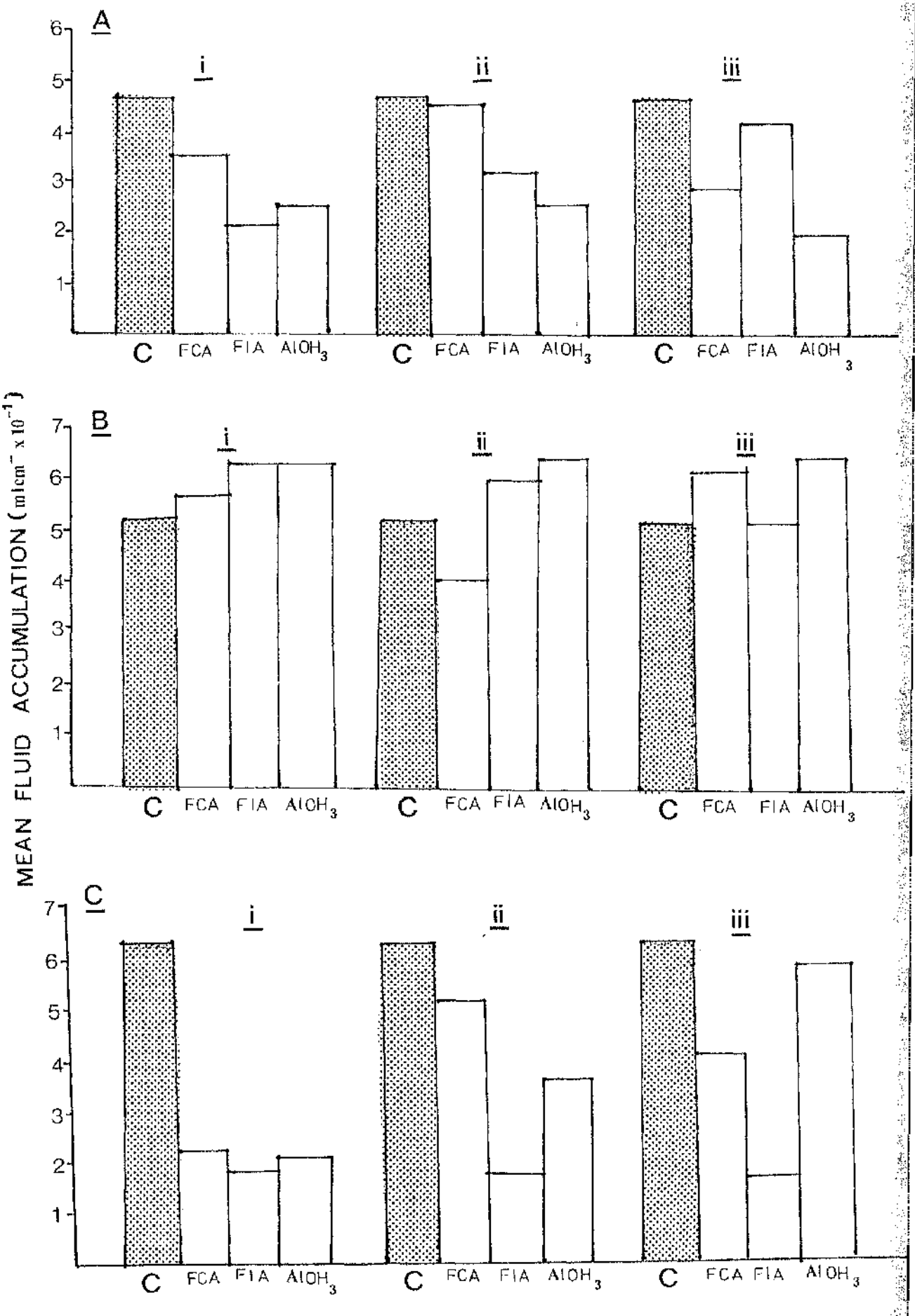
FIGURE 31 Histograms showing differences in fluid accumulation ligated ileal loops of control guinea-pigs and guinea-pigs given one parenteral vaccination and one parenteral booster dose

A (i) - vaccinated and boosted with protease, 80 µg
(ii) - " " " " " 40 µg
(iii) - " " " " " 20 µg

B (i) - vaccinated and boosted with neuraminidase, 0.04 I.U.
(ii) - " " " " " 0.02 I.U.
(iii) - " " " " " 0.01 I.U.

C (i) - vaccinated and boosted with protease, 80 µg and neuraminidase, 0.02 I.U.
(ii) - vaccinated and boosted with protease, 40 µg and neuraminidase, 0.02 I.U.
(iii) - vaccinated and boosted with protease, 20 µg and neuraminidase, 0.01 I.U.

C = control guinea-pigs (unvaccinated)
F.C.A. = Freunds complete adjuvant
F.I.A. = Freunds incomplete adjuvant
$Al(OH)_3$ = Aluminium hydroxide gel.


A
B
C
i
ii
iii
C
FCA
FIA
AlOH3
MEAN FLUID ACCUMULATION (ml cm⁻ x 10⁻¹)

concentrations used. The neuraminidase vaccination (Figure 31B) showed no decrease in fluid accumulation for any of the three concentrations tested. The combination vaccine of protease plus neuraminidase (Figure 31C) showed decreased fluid accumulation when compared to the mean control value, with 80 μg of protease and 0.02 I.U. of neuraminidase (Figure 31C(i)), the decrease in fluid accumulation is greater than for the two lower protease concentrations with the neuraminidase (Figure 31C(ii) and (iii)). This suggested that this combination was providing the best protection against V. cholerae challenge.

The results of a t-test analysis of the fluid accumulation in each vaccinated guinea-pig compared to the control guinea-pigs are shown in Table 28. This table shows quite clearly that neither the protease nor neuraminidase alone, used as vaccines, produced significant protection although the protease alone was shown to cause a decrease in fluid accumulation. The protease plus neuraminidase was, however, protective and highly significant protection was obtained with all three adjuvants containing 80 μg of protease and 0.02 I.U. of neuraminidase. 20 μg of protease plus the neuraminidase were found to be less protective than 80 μg of protease plus 0.02 I.U. of neuraminidase.

8.3 Parenteral immunization followed by an oral booster dose

The effect of an oral booster dose followed three weeks after an i/m injection was considered using the 80 μg of protease and 0.02 I.U. of neuraminidase and also 80 μg of protease alone. Two weeks after the oral booster dose the guinea-pigs were challenged with V. cholerae using the ileal loop method. The mean fluid accumulation in each group of guinea-pigs is shown in Table 29. Both the protease plus neuraminidase and protease alone showed decreased fluid accumulation when compared to

TABLE 28 STATISTICAL ANALYSIS OF PARENTERAL PRIMARY PLUS PARENTERAL BOOSTER IMMUNIZATION OF GUINEA-PIGS

Guinea-pig Numbers	Vaccine Component	Adjuvant	P value	Significant Protection
1346-1354	Proteinase 80 µg	F.C.A.	0.50	∓ -
		F.I.A.	0.15	"
		$Al(OH)_3$	0.18	"
1355-1363	Proteinase 40 µg	F.C.A.	0.90	"
		F.I.A.	0.45	"
		$Al(OH)_3$	0.20	"
1364-1372	Proteinase 20 µg	F.C.A.	0.30	"
		F.I.A.	0.75	"
		$Al(OH)_3$	0.30	"
1419-1427	Neuraminidase 0.04 IU	F.C.A.	0.80	"
		F.I.A.	0.12	"
		$Al(OH)_3$	0.35	"
1428-1436	Neuraminidase 0.02 IU	F.C.A.	0.40	"
		F.I.A.	0.60	"
		$Al(OH)_3$	0.30	"
1437-1445	Neuraminidase 0.01 IU	F.C.A.	0.60	"
		F.I.A.	0.90	"
		$Al(OH)_3$	0.40	"
1383-1391	Proteinase 80 µg + Neuraminidase 0.02 IU	F.C.A.	< 0.001	+ + +
		F.I.A.	< 0.001	+ + +
		$Al(OH)_3$	< 0.001	+ + +
1392-1400	Proteinase 40 µg + Neuraminidase 0.02 IU	F.C.A.	0.07	+
		F.I.A.	< 0.001	+ + +
		$Al(OH)_3$	< 0.001	+ + +
1401-1409	Proteinase 20 µg + Neuraminidase 0.02 IU	F.C.A.	0.10	∓ -
		F.I.A.	< 0.001	+ + +
		$Al(OH)_3$	0.60	∓ -

∓ - - not found to be protective
\+ - protective at $P \leqslant 0.05$ but $\geqslant 0.01$
++ - protective at $p \leqslant 0.01$ but $\geqslant 0.001$
+++ - protective at $P \leqslant 0.001$.

TABLE 29 FLUID ACCUMULATED IN LIGATED ILEAL LOOPS MEASURED IN $ml\,cm^{-1}$ FOLLOWING PARENTERAL IMMUNIZATION AND AN ORAL BOOSTER DOSE OF PROTEASE (G100/P) AND NEURAMINIDASE IN GUINEA-PIGS

Guinea-pig Numbers	Vaccination	Adjuvant	$ml\,cm^{-1}$
1455-1453	Protease (80 µg)	F.C.A.	0.503
		F.I.A.	0.280
		$Al(OH)_3$	0.325
1464-1472	Protease (80 µg)	F.C.A.	0.293
	+	F.I.A.	0.405
	Neuraminidase (0.02, I.U.)	$Al(OH)_3$	0.297
1473-1477	Controls - no vaccination		0.752

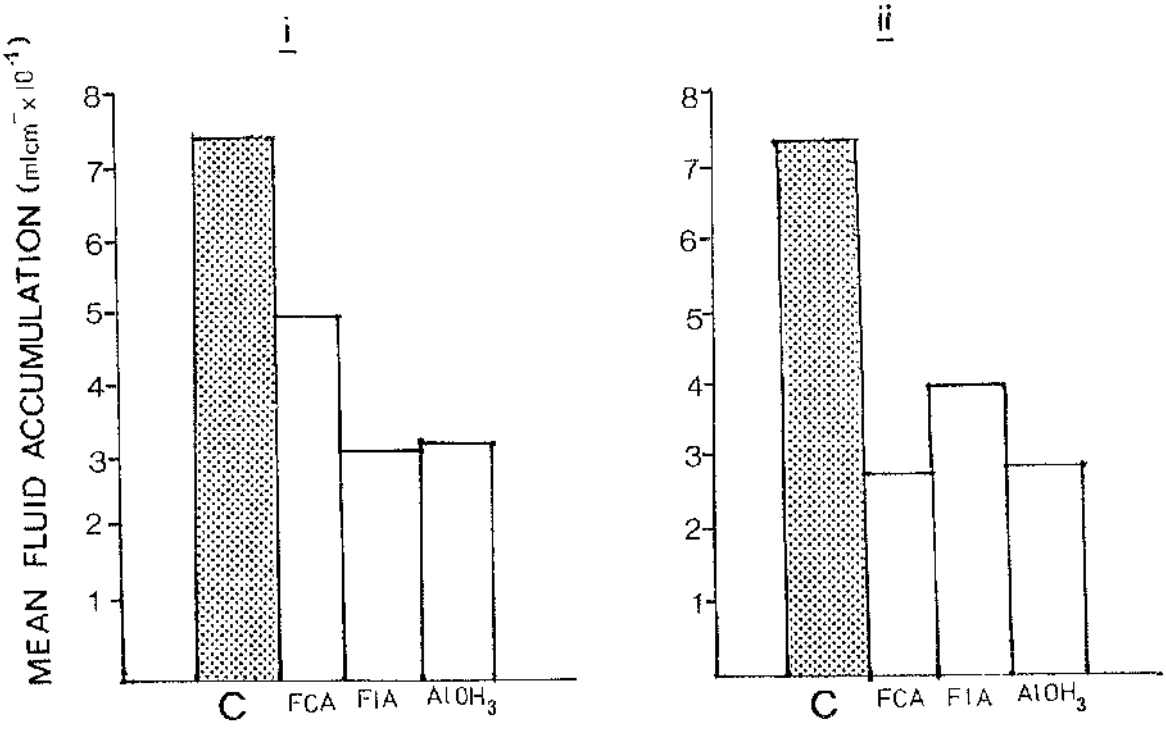
the control value of 0.752 $ml cm^{-1}$. Histograms of these values showed quite clearly this decrease in fluid accumulation (Figure 32). Statistically significant protection was found with all three adjuvants containing protease plus neuraminidase (Table 30) and in F.I.A. and $Al(OH)_3$ containing protease alone.

The protease and neuraminidase preparations used for the vaccines were tested for endotoxin and enterotoxin using the Limulus ameobocyte lysate (LAL) assay and the rabbit blueing dose assay respectively. The LAL assay showed that the protease preparation contained no detectable endotoxin (Table 31). The neuraminidase gave a positive result with a 1/10 dilution but a negative result with a 1/100 dilution. Since the vials are standardized to detect 0.25 $ng ml^{-1}$ of endotoxin, the neuraminidase contained somewhere between 2.5 - 25 $ng ml^{-1}$ of endotoxin.

Protease, 12.5 - 200 $\mu g ml^{-1}$ and neuraminidase, 0.025 - 0.5 $I.U. ml^{-1}$ showed no detectable enterotoxin by using the rabbit blueing dose assay.

FIGURE 32

Histograms of mean fluid accumulation in control guinea-pigs and guinea-pigs vaccinated parenterally followed by an oral booster dose



C - control guinea-pigs (unvaccinated)
F.C.A. - Freunds complete adjuvant
F.I.A. - Freunds incomplete adjuvant
$Al(OH)_3$ - Aluminium hydroxide gel

(i) - Vaccine component - protease (80 µg)

(ii) - Vaccine components - protease (80 µg) + neuraminidase (0.02 I.U.).

TABLE 30 PARENTERAL PRIMARY PLUS ORAL BOOSTER IMMUNIZATION OF GUINEA PIGS

Guinea-pig Numbers	Vaccine component	Adjuvant	P value	Significant Protection
1455-1463	Proteinase 80 µg	F.C.A.	0.2	∓ -
		F.I.A.	0.025	+
		$Al(OH)_3$	0.015	+ +
1464-1472	Proteinase 80 µg +	F.C.A.	0.01	+ +
	Neuraminidase	F.I.A.	0.005	+ +
	0.02 IU	$Al(OH)_3$	0.001	+ + +

∓ - - not found to be protective

\+ - protective at $P \leqslant 0.5$ but $\geqslant 0.1$

\+ + - protective at $P \leqslant 0.1$ but $\geqslant 0.001$

\+ + + - protective at $P \leqslant 0.001$.

TABLE 31 LIMULUS AMEBOCYTE LYSATE ASSAY ON PROTEASE AND NEURAMINIDASE USED IN VACCINE PREPARATION

SAMPLE	REACTION
Negative Control (pyrogen free water)	-
Positive Control	
10 $ngml^{-1}$	+
1 $mgml^{-1}$ *	+
Protease	
200 μgml^{-1}	-
20 μgml^{-1}	-
2 μgml^{-1}	-
Neuraminidase	
0.05 IU	+
0.005 IU	+
0.0005 IU	-
Inhibitor Control **	
+ Protease (200 µg)	+
Inhibitor Control **	
+ Neuraminidase (0.05 IU)	+

* *E. coli* endotoxin

\+ = solid gel formed

\- = no gel or clot formed

** = This must be positive for the assay to be viable.

DISCUSSION

DISCUSSION

The initial aims of the project were directed at the production and purification of V. cholerae proteases, the characterization of these enzymes and their role in adherence and as vaccine additives.

In 1981, at the beginning of this study, while there was a substantial literature on V. cholerae mucinase (Burnet and Stone, 1947; Singer, Wei and Hoa, 1948; Ada and French, 1957; Finkelstein and Lankford, 1955; Finkelstein et al, 1966) but almost nothing was published on the protease(s) of this organism. Protease activity was detected in the culture fluid of V. cholerae in 1970 (Hsieh and Liu, 1970) and there seems to have been little further interest for a decade. However, during the last two years of this research project it became apparent that several research groups have been active in this field (Schneider and Parker, 1982; Young and Broadbent, 1982; Finkelstein and Hanne, 1982; Finkelstein, Boesman—Finkelstein and Hanne, 1983; Booth, Boesman—Finkelstein and Finkelstein, 1983).

1. Production of enzymes

Extracellular protease production milligram^{-1} dry weight of cells was greatest when V. cholerae was grown in trypticase soy broth (BBL) (Table 7). When V. cholerae was grown in 2% (w/v) protease peptone (Difco) or in the minimal medium, syncase, this resulted in a slightly lower yield of protease. However, when the vibrios were grown in tryptic soy broth (Gibco) there was a 70% reduction in the protease activity milligram^{-1} dry weight of cells compared to that produced in trypticase soy broth (BBL). Young and Broadbent (1982) confirmed these

differences in protease production by V. cholerae grown in a variety of media. Protease production was greater in nutrient broth than in the minimal medium and the addition of glucose, although enhancing the growth rate of the organism, decreased the protease production in terms of specific enzyme activity. The composition, as stated by the manufacturers of trypticase soy broth (BBL) and tryptic soy broth (Gibco) (Appendix 1), show no major differences in composition, but in both instances the manufacturers state "this is a typical formula; each product lot is adjusted to compensate for variables in raw materials or supplemented to meet performance criteria". This suggests that there may be subtle differences in the two media although these are unknown. Also both products contain pancreatic digests of casein and papain digests of soyabean protein, the components of which may vary between the two media.

Protease production was detected from the early exponential phase of growth and reached a maximum level between 23-25 h (Figure 11). Maximum protease production during late stationary phase is not unique to V. cholerae. It is also true of Serratia marcescens (Lyerly and Kreger, 1979), Pseudomonas aeruginosa (Kreger and Gray, 1978) and Streptococcus sanguis (Straus, 1972). This late protease production is an in vitro characteristic and may not be true of the in vivo situation. It would therefore be interesting to determine when V. cholerae proteases are produced in vivo, as the time of protease production during in vivo colonization would help in understanding the role of proteases in the pathogenesis of cholera.

2. Purification of proteases

Gel filtration of the crude material through Sephadex G100

resulted in the loss of a great deal of contaminatory protein material as the major peak of protease activity (G100/P) did not coincide with the E_{280nm} peak (Figure 13). The SDS-PAGE of the crude material and G100/P (Figure 12, lanes 2 and 6 respectively) showed the loss of numerous bands after gel filtration leaving only five distinct peptide bands. Following gel filtration the specific activity of the crude protease increased from 17.2 to 120 EU ml^{-1} mg protein (Table 9) which indicated that this purification step had increased the specific activity of the preparation some seven-fold. The G100/P was also shown to have mucinase and haemagglutinating activities (Tables 16 and 17 respectively).

The presence of mucinase activity in the protease preparation was confirmed by the recent work of Schneider and Parker (1982), who found that their mucinase was also a protease. Various authors have suggested that mucinase, although not diarrhoeagenic (Burnet and Stone, 1947; Freter, 1955; Lam, Mandle and Goodner, 1955) may play a role in the pathogenesis of cholera. The mucinase activity is likely to be important in facilitating vibrio penetration of the mucous layer to reach buried epithelial cells on the brush-border surface.

The G100/P was shown to have low levels of haemagglutinating activity with a maximum reciprocal titre of 16.0 obtained with sheep red blood cells. Neither the use of chicken red blood cells nor the presence of calcium ions enhanced the titre of haemagglutinating activity which other workers (Finkelstein and Hanne, 1982) found increased haemagglutinin titres.

Finkelstein and Hanne (1982) purified the "soluble" haemagglutinin of V. cholerae and found that it had significant protease activity. Although the titre of haemagglutinating activity obtained

with the G100/P might be considered to be low it compares favourably with the titres obtained by Finkelstein and Hanne (1982) using the same red blood cells, with the "soluble" haemagglutinin. However, Hanne and Finkelstein (1982) found that mixed glycosidase treatment of chicken red blood cells made them more responsive to haemagglutination, presumably by the creation of more receptors on the cell surface by their enzyme action.

Isoelectric focussing of the crude protease produced three peaks of protease activity, IEF-1 (pI of 4.5-5.5), IEF-2 (pI of 6.5) and IEF-3 (pI of 9.0). Batch and fermenter-grown cultures both produced good yields of protease. Although their gel filtration elution profiles were similar to that shown in Figure 13, it was noticeable that their isoelectric focussing elution profile showed differences in the proportions of IEF-1 and IEF-2. The fermenter-grown cultures showed lower levels of IEF-1 than the batch-grown cultures. The only difference in the fermenter vessel from the 2 litre flasks used for batch production was the aeration rate; the temperature and time of growth were similar. This slightly higher aeration rate may have increased the autocatalytic activity of the protease resulting in the loss of protease activity in IEF-1. Autodigestion is a common property among proteases (Schroader and Shaw, 1968) and is the main reason for their instability at room temperature and the need to store the proteases at -20^{o}C (Table 8).

SDS-PAGE of IEF-1, IEF-2 and IEF-3 (Figure 17, lanes 5, 4 and 3 respectively) showed a considerable degree of purification when compared to the crude protease (Figure 17, lane 2). Purification of the crude protease following isoelectric focussing was also apparent from the levels of specific enzyme activity for the crude protease compared to the

IEF-proteases; IEF-1 and IEF-2 both showed a ten-fold increase in specific activity and IEF-3 showed a seven-fold increase in specific activity.

The IEF-1, IEF-2 and IEF-3 protease fractions were tested for mucinase and haemagglutinating activity. Neither activity was detected in the IEF-1 and IEF-2 fractions but IEF-3 showed both mucinase and haemagglutinating activity. Again, as for G100/P, the haemagglutinating activity in IEF-3 was low with a titre of 8.0.

IEF-3 possessed protease, mucinase and haemagglutinating activities. This fraction appears to be very similar to the "soluble" haemagglutinin recently described by Finkelstein and Hanne (1982) since this also had haemagglutinating, mucinase and protease activities.

The discovery that a cholera protease had haemagglutinating activity is reminiscent of the haemagglutinin-neuraminidase protein spikes of paramyxoviruses (Schneid and Choppin, 1974) and the haemagglutinin-neuraminidase protein of Cl. perfringens (Rood and Wilkinson, 1976a,b). One can only speculate as to why attachment and enzyme functions are present in a single protein in some pathogens. Paramyxoviruses, Cl. perfringens and V. cholerae share a common feature in that they can parasitize epithelial surfaces. Several authors have suggested that such parasites use a dual function protein both to hydrolyze the surface mucus layer and to interact with it to prevent being cleared from the intestine or respiratory tract.

Further attempts to purify individual proteases could possibly be achieved by raising monoclonal antibodies to each peptide band in the SDS-PAGE. These monoclonal antibodies could then be used in an immunosorbent column to separate single proteases. Although this would

allow purification of crude material, with no initial purification step being necessary, it would be cost inhibitory for large-scale production. Therefore, using an immunosorbent column, although useful in basic research, would not be as important for vaccine production due to the high cost.

Less expensive procedures for the isolation of proteases might be considered for vaccine production since the work of this thesis has shown that even isoelectric focussing does not yield individual proteases. As discussed later positive protection was achieved with a mixture of proteases (see page 185) and therefore a partially purified preparation of proteases may be sufficient.

The "soluble" haemagglutinin isolated by Finkelstein and Hanne (1982) was shown to exhibit hydrophobic properties similar to other microbial adhesins (Robertson, Vincent and Ward, 1977; Smyth et al, 1978; Wadström et al, 1979). Since the "soluble" haemagglutinin has similar properties to cholera proteases, especially IEF-3, the use of a hydrophobic matrix such as phenylsepharose could also be useful in further purification attempts.

Affinity chromatography, using haemaglobin-Sepharose, may be useful as this has been used in the purification of wheat proteases and proteases of Nocardia brasiliensis (Chua and Bushuk, 1969; Zlotnik, Schramm and Buckley, 1984).

3. Biochemical Properties of Cholera Proteases

The small intestine has a pH range of 6.5 at the upper end of the duodenum to 8.0 at the lower end of the ileum, with an average pH of 7.2. The V. cholerae crude protease had an optimum activity at pH

8.9-9.0, markedly higher than the pH of the small intestine. It was shown (Figure 12) that the crude protease had an activity of 30 $EUml^{-1}$ at pH 7.2 and 36.5 $EUml^{-1}$ at pH 9.0. This showed only an 18% decrease in activity from pH 9.0 to 7.2. Therefore, cholera proteases would still be highly active in the small intestine, although not at the experimental optimum pH for the activity.

In view of the variety of protease activities it is often difficult to characterize these enzymes biochemically. A single protease may display heterogeneity in molecular weight and charge, this may be due to autocatalytic degradation during purification. A single enzyme may also display multiple enzyme activities which represent catalytically identical but electrophoretically heterogeneous enzymes.

Peptide digest analysis was used to compare the specific catalytic activities of IEF-1, IEF-2 and IEF-3. The three proteases were shown to have different peptide patterns after SDS-PAGE of a 2 h digest of BSA by each of the proteases (Figure 21). This suggested that each of the IEF proteases had different catalytic specificities.

The presence of multiple activities in each of the IEF proteases, G100/P and the crude protease was shown by gelatin-PAGE (Figure 20). Two bands of protease activity, A and B, were present in IEF-2 and IEF-3. IEF-1 also showed these activities plus one other protease activity, band C. The crude protease showed activities A, B and C plus a protease activity, band D, which was not present in any of the semi-purified preparations. The loss of activity D during purification may have been due to autocatalytic degradation. The gel filtration elution profile of the crude protease (Figure 13) showed one major peak of protease activity (peak B, G100/P) and one minor peak,

peak A. The latter was not investigated as it coincided with the E_{280nm} peak which contained a lot of contaminating material. This meant that further purification of peak A would have resulted in a very low yield. Therefore, the crude preparation of protease contained this peak A protease which was absent from all semi-purified proteases and so this may be equivalent to the band D component which had protease activity shown by the gelatin-PAGE.

The presence of several protease activities in the gelatin-PAGE may be due to the presence of several distinct catalytic proteases in each preparation or a single enzyme has produced multiple protease activities which represent a catalytically identical protease that is showing electrophoretic heterogeneity due to autocatalytic degradation.

The use of inhibitors to classify further the IEF proteases suggested that more than one type of protease was present in each preparation. This conclusion was reached as IEF-1 was inhibited by the known metalloprotease inhibitors, 8-HQ and EDTA, and also by serine protease inhibitors. IEF-2 was inhibited by metalloprotease inhibitors, serine protease inhibitors and a sulphydryl protease inhibitor. IEF-3 was only inhibited by metalloprotease inhibitors (Table 14).

It is not certain that one protease can be inhibited by more than one class of inhibitor (Dr. Michael North, personal communication). This suggests that more than one protease is present in IEF-1 and IEF-2.

An unexpected finding was that the three serine protease inhibitors soyabean trypsin, anti-trypsin and egg-white anti-trypsin, did not all inhibit IEF-1 and IEF-2. Eggwhite anti-trypsin and anti-trypsin inhibited IEF-1 but only anti-trypsin inhibited IEF-2. This appears to be a rather unusual phenomenon and may be due to the different action of the

inhibitors on the proteases. Perhaps the optimum concentration of inhibitor and protease was not used which may have produced this anomaly.

The results from the inhibition studies confirm the presence of several proteases in IEF-1 and IEF-2 and disagrees with the idea of a single enzyme which produced multiple activity bands due to auto-catalytic degradation. However, the results obtained for IEF-3 disagree with this conclusion. IEF-3 has at least three activity bands on gelatin-PAGE, three peptide bands on SDS-PAGE, but was only inhibited by inhibitors specific for a metalloprotease. This suggests the presence of only one catalytic enzyme with electrophoretic heterogeneity or the presence of several metalloproteases in IEF-3.

Table 32 was compiled to allow comparison of the biochemical properties of the proteases isolated in this study with recently published work. This table shows quite clearly the complicated nature of the proteases. Comparisons can be made between the proteases although different strains and purification procedures were used by each group of workers. The most common feature appears to be a protease with an alkaline pH optimum and the inhibition by metallo-protease inhibitors.

The number of inhibitors used in this study were three times as many as used by Schneider and Parker (1982) or Young and Broadbent (1982) which is likely to account for the more complicated inhibition pattern seen. Perhaps if the other workers had used more inhibitors they would have found the presence of more than one protease in their preparations.

Schneider and Parker (1982) used ammonium sulphate precipitation followed by gel filtration for purification of their proteases. They were therefore working with a protease preparation similar to G100/P used in

TABLE 32 COMPARISON OF THE BIOCHEMICAL PROPERTIES OF *V. cholerae* PROTEASES

Reference	Strain	Growth Conditions	Purification Procedures	Protease Nomenclature	Molecular weight (k.Dal)	pH optima	Protease Inhibitors Inhibited by:
This Thesis	*V.cholerae* 10732	37°C, 24 h Fermenter or shaking. Trypticase soy broth.	85% $(NH_4)_2SO_4$ Sephadex G100 Isoelectric-focussing	G100/P, Pooled G100 protease IEF-1, pI 4.5-5.5. IEF-2, pI 6.5. IEF-3, pI 9.0.	G100/P, Sephadex: 21.5-31 PAGE: 42-57	9.0	IEF-1, Metallo-Serine IEF-2, Metallo-Serine Sulphydryl IEF-3, Metallo
Schneider and Parker (1982)	*V.cholerae* CA401	37°C, 24 h Shaking Syncase	70% $(NH_4)_2SO_4$ BioGel P100 or Sephadex G100	Fraction 3 Fraction 4	BioGel; Fraction 3-38 " 4-18 Sephadex: Fraction 3-22 " 4-10 PAGE: Fraction 3-36	9.0	Fraction 3, Metallo
Young and Broadbent (1982)	*V.cholerae el tor* 1621	37°C. No time stated Shaking Nutrient broth	Concentration (no method stated) Isoelectric focussing	I pI 4.2 II " 5.9	N.S.	I 8.0 II N.S.	I: Serine IIA) Not IIB) inhibited by any tested.
	V.cholerae el tor 1621 hip	As above	As above	IIA pI 4.7 IIB " 5.9 IIIA " 8.9 IIIB " 9.2	34.5 36 43	IIA 8.5 IIB 8.0 IIIA 6.0-6.5 IIIB 9.0-9.5	IIIA) IIIB) Metallo.
Finkelstein and Hanne (1982)	*V.cholerae* CA401	37°C, 24 h Fermenter (9L) Trypticase soy broth	PM 10 25-50% $(NH_4)_2SO_4$ Sephadex G75 Isoelectric focussing	pI 6.3 pI 5.3 pI 4.7	32)) N.S.	N.S.	N.S.

N.S. = not studied

this study. It seems probable that their protease preparation contained a mixture of proteases which would have been noticed if further purification was attempted.

4. Immunological Properties

The production of antisera against IEF-2 and IEF-3 appeared not to be successful. Cholera proteases are very unstable in culture supernates and may lose virtually all activity in a matter of hours at room temperature. The prolonged incubation required for the gel filtration technique may result in loss of antigenicity of protease, perhaps by autodegradation. This may explain why no antiserum was detected against IEF-2 and IEF-3.

In an effort to counteract autodigestion, 25 mM mercuric chloride was incorporated into the gel diffusion medium. This however had no effect and no reactions to IEF-2 or IEF-3 were detected with immune rabbit sera. Specific antibodies against IEF-1 and crude protease were detected by this technique which would seem to disagree with the phenomenon of autodigestion causing loss of antigenicity of the proteases in IEF-2 and IEF-3.

It is noteworthy that IEF-2 and IEF-3 had higher specific enzyme activities than IEF-1 or the crude protease. The sensitivity of the gel diffusion technique is not high, therefore, if the protease has a very high specific activity it may possess equivalent enzymatic activity but insufficient antigenicity to react in the Ouchterlony test.

5. Properties that may be associated with the *in vivo* colonization of *V. cholerae*

The mucosal secretory immune system is dominated by the secretion of antibody of the IgA isotype. Lubricating fluids issuing from mucous membranes contain relatively large amounts of this immunoglobulin in comparison to the amounts of IgG and IgM (Tomasi, 1972). In human beings, approximately 80% of all antibody secreting plasma cells within mucosal tissues synthesize IgA. The precise mechanism by which antigen disposal at mucosal sites takes place is unclear because secretory IgA apparently does not utilize complement for disposal of antigens and is not opsonic (Wilson, 1972; Van Epps and Williams, 1976). However, it has been suggested that secretory IgA functions by preventing adherence of bacteria to relevant tissues (Williams and Gibbons, 1972; Brandtzaeg, 1977). Since such adherence is a pre-requisite to colonization and disease, in theory, inhibition would be an adequate means by which secretory IgA could function in mucosal defence. Polymorphonuclear leukocytes and lymphocytes have been shown specifically to bind IgA proteins (Lawrence, Weigle and Spiegelberg, 1975), although the identification of these cellular receptors has as yet not yielded a coherent explanation for the role of cellular elements in the effector functions of IgA antibodies.

Many microbial pathogens have been shown to possess a proteolytic activity that can specifically digest IgA. These enzymes have been termed IgA proteases (see page 43). These typical IgA proteases cleave IgA1 at the hinge region of the molecule leaving intact Fc and Fab fragments (Figure 6). The *V. cholerae* proteases did not appear to yield

intact Fc and Fab fragments of IgA by their proteolytic activity as shown by immunoelectrophoresis (Figure 23). A typical IgA protease would yield fragments which would produce two distinct arcs after immunoelectrophoresis and interaction with specific antiserum. This was not found in this study with the various cholera proteases and it was concluded that none of the isolated proteases were typical IgA proteases. However, the cholera protease did effect some digestion of IgA although this did not destroy the antigenicity of the molecule as a single, shortened immunoelectrophoretic arc was observed. Previously, when trypsin and chymotrypsin were used to cleave IgA proteins an intact Fc fragment was not found as a product of digestion but instead they caused a progressive non-specific degradation of the Fc region of IgA, thereby rendering the IgA antigenically undetectable (Kornfield and Plaut, 1981). The digestion of IgA by cholera proteases was shown to be a very specific event, as shown by SDS-PAGE (Figure 22). The fact that cholera proteases do digest IgA in a specific manner suggests that this activity may be of importance in the evasion of the host mucosal secretory immune response.

A recent paper by Finkelstein, Boesman—Finkelstein and Holt (1983) has confirmed these conclusions. They demonstrated some 10% degradation of IgA by their soluble haemagglutinin/protease using ^{125}I-labelled IgA. They also concluded that the enzyme was clearly not identical to the typical IgA proteases but it was not clear whether there may be some effect on the antigenic binding site of IgA. It is noteworthy that Schneider and Parker (1982) alluded to the observation that V. cholerae mucinase was also active on immunoglobulins.

Polymorphonuclear leukocytes (PMN) play an important role in host defence against many infections. During the process of phagocytosis, PMN undergo a remarkable oxidative metabolism, resulting in emission of light or chemiluminescence (Allen, Stjernholm and Steel, 1972).

Ps. aeruginosa produces an alkaline protease and an elastase which have been shown to be involved in pathogenesis (Holder and Haidaris, 1979; Jagger, Bahner and Warren, 1983). Kharazmi et al (1984a) demonstrated that both the alkaline protease and the elastase were capable of inhibiting neutrophil chemotaxis and also interfering with the oxidative metabolism of these cells. Kharazmi et al (1984b) also showed that the alkaline protease and elastase were capable of inhibiting myeloperoxidase-mediated chemiluminescence.(*) These findings provided further evidence for the role of Ps. aeruginosa exoproteases as virulence factors.

It would be very interesting to determine if V. cholerae proteases have any effect on the PMN chemotaxis or the oxidative metabolism of these cells.

Lactoferrin is a host iron-binding protein found in mucosal secretions and has been assumed to play a role in host defence by withholding essential iron and thus reducing its availability to microbes in mucosal secretions (Bullen, 1981). The cholera proteases isolated in this study did not degrade lactoferrin (Figure 25) which contradicts the recent paper of Finkelstein, Boesman-Finkelstein and Holt (1983) who found their soluble haemagglutinin/protease degraded lactoferrin. They suggested that this may be a mechanism to counter iron sequestration by lactoferrin, although whether this digestion would have any effect on the iron binding capacity of lactoferrin is not known.

(*) (Rosen and Klebanoff, 1976; Webb, Keele and Johnston, 1974; Bender and VanEpps, 1983.)

Fibronectin is an adhesive high molecular weight glycoprotein that is present on the surface of mammalian cells (Yamada and Olden, 1978) and was shown to be synthesized by cells of the crypt of Lieberkühn in the intestine (Quaroni, Isselbacher and Ruaslahti, 1978). Whereas fibronectin is believed to facilitate attachment of Gram-positive bacteria to host cells and to participate in opsonization (Simpson, Courtney and Beachy, 1982), it has been reported to interfere with the adherence of Ps. aeruginosa to epithelial cells (Woods et al, 1981). The cholera protease(s) were found to degrade fibronectin after only 1 h, and further digestion occurred after 24 h (Figure 24). Finkelstein, Boesman-Finkelstein and Holt (1983) have recently stated that their soluble haemagglutinin/protease digested fibronectin, although their gel photographs of the digests were not convincing. In the published result electrophoretic bands were not readily detectable in the untreated fibronectin.

Woods et al (1981) presented evidence that suggested that trypsin-like proteases of Ps. aeruginosa facilitate attachment of these organisms to buccal epithelial cells by their action on host cell surface fibronectin. The fact that V. cholerae protease(s) have a fibronectinase activity does not permit conclusions as to whether the fibronectinase (a) is either responsible for the binding of the vibrios to the mucosal surface or possibly their subsequent detachment (Nelson, Clements and Finkelstein, 1976), (b) enables the vibrios to penetrate the fibronectin layer and then attach to another subtrate, or (c) is irrelevant to vibrio colonization of the intestinal epithelium. The V. cholerae G100/P and IEF-3 proteases were shown to have mucinase activity and, as has been mentioned, this is likely to be of importance

in the initial colonization of the small intestine by facilitating vibrio penetration of the mucous layer. Antibodies to mucinase have been reported in persons convalescing from cholera although formal cholera vaccine does not induce antibodies to mucinase. This suggests that the protease/mucinase is involved in pathogenesis.

The binding of the B-subunit of cholera enterotoxin to GM_1 gangliosides on the epithelial surface is a prerequisite for the insertion of the A-subunit into the cell membrane (Figure 3). This causes an increase in cyclic AMP by the activation of adenylate cyclase (Figure 4), the secretion of chloride ions into the bowel lumen and a reduction in the adsorption of sodium which leads to a net fluid deficit in the human body. Neuraminidase has been suggested to be involved in the binding of enterotoxin to the cell membrane (Durikhin, Popova and Korinskii, 1976). In this study neither protease, neuraminidase nor protease plus neuraminidase enhanced the binding of cholera toxin to ileal segments *in vitro* (Table 25).

E. coli heat-labile enterotoxin, which is related to cholera enterotoxin, was shown to produce two peptide fragments and have increased activity following trypsin treatment (Rappaport *et al*, 1976). Finkelstein, Boesman-Finkelstein and Holt (1983) found that their soluble haemagglutinin/protease was capable of nicking the A-subunit of the heat-labile enterotoxin of *E. coli*. Many authors (Young and Broadbent, 1982; Schneider and Parker, 1982; Finkelstein, Boesman-Finkelstein and Holt, 1983) have suggested that cholera enterotoxin may be activated in a similar manner to the *E. coli* enterotoxin but no work has been published which either agrees or disagrees with this suggestion.

The intact A-subunit was shown to be susceptible to proteolytic

cleavage producing A_1 and A_2 fragments of 21-24 k.Dal. and 5-7 k.Dal. respectively (Gill and Rapport, 1979). The cholera protease(s) were found to nick the A-subunit at a specific site producing one polypeptide with a very similar molecular weight to the intact A-subunit and one very low molecular weight peptide. These were comparable to the A_1 and A_2 fragments described by Gill and Rapport (1979). This proteolytic action appeared to increase the activity of the toxin but other factors present in the crude toxin may have been responsible for this effect. Protease activity of V. cholerae may be an important factor in the pathogenesis of cholera. It would be interesting and important to continue this work in an in vivo system. Ileal loops could be treated with the enzyme preparations and then injected with a known amount of toxin, any differences in fluid accumulation between treated and untreated loops could be compared. This in vivo approach would give valuable information on the involvement of proteases during cholera infection.

6. Adherence to the intestinal epithelium

Initially, when ileal tissue segments, either one square centimetre or cylinders of tissue, were treated with protease, neuraminidase or protease plus neuraminidase there was no demonstrable effect on the binding of V. cholerae organisms to the surface layer (Tables 19 and 21). Subsequent experiments showed that this was due to the fact that the V. cholerae organisms could bind to both the mucosal and serosal surface of the tissue. Vibrio binding to the serosal surface of ileal segments was shown by the experimental work with discs of ileal tissue exposed at the end of a specially prepared syringe (Table 23).

Of the number of vibrios in a suspension that adhered to the

mucosal surface, an equivalent 24% of this number also adhered to the serosal surface. Adherence of vibrios to the serosal surface was noticed also by Attridge and Rowley (1983a,b) but they found that some vibrio strains were able to adhere equally well to both the mucosal and serosal surface of the ileum. The tissue preparation used by Attridge and Rowley was the cylinder method initially used in this study, everted cylinders were used for mucosal adherence and non-everted segments were used to measure serosal adherence. This means that in both everted and non-everted tissue segments the mucosal and serosal surfaces of the tissue were still actually available for vibrio binding. Highly motile strains were used by Attridge and Rowley (1983a) and although one tissue surface was not always directly available for vibrio binding, it seems probable that these highly motile strains would be able to bind to either surface. This may be the reason why they found that some vibrio strains would adhere equally well to both mucosal and serosal surfaces whereas in the present study comparatively fewer organisms adhered to the serosal surface than the mucosal surface.

Although no difference was determined in the number of vibrios directly adhering to the epithelial cells of everted cylindrical tissue segments following protease treatment, it was noticed that following protease treatment of the tissue segment, the number of vibrios present in the washes of the adherence assay were greater than the number of vibrios present in the washes used for untreated tissue segments. Since the washings remove mucus and loosely bound vibrios, it seems likely that the protease treatment facilitated the movement of the vibrios through the mucus. This would agree with the finding that the protease had mucinase activity which is likely to aid movement of the vibrios through the

mucous gel apposed to the brush-border surface. To try to consider this movement through the mucus in more detail, paraformaldehyde vapour fixation was used to retain the mucus on the epithelial surface. However, this method of fixation was not used as the tissue segments were hardened and, although used in *in vitro* adherence assays, would be unrepresentative of the natural state.

Protease treatment caused a decrease in the number of vibrios binding to the mucosal surface but a proportional increase in the numbers associated with the serosal surface (Table 23). This explains why the protease produced no overall effect when both the mucosal and serosal surfaces were present in the assay system, ie. with one square centimetre and cylindrical tissue segments.

It might be argued that it would be disadvantageous to *V. cholerae* as a pathogen to elaborate a soluble protease that blocks attachment. Nelson, Clements and Finkelstein (1976) showed that cholera vibrios detach from the brush-border surface and suggested that this was due to enzyme action. This phenomenon of detachment may be an adaptation of *V. cholerae* to the parasitic mode of life allowing the vibrios to multiply and spread. If the cholera protease(s) was involved in the detachment from the brush-border surface this would be advantageous to the spread of the disease.

Partially purified soluble haemagglutinin, or "cholera lectin" as it was termed in the study of Finkelstein *et al* (1978), was isolated from an Inaba serovar. The "cholera lectin" was found to inhibit attachment of Ogawa serovars and *el tor* biovars in the infant rabbit. It was presumed that the haemagglutinin acted by occupying, or otherwise affecting the receptors on the surface of the epithelium. Finkelstein,

Boesman--Finkelstein and Holt (1983) also found that soluble haemagglutinin/protease inhibited adherence to the intestinal epithelium.

The cholera protease was isolated from late exponential phase cultures at a time when significant lysis and release of membrane fragments was occurring. To determine if the soluble nature of the protease was a peculiarity of *in vitro* cultivation, it was decided to assay *V. cholerae* cultures for cell-associated protease and also cell-associated haemagglutinating activities. Both protease and haemagglutinating activities were found in a cell-associated form but *V. cholerae* does produce at least four haemagglutinins (Finkelstein and Hanne, 1982), some of which would be expected to be present on the vibrio cell surface as three are cell-associated haemagglutinins.

It is conceivable that protease (which contains haemagglutinating activity) treatment of tissue segments is "masking" receptor sites which would otherwise be available for vibrio binding and so a decrease in the number of vibrios binding to the brush-border surface following protease treatment would be expected. The continuation of these adherence studies in an *in vivo* system is now necessary and perhaps a time-scale study of protease production with vibrio adherence *in vivo* would yield some important information.

It seems possible that the cholera protease(s), which has mucinase and haemagglutinating activity, serve a dual function of indirectly promoting attachment and detachment of *V. cholerae* to the intestinal brush-border surface. The time of production of *V. cholerae* proteases *in vivo* would confirm this hypothesis.

7. Protective activity of *V. cholerae* proteases

The G100/P mixture, hereinafter named protease, used in the vaccines contained a number of proteases and was haemagglutinating.

Active protection tests were initially carried out by giving one i/m injection of candidate vaccine to guinea-pigs (Table 25). A combination of protease plus neuraminidase was in all three adjuvants, protective against ileal loop challenge with virulent *V. cholerae*. This was rather unexpected so further experiments were done by giving one i/m injection followed by an i/m booster dose for protease, neuraminidase and protease plus neuraminidase at three different concentrations (Table 27). Once again neither protease nor neuraminidase alone elicited protection in guinea-pigs against ileal loop challenge. However, protease plus neuraminidase produced highly significant levels of protection against challenge with *V. cholerae*, with maximum protection in animals injected with 200 µg of protease and 0.05 I.U. of neuraminidase.

An ideal cholera vaccine would be one which stimulated prolonged antibody production at the mucosal surface of the intestine. However, results have indicated that oral or enteral vaccination is inferior to parenteral administration of vaccines, and requires multiple applications before a reasonable level of protection is obtained (Holmgren *et al*, 1975; Agarwal and Ganguly, 1972).

It was therefore decided to give guinea-pigs an initial parenteral immunization followed by an oral booster dose. Protease, 200 µg, plus neuraminidase, 0.05 I.U., and protease, 200 µg, alone were used for vaccine preparations (Table 31). Protease plus neuraminidase showed highly significant protection and for the first time protease alone was found to provide an effective vaccine.

Interestingly, Pierce and Gowans (1975) found that a maximum increase in the number of antitoxin-containing cells resulted when oral

immunization was preceded by a parenteral inoculation two weeks earlier. These workers suggested that the primary inoculation established immunological memory in the secretory IgA system of the intestine so that subsequent oral applications of antigen acted as a "booster".

In vivo, V. cholerae neuraminidase probably works synergistically with the protease to degrade epithelial mucins. Although neuraminidase was absent from culture fluids used for the purification of protease(s), it is possible that the protease(s) (mucinase/haemagglutinin) and neuraminidase are produced synchronously in vivo to form a "mucinase complex". The close association between the two enzymes is also shown by the fact that 90% of protease-deficient mutants isolated by Schneider and Parker (1978) were also deficient in neuraminidase. Neuraminidase mutants also showed a predominance of similar double neuraminidase-protease mutants suggesting that expression of protease and neuraminidase is inter-related. This may explain the requirement for both enzymes in a protective vaccine.

Active protection, using a protease vaccine, was also shown by Lyerly and Kreger (1983). They found that vaccination with a serratia protease stimulated active protection against experimental lethal serratia pneumonia.

It is noteworthy that neither the V. cholerae mucinase described by Schneider and Parker (1982) nor the haemagglutinin/protease described by Finkelstein and Hanne (1982) have been used in active protection tests. However, passive immunization with antibodies against mucinase or haemagglutinin/protease have been shown to be protective in the infant mouse model. Therefore, for the first time cholera protease(s) (mucinase/haemagglutinin) has been shown to produce active protection against V. cholerae using a guinea-pig ileal loop challenge.

The present study has shown the relatively cheap production of an extracellular enzyme capable of eliciting protection against V. cholerae and so it is worthy of consideration as part of a component vaccine. Until genetically engineered mutant strains that are protective but avirulent are accepted and production becomes cheap, vaccine work will continue along the lines of whole cell vaccines. Nevertheless, finding that proteases are protective may be important in understanding other infections of mucosal surfaces.

Throughout the work on the properties associated with the in vivo colonization of V. cholerae, adherence and protection all cholera protease preparations used were G100/P. It is now known that G100/P contains several proteases and it would be important, once these proteases were purified, to repeat some of this work using the individual protease preparations.

In section (5), for example, it is not known whether only one or several proteases are involved in toxin activation. In section 6 all adherence studies used G100/P and it would be interesting to determine if individual proteases would have different effects on adherence. Also important, although perhaps not cost efficient, would be to determine if one single protease would elicit protection or if a combination of several, as used in this work are necessary, to provide active protection against V. cholerae.

8. The role of *V. cholerae* proteases in the pathogenesis of cholera

It will be obvious to the reader that the major portion of the work presented in this thesis was done *in vitro*, summarized in Table 33. Therefore, there is more than an element of speculation involved when an attempt is made to translate the findings to the *in vivo* infection. There are a number of effects which may be due to the action of proteases (Figure 33) either acting alone or in concert with other enzymes in a "mucinase complex" which contribute to *V. cholerae* virulence:-

i) evasion of normal host clearance mechanisms

ii) movement through the mucus

iii) facilitate colonization

iv) exposure of receptor sites

v) activation of enterotoxin

vi) release of organisms into the lumen.

Positive evidence in this thesis would support the *in vivo* protease activities associated with i, ii, iii.

Although the proteases were shown to digest fibronectin (see page 127, Figure 24) *in vitro*, one cannot conclude that this is necessarily helping in the exposure of new receptor sites on the epithelial surface. In the gut environment it would seem unlikely that the initial adherence of *V. cholerae* would be conditioned by the chance production of an extracellular enzyme required to expose a specific receptor for attachment. Nevertheless, the exposure of new receptors may aid in the lateral spread of the organisms over the epithelial surface once the initial colonization has been achieved (Figure 34,a). Similarly, one could argue that the proteases aid in the adherence of the organisms to

TABLE 33

LIST OF ACTIVITIES OF THE V. cholerae MUCINASE COMPLEX

Degrades mucin	+ + +
Digests fibronectin	+
Digests IgA	+
Digests enterotoxin A-subunit into two peptide fragments	+
Activates cholera enterotoxin	?
Haemagglutinating activity	+
Blocks adherence	+ +
Protective	+ + +
Detachment	?

+ + + highly active to + weakly active.

Figure 33
POSSIBLE SCHEME OF ACTIVITY OF THE *V. cholerae* MUCINASE COMPLEX

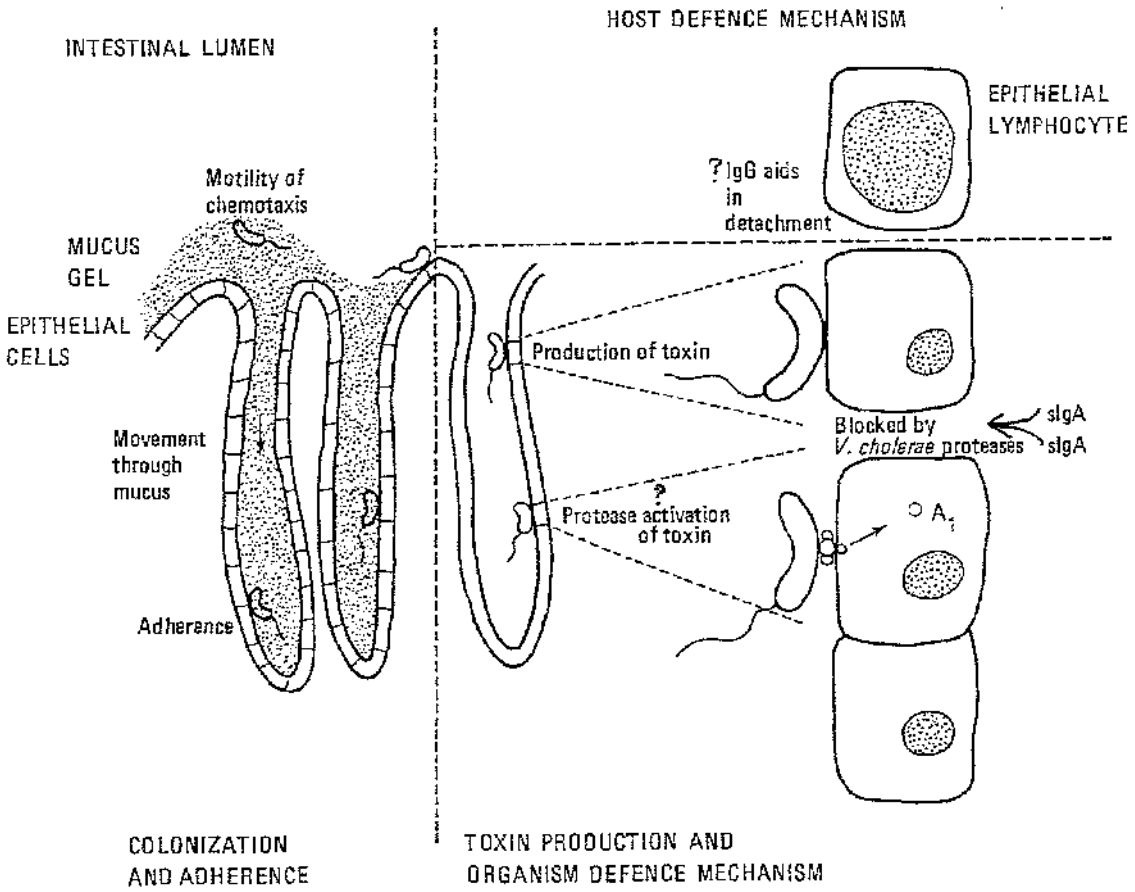
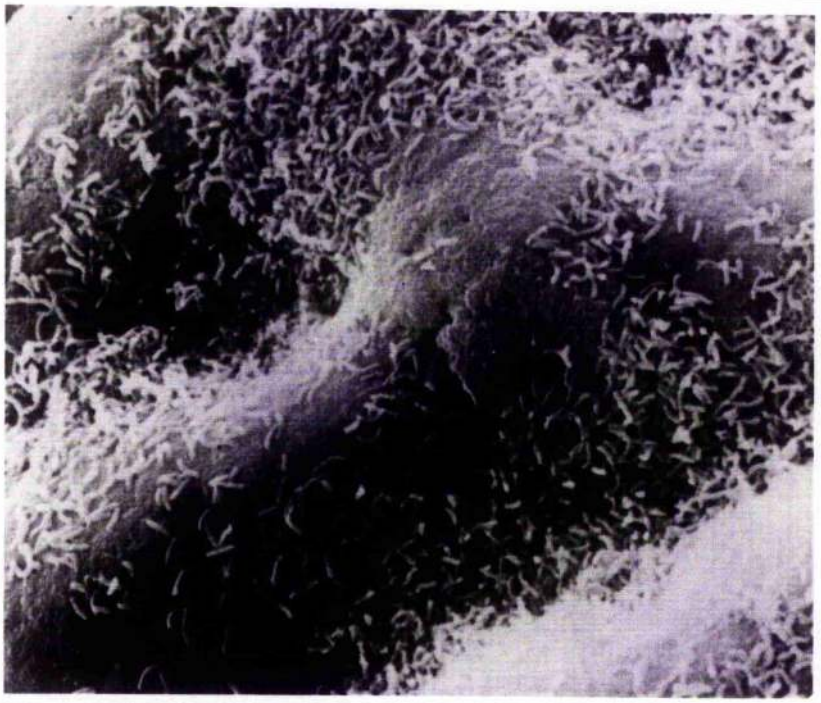


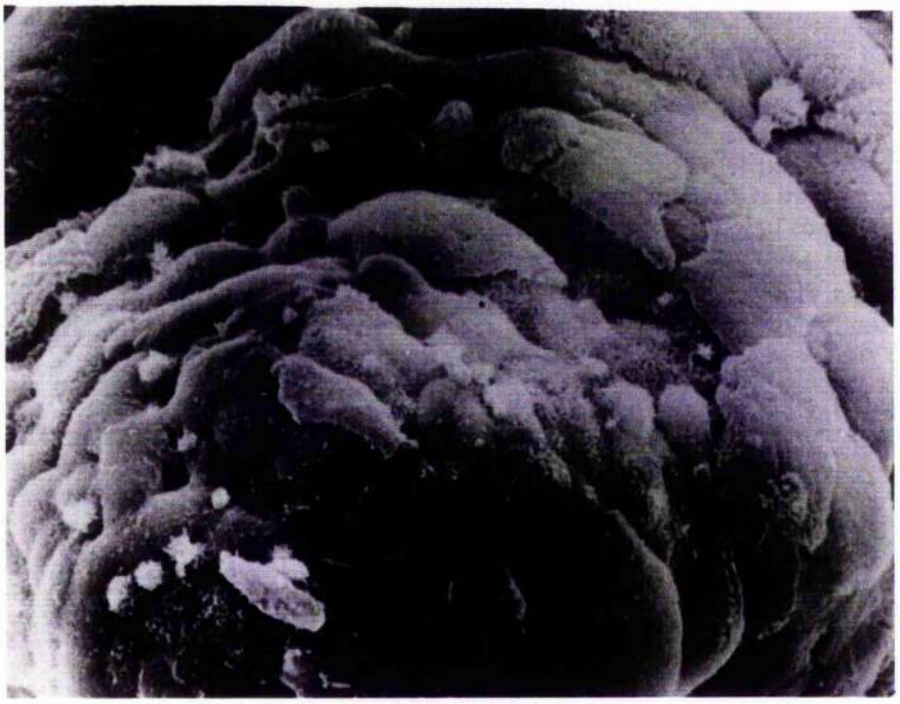
FIGURE 34 Adherence and detachment of V. cholerae to rabbit villi

a.



5 h sample showing large patches of V. cholerae on the adult villus

b.



12 h sample showing almost complete absence of vibrios.

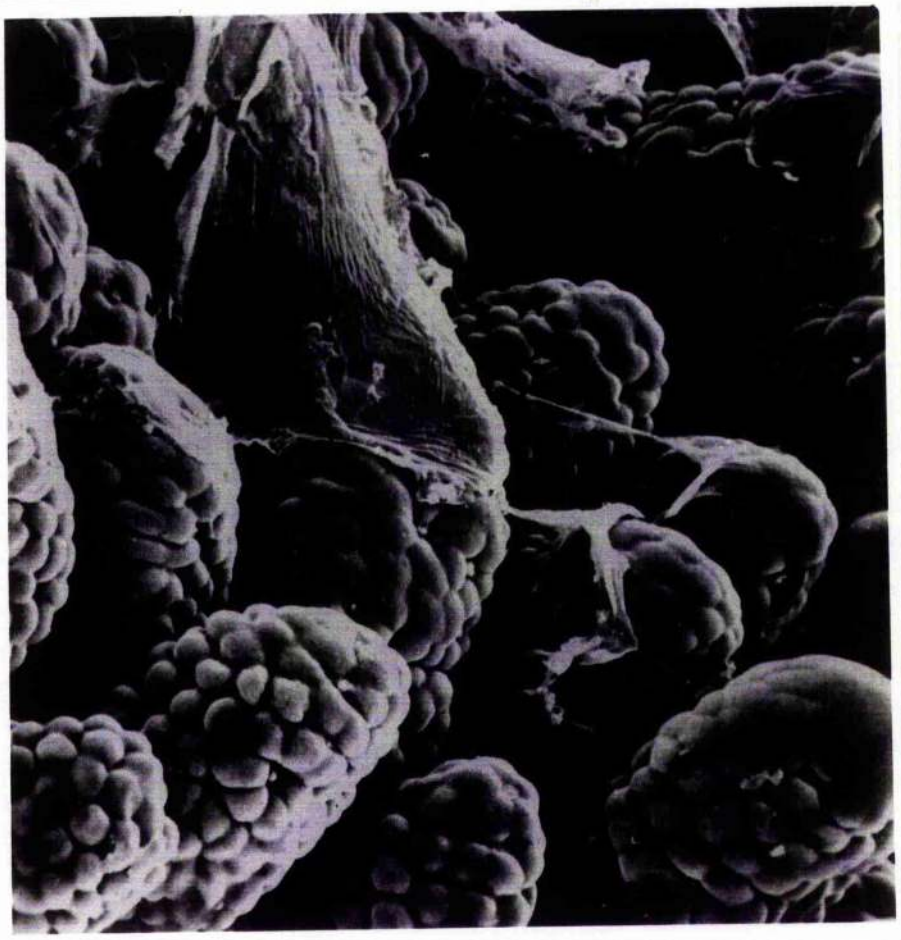
From Nelson, Clements and Finkelstein (1976).

the mucus, shown by Schrank and Verwey (1976) to be the initial event in colonization. However one must remember that this organism produces neuraminidase and an endoglycosidase which degrade mucin (R.A. Ollar and D.E.S. Stewart-Tull, personal communication). Consequently, it is more probable that these enzymes function to effect ii, the movement of the organisms through the mucus and thus allow contact with the epithelial surface. The work of Rozee et al (1982) showed that the mucus blanket was incomplete (Figure 35). However, in this laboratory histochemical staining has shown that mucus tends to be trapped between the villi and since V. cholerae organisms migrate into the crypts of Lieberkühn the mucinase complex would aid in the positive movement through the mucus. It does appear that once the organisms have attached themselves to receptors the IgA proteases may effect the continued colonization of the epithelial surface for a sufficient time to elapse for production of the effective exoenterotoxin. In addition, other V. cholerae proteases have been shown (see page 132) to activate the enterotoxin in vivo.

There is the characteristic phenomenon with infected patients on oral hydrotherapy that the disease symptoms regress within some 48 hours. The precise mechanism for this is still unexplained but may involve a) lack of nutrients for the organism to synthesize enterotoxin, b) increased water flow in the lumen causing wash-off of the organisms, or c) synchronized detachment. It is not possible to discuss a and b in relation to proteases but Nelson, Clements and Finkelstein (1976) have suggested that enzymes may ultimately be responsible for the synchronized detachment of V. cholerae (Figure 34a,b). It is difficult to envisage such a role for the proteases when one considers their presence during the whole process of adherence, colonization, organism defence and activation

FIGURE 35

Scanning electron micrograph of ileal epithelium of mouse showing fragmentation of mucous blanket



From Rozee et al (1982)

of enterotoxin. The IgG class of immunoglobulin has been shown to be the important protective antibody (Pierce and Reynolds, 1974; Pierce, Sack and Sircar, 1977; D.E.S. Stewart-Tull, personal communication) and one must also accept that the release of V. cholerae organisms may be due to a specific antigen:antibody reaction.

In conclusion it is apparent from this study that V. cholerae produces a variety of proteases which play important roles in the disease process. In addition, it is significant that the effects of the proteases can be negated by immune antibodies since experimental vaccines containing the enzymes stimulated protection against virulent challenge in laboratory animals. Further studies would be warranted to investigate whether such laboratory findings could be extended to provide an effective and long-lasting immunity in man.

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APPENDICES

APPENDIX 1

Growth Media

A. Trypticase soy broth (BBL)

30 g of powder were dissolved in 1 litre of distilled water and sterilized by autoclaving for 15 min at 121°C and 15 psi. Final pH, 7.8 ± 0.2.

Classical Formula per litre of distilled water:-

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0
Sodium Chloride	5.0
Dipotassium Phosphate	2.5
Dextrose	2.5

Adjusted and/or supplemented as required to meet performance criteria.

B. Tryptic soy broth (Gibco)

30 g of powder were dissolved in 1 litre of distilled water and sterilized by autoclaving for 15 min at 121°C and 15 psi. Final pH, 7.2 ± 0.2.

Classical formula per litre of distilled water:-

Ingredients per litre of chemically pure water	
Peptone 140 (Pancreatic Digest of Casein)	17.0 g
Peptone 110 IPapaic Digest of Soy Protein)	3.0 g
Sodium Chloride (NaCl)	5.0 g
Potassium Phsophate Dibasic (K_2HPO_4)	2.5 g
Dextrose	2.5 g

The above formula is typical, each production lot is adjusted to compensate for variables in raw materials.

Typical formula per litre of distilled water:-

Pancreatic digest of casein	15.0 g
Papain digest of soyabean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

Final pH, 7.3 ± 0.2.

APPENDIX 2

SYNCASE MINIMAL MEDIUM

A. SYNCASE

	Grams/litre distilled water
Na_2HPO_4	5.0
K_2HPO_4	5.0
Sucrose	5.0
NH_4Cl	1.18
Na_2SO_4	0.089
$MgCl_2.6H_2O$	0.042
$MnCl_2.4H_2O$	0.004
$FeCl_3.6H_2O$	0.005
Casamino acids	10.0

Final pH 7.2.

B. As above but substitute glucose for sucrose.

C. As A but with no sucrose present.

The above media were sterilized by autoclaving for 15 min at 121.^{o}C and 15 psi.

APPENDIX 3

BUFFERS AND SALINE

1. Physiological Saline (0.15M)

Sodium chloride (34.0 g) was dissolved in distilled water and the volume made up to 4.0 litres.

2. Phosphate Buffered Saline (P.B.S.) pH 7.2

(0.01M phosphate buffer, 0.15M NaCl)

Solution A : 3.58 g $Na_2HPO_4.2H_2O$ were dissolved in litre distilled water.

Solution B : 1.56 g $NaH_2PO_4.2H_2O$ were dissolved in 1 litre distilled water.

For use 72.0 ml of solution A and 28 ml of solution B were mixed and the volume made up to 200 ml with distilled water to give 0.01M phosphate buffer. Sodium chloride (8.766 g) was added to each litre of phosphate buffer.

3. Borate Buffer (0.05M, pH 9.0)

50 ml of 0.05M $Na_2B_4O_3.10H_2O$ were added to 4.6 ml of 0.2M HCl, and this diluted to 100 ml with H_2O.

4. Phosphate buffer (0.1M, pH 5.9)

5.07 ml of 0.1M Na_2HPO_4 were added to 44.92 ml of 0.1M NaH_2PO_4 and this diluted to 100 ml with H_2O.

APPENDIX 4. REAGENTS FOR ISOELECTRIC FOCUSSING

1. Anode Solution

Sucrose (B.D.H.)	12.0 g
Distilled water	14.0 ml
Sulphuric acid (Analar)	0.2 ml

Dissolve sucrose in distilled water. Add sulphuric acid dropwise with vigorous stirring to prevent caramelisation of the sucrose.

2. Cathode Solution

Sodium hydroxide	0.1 g
Distilled water	20.0 ml

3. Diluted Ampholines (10% v/v)

Ampholines 40% (w/v)	2.5 ml
Distilled water	7.5 ml

4. Dense Solution

Sucrose	25.0 g
Ampholine 10% (v/v)	7.5 g
Distilled water	32.0 ml
Total volume	55.0 ml

5. Light Solution

Sample *	x ml
Ampholine (10% v/v)	2.5 ml
Distilled water	(52.5 - x) ml

* This sample can contain up to 1 g of protein.

APPENDIX 5 GELS AND BUFFERS FOR SDS-PAGE

(a) STOCK SOLUTIONS

1. Acrylamide (B.D.H.) 30.0 g
   NN'methylenebisacrylamide (NN'bis) 0.8 g
) made up to 100 ml

2. Running buffer (10x) pH 8.8

Glycine	144.13 g	) made up to 1 litre
SDS	1.0 g	)
tris	30.28 g	)

3. Solubilizing buffer

0.5M Tris/HCl pH 6.8	25 ml
20% SDS w/v	20 ml
β mercaptoethanol	10 ml
glycerol	20 ml
0.1% bromophenol blue w/v	2 ml
H_2O	23 ml

4. Staining solution

Coomassie Brilliant Blue R (Sigma)	1.25 g
50% methanol	454 ml
glacial acetic acid	46 ml

5. Destaining solution

methanol	50 ml
acetic acid	75 ml
H_2O	875 ml

(b) GEL PREPARATION

(i) Separating gel (11% acrylamide)

Acrylamide/NN'bis	36.7 ml
1M Tris/HCl buffer, pH 8.8	37.5 ml
20% (w/v) SDS	0.5 ml
TEMED	25 µl
Ammonium persulphate 0.8% (w/v) (freshly prepared)	10 ml
H_2O	15.3 ml

(ii) Stacking gel (5% acrylamide)

Acrylamide/NN'bis	16.7 ml
0.5M Tris/HCl buffer, pH 6.8	25 ml
20% w/v SDS	0.5 ml
TEMED	25 µl
Ammonium persulphate, 0.8% w/v	10 ml
H_2O	47.8 ml

(c) SAMPLE PREPARATION

Protein samples (approximately 200 $\mu g ml^{-1}$*) were mixed 1:1 with solubilizing buffer and heated at 100°C for 5 min.

*with protease samples this value was much lower, ie 50 $\mu g ml^{-1}$.

APPENDIX 6 GELS AND BUFFERS FOR GELATIN-PAGE

(a) STOCK SOLUTIONS

1. Acrylamide 30.0 g
   NN'methylenebisacrylamide 0.8 g
) made up to 100 ml

2. Running buffer (1)x), pH 8.8

glycine	144.13 g	)
sodium dodecyl sulphate	1.0 g	) made up to 1 litre
tris	30.28 g	)

3. Staining solution

Amido Black	1 g
methanol	300 ml
acetic acid	100 ml
H_2O	600 ml

4. Destaining solution

methanol	300 ml
acetic acid	100 ml
H_2O	600 ml

(b) GEL PREPARATION

Separating gel

Acrylamide/NN'bis	19.8 ml
1.5M Tris/HCl, pH 8.8	
containing 0.4% (w/v) SDS	13.5 ml
Gelatin, 1% (w/v)	5.4 ml
Ammonium persulphate 100 $mgml^{-1}$	0.12 ml
(prepared freshly)	
TEMED (N,N,N,N'-Tetramethylethylenediamine)	50 µl
H_2O	14.7 ml

Stacking gel

Acrylamide/NN'bis	2.4 ml
0.5M Tris/HCl, pH 8.6	3.0 ml
containing 0.4% (w/v) SDS	
Ammonium persulphate, 100 $mgml^{-1}$	0.24 ml
TEMED	25 µl
H_2O	19.0 ml

(c) Sample Preparation

Samples were prepared in solutions containing 2.5% (w/v) SDS, 1% (w/v) sucrose and 4 μgml^{-1} phenol red. The samples used all contained approximately 10 $EUml^{-1}$ of protease activity.

APPENDIX 7

PAGE gel with gelatin overlay

1. PAGE gel

Cyanogum	7 g	make to 100 ml with H_2O
Tris	0.46 g	
Citric acid	0.08 g	

Add 1 ml of 10% (w/v) ammonium sulphate and 1 ml of 10% (w/v) ammonium dimethylaminoethyl cyanide, degas the solution and pour into gel mould.

2. Running buffer

Boric acid	18.5 g	make up to 1 litre
NaOH (pellets)	2.0 g	

3. Gelatin gel

Purified agar (Oxoid)	1.2 g
Gelatin (Oxoid)	0.25 g

This was added to 100 ml of 0.2M tris HCl buffer, pH 7.0. The mixture was autoclaved (15 psi for 15 min) and then poured into the gel mould.

4. Acid sublimate solution (Mercuric chloride solution)

$HgCl_2$	15 g
Concentrated HCl	20 ml
H_2O	80 ml

APPENDIX 8

Reagents for Immunoelectrophoresis

1. Barbitone buffer, 0.05M, pH 8.4.

Sodium barbitone	10.3 g
Barbitone	1.84 g
H_2O	1 litre

2. Barbitone agar

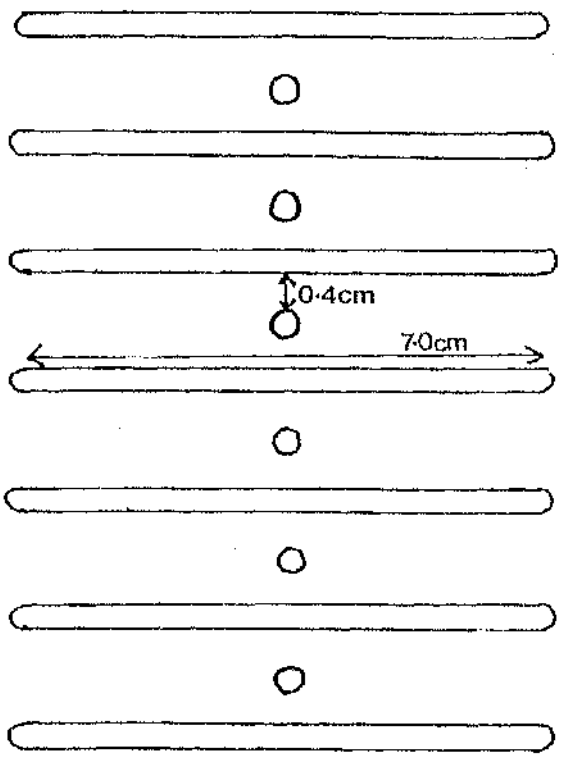
Purified agar	8 g	)
		) dissolve by steaming
Barbitone buffer	1 litre	)
Merthiolate	0.01 g	

3. Amido Black stain

Amido Black	0.5 g
methanol	90 ml
acetic acid	10 ml

APPENDIX 9

Template for Immunoelectrophoresis Plate



APPENDIX 10

Adjuvants used in protection studies and for antiserum production

A. Freunds complete adjuvant (F.C.A.)

1.0 ml Bayol F (Esso: provided by Dr. D.E.S. Stewart-Tull)

0.4 ml Arlacel A (I.C.I. America)

1.0 ml Antigen

250 µg freeze-dried Mycobacterium tuberculosis.

B. Freunds incomplete adjuvant (F.I.A.)

1.0 ml Bayol F (Esso: provided by Dr. D.E.S. Stewart-Tull)

0.4 ml Arlacel A (I.C.I. America)

1.0 ml Antigen

C. Aluminium hydroxide gel ($Al(OH)_3$)

1.4 ml $Al(OH)_3$ (prepared by method of Levine and Vaz, 1970)

1.0 ml Antigen

APPENDIX 11

TRITON-TOLUENE SCINTILLANT

Triton X-100	1 litre
Toluene (technical grade)	2 litres
2,5-Diphenyloxazole	8 g
1,4-Di-2-(e-methyl-5 phenyl oxazolyl benzene)	0.2 g

PUBLICATIONS

Society for General Microbiology

100th ORDINARY MEETING

Warwick, 9–12 April 1984

PATHOGENICITY GROUP: OFFERED PAPERS

1015 *Some Characteristics of Vibrio cholerae Proteinases*

C.R. BLEAKLEY and D.E.S. STEWART-TULL (Department of Microbiology, University of Glasgow)

Three specific proteinases were isolated from the culture fluid of *V. cholerae* by isoelectric focussing. The specificity of these was determined by the use of inhibitors. Their role in adhesion and colonization, examined with ^{14}C-labelled organisms, will be discussed. Protection tests showed that one or two injections of proteinase caused a significant reduction in fluid accumulated in guinea-pig ileal loops.