The Neuraminidase of *Vibrio cholerae*

Robert-A. Ollar

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Department of Microbiology

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

In memory of my beloved wife Elena, and to the love and unfailing devotion of my parents.

"Je n'y aurais jamais pensé possible"

Louis Pasteur

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ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
B.D.	Blueing Dose
B.G.B.	Borate Gelatin Buffer pH 7.5
B.S.A	Bovine Serum Albumin
Ca^{++}	Calcium ions
C.A.Y.E.	Casamino Yeast Extract Broth
CFU	Colony Forming Units
CM	Carboxymethyl Cellulose
ConA	Concanavalin-A
CTAB	Cetyl Trimethyl Ammonium Bromide
D	Dextrorotatory
DEAE	Diethylaminoethyl Cellulose
d.f.	degrees of freedom
diam.	diameter
DS-ASNB	Diazonium Salt of 4Amino-2'5 Di methoxy 4' Nitroazobenzene
EBSS	Earle's Balanced Salt Solution
e.g.	for example
EDTA	Ethylenediaminetetra-acetic acid
enz.	enzyme
Fig.	Figure
FITC	Fluorescein Isothiocyanate
FSH	Follicle Stimulating Hormone
g	gravity, centrifugal

G_{A_1}	Galactose(Beta)- 3Nacetylgalactosamine(Beta)1-4Glucose(Beta)-ceramide
$G_{D_{1a}}$	disialoganglioside
Geom.mean	geometric mean
G_{M_1}	monosialoganglioside
G_{T_1}	trisialoganglioside
GDH	Glycerol-3-phosphate dehydrogenase
gly	glycine
i.d.	identical ; intradermal
i.p.	intraperitoneal
ImU	International milli Units
I.U.	International Units
k	constant
k.Dal.	kilodalton
l	litre
L	Levorotatory
L.B.	Limit of Blueing
LDH	Lactate Dehydrogenase
LH	Luteinising Hormone
mM	millimolar
mmole	millimole
m.u.	milliunits
M.P.	3-Methoxyphenol
M.P.N.	2-(3'methoxyphenyl)-N-acetyl-α-neuraminic acid
N	Normal
NaAc	Sodium Acetate

NAD	Nicotinamide-adenine dinucleotide
NADH	Nicotinamide-Adenine Dinucleotide, reduced
NANA	N-acetylneuraminic acid
NCTC	National Collection Type Culture
n.gram	nanogram
nmole	nanomole
n.d. ; n.t.	not done
oval.	ovalbumin
PAGE	Polyacrylamide Gel Electrophoresis
PAS	Periodic Acid Schiff
P.B.S.	Phosphate Buffered Saline
P.E.P.	Phosphoenolpyruvate
pI	isoelectric point
PO_4	Phosphate
Pr.	Protein
R.D.E.	Receptor Destroying Enzyme
rev	revolutions
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
sIgA	secretory IgA
std	standard
TAF	T-lymphocyte Activating Factor
T.B.A.	Thiobarbituric Acid Assay
T.C.A	Trichloroacetic Acid
T.C.B.S.	Thiosulphate-Citrate Bile Salt Sucrose Agar
TIM	Triosephosphate Isomerase

tyr	tyrosine
u	unit
v	volume
w	weight

SUMMARY

Over the past 25 years research on the pathogenesis of cholera has focused on the role of enterotoxin, largely to the neglect of the other extracellular components such as neuraminidase, mucinase and proteinase. The main object of this thesis has been to study the effect of Vibrio cholerae neuraminidase on the histochemistry of the sialomucin layer, and the binding of the bacteria and of the enterotoxin to gut mucosa.

Both laboratory-prepared and commercial sources of neuraminidase were utilised. For the former, V. cholerae NCTC 10732 was grown in proteose peptone broth containing bovine colostrum. The culture supernate, which contained the enzyme, was precipitated with methanol and the methanolic precipitate filtered on Sephadex G 100. The peak of neuraminidase activity in the eluate from the gel column did not coincide with the main protein peak as measured by either 280 nm absorption or the Lowry method. The partially purified neuraminidase preparations contained endoglycosidase, phospholipase-C and proteinase activity (as expected from previous studies) but lacked aldolase activity.

Neuraminidase activity was determined both by the thiobarbituric acid assay (T.B.A.) and also by the methoxyphenyl neuraminic acid method (M.P.N.). Both procedures had advantages and limitations. The advantages of the M.P.N. method were greater sensitivity and better stability of the colour complex, especially at low enzyme concentrations. The main drawback of the method was its dependence on the synthetic

substrate M.P.N. which is not part of the mucin of the small-gut. The main virtue of the T.B.A. assay was its applicability to detecting N-acetylneuraminic acid (NANA)-containing moieties liberated from a variety of natural substrates. However, the procedure could not determine whether the NANA was liberated as monosaccharides (indicative of neuraminidase activity) or as more complex NANA-containing entities (indicative either of endoglycosidase activity or simple solubilisation of sialomucin). A further drawback of the T.B.A. method is that its colour complex is generated not only by NANA but by 2-oxo-3-deoxy sugar acids.

In order to investigate the possible effect of neuraminidase on rat intestinal mucosa in vitro, it was first necessary to find a tissue fixation procedure which preserved the sialomucin layer in tissue segments and frozen sections against loss during exposure to aqueous environments. Neither formal-saline nor Bouin's fluid were satisfactory but the paraformaldehyde vapour method cited by Culling gave adequate retention of the mucin layer.

The susceptibility of the NANA moiety of mucin in paraformaldehyde vapour-fixed frozen sections to neuraminidase activity was monitored by Alcian Blue-Periodic Acid Schiff (PAS) staining. In agreement with previous published observations, it was noted that treatment of the sections with V. cholerae neuraminidase caused a general reduction in Alcian blue staining and a corresponding increase in PAS staining. This was interpreted as being due to loss of NANA (detected by Alcian blue) coupled with retention of the general mucosubstance (detected by PAS). Quantitation of this phenomenon was obtained by determining the proportion of goblet cells staining with

Alcian blue or PAS. The proportion of PAS-staining goblet cells was related to enzyme activity and thus provided a histochemical assay for neuraminidase.

Although V. cholerae neuraminidase pretreatment of fixed rat ileal tissue caused significant histochemical change in the sialomucin layer, as revealed by the Alcian blue/PAS staining, it did not affect the adherence capacity of the tissue for live V. cholerae organisms. This result emerged from 8 experiments in which the numbers of these bacteria adhering to standardised tissue segments showed no significant difference when exposed to highly purified commercial V. cholerae neuraminidase compared with enzyme-free buffer. By contrast, the neuraminidase pretreatments of these tissue segments caused a highly significant increase in the binding of cholera enterotoxin (in agreement with previous findings). This was demonstrated by the rabbit skin-blueing test of solutions of enterotoxin which had been previously exposed to ileal segments which in turn had been either treated with neuraminidase or buffer.

Initial attempts to study the neutralisation of V. cholerae neuraminidase by specific antibody were frustrated by the presence of endogenous neuraminidase in the mammalian serum. This problem was overcome by using the IgG fraction of the anti-serum, with a similar fraction from normal serum as control. Immune serum IgG had definite neuraminidase-neutralising activity as measured by a variety of procedures: MPN, TBA and goblet cell assays. Normal serum IgG lacked these neutralising activities.

In regard to the pathogenesis of cholera, the observations in this thesis suggest that the role of neuraminidase may be to enhance the effect of enterotoxin through promotion of its binding to tissue,

but without affecting the initial adherence of the vibrios to the mucosa. It must be recognised, however, that neuraminidase is only one component of the ensemble of enzymes produced by V. cholerae and currently known under the collective heading of "mucinase". Therefore the exact role of neuraminidase in pathogenesis must necessarily be defined in relation to the activities of the other extracellular enzymes.

INTRODUCTION

I HISTORICAL PERSPECTIVE

1.1 Definition of Cholera

The Shorter Oxford English Dictionary On Historical Principles described cholera as having been derived from the Latin word for bile (Onions, 1973). The ancient medical literature described bile as one of the "four humours" of the classical physiologists. In line with the notion of bile, it was stated in 1634 that cholera was a malady associated with bilious diarrhoea, vomiting, stomach-ache and cramps (Onions, 1973). Cholera is now known to be a disease that is an acute (frequently severe) and dehydrating diarrhoeal infection which usually occurs with epidemic incidence and is caused by infection with vibrios (Finkelstein, 1975).

1.2 Endemic Indian Infection to Pandemic

In a classic monograph on the History of Asiatic Cholera, Macnamara (1876) stated that the greats of the classical period of medicine in Western Europe and China (Hippocrates, Galen and Wang-shooho) had never seen cholera in its epidemic or Asiatic form. The disease, however, was well known to the Indian subcontinent, and ancient Indian Medical literature (802-849 A.D.) noted that the disease was so widespread and feared, that the local people of lower Bengal actually prayed to a goddess of cholera in order to save themselves from this dreaded disease (Macnamara, 1876).

The disease did not spread throughout the rest of the world until 1817 (Macnamara, 1876; Pollitzer, 1959; Rosenberg, 1962; Longmath, 1966), and from then on became world-wide. Since the first world-wide epidemic or pandemic in 1817, there have been 6 other

pandemics commencing in 1829, 1852, 1863, 1881, 1899, 1961 (Pollitzer, 1959; Kamal, 1974).

1.3 Finding the Causative Agent

The causative agent of Asiatic cholera was first observed microscopically by Pacini (Pacini, 1854; Hugh, 1964) and first isolated by Robert Koch (Koch, 1883; Pollitzer, 1959) but the notion that it was due to a specific intestinal infection was shared by many of the 19th century cholera investigators. The Bengal Medical Board that reviewed the reports of the 1817-1819 India Cholera Epidemic, stated that the disease was due to a "pestilential virus" which acted upon stomach and small intestines but was not contagious. It was not until 1831 that Neale proposed that the disease was due to a contagium vivum. In 1838 Boehm made the first claim to have observed the causative agent from the faeces of cholera patients and described these organisms as "spherical organic particles". While microscopic investigation as to the nature of the causative agent was being pursued, epidemiological observations on the infectious nature of the disease were being made. A monumental epidemiological observation was made by Dr. John Snow in 1849. Snow traced the origin of a London cholera outbreak to a water pump in Broad Street. He demanded that the water pump handle be removed, and the outbreak subsided. It was concluded from this Broad Street water-pump cholera outbreak that the disease was a contagious infection which was the result of a poison that reproduced itself in the bodies of its victims. In addition, Snow further stated that it was these poisonous substances that spread the disease and were to be found in the excreta and vomitus of the cholera patients. The most

important observation made by Snow, however, was that the disease was spread to others by a contaminated water supply.

In 1854 when London was again gripped by a cholera outbreak, Snow noted that the infection was much more frequent among Londoners who utilized water from the sewage-contaminated lower Thames rather than those who utilized water from the Thames above the city of London (Macnamara, 1876). The views of Snow were further supported by the German investigator Max von Pettenkofer (1855), who held the view that the development of cholera took place in the soil and thus cholera and typhoid were dependent on the level of the ground water of a given region. While the epidemiological theories of Snow were being confirmed in the 1854-1855 London Cholera Epidemic, progress in the microscopic identification of the causative agent was being made. Pacini described comma-shaped organisms (microbio colerigentio) which he had observed microscopically from the infected intestinal tissues of cholera victims of the 1854 epidemic in Florence (Pacini, 1854). This finding has been cited as the earliest observation of the cholera vibrio (Hugh, 1964). At the same time that Pacini had described comma-shaped organisms in infected tissues, Hassall (1855) in London, also observed such organisms in the microscopic preparations of ricewater discharge from cholera patients. These organisms were described as vibriones and as such marked the first time that the causative agent was so described (Sticker, 1912).

The sighting of comma-shaped organisms in infected tissues and faeces were reported again by numerous other investigators after Pacini and Hassall (Leyden, 1866; from Sticker, 1912; Klob, 1867). It was, however, Robert Koch (1883) who first isolated motile comma-shaped

bacilli from tissues and faeces of cholera patients during an outbreak of cholera in Alexandria, Egypt, and named the organism Vibrio cholerae.

Though Koch (1883) isolated and described the causative agent, there still persisted some confusion as to non-cholera vibrios also capable of causing gastrointestinal infection. This confusion first appeared in 1884 when Finkler and Prior discovered a cholera nostras during a cholera outbreak in the city of Bonn. The first basic key to resolve this problem came from Koch himself, namely, that blood containing stools used to make gelatin plates showed haemolytic zones around V. cholerae colonies (Koch, 1884a). The actual utilization of the phenomenon haemolysis or the lack of it was put into proper perspective by Kraus in 1903. After experimenting with 12 cholera-like and 9 true cholera organisms, Kraus found that only the cholera-like organisms were capable of producing zones of haemolysis on blood agar. Thus it was recommended that haemolysis or the lack of it be utilized as a means of differentiating the cholera-like from the true cholera vibrios.

The whole concept of haemolytic versus non-haemolytic vibrios became of critical importance after 1905, with the isolation by Gotschlich of vibrios from the intestines of Mecca pilgrims who died at the El Tor quarantine station but did not show signs of cholera while alive and at post-mortem. These vibrios taken from the Mecca pilgrims did, however, show agglutination with cholera-immune serum. When these strains (eltor isolates) were again examined by Kraus and Pribram (1905), it was found that the strains produced a soluble haemotoxin and an exotoxin which was lethal to experimental animals. There followed a period of uncertainty concerning the role of El Tor vibrios and cholera infection. Kraus (1922) maintained the view that the El Tor vibrios were a class of

organisms that were distinct from the non-haemolytic V. cholerae organism, while other investigators (Kolle and Prigge, 1928) held the view that cholera vibrios could show a variable reaction concerning their haemolytic ability, and therefore such tests were not suitable for characterising the organisms.

The tragedy of this whole confusing state of affairs was that the world health authorities responsible for dealing with cholera declared that V. cholerae biovar eltor was free of any choleragenic capacity (Kamal, 1974). The refusal to accept V. cholerae biovar eltor as a causative agent of cholera infection persisted despite the fact that outbreaks of cholera had been reported in the Celebes (Indonesia) in the late 1930s, the mid-1940s and late 1950s (Van Loghem, 1938; Tanamal, 1959) and V. cholerae biovar eltor was isolated in each case.

The World Health Organisation was finally forced by the weight of mounting laboratory and epidemiological evidence (Felsenfeld, 1963, 1966; World Health Organisation, 1965) that V. cholerae eltor was also a causative agent of cholera infection. The agent (V. cholerae eltor) was declared responsible for causing the Seventh Pandemic of cholera which began in 1961 on the island of Sulawesi, and spread throughout the world (Kamal, 1974).

II TOXIN

2.1 Cholera a Toxicosis

With the discovery and isolation of the causative agent(s) (V. cholerae, biovar Classical; V. cholerae biovar eltor) of cholera infection, the question that followed was what was the pathogenesis

of the disease? It was suggested by Koch (1884b) that the disease was a toxicosis, but unfortunately the leading pathologist of the time, Virchow (1879), believed that the primary lesion in cholera infection was denudation of the intestinal epithelium resulting in exudation and fluid loss. It would take 82 years to establish firmly that Koch was correct in his assumption that cholera was indeed a toxicosis.

Virchow's view was first challenged by Cohnheim (1882), who refuted the entire concept of desquamation as being nothing more than post-mortem maceration. The notion of an intact membrane was further supported by later investigators (Goodpasture, 1922; Dutta, Panse and Kulkarni, 1959; Gangarosa et al, 1960; Fresh, Versage and Reyes, 1964). The first investigation to show that a toxin was responsible for water and ion loss was done by De in 1959. However, it was Benyajata in 1966, who established the role of cholera enterotoxin in man by instilling a sterile preparation into the small intestine of a volunteer, who later manifested diarrhoea, which resembled that found in cholera infection. With the aid of both light microscopy and electron microscopy, it was observed that the jejunum and ileal tissue sections from dogs orally infected with live V. cholerae organisms did not differ from the tissue sections of control dogs (non-infected), with the exception of a slight inflammation of the Crypts of Lieberkühn in infected tissues (Elliott et al, 1970). Thus the production of rice-water stools found in cholera infection was due to hypersecretion in the region of the Crypts of Lieberkühn (Elliott et al, 1970).

2.2 Molecular Structure of Cholera Enterotoxin

The enterotoxin of V. cholerae was first isolated and purified

by Finkelstein and Lo Spalluto in 1969. In a subsequent investigation, a protein that was immunologically identical to enterotoxin but biologically inactive was isolated (Finkelstein, Fujita and Lo Spalluto, 1971). This biologically inactive substance was later called toxoid (enterotoxoid) or choleragenoid. The intact and biologically active enterotoxin molecule was a protein of about 84 k. Dal. and did not contain any detectable lipid or carbohydrate (Lo Spalluto and Finkelstein, 1972). The molecular weights of the enterotoxin and enterotoxoid differed, in that the former had a molecular weight of 84 k. Dal. whereas the latter had a molecular weight of 58 k. Dal.. Both the enterotoxin and the enterotoxoid reversibly dissociated into 15 k. Dal. sub-units when treated with Tris + 10^{-3} M dithiothreitol or glycine (up to pH 6.8). If the enterotoxin molecule was electrophoresed in gel containing SDS, two bands were seen with molecular mass of 56 k. Dal., and 28 k. Dal. (Finkelstein, La Rue and Lo Spalluto, 1972). By contrast, when enterotoxoid was electrophoresed by the same method, only a single band of 56 k. Dal. was seen. Finkelstein (1973) stated that his pure enterotoxin did not have any of the following enzymic activities: neuraminidase, proteinase, lyase, lecithinase, hyaluronidase, chondroitin sulphatase, RNAase, DNAase and mucinase.

Enterotoxin was later found to be composed of two oligomeric sub-units which were noncovalently bound; one of about 60 k. Dal. (an aggregate of 8-10 k. Dal. peptides) and another major sub-unit of about 30 k. Dal. which contained 25 k. Dal. and 5 k. Dal. components (Cuatrecasas, Parikh and Hollenberg, 1973). The 60 k. Dal. enterotoxin unit could be dissociated into 10 k. Dal. peptides (B-sub-unit) and recombined with the 30 k. Dal. component (A-sub-unit) to restore the biological

activity of the parent molecule. When the intact toxin molecule was heated in 1.0-2.0% SDS, the enterotoxin components were consistently 36 k. Dal. and 8 k. Dal.. It was further found that after reduction and carboxymethylation, the 36 k. Dal. sub-unit was reduced to 27 k. Dal.. A smaller peptide was released by reduction and carboxymethylation, but this did not prove that the components were covalently coupled by disulphide linkage to the larger component because boiling for 50 min without reducing agents also brought about dissociation. It was therefore concluded that the two components were associated by 'extraordinarily tight non-covalent forces'. The 36 k. Dal., as well as the 27 k. Dal. components, were not soluble in aqueous media. Antisera against the 36 k. Dal. component gave rise to antibodies which cross-reacted with enterotoxin components (Table 1). This suggested that the 36 k. Dal. unit was composed of a unique peptide, the 27 k. Dal., which was associated with a smaller sub-unit (~8 k. Dal.) similar or identical to the B sub-unit. The 36 k. Dal. sub-unit was called the active sub-unit, with the 27 k. Dal. component being the unique, biologically-active portion.

Enterotoxoid proved to be a powerful antagonist of enterotoxin in intestinal loops (Pierce, 1973; Holmgren, 1973). The role of enterotoxoid as an antagonist of the action of enterotoxin was also *produced in experiments which utilized isolated rat fat cells (Cuatrecasas, 1973a), and with human neutrophils (Bourne et al, 1973). Enterotoxoid bound to the identical membrane receptor with the same avidity as did enterotoxin and therefore was a competitive antagonist of the binding and action of enterotoxin.

*exhibited

Antigen	Antiserum against toxin components 36 k. Dal.	27 k. Dal.
36 k. Dal.	++	++
enterotoxin	++	-
enterotoxoid	++	-
27 k. Dal.	+	++

Table 1: Reaction of antiserum against enterotoxin components

Legend:

- = no reaction

+ = weak reaction

++ = strong reaction

It was proposed that the B sub-unit was responsible for the initial interaction with cell surface, while the A sub-unit represented the biologically-active portion of the molecule (Cuatrecasas, Parikh and Hollenberg, 1973; Van Heyningen, 1974; Finkelstein et al, 1974; Holmgren and Lonnroth, 1975). The molecular difference between enterotoxin and enterotoxoid was the presence of a toxic sub-unit A in the former, and the A sub-unit contained two disulphide linked peptides of unequal sizes which were called A_1 (28 k. Dal.) and A_2 (5 k. Dal.) (Finkelstein et al, 1974). By contrast, the B sub-unit was common to both enterotoxin and enterotoxoid. The A sub-unit was linked to five B units (11 k. Dal. each) in a noncovalent fashion (Figures 1a,b). The A sub-unit alone activated adenylate cyclase to a similar extent as enterotoxin but higher concentrations were required than that necessary for the intact molecule, and this activity was not blocked by the presence of gangliosides or enterotoxoid (Figure 1b) (Van Heyningen and King, 1975; Sahyoun and Cuatrecasas, 1975; Gill and King, 1975). Direct measurement of the interaction of ^{125}I labelled A sub-unit with fat cell membranes provided additional evidence that this molecule did not react with the membrane receptors for enterotoxin or enterotoxoid (Sahyoun and Cuatrecasas, 1975).

2.3 Binding of Enterotoxin to Monosialoganglioside (G_{M_1})

Gangliosides have been defined as being sialomucolipids which are soluble in water and consist of a non-polar ceramide residue linked to a polar residue which is composed of a polysaccharide chain with one or more N-acetylneuraminic acid (NANA) moieties (Figure 2) (Bizzini, 1977). At present those gangliosides that have been described differ

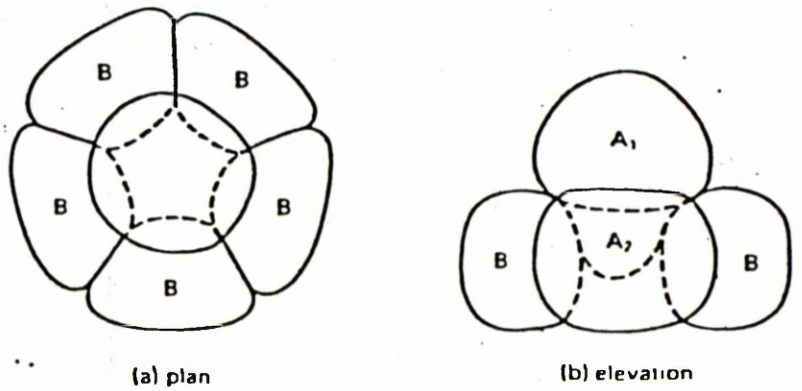


Figure 1a: The arrangement of the sub-units of cholera toxin. The five B sub-units form a ring structure (a) into which the A sub-unit partially inserts (b)

(Stephen and Pietrowski, 1981b).

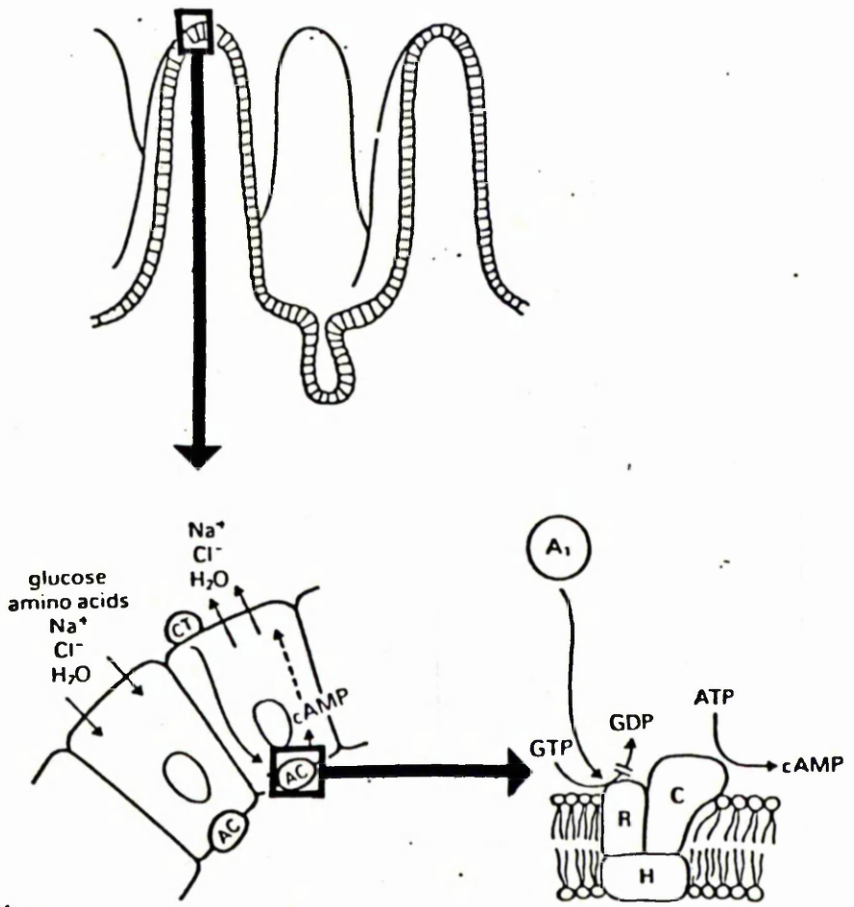


Figure 1b: Mechanism by which cholera toxin causes diarrhoea. Binding of toxin to receptors on the lumen surface of ileal mucosal cells is followed by entry of fragment A_1, which interacts with the adenylate cyclase complex on the basal membrane, inhibiting the GTPase-mediated turn-off the cyclase (probably by ADP-ribosylation of the GTP-dependent regulator protein). Increased intracellular cyclic AMP levels cause, by some as yet unknown mechanism, efflux of Na^+ and Cl^- ions, and hence also water.

(Stephen and Pietrowski, 1981b).

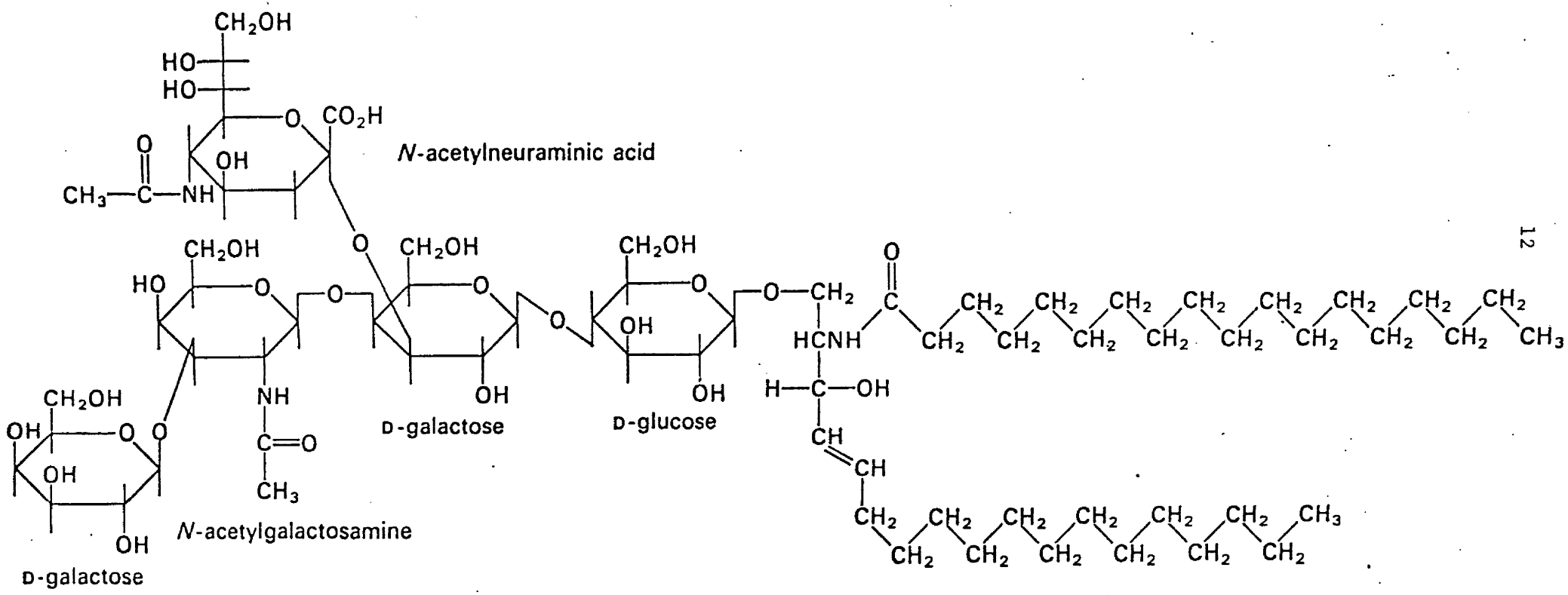


Figure 2 : Molecular structure of monosialoganglioside, G_{M_1} (Van Heyningen, 1977).

from each other in the composition of the polysaccharide residue (Bizzini, 1977). In nervous tissue, four types of gangliosides are commonly found. The polysaccharide chains of these gangliosides found in nervous tissue contains two galactose, one N-acetylglucosamine and from one to three NANA groups (Bizzini, 1977).

The effect of cholera enterotoxin in intestinal loops of rabbits and on epididymal fat cells of rats was neutralised by purified gangliosides (Van Heyningen et al, 1971). This finding suggested that fixation of enterotoxin to gangliosides might be involved in the binding of cholera enterotoxin to cell membranes. Holmgren (1973) found that monosialoganglioside (G_{M_1}) in contrast to other gangliosides tested, fixed cholera enterotoxin in vivo, and that one mole of G_{M_1} ganglioside (16 k. Dal.) could fix as well as inactivate about one mole of enterotoxin (84 k. Dal.), indicating specific 1:1 stoichiometric proportions. This finding strongly suggested that G_{M_1} ganglioside could be the tissue receptor for cholera enterotoxin. The fixation of tetanus toxin and the de-activation of cholera enterotoxin, was described as being toxin specific by Van Heyningen and Mellanby (1973). In addition, these investigators further stated that the de-activation of cholera enterotoxin was like the fixation of tetanus toxin because: (a) it was apparently limited to a particular enterotoxin, (b) it was specific to a particular ganglioside and (c) it took place at very low concentrations of ganglioside. De-activation of cholera enterotoxin and the inactivation of a number of other enterotoxins was distinguished on the grounds that de-activation was: (a) specific with respect to both enterotoxin and ganglioside, (b) occurred at much lower concentrations of ganglioside than required for inactivation and (c) occurred relatively quickly.

Neuraminidase-resistant monosialoganglioside (G_{M_1}) or GG_NSIC, was able to prevent cholera enterotoxin from acting in rabbit skin, and also prevented the adenyl cyclase system from acting in the small intestine of guinea-pigs. In addition, it was stated that the deactivation of cholera enterotoxin by a specific ganglioside could be due to its presence in the cell membrane, or to the presence of molecular structures in the cell membrane which closely resembled that of G_{M_1} ganglioside (King and Van Heyningen, 1973). Inhibition of the biological activity of enterotoxin with G_{M_1} was confirmed by other investigators (Cuatrecasas, 1973b,c,d; Holmgren, Lonnroth and Svennerholm, 1973a,b). A variety of gangliosides blocked the binding of enterotoxin to cell membranes but the most potent inhibitor of these was the G_{M_1} ganglioside (Cuatrecasas, 1973c,d). The enterotoxin showed an affinity for the carbohydrate region of the G_{M_1} ganglioside (King and Van Heyningen, 1973; Holmgren, Lonnroth and Svennerholm, 1973b). Derivatives of the G_{M_1} ganglioside which had only one hydrocarbon chain were shown to bind enterotoxin (Stäerk et al, 1974; Holmgren, Månsson and Svennerholm, 1974), whereas the oligosaccharide fragments of the G_{M_1} ganglioside were shown to bind to enterotoxin with somewhat reduced affinity. ^{125}I-labelled enterotoxin was bound to all regions of the gastrointestinal tract of adult guinea-pigs with equal avidity (Peterson, 1974). The conclusion drawn from this finding was that the enterotoxin receptor was a universal component of many mammalian cell membranes. ^{125}I-labelled enterotoxin binding was inhibited by preincubation of the tissue homogenates with unlabelled enterotoxin but not by comparable concentrations of normal rabbit serum (Peterson, 1974). The binding of enterotoxin to three transformed

mouse epithelial cell lines derived from the parent strain, the effect of enterotoxin on DNA synthesis and adenylate cyclase activity varied in parallel with the ganglioside composition of the cells (Hollenberg et al, 1974).

The binding of enterotoxin by brain tissue increased during incubation for 1 h at 37°C. However, the binding of cholera enterotoxin to rabbit small intestinal epithelial tissue was less affected by the same conditions of exposure to enterotoxin. In addition, the epithelial tissue in guinea-pigs was found to bind enterotoxin (Gascoyne and Van Heyningen, 1975), thus confirming an earlier finding made by Peterson (1974). Rabbit liver tissue was also capable of binding enterotoxin at levels comparable to that bound by the epithelial tissue of rabbit small intestine (Gascoyne and Van Heyningen, 1975). The binding capacity of the tissues tested with the exception of the brain was found to vary when tissues taken from guinea-pig were compared with those taken from rabbit. A variation in binding capacity was even found to exist when different individuals of the same species were compared. A slight difference existed between the enterotoxin binding capacity of various tissues and their content of enterotoxin-binding ganglioside. Enterotoxoid bound more slowly and less strongly than enterotoxin to the intestinal mucosa. It was suggested that both the binding and the active components of the enterotoxin molecule may be necessary for optimal binding of the enterotoxin to the intact cell. In ruptured cells enterotoxoid only partially blocked the action of the enterotoxin (King and Van Heyningen, 1975). This suggested that while binding to a membrane receptor was necessary for the action of the

enterotoxin on the whole cell, it was possible to activate adenyl cyclase in a perforated cell by a process which was apparently independent of membrane binding. It was noted, however, that this activation of adenyl cyclase could only be possible if the A sub-unit dissociated from enterotoxoid, B sub-unit. It was demonstrated by immunofluorescence microscopy using normal human lymphocyte membranes and C 1210 lymphoma cells, that when enterotoxin and fluorescein-labelled antibodies were employed as ligands, the G_{M_1} gangliosides on the lymphocytes were redistributed into aggregates and caps (Révész and Greaves, 1975).

G_{M_1}-deficient cells also showed by immunofluorescence microscopy a similar pattern of ligand-induced redistribution when exogenous G_{M_1} ganglioside was inserted. This observation was later confirmed by other investigators (Craig and Cuatrecasas, 1975; Bennett, O'Keefe and Cuatrecasas, 1975; Sedlacek et al, 1976) who also utilised immunofluorescence microscopy. The effect of enterotoxin binding caused by the addition of exogenous G_{M_1} ganglioside upon ganglioside-deficient guinea-pig tumour (104Cl) cells was further examined with ^{125}I-labelled enterotoxin or Dolichos biflorus lectin (specific for the terminal nonreducing α-linked N-acetylgalactosamine moieties of glycoproteins and glycosphingolipids) (Basu et al, 1976). Increased binding of ^{125}I-labelled Dolichos biflorus lectin and enterotoxin to the cell surfaces of those tumour cells occurred after the exposure of the cells to exogenous G_{M_1} ganglioside. Normal human fibroblasts were stated to contain approximately 8.0×10^6 molecules of G_{M_1} ganglioside per cell (Moss, Mangiello and Fishman, 1977).

It was proposed that there existed multiple sites on the enterotoxin molecule which enabled it to bind to the oligosaccharide portion of the G_{M_1} ganglioside (Fishman, Moss and Osborne, 1978). Equilibrium dialysis and gel permeation chromatography confirmed that each enterotoxin molecule was able to bind between 5 and 6 molecules of oligosaccharide. This finding supported the earlier findings of King and Van Heyningen (1973) and Holmgren, Lönnroth and Svennerholm (1973b), who also found that the enterotoxin was bound to the G_{M_1} ganglioside by the carbohydrate region. Studies conducted by O'Keefe and Cuatrecasas (1978) which utilised mouse fibroblasts (KBALB/3T3) which had been transformed by sarcoma virus (transformation caused a marked loss in G_{M_1} ganglioside to that of the original parent strain of fibroblasts (BALB/C3T3)), and rat fat cells supported the findings of earlier investigation (Basu et al, 1976), namely, that preincubation of transformed, and rat fat cells with exogenous G_{M_1} gangliosides did enhance the binding of ^{125}I-labelled enterotoxin. However, despite the enhanced binding of enterotoxin to the transformed cell line, there did not occur any enhanced biological response (stimulation of adenylate cyclase). By contrast, preincubation of the rat fat cells with exogenous G_{M_1} gangliosides did enhance the stimulation of adenylate cyclase. The failure of the transformed cell to manifest an increase in biological response despite having bound increased amounts of enterotoxin, was explained by the fact that the addition of exogenous G_{M_1} would not be expected to alter sensitivity to enterotoxin in a cell which already contained more enterotoxin binding sites than normally required for maximal stimulation of adenylate cyclase (O'Keefe and Cuatrecasas, 1978).

It was found that endogenous neuraminidases were capable of unmasking neuraminidase-stable and neuraminidase-labile gangliosides in rabbit intestinal homogenates by attacking glycoproteins (Gascoyne and Van Heyningen, 1979). In addition, exogenous neuraminidase caused neuraminidase-labile gangliosides to be converted into cholera enterotoxin-binding, neuraminidase-stable G_{M_1} ganglioside. When the cell surfaces of human neutrophils were first pretreated with V. cholerae neuraminidase, there occurred an increased binding of fluorescein-labelled cholera enterotoxin (Ackerman, Wolken and Gelder, 1980). This finding was attributed to conversion of the membrane gangliosides to enterotoxin-binding gangliosides, as had been previously stated (Gascoyne and Van Heyningen, 1979). However, the findings of Gascoyne and Van Heyningen (1979), and Ackerman, Wolker and Gelder (1980) conflicted with those of Holmgren (1981) who found that the treatment of rabbit intestinal epithelium with V. cholerae neuraminidase failed to create new receptors for enterotoxin, despite the fact that neuraminidase pretreatment created new receptor sites in the epithelium of other tissues. Vertiev, Shaginiyan and Ezepchuk (1981) stated that it was difficult to prove that neuraminidase treatment increased the number of specific cell receptors for enterotoxin because both experimental and commercial preparations of V. cholerae neuraminidase were contaminated by proteinase and glycosidase activities.

Thus, with the binding of cholera enterotoxin to its receptor site on the epithelial cell membrane the initial process in cholera pathogenesis has been established.

III INTESTINAL COLONISATION AND RELATED ASPECTS

With the finding in the mid-1960s (Benyajata, 1966) that cholera pathogensis was indeed due to a toxin, the greater part of scientific investigation on this subject was dominated in one way or other by enterotoxin. By the mid-1970s, however, researchers began to realise that an understanding of the manner in which the vibrios associated themselves with the host mucosa was equally important. This new focus of research endeavour was put forth as follows: If V. cholerae was to be a successful pathogen several conditions must be satisfied, (a) survival through the stomach's acid barrier, (b) colonisation of the ileum and (c) secretion of its enterotoxin (Nelson, Clements and Finkelstein, 1976).

3.1 Motility

Motile strains of V. cholerae were more virulent for suckling pigs, because the chance of the organism coming into contact with the small intestinal mucosa cells was greater (Guentzel and Berry, 1975). Non-motile cholera vibrios were found to be unable to attach to rabbit brush border membranes (Jones and Freter, 1976a,b). When thin sections of rabbit ileum were exposed to vibrios, 8 h and 12 h post-infection, microscopic (fluorescence) observations with fluorescein-labelled anti-vibrio antibody showed that only motile organisms had penetrated into the intervillous areas (Guentzel et al, 1977). Fluorescence microscopy again revealed that while nonmotile organisms remained in the gut, motile organisms were able to penetrate into the intervillous spaces and deep within the Crypts of Lieberkühn (Yancey,

Willes and Berry, 1978). In addition to motility, Allweiss et al (1977) proposed that V. cholerae organisms must also possess chemotactic factors that enable them to interact with small intestinal mucosal surfaces (see section on Adhesion).

These investigations have revealed that those V. cholerae organisms which were highly motile and also possessed a directed chemotactic response were better able to associate with the intestinal mucosa.

3.2 Mucin

Perhaps one of the most formidable barriers preventing the association of V. cholerae organisms with the small intestinal mucosa is the mucin layer. As early as 1933, Florey noted that mucus was seen as lace-like strands under the light-microscope. He had found that the mucus which clung to the villi in the cat small intestine, was able to be cleansed of foreign particles (inert hydrokollog particles) by contractions of the underlying villi. Since Florey, other investigators have examined the mucin layer more closely with electron microscopy (Nelson, Clements and Finkelstein, 1976; Rozee et al, 1982) and have confirmed that the mucus existed as lace-like strands (Plate 1).

The mucus in the rabbit small intestine was described as a gel-like zone which separated the villi of the ileum from particulate intestinal contents (Schrank and Verwey, 1976). This region of ileal mucus extended 100 μm above the villi tips, and when stained by either Toluidine blue or Theonine, it stained the characteristic reddish colour. The recent studies of the development of the blanket in ilea of 1-44 day old mice were carried out with both scanning and transmission electron microscopy (Rozee et al, 1982). Electron micrographs of day

old mice revealed that the mucus was only seen at the depressed openings of the goblet cells. At a period of 4 days after birth the mucus blanket coated a few areas of the epithelial surface of the ileum (Plate 1). Mice that were 12-14 days old possessed a much thicker blanket which was seen microscopically as a discontinuous balled and rolled up layered structure. Mice that were 24-30 days old possessed a mucus layer 10-20 μm in thickness which was described as being a continuous blanket which covered the villi of the ileum. It was noted that there sometimes occurred cracks in this layer. By a period of 44 days after birth, the mucin was estimated to be 30-50 μm in thickness. From the period of 24 days after birth, it was observed that there was present on the mucus a complete community of bacteria and protozoa.

A variety of mucosubstances of the human alimentary tract were studied utilising the Alcian blue/PAS staining method at pH 2.5 (Gad, 1969). The Alcian blue/PAS staining method enabled a distinction to be made between periodate unreactive and periodate reactive acid mucosubstances, from neutral periodate reactive mucosubstances. Periodate-unreactive acid mucosubstances stained blue, periodate-reactive mucosubstances stained blue, and neutral periodate mucosubstances stained magenta. At pH 1.0 the Alcian blue/PAS method differentiated between sulphomucins which stained blue, and neutral and sialomucins which stained red. The striated border of the small intestine gave a reaction which indicated the presence of sialomucin. In contrast, the colon and rectum demonstrated the presence of sulphomucin. Pearse (1968a) stated that the only possible way in

Electron micrograph of mouse ileum

Plate 1: Unwashed ileal epithelium from a 4 day old mouse showing the degree of fragmentation and condensation of the mucus blanket.

(Bar = 50 μm).

(Rozee et al, 1982).

which mucosubstances can be defined histochemically was in terms of their response to the application of localising reagents used under rigorously defined conditions (Tables 2, 3). A distinction was found between submucosal gland mucin and goblet cell mucin, namely, that the former was rich in sulphate while goblet cell mucin showed reduced sulphation and the levels of sialic acid were increased at the expense of fucose moieties (Gallagher and Corfield, 1978). The presence of sialic acid in the goblet cell mucin was clearly revealed by localising its presence in microscopic sections using a peroxidase-labelled Limulus polyphemus lectin which has affinity for sialic acid moieties (Yamada and Shimazu, 1979). When the goblet cell mucin was analysed, protein accounted for 12% of the dry weight, with the most abundant amino acids being threonine, serine and proline (Forstner, Jabbal and Forstner, 1973). In goblet cell mucus, the acidic amino acids were more numerous than the basic amino acids and sulphur-containing amino acids were low in concentration. The analysis of the sugar composition of the goblet cell mucus revealed that hexose, hexosamine, sialic acid and fucose were present. The composition of sugar per dry weight of goblet cell mucus was, total hexose 23%, hexosamine 22.4%, sialic acid 10% and fucose 6.6%. The sulphur content of goblet cell mucus was less than 1.0% of the dry weight and there was an absence of lipid.

In two separate investigations (Finkelstein, 1973; Holmgren and Svennerholm, 1977) it was observed that the mucus layer must be penetrated before there could be a large established number of cholera vibrios in the intervillous spaces and the crypts of the small intestine (see section on adhesion). When live V. cholerae organisms were injected into cat small intestine, there appeared antibodies in the

(I) NEUTRAL MUCOSUBSTANCES

Neutral glycoproteins
Immunoglobulins
Fuco-mucoids
Manno-mucoids
} (All PAS-positive) (Periodate-reactive)

SYMBOL. *G*-mucosubstance

(II) ACID MUCOSUBSTANCES

A. Sulphated

(1) *Connective Tissue Mucopolysaccharides.* (PAS-negative) (Periodate unreactive)
(*a*) Stable to testicular hyaluronidase
(i) Alcianophilic in 1·0M-$MgCl_2$-keratan sulphate, heparin.
SYMBOL. S-mucopolysaccharide A(1·0$MgCl_2$)
(ii) Alcianophilic in 0·7M-$MgCl_2$-dermatan sulphate.
SYMBOL. S-mucopolysaccharide A(0·7$MgCl_2$)
(*b*) Labile to testicular hyaluronidase
(i) Alcohol-resistant azurophilia (0·02 per cent.) at or above pH 2·0—chondroitin sulphates in cartilage.
SYMBOL. S-mucopolysaccharide B2·0T
(ii) Alcohol-resistant azurophilia (0·02 per cent.) at or above pH 4·0—chondroitin sulphates in vascular walls.
SYMBOL. S-mucopolysaccharide B4·0T

(2) *Epithelial Sulphomucins* (testicular hyaluronidase-resistant)
(*a*) PAS-negative
(i) Sulphate esters on *vic*-glycols.
SYMBOL. VGS-mucin
(ii) Sulphate esters not on *vic*-glycols.
SYMBOL. S-mucin
(*a*) Alcohol-resistant azurophilia (0·02 per cent.) at or above pH 2·0.
SYMBOL. S-mucin B2·0
(*b*) Alcohol-resistant azurophilia (0·02 per cent.) at or above pH 4·5.
SYMBOL. S-mucin B4·5

B. Non-sulphated
(1) *Hexuronic acid-rich mucopolysaccharides*—hyaluronic acid.
SYMBOL. U-mucopolysaccharide

(2) *Sialic acid-rich mucosubstances*
(*a*) Connective Tissue Sialomucins
SYMBOL. C-mucopolysaccharide
(*b*) Epithelial Sialomucins
(i) Labile to Neuraminidase, PAS-positive, metachromatic with Azure A.
SYMBOL. CG mucin BN
(ii) Slowly digested by Neuraminidase
(*a*) PAS-positive.
SYMBOL. CG-mucin N±
(*b*) PAS-negative.
SYMBOL. C-mucin N±
(iii) Stable to Neuraminidase.
(*a*) Rendered metachromatic and susceptible to enzyme by prior saponification.
SYMBOL. S-mucin (Sap)BN
(*b*) Stable to Neuraminidase after saponification.
(i) PAS-positive.
SYMBOL. GC-mucin
(ii) PAS-negative.
SYMBOL. C-mucin

Table 2 : Classification of mucosubstances (Pearse, 1968b).

Symbol Equivalents of Some Characteristic Mucosubstances

Symbol	Localization and Description of Tissue Mucosubstance
G-mucosubstance	Gastric surface epithelia Thyroid Colloid (Man, Guinea pig, Rabbit) Coagulating Gland Fluid (Rat, Mouse)
S-mucopolysaccharide A(1·0$MgCl_2$)	Cornea and Mast Cells
S-mucopolysaccharide B2·0T	Cartilage Ovarian Follicle Fluid
S-mucopolysaccharide B4·0T	Aorta, Heart valves, Renal papilla, Some areas of Cartilage
VGS-mucin	Sublingual glands (Hamster), Glossal mucous glands (Rabbit), Colonic Goblets (Rabbit)
S-mucin B2·0	Colon (Guinea pig)
S-mucin B4·5	Exorbital Lacrimal Gland (Mouse)
SG-mucin B2·0	Glossal mucous glands (Rat, Mouse) Recto-sigmoid colonic goblets (Rat, Mouse)
SG-mucin B4·5	Duodenal Goblets (Rat, Mouse) Pyloric Glands (Rat, Mouse)
U-mucopolysaccharide	Ganglion cysts of Synovia (Man), Vitreous (Man), Cock's comb. Oestrogen-treated cervix uteri (Mouse)
C-mucopolysaccharide	? Cartilage
CG-mucin BN	Sublingual glands (Mouse, Hamster, Guinea pig)
CG-mucin N±	Vaginal epithelium (Pregnant Mouse)
C-mucin N±	Rectosigmoid mucous cells (Mouse)
S-mucin (Sap) BN	Sublingual Gland (Rat)
GC-mucin	Sublingual Gland (Man, Monkey)
C-mucin	Mammary gland secretion (Mouse)

Table 3 : Symbol equivalents of some characteristic mucosubstances

(Pearse, 1968b).

mucus secretions (Cooper and Jackson, 1981). The intestinal route of injection favoured the local formation of agglutinating antibodies that were directly transferred to mucus secretions and were of the s-IgA class (see sections on Adhesion and Immunological factors).

The presence of a mucus blanket and its associated flora, prevented colonisation of the intestinal epithelium by potential pathogenic organisms (Rozee et al, 1982). The presence of agglutinating antibodies of the s-IgA class in the mucus secretions of a host that has been orally immunised further facilitate the effectiveness of the mucin barrier to clear itself of foreign organisms (Williams and Gibbons, 1972; Freter, 1972). The removal of this mucus blanket and its normal associated flora by dietary changes, antibiotic treatment, or exposure to microbial toxins would effectively neutralise the mucin barrier and therefore facilitated tissue colonisation to invading organisms with the establishment of their associated pathology (Rozee et al, 1982; see section on Non-Immunological Factors). Allen (1983) has stated that progress in the study and better understanding of mucus has been slow mainly because of numerous technical problems encountered in isolating it as a soluble pure, native and undegraded mucus glycoprotein that retains both the viscous and gel-forming properties of the native secretion.

3.3 Extracellular Enzymes

Though the intestinal sialomucin layer presents a physical barrier to the V. cholerae organisms associating with the intestinal epithelial cell surface, it has been proposed (Arbuthnott and Smyth, 1979; Savage, 1980) that the major extracellular enzymes (mucinase, neura-

minidase and proteinase) produced by the organisms could provide an effective means of hydrolysing the mucus. The final outcome of such extracellular enzymic activity could enable the organisms to create random tracks in the sialomucin which would permit penetration to the columnar epithelial surface (Arbuthnott and Smyth, 1979). Thus, the V. cholerae organisms could then colonise a habitat on the surface of the small intestinal epithelium (Savage, 1980).

3.3a Mucinase

The mucinase (glycosidase) of V. cholerae was first mentioned by Burnet and Stone (1947a) who noted that when guinea-pig ileal segments were exposed to seitz-filtrates of 16 h old V. cholerae for 3 h at 37°C, there occurred desquamation of the ileal tissue. The filtrates of V. cholerae also possessed an enzyme which removed the influenza virus-receptor from red blood cells and designated it as Receptor Destroying Enzyme or R.D.E. (Burnet and Stone, 1947b). To establish whether the desquamation of guinea-pig epithelium was due to the R.D.E., the V. cholerae filtrate was heated for 30 min at 56°C and it was found that the receptor destroying activity was destroyed, but the desquamating activity was still present. Purified V. cholerae mucinase could be precipitated in the cold with ammonium sulphate, and after dialysis with borate buffered saline pH 7.0, could be precipitated further by two volumes of acetone in the cold (Burnet, 1948). The mucinase activity was not significantly affected between pH 7.0 - 8.6 but was ill-defined below pH 6.5. The mucinase activity was slightly higher in the presence of Ca^{++} ions than in their absence. If, however, the V. cholerae filtrate was treated with excess $CaCl_2$, at

pH 6.0 and heated for 30 min at 55°C, R.D.E. was fully active but mucinase activity was destroyed (Burnet, 1949). Mucinase activity was present in non-cholera vibrios as well as in cholera vibrios (Singh and Ahuja, 1953).

Jensen (1953) observed that immunisation of rabbits with mucinase from culture filtrates of 24-h-old V. cholerae (93A) grown on brain heart infusion agar gave rise not only to relatively high titres of antimucinase antibody but to agglutinins as well. The antigenic properties of mucinase were found not to be impaired by lyophilisation. When rabbits were immunised with washed, viable cholera vibrios, agglutinins were produced but, in contrast, the level of antimucinase antibody was very low. Immunisation of rabbits with mucinase filtrates that were heated or treated with phenol or formalin gave agglutinins and little or no antimucinase activity. Antimucinase immunity was unable to prevent desquamation (Singh and Ahuja, 1953). It was not possible, however, to serologically differentiate the mucinase produced by non-cholera from that of cholera vibrios (Freter, 1955).

After a period of 35 years since Burnet's first paper on mucinase in 1947, Schneider and Parker (1982) re-examined the purification and characterisation of V. cholerae mucinase. The crude mucinase was purified on a Bio-Gel P-100 column and two peaks were obtained. These peaks were re-chromatographed on the same column and the peaks were termed fraction 3 and 4. The G 100 Sephadex column gave weights for fractions 3 and 4 which were respectively 38 k. Dal. and 22 k. Dal.. If, however, the estimation procedure was carried out on the Bio-Gel P-100 column the weights of fractions 3 and 4 were 18 k. Dal. and 10 k. Dal. respectively. These findings were consistently obtained with ammonium

sulphate-precipitated material or with highly purified material which was chromatographed.

These peaks (fractions 3 and 4) were assayed for proteinase, intestinal mucinase and ovomucinase enzyme activities. The re-chromatographed fraction 3 had a protein content of 1.14 mg (mucinase active fraction)$^{-1}$ and a proteinase specific activity of 7.3 units (mg mucinase active fraction)$^{-1}$, and an intestinal mucinase specific activity of 0.554 units (mg mucinase active fraction)$^{-1}$. The re-chromatographed fraction 4 had a protein content of 0.37 mg (mucinase active fraction)$^{-1}$, a proteinase specific activity of 5.9 units (mg mucinase active fraction)$^{-1}$ and an intestinal mucinase specific activity of 0.397 units (mg mucinase active fraction)$^{-1}$. The ratio of proteinase to mucinase in fraction 3 was 13.2 and 14.9 in fraction 4. Fraction 3 when tested on SDS-PAGE and non-denaturing PAGE gels showed only a single band and in both gel systems had a molecular weight of approximately 36 k. Dal.. Electrophoresis in high pH nondenaturing gels was performed on fractions 3 and 4. Assays of gel slices demonstrated that proteinase, intestinal mucinase and ovomucinase activity occurred at the same position of the fraction 3 protein. Activity stains on casein agar revealed only a single band of proteinase activity at the position of the fraction 3. The optimum pH for proteinase activity was 9.0 but the preparation showed an activity pH range of 6.2-9.6. The proteinase activity was destroyed when the enzyme preparation was heated to 65^{o}C for 35 min. The enzyme was active against such substrates as immunoglobulins, bovine serum albumin and bovine catalase. When enzyme inhibitors of ovomucinase, intestinal mucinase and proteinase

activity were investigated, only heavy metals were highly inhibitory.

Antiserum produced in New Zealand rabbits against fraction 3 inhibited proteinase, intestinal mucinase and ovomucinase activity as well as fraction 4 enzyme activity. The anti-fraction 3 serum possessed both vibriocidal and agglutinating activity but it did not neutralise cholera enterotoxin in tissue studies.

When the antiserum was passed through a lipopolysaccharide-Sepharose column, the resulting antibody had a low titre of vibriocidal activity, and the agglutination activity titre was reduced from 256 to 32. The adsorbed antibody gave a single line of identity with fraction 3 and 4 in an Ouchterlony double diffusion assay. The protective capacity of the anti-sera preparations was determined by the "passive protection of infant mice". In this assay suckling mice which had not been fed for 4 h were inoculated perorally with 0.05 ml of the antiserum to mucinase or the adsorbed antisera to mucinase, and within 30 min were challenged with 10^7-10^8 CFU of V. cholerae strain GA401, and killed after 16 h. The small intestine was removed, weighed, and the remaining body weight determined. A ratio of less than or equal to 0.060 was considered to signify a negative response while a ratio of greater than or equal to 0.065 indicated fluid secretion. The adsorbed antiserum was protective against mucinase (ratio of 0.060) even at dilutions where it was no longer vibriocidal. The unadsorbed antiserum which possessed antibody to somatic antigens and mucinase gave protection (ratio = 0.061) even when diluted to a low titre of vibriocidal activity. The protection afforded by this vaccine against fraction 3 was not directed against vibrio somatic antigen or cholera enterotoxin.

Culture fluids used for the purification of mucinase lacked

neuraminidase activity. This lack of neuraminidase activity, however, could perhaps have been due to the fact that the buffer (pH 8.0) used was not at the optimum pH for V. cholerae neuraminidase activity (5.5-6.2; Ada, French and Lind, 1961; Schick and Zilg, 1977).

The role of mucinase, however, in V. cholerae still remains unclear.

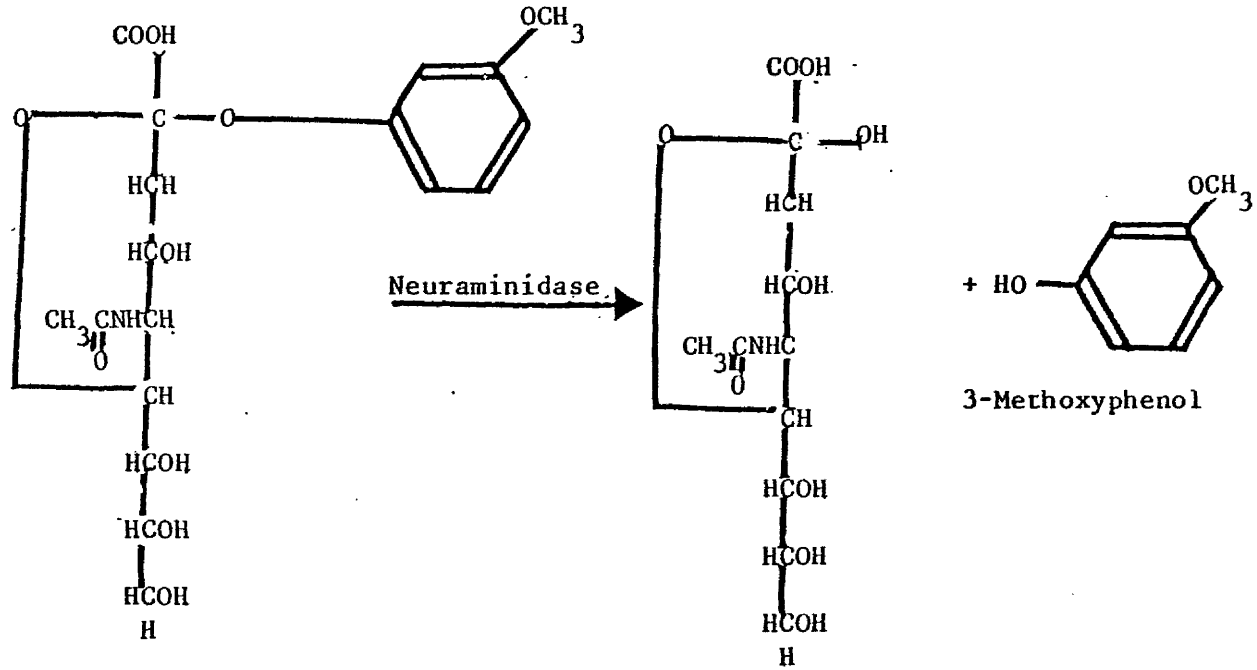
3.3b Neuraminidase

In 1956 R.D.E. was characterised as a glycosidase by Gottschalk. The R.D.E. was termed sialidase by Heimer and Meyer (1956), and later neuraminidase (Gottschalk, 1957). V. cholerae neuraminidase caused the removal of the terminal neuraminic acid unit from a neuramin-lactose substrate by hydrolytic cleavage of the glycosidic bond joining the keto group of N-acetylneuraminic acid to D-galactose or galactosamine (Figure 4a,b). Sialic acid moieties liberated as a result of the enzymic activity of neuraminidase were measured by the use of periodate oxidation, the development of a colour complex with thiobarbituric acid, and extraction of the colour complex in an organic phase, cyclohexanone (Warren, 1959). The colour complex was read in a spectrophotometer at $E_{532\ nm}$, $E_{549\ nm}$, $E_{562\ nm}$. The maximal absorption for sialic acid was at $E_{549\ nm}$, and the second absorption at $E_{532\ nm}$ was due to 2-deoxyribose. Aminoff (1961) noted that the N-acetylneuraminic acid coloured complex of the thiobarbituric acid procedure was identical to the colour complex obtained with 2-oxo-3-deoxy sugar acids. It was emphasised therefore that there existed a danger in relying solely on the thiobarbituric acid procedure to determine the sialic acid content of mucin.

V. cholerae neuraminidase was successfully purified from culture filtrates of V. cholerae strain 4 Z (Ada, French and Lind, 1961). The purification involved a five step procedure, namely, fractionation with concentrated cold methanol, adsorption to and elution from human red blood cells, fractionation with aqueous saturated ammonium sulphate, chromatography on columns of hydroxyl apatite and finally crystallisation. Enzyme activity was stimulated with Ca^{++} ions and was inhibited by ethylenediaminetetra-acetate. The enzyme showed maximal activity at pH 5.6. Moving boundary electrophoresis carried out on the crystalline V. cholerae neuraminidase (the crystalline neuraminidase prepared by Ada, French and Lind, 1961) revealed electrophoretic patterns at pH 6.7, 8.5 and 5.1 (Pye and Curtain, 1961). At each pH a corresponding single band was seen migrating in the direction of the anode. The enzyme sedimented as a single symmetrical boundary at pH 6.7 and 8.5 (Pye and Curtain, 1961). Neuraminidase from V. cholerae, Cl. perfringens and influenza virus had different isoelectric points, namely, 4.8, 4.95 and 8.9 respectively (Neurath, Hartzell and Rubin, 1970).

When V. cholerae strains Inaba 569B and Ogawa B 1307 were utilised for the production of neuraminidase (Kusama and Craig, 1970), strain 569B produced greater quantities of neuraminidase after 12 hr growth at 37°C. At 48 h and a temperature of 37°C, both strains produced equal quantities of neuraminidase but, after 48 h neuraminidase production by strain B 1307 continued to increase whereas that of strain 569B decreased. Affinity chromatography was successfully utilised to purify V. cholerae and Cl. perfringens neuraminidase (Cuatrecasas and Illiano, 1971). The affinity column consisted of

agarose beads which were linked to glyc-glyc-tyr by cyanogen bromide, and which in turn were linked by azo linkage to an inhibitor of neuraminidase, namely, N-(p-aminophenyl) oxamic acid. The neuraminidase activity present in extracts of Cl. perfringens and V. cholerae was completely adsorbed by this affinity column. An improved assay was developed which measured neuraminidase activity by the liberation of blue methoxyphenyl (Figure 3) from an artificial substrate (2-(3' methoxyphenyl-N-acetylαneuraminic acid - M.P.N.) (Palese, Ducher and Kilbourne, 1973). The assay was both rapid and precise and, in addition, the artificial substrate could be used for standardising the activity of neuraminidase from viral, bacterial and mammalian sources. Subsequent investigators (Sedmak and Grossberg, 1973) studied the enzyme kinetics of influenza virus and V. cholerae neuraminidase with the M.P.N. assay, and found the assay to be a rapid and precise measurement of neuraminidase activity but suggested that its use be limited to highly purified enzyme preparations, because contaminating proteins gave high background readings. Neuraminidase was also purified by Schick and Zilg (1978) who utilised a simplified procedure wherein crude neuraminidase obtained from culture fluid of V. cholerae organisms was passed two times through a DEAE cellulose column and eluted by an ionic strength gradient (0.1-0.3 M sodium acetate buffer pH 5.5). The specificity of neuraminidase action was related to the bacterial origin of the enzyme (Finné, 1978a). V. cholerae neuraminidase cleaved sialic acid from follicle stimulating hormone (FSH) but did not cleave sialic acid from luteinising hormone (LH) and therefore did not influence the biological activity of the hormone (Finné, 1978a). By contrast,



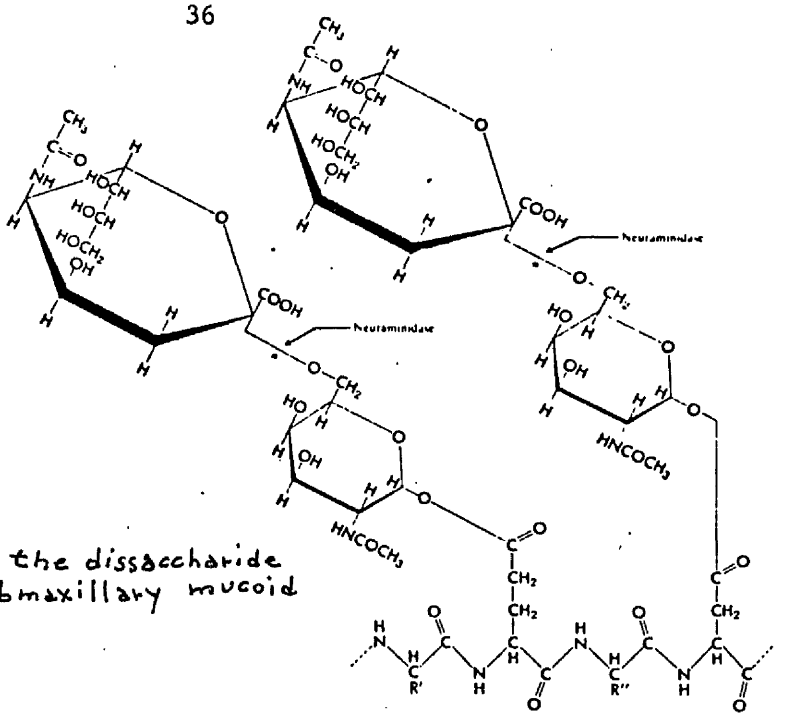
2-(3'-Methoxyphenyl)-N-acetyl-alpha neuraminic acid (M.P.N.)

N-acetylneuraminic acid

Figure 3 : Enzymatic hydrolysis of M.P.N. by neuraminidase (Palese, Bucher and Kilbourne, 1973).

Cl. perfringens neuraminidase did not possess the selective activity of the V. cholerae neuraminidase and therefore it destroyed the LH activity.

The effect that neuraminidase pretreatment had upon tissue sections was studied by Spicer and Warren (1960) who utilised Alcian blue stain followed by periodic acid schiff to histologically localise sialomucin. Histologically such neuraminidase pretreatment produced a loss of metachromasia. N-acetylneuraminic acid containing mucins of bovine submaxillary and rat sublingual glands pretreated with 0.1N H_2SO_4 (1 h at 80°C) or influenza neuraminidase (24 h at 37°C), showed a loss in Alcian blue staining in the mucus acini of the bovine submaxillary gland (Quintarelli et al, 1961). This loss of Alcian blue staining after pretreatment with either the acid or neuraminidase was attributed to the loss of sialic acid groups (Figures 4a, b). Another investigator (Kent, 1963) noted that "since chemically active groups in polysaccharide antigens in sections (e.g. hydroxyl, carboxyl) can be readily altered, the effect of these alterations can be investigated". Neuraminidase treatment of pig submaxillary gland for 24 h caused a complete removal of sialic acid as seen by a loss in the Alcian blue staining reaction. When the cow and pig submaxillary mucin were submitted to neuraminidase digestion, the loss of sialic acid was seen histochemically as an absence of Alcian blue staining. Neuraminidase digestion did not remove the whole mucin molecule, because mucinous areas continued to give positive PAS staining reactions (Figure 4a,b). In addition, when the cow submaxillary mucin failed to react with fluorescein labelled antibody after neuraminidase digestion, this



Structure of the dissaccharide unit of submaxillary mucoid

Figure 4a :

Diagrammatic segment of bovine submaxillary gland glycoprotein, showing (1) the structure of the prosthetic group; (2) the glycosidic ester linkages joining the prosthetic groups to the β- and γ-carboxyl groups, respectively, of aspartic and glutamic acid residues; (3) the neuraminidase-susceptible α-ketosidic linkage within the prosthetic group.

(White, Handler and Smith, 1968).

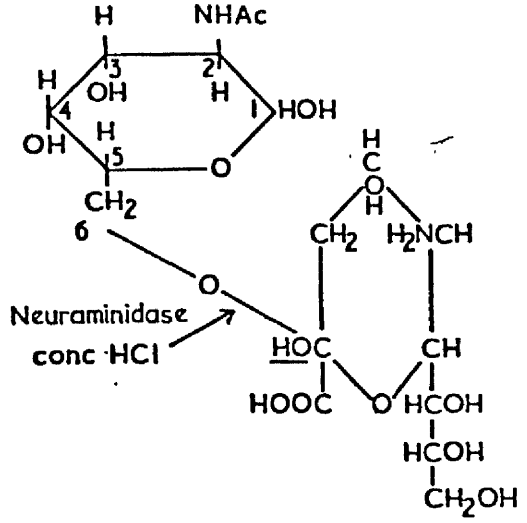


Figure 4b : Structure of the Disaccharide O-α-D-sialyl (2-6)-N-acetyl-D-galactosamine

(Pearse, 1968b).

suggested that sialic acid (NANA) was an essential antigenic determinant of cow submaxillary mucin, because when it was removed by neuraminidase digestion, there occurred a corresponding failure of cow submaxillary mucin to react with specific conjugated antibody to cow submaxillary mucin. The earlier findings of Spicer and Warren (1960) and Quintarelli et al (1961) concerning loss of Alcian blue staining following neuraminidase pretreatment were confirmed by Gad (1969). A more recent histological investigation that dealt with the effects of neuraminidase pretreatment utilised a peroxidase-labelled lectin from Limulus polyphemus (specific for sialic acid moieties) (Yamada and Shimazu, 1979). The loss of sialic acid from tissue sections due to enzymatic pretreatment seen by earlier investigators (Spicer and Warren, 1960; Quintarelli et al, 1961; Kent, 1963; Gad, 1969) as a loss in Alcian blue, was observed more recently as a loss in peroxidase-labelled Limulus polyphemus lectin (Yamada and Shimazu, 1979).

Neuraminidase has been utilised in a variety of biological and biochemical applications. One of the earliest applications was to remove influenza virus receptors from red blood cells (Burnet and Stone, 1947b). The enzyme was utilised to bring an increase in the production of corticosterone and cyclic AMP when adrenal cells were first pretreated with it before being exposed to cholera enterotoxin (Haksar, Maudsley and Person, 1974). When V. cholerae neuraminidase was added to a weakly enterotoxigenic strain of V. cholerae biovar eltor, the enterotoxigenic activity of the organism was intensified (Soloviev et al, 1976). Inactivation of endogenous follicle stimulating hormone (FSH) was brought about by pretreating the animal ovaries with neuraminidase (Finné, 1978b).

Pretreatment of frozen sections of mouse spleen cell follicle with V. cholerae neuraminidase amplified the adhesion reaction of erythrocytes to the tissue (Radaszkiewicz, Weirich and Denk, 1979). Pretreatment of unfixed human neutrophils with V. cholerae neuraminidase caused increased binding of fluorescein-labelled cholera enterotoxin (Figure 5) (Ackerman, Wolken and Gelder, 1980). Knop (1980a,b) found that V. cholerae neuraminidase affected Con-A induced T-cell proliferation by increasing the efficiency of macrophage T-cell cooperation.

A review of the current concepts on neuraminidases and their activity was made by Hutchinson and Kabayo (1977). Neuraminidase has been found in most myxo-viruses but not in other classes of virus, and so myxovirus(es) were formerly defined as having an affinity for mucin (Hutchinson and Kabayo, 1977). It was noted that in budding viruses such as influenza which possess surface neuraminidase, there was a lack of surface sialic acids. By contrast, those viruses such as Sindbis or Rabies which lack surface neuraminidase possess surface sialic acid. In addition, neuraminidases account for as much as 10% of the total virus protein (Hutchinson and Kabayo, 1977). In bacteria, the neuraminidases were either cell bound components or were excreted into culture filtrates. In higher vertebrates, neuraminidases were associated with lysosomes and other cellular organelles. These cellular neuraminidases could perhaps regulate the survival of glycoproteins circulating in the serum. The survey reached the conclusion that little was known about the function of bacterial neuraminidase, and proposed that when V. cholerae neuraminidase cleaved sialic acid residues from complex glycoproteins it prevented non-specific interactions with enterotoxin from taking place. Other investigators

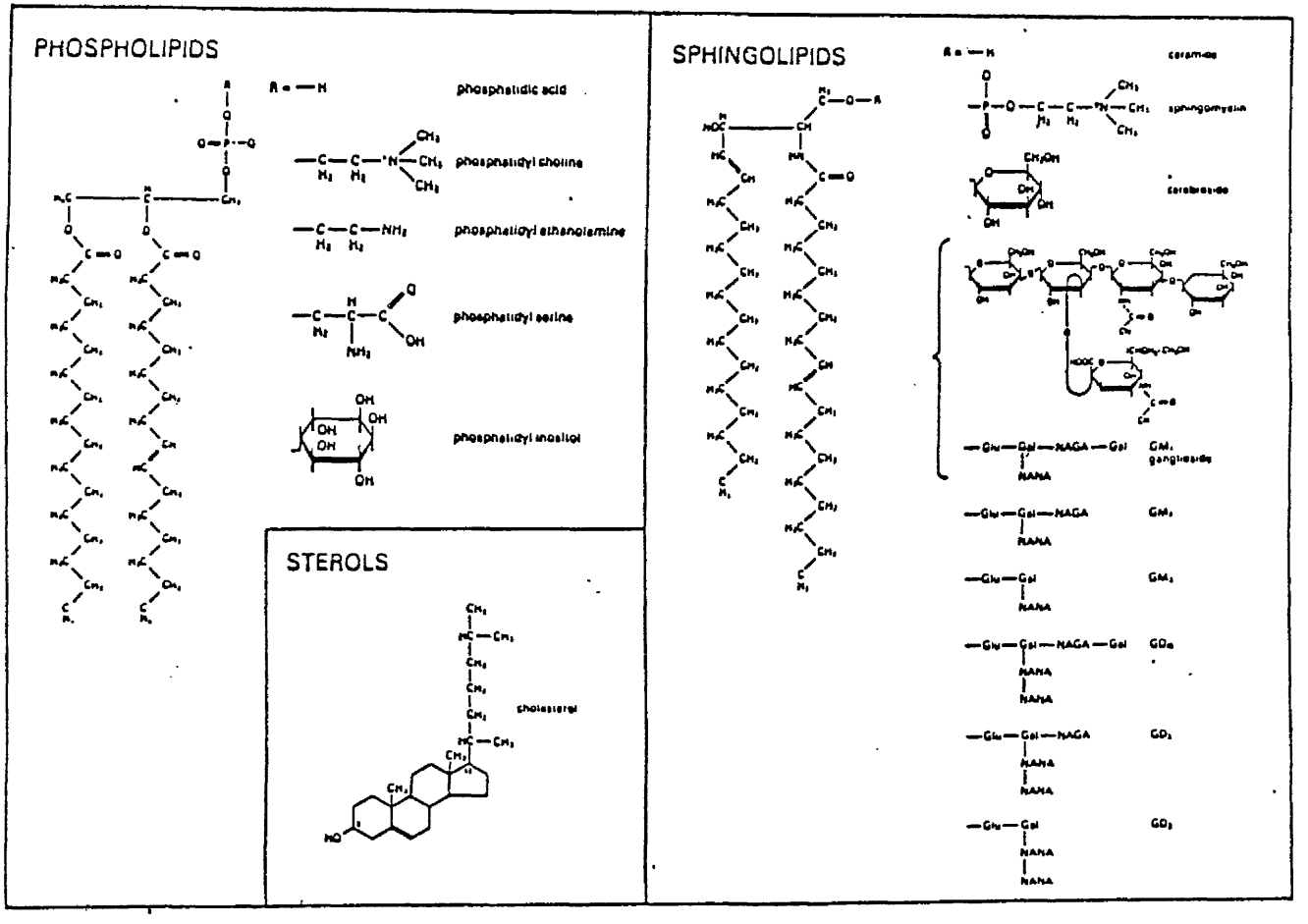


Figure 5 : Membrane constituents (Stephen and Pietrowski, 1981a).

(Soloviev et al, 1975) held the view that V. cholerae neuraminidase might play an important role during the early phase of V. cholerae pathogenesis, namely, in the adhesion process.

3.3c Proteinase

The proteinases make up the third major category of extracellular enzymes produced by V. cholerae organisms. When Inaba 569B and Ogawa B 1307 strains were grown in shaken Casamino acid-yeast extract glucose medium for 4 days at 30°C and 37°C (Kusama and Craig, 1970), no detectable proteinase activity was measured for filtrates of strain 569B with the proteinase assay procedure devised by Kunitz (1947). In filtrates from strain B 1307, however, detectable levels of proteinase activity were measured with the Kunitz procedure at 10 h post-inoculation at 37°C and at 24 h post-inoculation at 30°C. In addition, strain B 1307 produced gradual increases in detectable proteinase activity. The levels of detectable proteinase were found to be higher at 37°C than 30°C during the period of observation (Kusama and Craig, 1970).

There occurred one or two proteinase fractions when 5 strains of V. comma organisms were examined with the zymogram technique for proteinase activity (Dahle and Sandvik, 1971). Most of the proteinase fractions studied tended to move more to the cathode but some did, however, migrate in the direction of the anode at pH 6.2 (Dahle and Sandvik, 1971). There occurred serological cross-reactions between the proteinase A fractions by a strain of Aeromonas liquefaciens and 2 strains of V. comma. In addition, proteinase A fraction from Ae. liquefaciens was found to be enzymoserologically identical or closely related to one of the proteinase fractions of V. comma. The

proteinase B fraction of Ae. liquefaciens, however, was not enzymo-serologically related to that of V. comma (Dahle and Sandvik, 1971).

Schneider and Parker (1978) isolated mutants of strains Inaba CA401 and Ogawa CA411 of V. cholerae which were deficient in proteinase production. This deficient proteinase production was linked with a reduction in neuraminidase production. These deficiencies in proteinase and neuraminidase production were associated with a marked loss of virulence for infant mice despite the fact that enterotoxin was produced. When partial reversion to proteinase production occurred in the mutants, there was a corresponding increase in neuraminidase activity and an increased virulence for infant mice.

A single procedure for the analysis of V. cholerae proteinases was developed in which the catalytic specificity of the enzyme served as a basis for typing of V. cholerae strains (Schneider, Sigel and Parker, 1981). The procedure for analysis of proteinase typing utilised a standard protein (serum albumin) as a substrate for the enzyme which was found in the culture supernate. The peptides which were generated due to the action of the enzyme were separated on the basis of relative molecular weight by SDS-PAGE gel electrophoresis. The resulting bonding patterns in the gel were then used as the basis for the catalytic typing of proteinases.

When the culture supernate of V. cholerae biovar eltor 1621 and a mutant strain 1621 hip were subjected to isoelectric focusing, 3 different types of extracellular proteinases were revealed (Young and Broadbent, 1982). The first type was found to be the major activity in the parent strain, and was inhibited by phenylmethylsulfonyl fluoride and lima bean trypsin inhibitor. The second type was also

present in the parent strain but was also present in the mutant strain as well. This second type of proteinase was resistant to the inhibitors of metalloproteins and serine proteinases. A third type of proteinase was found to be present in the mutant strain, and was inhibited by EDTA.

Recent studies by Schneider and Parker (1982) have revealed that when mucinase was purified from V. cholerae strain CA401 by ammonium sulphate precipitation and column chromatography on Bio Gel P-100, proteinase activity was present, with the optimum pH at 9.0, and the activity occurring over a pH range of 6.2 to 9.6.

As has been the case for the other exocellular enzymes produced by V. cholerae organisms, the role of the proteinases still remains unclear as regards cholera pathology.

3.4 Adhesion

The outcome of the interaction between the host and the invading microbe depends on a combination of both virulence and the susceptibility of the host (Arbuthnott and Smyth, 1979). A correlation exists between the ability of organisms to adhere and their distribution on the epithelial surface (Gibbons and Van Houte, 1971). In a pathological process involving the small intestine the first event would be the attachment of the organism to the host epithelial surface in order to give it resistance to the mechanical flushing in the lumen (La Brec et al, 1964; Reed and Williams, 1978; Arbuthnott and Smyth, 1979). By achieving attachment, the pathogenic organism would survive and multiply on the surface of the host tissue in competition with commensal

organisms (La Brec et al, 1964; Arbuthnott and Smyth, 1979). Most often these organisms which had lost virulence (e.g. mutant strains) were no longer able to penetrate the epithelial barrier of the intestinal mucosa (La Brec et al, 1964).

Most enteric organisms have to overcome the effective barrier of the epithelial layer by being able to colonise various regions of the intestine (Erlandsen and Chase, 1974). The intestinal protozoan of rats Hexamita muris occupied the blind invaginations of the small intestine, while certain spiral-shaped organisms succeeded in establishing contact with the epithelium in the region of the crypts of Lieberkühn. In the case of the lactobacilli, the organisms adhered to the keratin layers of the stratified squamous epithelium of the rat stomach. Segmented filamentous bacteria have a more direct physical association between the host microvillous border of the intestinal epithelial cells and a specialised attachment segment or holdfast of the organism. In E. coli a pilus-like structure called the K-88 antigen has been found to be responsible for adhesion (Nagy, Moon and Issaacson, 1977).

Freter (1969) found that cholera vibrios in infected ileal loops of rabbits were distributed such that approximately one half were in the lumen while the other half were adsorbed to the mucosa. When rabbit anti-cholera serum was given to the infected loops there was a marked decrease in the percentage of vibrios adsorbed to the mucosa. A hypothesis formulated to explain this decrease in adherent organisms stated that the mechanism by which intestinal antibodies afford protection was by decreasing the adsorption of V. cholerae organisms (Freter, 1970). The principal immunoglobulin in the small intestine s-IgA (secretory IgA) agglutinated bacteria but was considered not to

be bactericidal, able to mediate complement dependent lysis, bind macrophages, or enhance phagocytosis (Williams and Gibbons, 1972). It was therefore postulated that the protective role of s-IgA was expressed by its ability to bind and aggregate bacteria (Williams and Gibbons, 1972). The hypothesis first formulated by Freter (1970) which proposed that intestinal antibodies afford protection to the intestinal mucosa by decreasing the number of adherent organisms, was confirmed by other investigations (Freter, 1972; Fubara and Freter, 1972; Freter, 1974; Freter and Jones, 1976).

Studies with frozen sections (Schrank and Verwey, 1976) observed cholera vibrios to be in close contact with the mucosa of the small intestine (Schrank and Verwey, 1976; Allweiss et al, 1977). A clumping of the cholera vibrios was observed within the mucus layer (Schrank and Verwey, 1976; Jones and Freter, 1976a). The V. cholerae organisms were found to move along tracks which were parallel to the lines of strain created within the stretched mucus layer of rabbit small intestine (Jones and Freter, 1976a). The presence of calcium ions was required for the adhesion of V. cholerae vibrios to rabbit brush border membranes but was not required for adhesion to take place in intact rabbit intestinal slices (Jones and Freter, 1976a). It was suggested that the calcium ions required for adhesion to occur in the rabbit brush border membrane were not needed in the intact rabbit intestinal slices simply because calcium ions which were already present in the buffer solution had diffused into these intact tissue slices. The colonisation of rabbit intestinal epithelium by V. cholerae organisms in experimental systems which utilised ligated loops of adult rabbits and non-ligated intestine of infant rabbits was done with scanning and

transmission electron microscopy (Nelson, Clements and Finkelstein, 1976). The investigators noted that there was a time lag period of up to 1 h before a significant degree of attachment of large numbers of vibrios occurred. The organisms were initially on the sides of the villi adhering by their surface coats directly to the tips of the microvilli; a few organisms were partially embedded in the brush border. Cultures of non-motile strains of V. cholerae were not able to adhere to rabbit brush border membranes (Jones and Freter, 1976b). Non-chemotactic mutant strains of V. cholerae also did not accumulate at the surface of intestinal tissue (Allweiss et al, 1977).

A study was made to examine the role of mannose-sensitive haemagglutinins in the adherence of V. cholerae strain KB207, biovar eltor, serotype Ogawa to rabbit small intestinal discs (Bhattacharjee and Srivastava, 1978). The haemagglutinins from agar cultures were mannose-sensitive, while the shaken or static broth cultures consistently gave mannose-resistant haemagglutination. Organisms from the broth culture of strain KB207 were less adhesive to freshly isolated rabbit intestinal discs (10 mm diam) than those from agar culture, despite the fact that haemagglutinins were present in both. D-mannose inhibited the adherence of organisms from the agar culture to rabbit intestinal discs, while the organisms from broth cultures which were less adhesive, and produced mannose-resistant haemagglutinins were not affected by D-mannose. When the intestinal discs were first exposed to D-mannose for 15 min prior to exposure to vibrios, there was no inhibition of adherence. Several non-motile strains produced haemagglutinins, and those non-adhesive strains derived from KB 207, both motile and non-motile, possessed haemagglutinating activity. Therefore

it was concluded that there was no relationship between haemagglutinins and adherence of V. cholerae to rabbit small intestine.

The role of chemotaxis in bacterial association to rabbit ileal tissue was further investigated by Freter, O'Brien and Halstead (1978). When slices of rabbit ileum were incubated in a suspension of V. cholerae organisms (in 0.1 M Krebs-Ringer-Tris buffer pH 7.4) for a period of 8 min, the non-chemotactic mutant strains were less adhesive when compared with parent strains. However, when slices of ileal tissue were placed directly against a thick paste of non-chemotactic vibrios, the advantage of the chemotactic parent strain over the mutant non-chemotactic strain was abolished. Therefore, the importance of chemotaxis was only in the process of delivering the vibrios to the surface of the mucus gel (Freter, O'Brien and Halstead, 1978).

Additional studies were made on the effects of chemotaxis on interaction of cholera vibrios with intestinal mucosa (Freter, O'Brien and Macsai, 1979). Rabbit intestinal slices were exposed to a suspension (in 0.01 M Krebs-Ringer-Tris buffer pH 7.4) which contained a mixture of polystyrene particles (1.1 μm diam), S. cerevisiae and vibrios of either parent or nonchemotactic mutant 31 strains for a period of 8 min. The intestinal slices were washed in 0.01 M Krebs-Ringer-Tris buffer pH 7.4, and frozen in liquid nitrogen. Sections of the slices were prepared in a cryostat at 12μ thickness. The sections were stained by 1.0% (w/v) giemsa stain, in order to determine the penetration of vibrios and other particles into the mucus gel of the rabbit intestinal slices. A count of the micro-organisms in 40 areas (as defined by the partially closed microscope diaphragm) was made.

Because of the difficulty in exactly quantitating the bacteria histologically, a ratio between the number of vibrios and polystyrene particles was utilised as a parameter for comparing the number of parent or non-chemotactic mutant 31 vibrios that had penetrated into the mucus gel. The findings shown in Table 4 indicated that while the chemotactic strain was relatively efficient in deeply penetrating the mucus gel of the rabbit intestinal slice (as seen by an increasing vibrio : particle ratio value), the nonchemotactic mutant 31 was, by contrast, inefficient in deeply penetrating the mucus gel (as seen by a decreasing vibrio : particle ratio value).

Both wild-type and mutant strains of V. cholerae were utilised in order to determine their ability to adhere to freshly isolated rabbit intestinal discs (Bhattacharjee and Srivastava, 1979). The optimum time for the exposure of organisms to tissue was 30 min at 37°C, and the optimum concentration of organisms per 5.0 ml phosphate buffered saline suspension was 10^5-10^7 vibrios. Non-motile, and weakly motile organisms were incapable of adhesion, but one of the motile strains (KB381) also showed limited adherent capacity. When phosphate buffered saline suspensions of cholera vibrios were first pretreated with 100 μg chloramphenicol ml^{-1}, and then exposed to rabbit intestinal tissue discs in the presence of the antibiotic (chloramphenicol), a significant reduction in the number of adherent wild-type organisms occurred. By contrast, the same pretreatment did not have any effect on the number of adherent organisms of a non-motile strain or a poorly adherent strain. When the V. cholerae organisms were exposed to rabbit intestinal tissue discs which had been previously immunised either parenterally (heat-killed strains KB92 and CD 1; live or

Vibrio strain	Initial suspension	Mucus gel		
		Above villi	Between villi (luminal half)	Between villi (basal half)
Parent strain	1.03	1.16	4.00	11.34
Nonchemotactic mutant 31	1.19	0.99	2.38	1.75

Table 4 : Penetration of parent strain and nonchemotactic mutant into mucus gel.
(Freter, O'Brien and Macsai, 1979).

Legend: numbers indicated in the table are that of a ratio;
no. of vibrios/no. polystyrene particles.

attenuated strain CD 1) or orally immunised (live strain CD 1), there was a significant reduction in the numbers of organisms which adhered to the immunised tissue.

A non-cholera vibrio, Kanagawa-Positive V. parahaemolyticus was examined in order to ascertain how its adhesion to human foetal intestinal cell monolayers would be affected by potential inhibitors of adherence (negatively charged glycoproteins, mono, di, tri, oligo and polysaccharides, lectins, enzymes, mono and dissaccharide components of chondroitin sulphate and xanthan; Carruthers and Anderson, 1979). When the monolayers were first exposed to 500 μg of the potential inhibitor in 0.5 ml of Earle's Balanced Salt Solution (EBSS) pH 7.2 for 15 min at 37°C, and then exposed to a suspension of 5.0×10^8 V. parahaemolyticus organisms, there occurred differences in the bacterial adherence when compared to the control or nontreated cells (Table 5). The results cited in Table 5 demonstrated that alterations in surface charge may contribute to the varying degrees of adherence by V. parahaemolyticus.

Srivastava, Sinha and Srivastava (1980) inoculated pathogenic, laboratory (non-adherent, streptomycin-dependent), and attenuated strains of V. cholerae into the ligated loops of rabbit (immunised and non-immunised against V. cholerae organisms) at a concentration of 10^5 cells ml^{-1} for a period of 18 h. No trace of cholera enterotoxin was found in those loops (non-immunised) inoculated with poorly adherent strains. The adhesive strains adhered quite well in the ligated intestinal loops of those rabbits that had not been immunised against V. cholerae organisms, but poorly in the ligated loops of immunised rabbits.

Inhibitor	Percentage adherence
None	100
Chrondroitin sulphate	24
Bovine proteoglycan preparations	
Cartilage	
Aggregate	21
Monomer	20
Nucleus pulposus	30
Skin	34
Aorta	27
Chondroitinase ABC digest	
Chondroitin sulphate	53
Proteoglycan aggregate	54
Heparin	51
Heparin sulphate	62
Pentosan sulphate	25
Dextran sulphate (mo. wt. 500 k. Dal.)	12
Xanthan	10
Inositol hexasulphate	1
Polygalacturonic acid	41
Glucose-6-phosphate	18

Table 5 : Inhibition of adherence of Kanagawa-positive strains by potential inhibitors (Carruthers and Anderson, 1979).

Legend: Bacterial adherence in the presence of a potential inhibitor calculated as a percentage of the adherence in its absence (control).

The adhesive properties of two V. cholerae strains, a wild-type pathogen KB207 and a mutant strain CD11 (derived from the wild-type pathogen by N-methyl-N'nitro-N-nitrosoguanidine treatment), were compared (Srivastava and Srivastava, 1980). The mutant strain CD 11 was both chemotactic and motile but was poorly adherent to rabbit intestinal mucosa. Exposure of the wild-type strain KB207 and the mutant strain CD 11 to D-mannose (10 mg ml^{-1}), followed by exposure to freshly isolated rabbit intestinal discs (previously treated with 100 m mol of sodium metaperiodate for 15 min) inhibited the adherence of the wild-type strain but did not affect the mutant strain.

Chemotactic and non-chemotactic strains of V. cholerae were exposed to a variety of substances to determine whether these reagents were chemotactically active for the vibrios (Freter and O'Brien, 1981a). Test tubes which contained bacterial suspensions (in 0.01 M Krebs-Ringer-Tris buffer pH 7.4) composed of mixtures of V. cholerae parent strain (6.0×10^3 cells ml^{-1}) and V. cholerae nonchemotactic mutant strain 31 (2.4×10^5 cells ml^{-1}), were exposed to capillary tubes (0.27 mm diam) each filled with one of 20 L-amino acids (Alanine, Arginine, Asparagine, Cysteine, Glutamic acid, Glutamine, Histidine, Isoleucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine and Valine). Vibrio suspensions were exposed to concentrations of the L-amino acids (varied from 10^{-4} - 10^{-6} M) for a period of 1 h at 37°C in order to determine if these L-amino acids were taxins (chemotactically active substances). The criteria for taxins were: (a) the substance attracted an average of 10 chemotactic parent strain vibrios in the taxin filled capillary and (b) the ratio of chemotactic parent strain : non-chemotactic strain vibrios in the capillary was 3

times higher than in the control capillary which contained only buffer. The chemotactic parent strain was found to be attracted to all 20 L-amino acids. When the experiment was performed with 8 different carbohydrates (D-Fucose, L-Fucose, D-Glucose, D-Galactose, D-Mannose, N-Acetylneuraminic acid, N-acetyl-D-glucosamine and N-Acetyl-galactosamine) in place of the L-amino acids, the chemotactic parent strain was attracted to only D-glucose, D-mannose, N-Acetylneuraminic acid, N-acetyl-D-glucosamine and N-Acetyl-D-galactosamine. The results obtained from the experiments with the carbohydrates contrasted with those obtained for the L-amino acids, namely, that the maximum number of vibrios which entered into the capillary was much lower for the capillaries containing carbohydrates than those containing amino acids (e.g. response to proline was 220 vibrios as opposed to 12 vibrios for galactose). When the experiment was performed using pepsin digest of rabbit mucosal scrappings or tryptone, the vibrios were attracted as strongly as was the case with amino acids. Finally, when bacterial suspensions of chemotactic parent or non-chemotactic strains were exposed to isolated rabbit brush border membranes for 15 min at $37^{o}C$, the nonchemotactic mutant could not be distinguished from the chemotactic strain with regard to their ability to adhere in vitro to rabbit brush border membranes.

The role of chemotaxis in the association of V. cholerae organisms to tissues was re-assessed (Freter and O'Brien, 1981b) by injecting 0.05 ml vibrios (4.0×10^5 vibrios each of parent or non-chemotactic mutant strain 31 suspended in Trypticase Soy Broth plus 0.01% (w/v) Evans Blue) into the stomach of 5-6 day old BALB-Cwm mice.

The mice were sacrificed at an interval of 4 h after infection, and at intervals of 18-22 h after infection. The entire small intestine was then removed and homogenized in 25 ml of sterile Trypticase Soy Broth, and dilutions of the homogenates were plated out in pour plates of semisolid agar (1.0% (w/v) tryptone, 0.5% (w/v) NaCl, 0.4% (w/v) agar, 0.01% (w/v) 2,3,5-triphenyl tetrazolium chloride and 1.0 mg streptomycin ml^{-1}). Despite previous *in vivo* and *in vitro* studies with cholera vibrios, the non-chemotactic mutant strain showed a greater fitness in 5 day old mice as seen by a higher percentage of nonchemotactic mutants recovered from the small intestine (after 4 h parent strain : 0.0299%; mutant strain : 0.167%, and after 18-22 h parent strain : 26.2%; mutant strain : 132.2%). The histological studies showed, however, that there occurred a greater penetration of the chemotactic parent vibrios into the mucus gel.

In experiments where radioactively labelled (either ^{32}P, ^{35}S, or ^{3}H thymidine) chemotactic parent or non-chemotactic strains of *V. cholerae* were injected into mouse small intestine, the ^{32}P labelled organisms gave results that were in accord with the notion of an accelerated rate of death of the chemotactic parent strain in the infant mouse small intestine (Freter and O'Brien, 1981b). Where the vibrios (both chemotactic and non-chemotactic) were labelled with ^{35}S or ^{3}H thymidine, however, the amount of radioactivity retained in the small intestine after the feeding of radioactive labelled *V. cholerae* organisms, more closely correlated with the isotope rather than with the strain of vibrio under scrutiny. Therefore, Freter and O'Brien (1981b) expressed grave misgivings on the validity of experimental techniques which solely relied on radioisotopes to determine the

in vivo location and the death or survival of radioactively labelled bacteria.

A follow up study was made to focus more clearly upon the role of chemotaxis in rabbit ileal loops with chemotactic parent, non-chemotactic mutant 31, and nonmotile strains of V. cholerae organisms (Freter, O'Brien and Macsai, 1981). As in an earlier investigation (Freter, O'Brien and Macsai, 1979), a suspension which contained a mixture of three components (polystyrene particles, S. cerevisiae and V. cholerae chemotactic parent, or nonchemotactic mutant 31 or nonmotile mutant organisms) was utilised for adhesion experiments. A volume of 0.25 ml of the suspension mixture (contained 7.5×10^8 organisms of each of the three major components) was injected into the ligated small intestinal loops of Dutch belted male rabbits. The loops were exposed for 15 min to the suspension mixtures and were then removed from the animals. Frozen sections were stained (1.0% (w/v) giemsa) without fixation, and a count (40 fields) of the microorganisms and particles in mucus gel, the overlying villi and the spaces between the villi (Table 6) of the frozen sections at 500 X under the optical microscope. The chemotactic parent had a vibrio : particle ratio of 1 in the lumen of the rabbit intestinal loop, which increased to 10 in the deep intervillous spaces. Both the nonchemotactic mutant and the nonmotile mutant, however, showed no changes in the vibrio : particle ratio. It was therefore stated that the superior ability of the parent strain to penetrate the intestinal mucus gel in vivo was related to its active motility which was guided by chemotaxis along a taxin gradient (a gradient of chemotactically active substances) which extended deeply into the mucus gel.

The inhibitory activity of G15 Sephadex purified mucosal extracts was tested on 1.0 ml (0.01 M Krebs-Ringer-Tris buffer pH 7.4 suspended

		Mucus gel		
Strain	Initial suspension	Above villi	Between villi (luminal half)	Between villi (basal half)
Parent (chemotactic)	1.03	1.22	4.62	9.99
Mutant 31 (nonchemotactic)	1.07	1.00	3.08	1.72
Mutant (nonmotile)	0.97	1.08	1.25	1.09

Table 6 : Comparison of penetration of vibrios into mucus gel (Freter, O'Brien and Macsai, 1981).

Legend: numbers in the table indicate vibrio/particle ratio.

organisms) suspensions of V. cholerae chemotactic parent strain (5.0 x 10^5 vibrios labelled with ^3H thymidine) plus rabbit ileal slices for 15 min at 37°C (Freter et al, 1981). The slices were rinsed twice in sterile saline and finally digested with protosol. Radioactivity was counted by a liquid scintillation counter. Exposure to the G15 Sephadex purified mucosal extracts brought about inhibition of V. cholerae chemotactic strains in the in vitro adherence to rabbit ileal slices. This supported the theory that inhibition of mucosal association by the pepsin digested mucosal extracts was the result of the blocking of taxin receptors on the surface of the vibrio.

The penetration of bacteria and inert particles into mucus gel was once more investigated by exposing a 1.0 ml mixture containing polystyrene particles (1.0 x 10^9) plus S. cerevisiae cells (1.0 x 10^9) plus V. cholerae chemotactic parent or non-chemotactic mutant vibrios (1.0 x 10^9) to slices of rabbit ileum for 15 min at 37°C. These were rinsed in buffer (0.01 M Krebs-Ringer-Tris buffer pH 7.4), frozen in liquid nitrogen, sectioned, and finally stained in 1.0% (w/v) Giemsa stain. The sections were viewed under the optical microscope at 500X magnification, and the particles and microorganisms were counted (in 40 areas which were defined by the partially closed microscope diaphragm). The vast majority of the organisms predominantly associated with the mucus gel of the rabbit ileal slices rather than the mucosal epithelium. The polystyrene particles and the non-chemotactic mutant vibrios penetrated the mucus gel of the rabbit ileal slice poorly, but the chemotactic parent vibrios did, by contrast, reach the deep intervillous spaces in significantly higher numbers. From this result it was stated that though the mucus gel represented a

barrier, it could be penetrated by both inert particles and non-chemotactic mutants rather inefficiently, but, where the vibrio is both motile and guided by chemotactic gradients, it could efficiently penetrate into the deeper layers of the intervillous spaces within a period of minutes. It was further stated that the association between mucus gel and organisms is reversible to some degree, and that the motile organisms must keep moving rapidly just to stay in place against the natural flow of mucus.

The relationship between bacterial adhesion and pathogenesis of cholera was recently examined again by Chitnis, Sharma and Kamat (1982). Anti-serum to V. cholerae lipopolysaccharide was raised in rabbits by intravenous injections (0.5, 1.0, 1.5 and 2.0 ml volumes) of a suspension of boiled cells (1.0×10^9) V. cholerae strain 569B at intervals of 3 to 4 days. The rabbits were then bled 1 week after the final injection. To test the effect of antibacterial antibody on in vivo adhesion and toxin production, ligated small intestinal loops of 10.0 cm in length made in adult rabbits were utilised. Alternate ligated loops were given 1:80 dilution of normal rabbit serum (decomplemented) or 1:80 dilution of the anti-serum against boiled cells of V. cholerae strain 569B. After a period of 30 min the loops were challenged with 1.0×10^5 cells of V. cholerae strain 569B, and after an interval of 8 h the loops were opened and the volume of each loop was measured. The contents of the loops were subjected to viable vibrio counts and assayed for cholera enterotoxin (cutaneous vascular permeability procedure of Craig, 1965). The intestinal loops were rinsed in sterile phosphate buffered saline, homogenised, and the homogenate was

plated out on nutrient agar in order to determine the number of organisms adherent to the mucus membrane by viable bacterial count (Table 7). The loops treated with the anti-serum had fluid accumulation that varied from between 0-1.5 ml, whereas those loops that received the normal serum showed fluid accumulations of 5.0-7.0 ml. Those loops that were treated with either anti-serum or normal serum showed approximately the same number of organisms (organisms in the lumen plus organisms adhering to the mucosa). The total amount of biologically-active cholera enterotoxin produced in both anti-serum and normal serum treated loops were again virtually the same, namely 1600-3200 units. The same amounts of cholera enterotoxin, however, produced greater fluid accumulations in the normal serum treated loops where the numbers of adherent vibrios rose 10-15 fold. The findings of this investigation presented evidence to support the view that adherence of cholera vibrios to the mucus membrane is an important step in V. cholerae pathogenesis. The reason for this is that the level of cholera enterotoxin produced by the vibrio is not adequate to induce fluid section, unless the enterotoxin is released by the adherent organisms in the highest concentration at the correct site on the small intestinal epithelium.

Holmgren, Svennerholm and Lindblad (1983) stated that 12 Classical biovar strains of V. cholerae (either serotype Inaba or Ogawa) caused agglutination (bacterial cell adhesion) of human erythrocytes, and that the addition of L-fucose brought about an inhibition of agglutination. By contrast, the agglutination of chicken erythrocytes by 12 eltor biovar strains of V. cholerae, was reversed by the addition of D-mannose or one of the following carbohydrates: D-fructose,

Serum	Starting bacterial suspension (vibrios ml^{-1})	Total number of vibrios in loop (after 8 h)	Number of vibrios in the lumen (after 8 h)	No. of vibrios adhering to the small intestine (after 8 h)	Total units entero-toxin	Fluid accumulated in gut ($ml\ loop^{-1}$)
Normal serum	1.2×10^6	1.293×10^9	1.28×10^9	1.3×10^7	3200	7.0
		5.675×10^8	5.6×10^8	7.5×10^6	1600	7.6
		1.437×10^9	1.43×10^9	7.1×10^6	1600	5.0
		7.84×10^8	7.65×10^8	1.87×10^7	1600	5.0
Antiserum to V. cholerae 569B Inaba boiled cells	1.2×10^6	8.66×10^8	8.65×10^8	9.65×10^5	2262	1.5
		7.67×10^8	7.6×10^8	7.45×10^5	1600	1.0
		1.53×10^9	1.53×10^9	4.45×10^5	1600	0.5
		1.36×10^9	1.36×10^9	9.55×10^5	1600	0.0

Table 7 : Effect of antibacterial antibody on *in vivo* adhesion and toxin production.

(Chitnis, Sharma and Kamat , 1982)

D-glucose, αmethyl mannoside and sucrose. In an earlier investigation (Holmgren, Svennerholm and Åhren, 1981), it was found that milk from Swedish women brought about an inhibition of cell adherence (haemagglutination) in both classical and eltor biovar strains of V. cholerae. It was also noted that the main inhibitory activity of human milk was separate from the immunoglobulin fraction of the human milk. Therefore it was hypothesised that the capacity of the non-immunoglobulin fraction of the human milk to bring about the inhibition of haemagglutination could be due to the presence of structure analogs (oligosaccharides or glycoconjugate cell receptors) for specific bacterial adhesins and enterotoxins. This hypothesis was re-examined in 1983 by Holmgren, Svennerholm and Lindblad who first fractionated human milk.

The human milk was first fractionated by ultrafiltration with a PM-10 membrane, which separated the milk into three fractions. The first of these milk fractions was the high molecular weight fraction A, the second fraction was a low molecular weight fraction B and the third fraction was also a low molecular weight fraction C. Fraction A was passed through an immunosorbent column which contained anti-human immunoglobulin α chain specific antibody, and anti-human total immunoglobulin to remove the immunoglobulin components. Caseins were removed from both whole human milk and fraction A by precipitation at pH 4.5 with 0.1 M NaAc. Both the A and B fractions were further fractionated by ammonium sulphate precipitation (40-100% saturation) in the cold for 60 min, followed by centrifugation at 10,000 g for 2 min. The precipitates were resuspended in 50 volumes of Krebs-Ringer-Tris buffer pH 7.4, and concentrated to their original volume

(fraction suspended in 10 volumes of Krebs-Ringer-Tris buffer pH 7.4). The addition of sodium periodate (to a final concentration of 0.05 M) followed by the boiling of the sample (fraction A or fraction B), and the incubation of the sample at 37°C for 24 h was necessary in order to destroy the haemagglutinating capacity of the oligosaccharides present in the human milk. To remove the structure analogs (glycocompounds) from the milk, 1.0 ml volumes of fraction A and B were added to a concanavalin A column. The glycocompounds which were bound to the column were eluted with 0.5M αmethyl-D-glycopyranoside. Fraction A was subjected to pronase digestion (2.3 mg (ml fraction A)$^{-1}$) to see if such a treatment had any effect on the high molecular weight haemagglutinating capacity of fraction A. Lipids were removed from pooled human milk by methanol-water extraction. The lipid extracts were further separated into acidic and neutral lipids by ion exchange.

The capacity of the glycocompounds to cause haemagglutination was tested by adding 10 µl of 3% erythrocyte suspension to 10 µl of two serial dilutions of *V. cholerae* suspension (either classical or *eltor* biovar) on a glass microscope slide in the presence of glycocompound (Holmgren, Svennerholm and Lindblad, 1983). These glycocompounds did possess the capacity to inhibit haemagglutination of erythrocytes by either the classical or *eltor* biovar of *V. cholerae* organisms.

The relevance of the investigation of Holmgren, Svennerholm and Lindblad (1983) was expressed in three ways: (a) that isolation and characterisation of the inhibitory carbohydrates from milk and other secretions could be useful to identify the natural cell bound

receptors for classical and eltor V. cholerae organisms, (b) that these structure analogs may be of relevance in relation to host defence in newborn infants because enteric infections were more prevalent in infants not weaned on human milk and finally, (c) if the inhibitory receptor-like glycoproteins are also present in saliva and gastro-intestinal juice, these could perhaps be utilised as the basis for a defence against mucosal infections.

Recently, Attridge and Rowley (1983) noted that V. cholerae strain 017, biovar eltor, attached equally well to the serosal and mucosal surfaces of 6 month old Swiss mouse intestinal tissue segments. In addition, the binding of cholera vibrios to these intestinal tissue segments remained undiminished even when the tissue segments were first boiled before being exposed to the organisms. Therefore Attridge and Rowley stated that receptor structures for cholera vibrios would have to be heat stable and abundant over both mucosal and serosal surfaces.

Despite the fact that many parameters have been found experimentally to increase, decrease and inhibit the adhesion of V. cholerae organisms to intestinal mucosa, there are still conflicting descriptions of vibrio adherence. These conflicting reports could be perhaps due to differences in methodology used to measure vibrio adhesion (Attridge and Rowley, 1983). The precise mechanism by which vibrio adhesion is achieved, however, still remains unresolved.

IV BASIC INTESTINAL ECOLOGY

Infections which are localised within, or invade by the gastro-intestinal tract are important causes of morbidity and mortality in humans and animals (McClelland, 1979). The relationships therefore between the gastrointestinal tract and pathogens cannot be easily separated from the relationship of host to its normal intestinal flora.

The composition, distribution and activity of the intestinal flora is dependent upon the composition of the intestinal contents in the various regions of the gut and on the antibacterial systems of the host (Drasar, 1974). Further factors affecting the antibacterial systems of the host are diet, physiology of the host and the interaction between bacteria in some parts of the intestine (Drasar, 1974; Table 8). The gut is able to control the normal and abnormal microbial flora by two major types of defence mechanism which utilise (a) Non Immunological Factors, and (b) Immunological Factors).

4.1 Non-Immunological Factors

The Non-Immunological Factors have an important role to play in determining the intestinal microflora (McClelland, 1979). The low pH of the stomach, peristalsis, movement of the villi and the toxicity of conjugated and deconjugated bile acids all serve as non-immunological factors which restrict bacterial growth in the intestine (Franklin and Skoryna, 1971; Binder, Filburn and Floch, 1975). Those bacteria which are part of the normal flora are able to deconjugate bile acids, and therefore able to restrict the growth of pathogenic bacteria

Table 8 : Local host factors influencing the intestinal flora

(1) Host physiology
  (a) Intestinal secretions
  (b) Intestinal mucosa
  (c) Immune mechanisms

(2) Environmental factors
  (a) Bacterial contamination
  (b) Diet
  (c) Antibacterial drugs

(3) Bacterial interactions

(Drasar, 1974)

(Binder, Filburn and Floch, 1975). Mucin acts as an important component of the non-immunological host resistance because of its ability to trap particles, and then with the peristaltic contraction in the stomach and the intestine to remove it (Florey, 1933; see section on Mucin).

The Paneth cells have been found to function as phagocytes (Erlandson and Chase, 1972), and perhaps these cells keep the crypts of Lieberkühn free from bacteria and other microbes. At present, however, there does not exist enough evidence to support the hypothesis that polymorphonuclear leukocytes and macrophages function as effective phagocytes either in the gut lumen or at the epithelial surface of the intestinal tract (Smith, 1977).

Indigenous microorganisms which are normally part of the intestinal flora prevent or perhaps limit the growth of pathogenic microorganisms in the gastrointestinal tract (Savage, 1972, 1977). The mechanism of microbial interference is complex, but it has been observed that when the ecosystem in the gut of the host has been disturbed by antimicrobial and other drugs, starvation or stress, the system of microbial interference does not function properly (Freter, 1956; Savage, 1972; Tannock and Savage, 1974; Holdeman, Good and Moore, 1976). The stress factor that travellers encounter would also disturb the gut ecosystem, thus making them susceptible to diarrhoeal diseases (Shore et al, 1974).

4.2 Immunological Factors

In a host which has had prior contact with antigens of a

particular microbial pathogen, the organisms would encounter immuno-specific as well as non-immunological host resistance factors (McClelland, 1979; Thomas and Jewell, 1979). An elicited immune response due to a particular antigen could have come about because it shared antigen similarity with another pathogen which the host had previously encountered (Myerowitz, Gordon and Robbins, 1973). In addition, it is often the case that the host elicited an immune response to a particular antigen because it shared antigens with non-pathogenic organisms which normally make up part of the intestinal flora (Myerowitz and Norden, 1977).

The antibodies that play an influential role in dealing with intestinal surfaces are those secreted onto these surfaces (Tomasi and Grey, 1973; Fubara and Freter, 1973). One of the tasks attributed to these secretory antibodies is to prevent bacterial pathogens from attaching to the intestinal epithelium (Fubara and Freter, 1973). The secretory antibodies which predominate in the normal small and large intestine are of the IgA class (Bienenstock, 1979; McClelland, 1979). This IgA molecule produced by the lymphoid system of the gastrointestinal tract differs from the IgA found in other parts of the mammalian body because it is associated with an additional structure called the secretory piece (Thomas and Jewell, 1979). This secretory piece is produced by the glandular epithelial cells of the intestinal tract but not by goblet cells (Poger and Lamm, 1974; Brandtzaeg, 1974). The secretory piece or secretory component is a glycoprotein which is attached to IgA dimers and which enable the IgA to be actively transported across the mucosa to the intestinal lumen,

and also serves to protect the IgA molecule from proteolysis in the intestinal lumen (Thomas and Jewell, 1979). Despite the extensive information about the physiological functions of s-IgA (secretory IgA) many functions are still in dispute (Table 9).

The ecological environment of the gastrointestinal tract is constantly being exposed to liquids which contain food, microbial antigens and pathogenic organisms, and thus exerts both physical and immunological mechanisms to maintain ecological stability.

Table 9 : Functions of IgA antibody

Viral neutralisation
Bacterial agglutination
Inhibition of bacterial motility
Bacterial killing (with co-factors)
Alteration of bacterial growth
Antitoxin activity
Inhibition of bacterial adherence to mucosal surfaces
Opsonisation (?)
Complement fixation (?)
Binding to mucin (?)
Non-antibody binding of proteins (antitrypsins)
Inhibition of bacterial enzymes
Inhibition of antigen uptake

(McClelland, 1979)

OBJECT OF RESEARCH

At present, the actual function or functions of Vibrio cholerae neuraminidase in cholera infection are not clear. This is despite the extensive knowledge of the biochemical properties of the enzyme itself.

The specific objects of this investigation were to determine the effects of V. cholerae neuraminidase on the sialomucin of the small intestine, on the ability of antineuraminidase antiserum to neutralise the activity, and finally to throw light on the possible functions of the enzyme in the pathological processes associated with cholera.

MATERIALS AND METHODS

I STRAINS OF VIBRIO CHOLERAE

V. cholerae NCTC 10732, Classical Biovar, Serotype Inaba, was obtained from the National Collection Type Culture (NCTC).

V. cholerae SL I, Classical Biovar, Serotype Inaba, was obtained from Dr. J.P. Craig, Downstate University and original was isolated by Dr. Rolf Freter, University of Michigan.

II PRODUCTION OF V. CHOLERAE NEURAMINIDASE

The 15 litres of Proteose peptone - Colostrum medium (pp215-216 Appendix II) in the fermenter vessel were inoculated with an overnight culture of V. cholerae 10732 grown in 250 ml of 2% proteose peptone broth. The overnight culture was introduced into the fermenter vessel by the filling port (pp 223-227, Appendix IV.) The anti-foam mixture was 1% Silcolapse. The fermenter was run at 37°C with an air-flow of 5 litres min^{-1} and stirred at 300 rev min^{-1}. The air outlet hose was connected to a condenser and to two 2 litre flasks in series which contained 1.0% (v/v) hibitane. After 24 h the organisms were killed in situ by increasing the temperature to 56°C for 2 h.

The culture was transferred to a presterilised 20 litre jar with positive pressure to force the culture through the sampling port hose into the jar. Calcium chloride ($CaCl_2$ anhydrous; 75.0 g) was added to flocculate the cells. In some experiments the presence of too much anti-foam prevented salting out and therefore it was necessary to centrifuge the culture fluid at 7000 rev min^{-1} (9000 g) to remove cells in an M.S.E. 25 centrifuge.

III METHANOLIC PRECIPITATION OF PROTEIN

The culture supernate (800 ml) was placed in each of 18 winchester bottles (2.5 litres) and 240 ml of methanol, precooled to -30°C, was added. Similar quantities were added each hour during a 5 h period, thus giving a total volume of 1200 ml methanol. Methanolic precipitation occurred within 48 h at 4°C. The supernate was decanted and discarded and the protein was harvested by centrifugation

for 15 min at 9000 rev min^{-1} (12000 g). The pellets from the 18 Winchesters were pooled and resuspended in 100 ml 0.05 M NaAc buffer pH 5.5. The suspension was dialysed against 0.05 M NaAc (pH 5.5) buffer for 2-3 days at 4°C. The dialysed suspension was centrifuged for 15 min at 9000 rev min^{-1} (12000 g) and the supernate was retained. The residue was washed once with buffer and the supernates containing the crude neuraminidase were pooled. The crude neuraminidase (200 ml) was concentrated with Carbowax (polyethylene glycol 4000; B.D.H., Poole, England) to a final volume of 50 ml. This neuraminidase concentrate was used as the starting material for further enzyme purification by gel filtration or ion exchange chromatography.

IV PURIFICATION OF NEURAMINIDASE

1. Gel filtration

The most suitable Sephadex for the semi-purification of crude enzyme preparation was determined. Sephadex G75, 100 and 200 (3.0 g) were allowed to swell for 5 h in a boiling-water bath. The gels were each resuspended in 0.05 M NaAc buffer pH 5.5. A column (20 x 2.0 cm) was poured with each grade and equilibrated with 0.05 M NaAc buffer pH 5.5. The columns were run at a flow rate of 1.4 ml min^{-1} and 2.0 ml fractions were collected. A sample of 1.0 ml of crude enzyme preparation was added to each column. Peak fractions ($E_{280\ nm}$) were pooled and concentrated down to the initial volume of 1.0 ml against Carbowax at 4°C. The pooled fractions were tested for neuraminidase activity by the thiobarbituric acid assay (T.B.A.). These peak samples were stored at -20°C.

The G100 (7.0 g) was allowed to swell for 5 h in a boiling-water bath. The gel was resuspended in 0.05 M NaAc buffer pH 5.5. A column 1.5 x 81 cm (Whatman I.E.C. Column of 1.5 x 100 cm dimensions) was poured and equilibrated with 0.05 M NaAc buffer pH 5.5. The crude enzyme preparation (2.0 ml) was pipetted onto the column. Fractions (2.0 ml) were eluted from the column at a flow of 0.8 ml min^{-1} with 0.05 M NaAc buffer pH 5.5. The $E_{280\ nm}$ value and Lowry protein value were determined for each fraction to detect the protein peaks. In addition, a T.B.A. assay and a methoxyphenyl-neuraminate assay (M.P.N.) were done to determine the peak of enzyme activity. Fractions with strong T.B.A. and M.P.N. neuraminidase activity were pooled, concentrated down to the initial volume of 2 ml with Carbowax at 4°C and stored at -20°C.

In order to obtain a supply of enzyme, activity peaks were pooled from groups of six gel filtration runs. These neuraminidase preparations were designated as follows:

N_1 - 8.0 ml

N_2 - 31.0 ml

N_3 - 14.0 ml

N_4 - 20.0 ml.

2. Ion-exchange chromatography

Both diethylaminoethyl cellulose (DEAE) and carboxymethyl cellulose (CM) (Whatman Ltd., Maidstone, Kent, England) were used in the purification of neuraminidase.

2a. CM-cellulose ion exchange chromatography

Fines were removed from 80 g CM cellulose (Whatman, Grade CM-32, micro-granular, cationic exchanger) by washing with water and decanting the fines at each wash. The cationic exchanger was pre-cycled with first 15 volumes of 0.5 N NaOH for 30 min and subsequently washed with 0.1 M NaAc buffer (pH 5.5) until the effluent reached the intermediate pH of 8.0. Secondly, 15 volumes of 0.5 N HCl were added for 30 min followed by repeated washing with 0.1 M NaAc buffer (pH 5.5) until the effluent reached pH 5.5. The CM-cellulose was suspended in this buffer to give a pourable slurry. A column (2 x 78 cm) was poured and equilibrated with 0.1 M NaAc buffer pH 5.5. Two ml of the crude neuraminidase preparation (N79625) was run through the column and 2.0 ml fractions were collected. Peak fractions were pooled, placed in dialysis tubing (Visking, Medicell Int. Ltd., Liverpool, England) and concentrated down to the initial volume of 2 ml with Carbowax. The peak sample was tested for neuraminidase activity by the T.B.A. assay and stored at -20°C.

2b. DEAE-cellulose ion exchange chromatography

Fines were removed from the DEAE-cellulose (Whatman, Grade DE-52, microgranular, anionic exchanger) and 80.0 g were suspended in 0.05 M NaAc buffer (pH 4.4) to form a slurry. The DE-52 DEAE-

cellulose was already precycled. A column (2 x 80 cm; Pharmacia, Uppsala, Sweden) was poured and equilibrated with 0.05 M NaAc buffer pH 4.4. The neuraminidase concentrate (2.0 ml) was pipetted onto the column and proteins were eluted from the column by stepwise gradient elution with 0.05 M and 0.1 M NaAc buffer pH 4.4, and 1.0 M NaCl. Fractions (2.0 ml) were eluted from the column at a flow rate of 0.8 ml min^{-1}. The $E_{280\ nm}$ values for each fraction were used to detect the protein peaks. Peak fractions were pooled, placed in dialysis tubing and concentrated with Carbowax to the original starting volume of 2 ml. Finally the concentrates were again placed in dialysis tubing and dialysed overnight at 4°C against 0.05 M NaAc buffer pH 5.5. A T.B.A. assay was done to detect concentrates enzymatic activity. The peak samples were stored at -20°C.

2c. Further purification of neuraminidase preparations by DEAE cellulose ion exchange chromatography

The column was equilibrated with 1.0 M NaCl. The neuraminidase-containing peak VI (2.0 ml) was pipetted onto the column and fractions (2.0 ml) were eluted from the column with 1.0 M NaCl at a flow rate of 0.8 ml min^{-1}. The peak fractions were pooled, placed in dialysis tubing and concentrated with Carbowax at 4°C to bring the volume down to the initial volume. The concentrates were dialysed overnight against 0.05 M NaAc buffer pH 5.5 and a T.B.A. assay was done to determine the level of neuraminidase activity. The peak samples were stored at -20°C.

Samples of 0.05 M NaAc buffer pH 5.5, Calbiochem V. cholerae neuraminidase 1.0 I.U. ml^{-1}, Borate Gelatin Buffer pH 7.5, and Sephadex G 100 1 x purified cholera enterotoxin (0.1 ml) were analysed for neuraminidase activity by the T.B.A. and M.P.N. assays.

V PROTEIN ESTIMATION

1. Lowry procedure (B.S.A. as protein standard) (Lowry et al, 1951)

A standard protein solution was prepared by dissolving 2 mg BSA ml^{-1} distilled water. For the standard curve 40, 80, 120, 160 and 200 µg BSA were used; the total volume was adjusted in each tube to 1.0 ml with distilled water. The semi-purified neuraminidase preparations N_1-N_4 from Sephadex G100 were analysed by taking 0.1 ml of neat preparation. To all preparations 5.0 ml Folin C reagent (p 218, Appendix III) was added. The preparations were allowed to stand at room temperature for 10 min. Folin D reagent (p 218, Appendix III; 0.5 ml) was added and the samples were allowed to stand for 30 min. The mixtures were read against a blank of distilled water at $E_{750\ nm}$. To obtain the protein content of the preparation expressed as µg B.S.A., the following calculation was utilised:

$$k_{B.S.A.} \times E_{750\ nm} \times 10 = \mu g \text{ protein } ml^{-1} \text{ as B.S.A.}$$

2. Micro-biuret procedure (Ovalbumin as protein standard)

(Modification of method of Leven and Brauer, 1951)

A protein standard solution was prepared by dissolving 1.5 mg ovalbumin ml^{-1} distilled water. The concentrations used for the preparation of the standard curve were, 150, 300, 450 and 600 micrograms and the total volume of the samples in distilled water was 1.0 ml. In addition, the protein content of semi-purified neuraminidase preparations N_1-N_4 from G100 Sephadex were analysed, 1.0 ml of the neat preparation was used. 3 N NaOH (0.3 ml) was added to tubes containing the samples, and the mixtures were placed in a heating block at 100°C for 5 min. The tubes were cooled to room temperature. A solution of 2.5% (w/v) $CuSO_4.5H_2O$ (0.2 ml) was added, the tubes were shaken and left to stand for 5 min at room temperature. The mixtures were centrifuged for 3 min at 3000 rev min^{-1} (1200 g) in a benchtop centrifuge (M.S.E. Minor) and the samples were read against a distilled water blank at $E_{555\ nm}$ in an SP 500 spectrophotometer.

To obtain the protein content of the preparation expressed as µg ovalbumin, the following calculation was utilised:

$$K_{oval} \times E_{555\ nm} = \mu g \text{ protein } ml^{-1} \text{ as ovalbumin.}$$

VI ENZYME ASSAYS

1. T.B.A. procedure for neuraminidase activity (Warren, 1959)

A stock solution of N-acetylneuraminic acid (Sigma) was prepared by suspending 6.18 mg in 10 ml of 0.05 M NaAc buffer, pH 5.5. The stock solution was diluted to give a series of concentrations from 6.18 μg to 37.116 μg (i.e. 0.02 μmoles - 0.12 μmoles ml^{-1}). It was necessary to double the initial concentrations to achieve final concentrations of 0.02 μmoles - 0.06 μmoles ml^{-1} as the assay measures 0.5 ml of the original 1 ml. These concentrations of N-acetylneuraminic acid were incubated in a water-bath at 37°C for 30 min. Phosphotungstic acid, 1.0 ml (5.0 g in 100 ml 2.0 N HCl) was then added to the tubes which were mixed in a Whirlimixer and then centrifuged in a benchtop centrifuge. A 0.5 ml volume of supernate was pipetted from each tube and placed into clean tubes. Periodate solution, 0.1 ml (4.27 g in 38.3 ml H_2O + 61.7 ml phosphoric acid (Analar)) was added and the tubes were allowed to stand for 20 min at room temperature. Subsequently, 1 ml of arsenite solution (17.75 g Na_2SO_4 in 250 ml H_2O + 5 ml 5 N H_2SO_4 + 25 g sodium arsenite) was added and the tubes were shaken until the yellow colour disappeared; this indicated that unreacted iodides had been removed. This was followed by the quick addition of 3.0 ml of thiobarbituric acid (4.5 g of 2 X recrystallised thiobarbituric acid (p 221, Appendix III) in 750 ml 0.5 M Na_2SO_4). The tubes were placed in a heating block at 100°C for 15 min and cooled for 5 min. Distilled cyclohexanone (4.6 ml, B.D.H.) was added to each tube, and after shaking, centrifuged for 3 min at 3000 rev min^{-1} (1200 g) in 30 ml glass bottles (Universals).

It was also possible to use n-butanol as an alternative organic phase to cyclohexanone. The n-butanol was less toxic to use. The organic phase was removed and read against a buffer blank at $E_{549\ nm}$ in an SP 500 spectrophotometer.

When testing the neuraminidase activity of actual enzyme preparations, a substrate of bovine submaxillary mucin was prepared by dissolving 20 mg in 5.0 ml of 0.05 M sodium acetate buffer pH 5.5 containing 0.1% $CaCl_2$ (w/v) and 1% NaCl (w/v); 0.4 ml of this mixture was used in the actual assay. Two commercial enzyme preparations were tested, as well as semi-purified G100 pooled column preparations N_1-N_4. The commercial enzymes were Clostridium perfringens neuraminidase (Sigma), and V. cholerae neuraminidase (Koch-Light). The Sigma enzyme was prepared by dissolving 1 mg in 5.0 ml of 0.05 M NaAc buffer pH 5.0. The Koch-Light enzyme was prepared by diluting the stock solution (200 ImU ml^{-1}) 1:5 in 0.05 M NaAc buffer pH 5.5 containing 0.1% $CaCl_2$ (w/v) and 1% NaCl (w/v). In the assay 0.1 ml samples were utilised thus, 3.4 ImU of the Sigma enzyme and 4.0 ImU of the Koch-Light enzyme were used in the assay. The semi-purified G100 pooled fractions N_1-N_4 were added to the substrate undiluted in 0.1 ml aliquots. The volume of the enzyme substrate preparations was made up to 1.0 ml by adding 0.5 M NaAc buffer (in the case of the V. cholerae neuraminidase at pH 5.5 + 0.1% $CaCl_2$ and 1% NaCl; in the case of the Cl. perfringens neuraminidase at pH 5.0 without additional ions). The preparations were incubated for 30 min. The assay procedure followed was the same as that cited in the standard curve procedure above.

One I.U. (1000 ImU) of neuraminidase liberates 309.3 μg NANA min^{-1} or 9279 μg (9.28 mg) NANA $(30\ min)^{-1}$. In obtaining the activity levels of neuraminidase the following calculations were utilised:

$$K_{NANA} \times E_{549\ nm} \times 10 = \mu g\ NANA\ released\ ml^{-1}$$

$$\frac{\mu g\ NANA\ ml^{-1} \times total\ volume}{total\ microbiuret\ protein} = \mu g\ NANA\ released\ (mg\ protein)^{-1}\ in\ sample = A$$

$$\text{Specific activity} = \frac{A}{9279} \times 1000 = ImU\ (mg\ protein)^{-1}.$$

2. M.P.N. procedure for neuraminidase activity (Palese, Bucher and Kilbourne, 1973)

3-Methoxyphenol (B.D.H.) was used to prepare a standard curve. One ml of the commercial preparation of 3-methoxyphenol (1.14 g ml^{-1} 0.1 M PO_4 buffer (B.D.H. Chemicals Ltd., Poole, England) (p 219, Appendix III) + 2 mM $CaCl_2$ pH 5.9) was diluted in 9.18 ml of 0.1 M PO_4 buffer (B.D.H.) + 2 mM $CaCl_2$ pH 5.9 (p 219, Appendix III), this gave 100 µmoles $(0.1\ m)^{-1}$. The final concentration of methoxyphenol used was 100 nm $(0.1\ ml)^{-1}$, and 20, 40, 60, 80 and 100 nm of 3-methoxyphenol were used for the standard curve, the volumes were adjusted to 0.1 ml with 0.1 M PO_4 buffer. For the actual assay of neuraminidase activity a substrate of 2-(3'methoxyphenol)-N-acetyl-α-neuraminic acid (Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland) was prepared by dissolving 50.0 mg (10^{-2} M) in 11.82 ml 0.1 M sodium phosphate buffer (B.D.H.) pH 5.9. The neuraminidase activity of Cl. perfringens neuraminidase (Sigma), V. cholerae neuraminidase (Koch-Light) and V. cholerae neuraminidase semi-purified Sephadex G100 preparations N_1-N_4 were tested.

The Cl. perfringens neuraminidase (Sigma) was prepared by dissolving 1.0 mg in 5.0 ml 0.05 M NaAc buffer pH 5.0 and the V. cholerae neuraminidase (Koch-Light) was prepared by diluting the stock solution (200 ImU ml^{-1}) 1:5 in 0.05 M NaAc buffer pH 5.5 containing 0.1% $CaCl_2$ (w/v) and 1.0% NaCl (w/v). Aliquots (0.1 ml) were taken from the above enzyme preparations, from N_2-N_4 preparations and from the methoxyphenol standards and 0.1 ml of 0.1 M PO_4 buffer pH 5.9 + 2 mM $CaCl_2$ was added. The methoxyphenol standards were placed in a waterbath at 37°C for 30 min. The enzyme preparations were preheated in the same waterbath for 2-3 min before the addition of 0.1 ml of 2-(3'methoxyphenyl)-N-acetyl-α-neuraminic acid. They were reincubated for 30 min and the reaction was stopped by adding 25 µl of 1 M di-sodium E.D.T.A. (B.D.H.). The tubes were placed in an ice-bath for 30 min and 0.2 ml of diazonium salt of 4 amino - 2.5 di-methoxy-4' nitroazobenzene (Koch-Light, prepared by adding 6.0 mg of diazonium salt in 1.0 ml 0.4 M sodium phosphate buffer pH 7.0 - this must be prepared on the day used and filtered through a Whatman filter paper) was added. After 30 min a precipitate formed and 4 ml 0.5 N NaOH was added to each tube to solubilise the precipitate. The tubes were centrifuged for 3 min at 3000 rev min^{-1} (1200 g) in a benchtop centrifuge (M.S.E. Minor). The samples were read against a blank that contained 0.1 M PO_4 buffer + 2 mM $CaCl_2$ pH 5.9 at $E_{580\ nm}$.

One I.U. of neuraminidase liberates 121.14 µg methoxyphenol, 1 µmole min^{-1} or 3634.2 µg (3.64 mg) methoxyphenol $(30\ min)^{-1}$, or 1457.3 µg = NANA (for 1 µmole MP in 30 min) when expressed as NANA. In obtaining the activity levels of neuraminidase the following calculations were utilised:

$K_{M.P.}$ x $E_{580\ nm}$ x 0.001 (conversion factor to convert nmole to µm) x 121.14 µg (conversion to convert µm to µg) = µg M.P. released.

µg M.P. x 0.401 (the conversion factor from the ratio of the molecular weight of 3-methoxyphenol 121.14 : the molecular weight of NANA 309.3) x 10 = µg NANA released ml^{-1}.

$$\frac{\text{µg NANA ml}^{-1} \times \text{total volume}}{\text{total microbiuret protein}} = \text{µg NANA released (mg protein)}^{-1}$$

in sample = A

$$\text{Specific activity} = \frac{A}{1457.3} \times 1000 = \text{ImU (mg protein)}^{-1}.$$

3. Proteinase activity (Kunitz, 1947)

A trypsin (Sigma) solution used in the standard curve was prepared by dissolving 2 mg ml^{-1} in 0.05 M Tris (hydroxymethyl) aminomethane (Tris) -hydrochloride (Sigma) buffer pH 9.0 (p 214, Appendix I). A standard curve was prepared with 20, 40, 60, 80 and 100 µg trypsin, the final volume was 1.0 ml in 0.05 M Tris-hydrochloride buffer pH 9.0. A stock solution of substrate consisted of 1.0 g casein (Hammarsten grade, B.D.H.) dissolved in 100 ml 0.1 M PO_4 buffer pH 7.6. The suspension was heated for 15 min in boiling water to bring about complete solution of the casein. Trypsin and the V. cholerae preparations N_1-N_4 were assayed for proteinase activity, by adding 1.0 ml of sample to 1.0 ml of 1.0% casein. A control tube contained 1.0 ml of diluted trypsin + 3.0 ml of 5% trichloroacetic acid (B.D.H.) before the 1.0 ml of 1.0% casein was added. The reaction for all tests was done in a 37°C waterbath for 30 min. After this time the reaction was stopped by adding 3.0 ml of 5.0% trichloroacetic acid (w/v) and incubating at 45°C for 15 min. Undigested casein was removed by centrifugation. The samples were read against a blank of 0.05 M Tris HCl buffer pH 9.0 at $E_{280\ nm}$. One unit of proteinase is the amount of enzyme that released 1 µmole of tyrosine per min. For determining the presence of proteinase in the semi-purified preparations N_1-N_4 the following calculations were utilised:

$$k_{trypsin} \times E_{280\ nm} = \mu g\ ml^{-1} \text{ proteinase (as trypsin)} = A$$

$$\frac{A \times \text{total volume}}{\text{total protein}} = \mu g \text{ proteinase (as trypsin)}$$

$$(\text{mg microbiuret protein})^{-1}.$$

4. Phospholipase-C activity (Boehringer Mannheim, 1983)

Phospholipase C (Boehringer-Mannheim, ex. Bacillus cereus; p 217, Appendix III) was obtained as a 2.0 ml ml^{-1} suspension in 3.2 M ammonium sulphate. The enzyme was diluted 1:100 with ice cold redistilled water before use in the assay. The standard concentrations of phospholipase C used were: 200, 240, 280, 320, 360 and 400 ngrams and represented 8.0, 9.6, 11.2, 12.8 and 14.4 ImU respectively. The total volume was adjusted to 20 µl with ice cold redistilled water. The semi-purified V. cholerae neuraminidase fractions N_1-N_4 were assayed for phospholipase C activity with 20 µl of neat fraction. The control consisted of a 20 µl aliquot which contained 240 ngrams phospholipase C + 0.3 ml trichloroacetic acid (1.5 M).

The following were pipetted into centrifuge tubes: 0.1 M triethanolamine buffer pH 7.5 + 20 mM $CaCl_2$ (1.0 ml; p 214 Appendix I), freshly prepared lecithin suspension (p 220, Appendix III), redistilled water (0.5 ml), lipase suspension (0.05 ml) and finally the 20 µl sample. The mixtures were incubated for 15 min in a 37°C waterbath. The reaction was stopped by adding 0.3 ml of 1.5 M trichloroacetic acid. The tubes were placed in a boiling water bath for 2 min, cooled and centrifuged at 3000 rev min^{-1} (1200 g) for 5 min. The supernate (0.25 ml) was pipetted into a cuvette with 2.5 ml 0.3 M triethanolamine buffer pH 7.6 + 4 mM magnesium sulphate (p 214, Appendix I) and 0.15 ml of a solution which consisted of ATP 32 mM/NADG 6 mM/PEP 45 mM (p 220, Appendix III), lactate dehydrogenase (0.01 ml of 5.0 mg ml^{-1} in 3.2 M ammonium sulphate) and pyruvate kinase (0.01 ml of 2.0 mg

ml^{-1} in 3.2 M ammonium sulphate). The reagents were mixed and allowed to stand for 2 min at room temperature before reading against a blank of redistilled water at $E_{340\ nm}$.

This reading was designated E_1 or the $E_{340\ nm}$ reading prior to the addition of glycerol kinase. Finally, 0.04 ml of glycerol kinase (1.0 mg ml^{-1} in 3.2 M ammonium sulphate) was added. An E_{340nm} reading was taken after 10 min against a blank of distilled water. This reading was designated E_2 or the $E_{340\ nm}$ after the addition of glycerol kinase. The value E_1-E_2 was equal to the relative phospholipase C activity.

One I.U. phospholipase-C hydrolyses 1 μmole lecithin (804.2 μg) min^{-1}, or 12063 μg (12.063 mg) lecithin $(15\ min)^{-1}$. The phospholipase-C activity of the N_1-N_4 preparations were determined as follows: $E_{340\ nm}$ prior to glycerol kinase treatment = E_1: $E_{340\ nm}$ after glycerol kinase treatment = E_2: $E_1-E_2 = \triangle E$

$k_{phospholipase-C} \times \triangle E \times 0.001$ (conversion factor to convert ngrams to μg) = μg lecithin hydrolysed

μg hydrolysed lecithin x 50 (factor to convert to ml since 0.02 ml used in the assay) = μg lecithin hydrolysed ml^{-1}

$$\frac{\mu g \text{ lecithin hydrolysed } ml^{-1}}{\text{total protein}} \times \text{total volume} = \mu g \text{ lecithin hydrolysed } (mg\ protein)^{-1} = A$$

$$\text{Specific activity} = \frac{A}{12063} \times 1000 = ImU\ (mg\ protein)^{-1}.$$

5. Endoglycosidase activity (Dubois, Gilles and Hamilton, 1956; Chien et al, 1975).

A standard curve was prepared for the phenol sulphuric acid assay for neutral sugar with glucose (1.0 mg ml^{-1} in distilled water) as the neutral sugar. The total volume in each test was brought to 2.0 ml with distilled water, 50 µl of 80% (w/v) phenol (reagent grade B.D.H.) and 0.5 ml conc. sulphuric acid (Analar) (p 217, Appendix III). The reagents were mixed with a Whirlimixer, and the tubes were placed in an ice bath for 30 min. The reaction was measured at $E_{490\ nm}$ against a distilled water blank.

The standard solution of endoglycosidase H (Seikagaku Kogyo Co. Ltd., Tokyo, Japan, freeze dried - ex Streptomyces griseus) was prepared by diluting 2.0 µl of enzyme with 100 µl of 0.1 M NaCl containing 0.1% bovine serum albumin. Aliquots of 10 and 20 µl of this stock solution were used. The semi-purified V. cholerae neuraminidase preparations N_2-N_4 were assayed as neat 20 µl volumes. The substrate for the endoglycosidase activity was ovalbumin (Sigma Grade V 5.0 mg in 0.5 ml of 0.05 M sodium acetate buffer pH 5.5 which contained 0.5% (w/v) sodium dodecyl sulphate (B.D.H.)). The control was prepared by adding 12.5% (w/v) trichloroacetic acid to the ovalbumin substrate before the addition of enzyme. The 20 µl sample was added to 0.5 ml of the ovalbumin substrate and incubated for 1 h in a 37°C waterbath. The reaction was stopped by adding an equal volume of cold 12.5% (w/v) trichloroacetic acid to inactivate endoglycosidase, the tubes were kept at 0°C for 15 min and centrifuged at 17,000 rev min^{-1} (35,000 g) in a 3 x 25 ml rotor with 3.0 ml tube adaptors in an

M.S.E. 65 centrifuge. Aliquots of 0.2 ml of the clear supernate were assayed for total neutral sugar to determine endoglycosidase activity. After the addition of 50 µl of 80% phenol and 0.5 ml of conc. sulphuric acid (Analar, B.D.H., Poole, England), the samples were allowed to stand at room temperature for 30 min to allow for colour development, and read against a distilled water blank at $E_{490\ nm}$.

One I.U. is that amount of enzyme capable of catalysing the hydrolysis of 1 µmole of (man_5 - $GlcNAc_2$ - Asn- ^{14}C - Acetyl) per minute at 37°C. The endoglycosidase activity (m.u.) of the N_1-N_4 preparations was measured by the release of neutral sugar (as glucose) from an ovalbumin substrate, and the level of activity was determined against the commercial enzyme preparation. The following calculations were utilised:

$K_{phenol}\text{-}H_2SO_4 \times E_{490\ nm}$ = µg glucose of preparation = A

$$\frac{A \times 6.0 \text{ mu of commercial enzyme}}{9.145 \text{ (µg glucose released by 6m.u.commercial enzyme)}} \times 50^{*} =$$

$m.u.ml^{-1}$ of preparation;

$$\frac{m.u.ml^{-1} \text{ of preparation}}{\text{total protein}} \times \text{total volume} \times 0.001^{**}$$

= enzyme units $(mg\ protein)^{-1}$

* conversion factor to express result ml^{-1}

**conversion factor to express results as units $(mg\ protein)^{-1}$.

6. Aldolase activity (Beisenherz et al, 1953)

Aldolase (Boehringer-Mannheim, ex. rabbit muscle - p 217, Appendix III) used in the preparation of the standard curve was obtained from the manufacturer as a 20 mg crystalline suspension in 2.0 ml 3.2 M ammonium sulphate. The working stock solution was diluted 1:100 in 0.1 M triethanolamine hydrochloride buffer (Sigma) pH 7.6 (0.9 ImU $(1.0\ \mu l)^{-1}$). The concentrations of aldolase in the standard curve were 0.1, 0.2, 0.3, 0.4 and 0.5 µg, the final volume was brought to 20 µl with 0.1 M triethanolamine hydrochloride buffer, pH 7.6. The V. cholerae neuraminidase preparations N_1-N_2 were assayed neat in 20 µl volumes because of the expected low aldolase activity. The control was 0.5 µg aldolase without substrate. Into a cuvette the following were added: 2.81 ml 0.1 M triethanolamine hydrochloride buffer pH 7.6, 0.05 ml β-nicotinamide-adenine dinucleotide solution, (12 mM), 0.1 ml fructose 1.6 di-phosphate solution (33 mM) and 0.02 ml glycerol-3-phosphate dehydrogenase/triosephosphate isomerase suspension (p 217, Appendix III). The reaction was started by the addition of the 20 microlitre enzyme sample to the cuvette. The $E_{340\ nm}$ was read at 1 min intervals for a period of 5 min and the extinction calculated at each time interval.

One I.U. will convert 1.0 µmole of fructose-1,6 diphosphate to dihydroxy-acetone phosphate and glyceraldehyde-3-phosphate per minute at 25°C. The $E_{340\ nm}$ was read at 1 min intervals for a period of 5 min, and $\triangle E$ extinction was calculated at each time interval. The mean of all the $\triangle E$ values was calculated. To obtain aldolase activity in each of the preparations the following calculations were carried out:

1) $\dfrac{\bar{x} \triangle E_{340\ nm} \text{ of commercial enzyme X ImU } (20\ \mu l)^{-1}}{\bar{x} \triangle E_{340\ nm} \text{ of commercial enzyme}} = \text{activity of preparation (ImU } (20\ \mu l)^{-1})$

2) ImU $(20\ \mu l)^{-1}$ X 50 = activity of preparation ml^{-1}

3) Specific activity of preparation = $\dfrac{\text{ImU of preparation } ml^{-1}}{\text{mg of protein } ml^{-1}}$

VII PRODUCTION OF ANTISERA

Anti-neuraminidase antisera used in this investigation were prepared by Dr. D.E. Stewart-Tull. Anti-neuraminidase antisera DST R76 and DST R77 were prepared by immunising rabbits intramuscularly with 1.0 ml of a mixture which contained: 0.5 ml of Sephadex G75 semi-purified *V. cholerae* neuraminidase, 1.0 ml saline, 1.5 ml Bayol F Oil and 0.6 ml Arlacel A. Mixtures for injection into rabbits DST R78 and DST R79 were similar but Koch-Light *V. cholerae* neuraminidase (100 ImU $(0.5\ ml)^{-1}$) was used. A booster injection of 2.0 ml was given intraperitoneally after one month. Ten days later a trial bleed was taken from the ear vein. The serum was tested for the presence of anti-neuraminidase antibodies by an Ouchterlony double diffusion in gel test. Animals were exsanguinated and the serum was collected and stored at $-20^{o}C$.

I. Purification of IgG (by DEAE)

DEAE cellulose (Whatman, Grade DE 52, wet microgranular) was equilibrated with 0.01 M PO_4 buffer pH 7.5. A Whatman glass column (1.5 x 45 cm) was filled with the DE 52 slurry such that the final bed dimensions were 1.5 x 25 cm and the bed volume was 51.25 cm^3. The column was connected to a buffer reservoir containing 0.01 M PO_4 buffer pH 7.5. The flow rate of the column was 0.8 ml min^{-1} and 2.0 ml fractions were collected. Rabbit anti-neuraminidase serum (DST R78 - 4.0 ml) was placed in Visking tubing and dialysed against 0.01 M PO_4 buffer pH 7.5 overnight at 4°C. The equilibrated serum was pipetted onto the column. The first protein peak, eluted with the starting buffer, was the IgG. The fractions containing IgG were pooled and concentrated with Carbowax to a volume of 4.0 ml. The column was washed with 0.3 M phosphate buffer and re-equilibrated with 0.01 M PO_4 buffer pH 7.5. The 1 X purified IgG from serum DST R78 was purified a second time on the DE 52 column in the same manner as cited above.

The IgG was also separated from normal rabbit serum by the procedure described above with one variation, namely, the serum was first fractionated with ammonium sulphate. In this procedure 4.0 ml of normal rabbit serum was added to 4.0 ml of 100% saturated (69.7g$(NH_4)_2SO_4$ in 100 ml distilled water). The precipitate was spun down by centrifugation, at 3000 rev min^{-1} (1200 g) for 10 min. The precipitate was washed 5-6 times with 50% saturated $(NH_4)_2SO_4$ solution until haemoglobin was removed. The precipitate was re-suspended in 4.0 ml of 0.01 M PO_4 buffer pH 7.5 and dialysed against 0.01 M PO_4 buffer pH 7.5 overnight at 4°C. This was added to the DE 52 anionic

exchanger column and IgG prepared as cited above.

Rabbit anti-neuraminidase (DST R79) was prepared as described above for normal rabbit IgG.

VIII PURIFICATION OF CHOLERA ENTEROTOXIN

Sephadex G100 (35 g) was suspended in 700 ml distilled water and placed in a boiling-water bath for 5 h to allow the gel to swell. The gel was re-suspended in borate gelatin buffer pH 7.5 (p 212, Appendix I) previously degassed under vacuum. A column (2.6 x 81 cm; L.K.B., Bromma, Sweden) was filled with the Sephadex G100. The column was equilibrated with borate gelatin buffer pH 7.5. The flow rate of this column was 0.8 ml min^{-1}. Crude cholera toxin (1.2 g N.I.A.I.D. Batch 001 in 2.0 ml borate gelatin buffer pH 7.5) was pipetted onto the column. The $E_{280\ nm}$ of each fraction was measured to detect the protein eluted. The first peak contained the toxin and a second larger peak contained non-toxic brown products of the medium and was discarded. The pooled peak toxin fractions were concentrated with Carbowax to 2 ml.

IX HISTOLOGICAL PROCEDURES

Rat small intestine was removed from sacrificed healthy rats. Segments of the ileum were wrapped in aluminium foil and quickly frozen in liquid nitrogen. The frozen tissue segments were stored at -70°C in universal bottles (30 ml). Subsequently, the tissue segment was quickly removed from the aluminium foil and was cut transversely into small lengths. Each segment was placed in a transverse orientation in an aluminium foil boat (2 x 3 cm) filled with Ames OCT freezing compound (Raymond Lamb Ltd., London). The aluminium foil boat was immersed in an isopentane (B.D.H.) slurry in a 250 ml beaker immersed in liquid nitrogen. The isopentane was kept in liquid nitrogen until it became viscous. The boat containing tissue was kept in the isopentane slurry until the tissue turned completely milky white. The aluminium foil boat was quickly removed from the tissue block, a drop of freezing compound was added to the back of the tissue block and the tissue was attached to a cryostat chuck face.

The cryostat (SLEE Medical Ltd., London) was set to -25°C. The chuck to which tissue block had been attached was precooled in liquid nitrogen before placing in the cryostat. A wedge-shaped microtome knife (SLEE), used to cut sections, was fitted with a Pearse knife cup which contained dry ice. The microtome knife was cooled for 10-15 min in the cryostat cabinet before sectioning the tissue block. Thick sections (10-12 μ) were cut until a tissue face was exposed. Serial tissue sections 8 μ in thickness were cut, placed on glass microscope slides (precleaned with 90% (v/v) ethanol and coated with egg albumin) and placed at 4°C to slowly thaw.

1. Preservation of mucosubstances by vapour fixation (Culling, 1974)

A litre desiccator was dried for 4-6 h at 80°C and a crucible containing 5.0 g paraformaldehyde powder (B.D.H.) was placed in the dessicator. The lid was closed and the vessel was heated in an oven at 80°C for 1 h. After 1 h at 80°C, the crucible was removed and replaced with frozen sections on glass slides. The lid was replaced and the desiccator was left at 60°C for 1 h.

X EFFECTS OF NEURAMINIDASE AND OTHER CHEMICAL TREATMENTS ON GOBLET CELL HISTOCHEMISTRY

Frozen transverse sections of rat ileum were cut 8 microns thick. These sections were fixed in paraformaldehyde vapour for 1 h at 60°C. In this series of experiments the fixed rat ileal sections were treated for 24 h at 37°C in a moistened chamber with 0.1 ml (or 0.2 ml) of the following: (1) Distilled water, (2) 0.05 M NaAc buffer pH 5.0 or 0.05 M NaAc buffer pH 5.5, (3) Cl. perfringens neuraminidase (Worthington: 680 ImU ml^{-1}), (4) Cl. perfringens neuraminidase (Sigma: 680 ImU ml^{-1}), (5) V. cholerae semi-purified neuraminidase (N_2 : 55 ImU ml^{-1}), (6) V. cholerae neuraminidase (Calbiochem: 1.0 ImU ml^{-1}), (7) Concentrated trypsin inhibitor (1.0 mg Sigma inhibitor; (10 ml 0.05 M NaAc buffer pH 5.5)$^{-1}$), (8) Concentrated trypsin inhibitor (2.0 ml) + neuraminidase (N_2; (0.1 ml) + 0.05 M NaAc buffer pH 5.5 (0.05 ml)), (9) Borate gelatin buffer pH 7.5, (10) Cholera enterotoxin, N.I.A.I.D. 001 (3.0 g (5.0 ml borate gelatin buffer pH 7.5)$^{-1}$), (11) mixture of 0.05 M NaAc buffer pH 5.5 (0.3 ml) + 0.01 M PO_4 buffer pH 7.5 (0.05 ml), (12) mixture of 0.05 M NaAc buffer pH 5.5 (0.05 ml) + 0.01 M PO_4 buffer pH 7.5 (0.3 ml).

*Note: Because the maximal effect of the enzyme upon the tissue section was required, each enzyme preparation was added undiluted.

Two frozen sections were utilised for each treatment and two frozen sections were left untreated to serve as additional controls. Where mixtures of whole rabbit serum or IgG + neuraminidase were utilised, the tubes that contained such mixtures were first incubated for 2 h at 37°C in a waterbath, allowed to stand overnight at 4°C, and finally centrifuged at 3000 rev min^{-1} (1200 g) in a bench top centrifuge for 5 min. After 24 h, sections were stained by Alcian Blue/PAS and Haematoxylin-Eosin staining procedures (p229, Appendix VI). The goblet cells were counted in 20 fields under the X 40 objective (i.e. a total area of 8.84 sq mm) as either Alcian Blue staining or PAS staining. The data were analysed statistically by a Student t-test (in its double-tailed form) which compared the goblet cell counts of the untreated or buffer treated sections with those treated with neuraminidase or other treatments. The values cited for the t-test throughout this thesis were obtained from the following formulae:

$$a)\quad s^2 = \left[\sum_{i=1}^{n_1} (x_{1i} - \bar{x}_1)^2 + \sum_{i=1}^{n_2} (x_{2i} - \bar{x}_2)^2\right] / \ (n_1 + n_2 - 2)$$

$$b)\quad t = (\bar{x}_1 - \bar{x}_2) \Big/ \left[S\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} \right]$$

where: s = sample standard deviation; t = t distribution; x_1 and x_2 respectively = the first and second samples; $\bar{x}_1$ and $\bar{x}_2$ respectively = the mean of first and second samples; $\sum_{i=1}^{n_1} (x_{1i} - \bar{x}_1)^2$ and $\sum_{i=1}^{n_2} (x_{2i} - \bar{x}_2)^2$ respectively = the sum of the squares for the first and second samples; and finally n_1 and n_2 respectively = the number of observations for the first and second samples. These formulae were obtained from Pearson and Hartley (1966).

The counts of goblet cells in 20 fields and the average number in each field (0.442 sq mm) were presented as histograms.

1. Effects of specific antisera

The effects of rabbit anti-neuraminidase sera, IgG fraction of rabbit anti-sera and normal rabbit sera were tested in order to find out their effects on neuraminidase activity and on the number of goblet cells staining Alcian blue and PAS. Constant amounts of neuraminidase were treated with a series of increasing volumes of sera in a final volume made up with 0.05 M NaAc buffer (pp 230-231, Appendix VII). Controls of enzyme plus buffer and sera plus buffer were included. Each of the mixtures of sera plus enzyme, and the controls, were incubated for 2 h at 37°C in a waterbath. These mixtures were then held overnight at 4°C. They were centrifuged at 3000 rev min^{-1} (1200 g) for 5 min in a bench top centrifuge. The supernates were removed to clean tubes.

Aliquots of 0.1 ml were taken from each of the mixtures and added to the tissue sections which were then handled as previously described (see pp 98, 99).

XI THE EFFECT ON NEURAMINIDASE ACTIVITY BROUGHT ABOUT BY ABSORPTION WITH SPECIFIC RABBIT SERA AS MEASURED BY THE T.B.A. AND M.P.N. ASSAYS

The neuraminidase activity* absorbing capacity of anti-neuraminidase sera, IgG fraction of anti-neuraminidase sera, normal serum and the IgG fraction of normal rabbit serum were tested by spectrophotometric assays (T.B.A. and M.P.N.). The mixtures of enzyme plus specific sera as well as the controls were prepared as previously described (p230-231, AppendixVII), and a volume of 0.2 ml was taken from each of the mixtures in order to perform T.B.A. and M.P.N. assays of neuraminidase activity.

* neutralising

XII DILUENT RELATED SIALOMUCIN LOSS AND NEURAMINIDASE INDUCED NANA LIBERATION

a) Release of sialomucin from unfixed tissue by diluent

Six rat ileum segments (0.5 cm^2) were placed in 1.0 ml of 0.05 M NaAc buffer pH 5.5 for 1 h at room temperature. The supernates were tested in the T.B.A. assay in order to detect the spontaneous release of sialomucin. The tissue segments were placed in 1.0 ml of sterile casamino yeast extract broth (C.A.Y.E.) for 1 h at 37°C, and the broth was tested in the T.B.A. assay. The tissue segments were homogenised in sterile tissue grinders in 1.0 ml of 0.05 M NaAc buffer pH 5.5. The homogenate was centrifuged for 3 min at 3000 rev min^{-1} (1200 g). The volume of each supernate was measured before NANA was measured by the T.B.A. assay. Three tissue segments served as controls and were washed with P.B.S. prior to washing in NaAc buffer.

b) Release of sialomucin from fixed tissue by diluent

In further experiments a similar procedure was used but the size of the segments was 1.0 cm^2 and the tissue segments were fixed in paraformaldehyde vapour for 1 h at 60°C. Fixed segments were placed in 1.0 ml of 0.05 M NaAc buffer pH 5.5 for 1 h at temperature and the liquid assayed as before. The segments were placed in 1.0 ml of sterile C.A.Y.E. medium for 1 h at 37°C and the broth assayed as before.

c) Release of NANA from ileal segments by V. cholerae neuraminidase

The effect of neuraminidase was tested on six fixed segments.

Each of the segments was placed in a tube which contained 1.0 ml of 0.05 M NaAc buffer for a period of 30 min at 37°C. The supernates were tested in the T.B.A. assay. Three segments were placed in 0.9 ml of NaAc buffer and 0.1 ml of V. cholerae neuraminidase (Calbiochem, 1.0 ImU ml^{-1}) was added before incubation at 37°C for 30 min. The supernates were tested in the T.B.A. assay. Three segments were treated with 1.0 ml of 0.05 M NaAc buffer pH 5.5 alone for 1 h at 37°C.

XIII EFFECT OF NEURAMINIDASE ON ADHESION OF V. CHOLERAE TO RAT ILEUM

T_1N_1 broth (p 216, Appendix II) was inoculated with V. cholerae strain 10732 and incubated at 37°C overnight. C.A.Y.E. broth (14.7 ml) was inoculated with 0.3 ml of this overnight growth (1:50 dilution of the overnight growth) and incubated for 6-8 h at 37°C. Nine fixed rat ileal segments (1.0 cm^2) were treated by the procedures shown in the flow diagram, Figure 6.

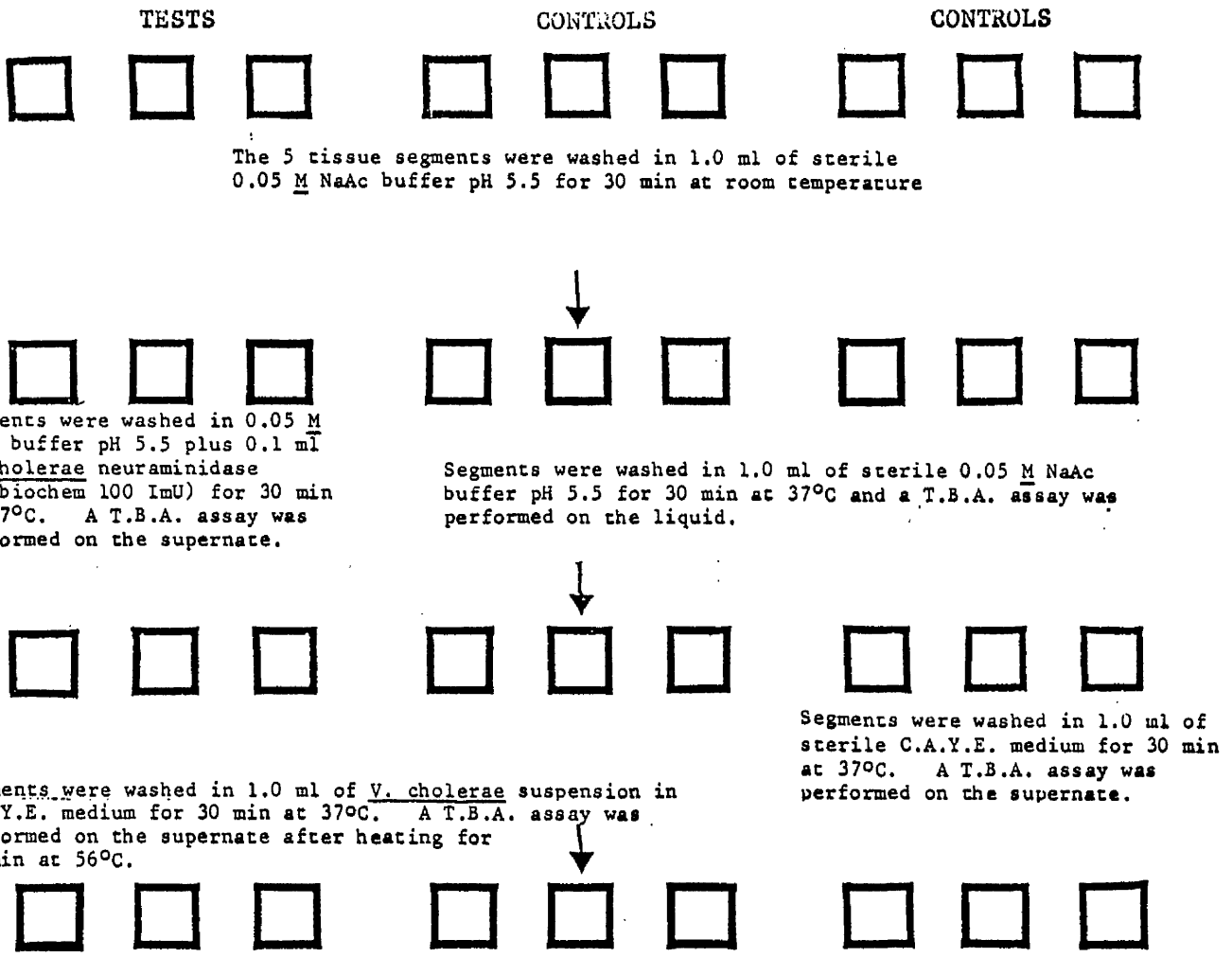


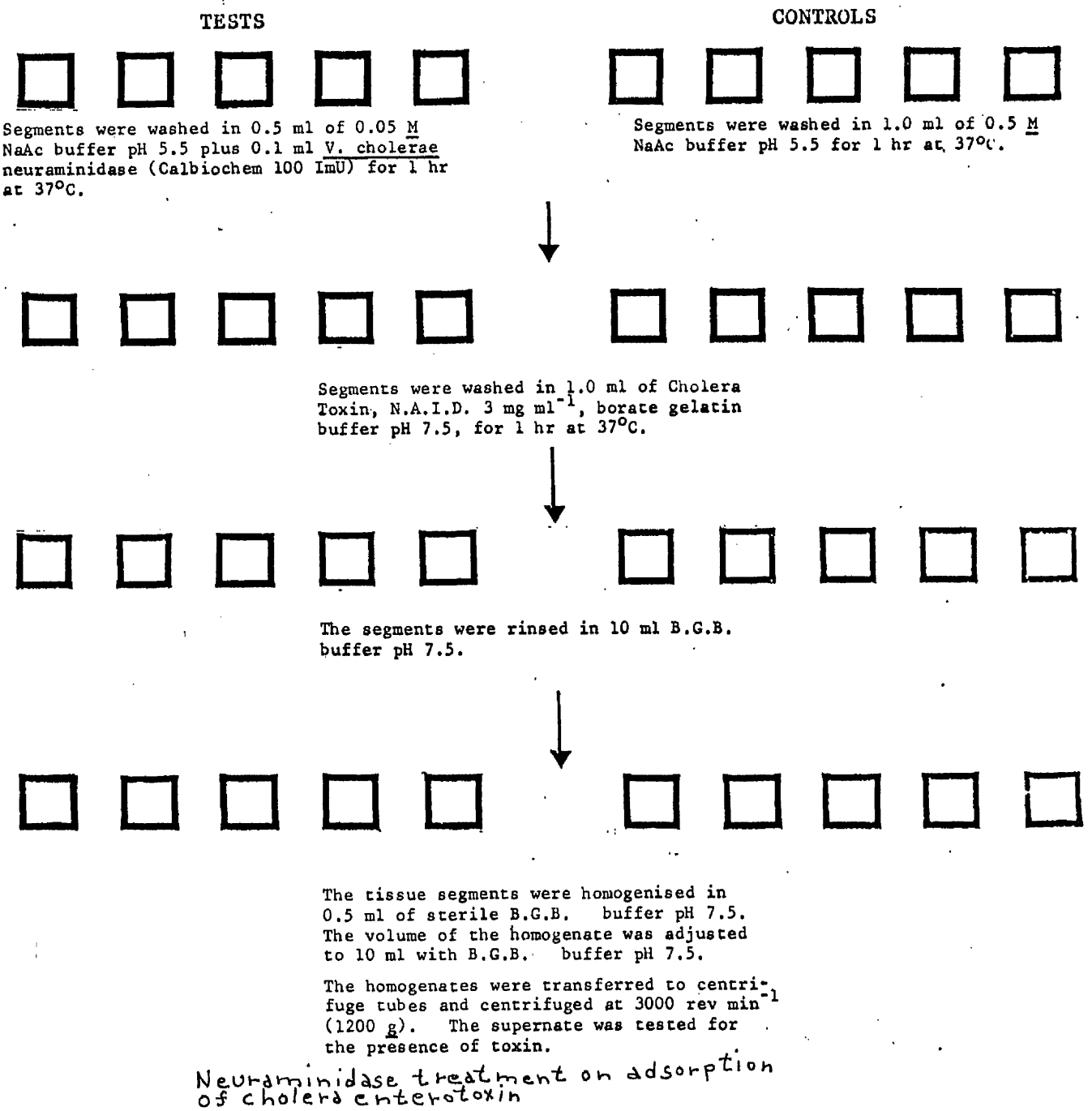
Figure 6. Enzymic treatment of rat ileal segments before *V. cholerae* adhesion

XIV EFFECT OF NEURAMINIDASE ON ADSORPTION OF CHOLERA ENTEROTOXIN TO RAT ILEUM

The effect of neuraminidase pretreatment on the adsorption of cholera enterotoxin was done by comparing neuraminidase pretreated with 0.05 M NaAc buffer pH 5.5 pretreated fixed rat ileal segments (Figure 7).

I. Testing for cholera enterotoxin

The cholera enterotoxin (600 BD) which had been exposed to the ileal segments was diluted 1:10 (300 $\mu g\ ml^{-1} \equiv 60$ BD), 1:20 (150 $\mu g\ ml^{-1} \equiv 30$ BD) and 1:40 (75 $\mu g\ ml^{-1} \equiv 15$ BD) in B.G.B.S. buffer pH 7.5. The Limit of Blueing Dose Assay was done by Dr. Stewart-Tull. Volumes of 0.1 ml of the enterotoxin dilutions were injected intradermally into the back of a rabbit, i.e. 6.0, 3.0 and 1.5 BD, respectively. After 24 h 3.0 ml of a 5% (w/v) Pontamine skyblue solution were injected into an ear vein. After 1 h, the zones of blueing at the site of enterotoxin injections were measured. One cholera enterotoxin Blueing Dose (B.D.) is equal to 64 mm^2. An exact number of Blueing doses cannot be obtained from the area of blueing (mm^2) because not all the enterotoxin injected i.d. into the shaved rabbit skin elicit a tissue reaction (Craig, 1965). Therefore, the approximate number of cholera enterotoxin B.D. adsorbed to ileal tissue was calculated as a range from the blueing reaction produced by the dilutions of toxin solution after exposure to the tissue.



Neuraminidase treatment on adsorption of cholera enterotoxin

Figure 7. Flow diagram to show the procedure for neuraminidase treatment of enterotoxin to rat ileal tissue.

RESULTS

I NEURAMINIDASE

1. Production of crude neuraminidase

Fifteen volumes of protease peptone-colostrum medium (Ada, French and Lind, 1961) in the batch fermenter yielded 186.35 I.U. of neuraminidase as measured by the thiobarbituric acid assay for the release of neuraminic acid (sialic acid) from bovine submaxillary mucin. In view of the high yield of enzyme, the batch fermenter was used in the preparation of all crude neuraminidase.

The neuraminidase was precipitated from the culture supernate with methanol. The precipitate was harvested by centrifugation and this material was designated 'crude neuraminidase'.

2. G 100 Gel filtration

Initial experiments indicated that two distinct protein peaks were separated on Sephadex G100. The second peak contained brown coloured residues from the methanolic precipitate of the proteose-peptone-colostrum culture fluid (Figure 8). The assays for neuraminidase activity showed that with G75 and G200 more protein and more enzyme were associated with the second brown peak fractions (Figure 8). Filtration on Sephadex G100 gave an $E_{280\ nm}$ elution profile shown in Figure 9. The first peak contained an enzyme activity of 5.4 ImU $(mg\ protein)^{-1}$ and the second $E_{280\ nm}$ peak contained an enzyme activity of 0.78 ImU $(mg\ protein)^{-1}$. The fractions between the two peaks contained 9.3 ImU $(mg\ protein)^{-1}$. The Lowry protein, T.B.A. and M.P.N. assays that were done on each fraction showed the location of the enzyme. It was found that the

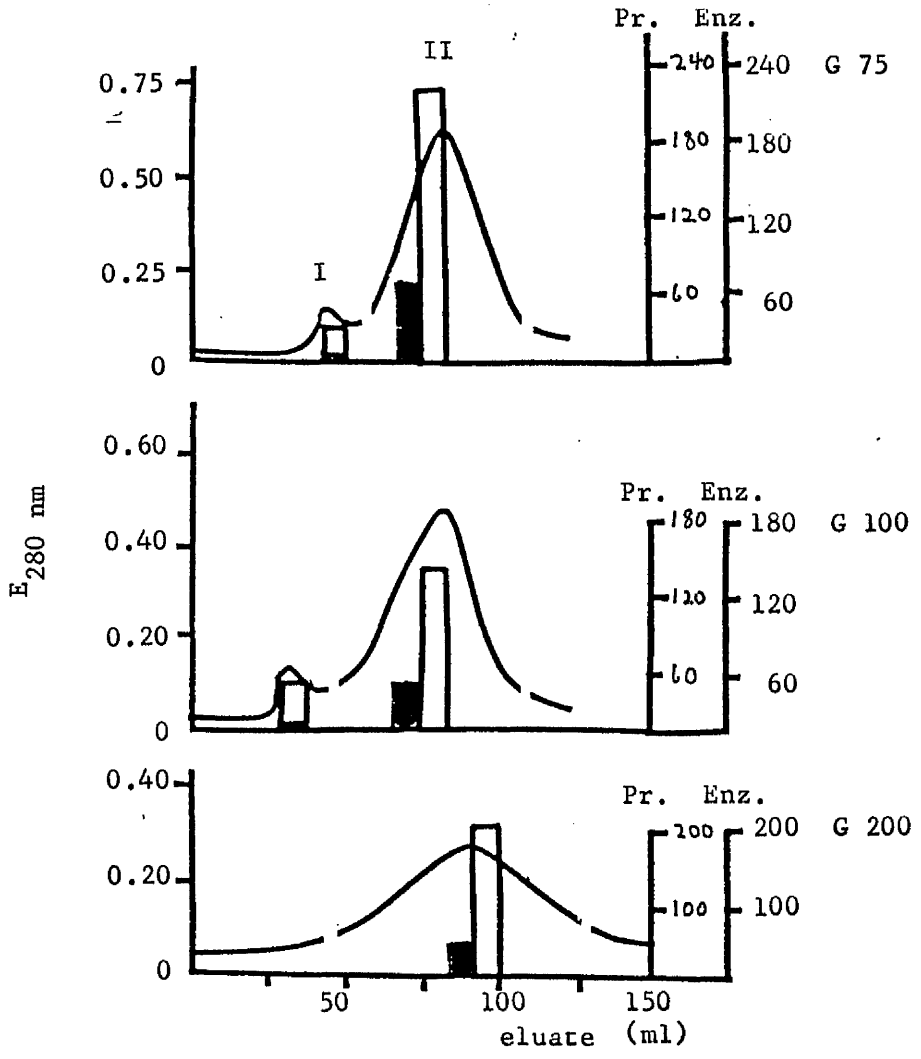


Figure 8 : Gel filtration of crude neuraminidase.

Legend: ●: Total protein value of peak in mg (based upon $E_{280\ nm}$ readings)

○: Total neuraminidase activity value in ImU (based upon T.B.A. assay).

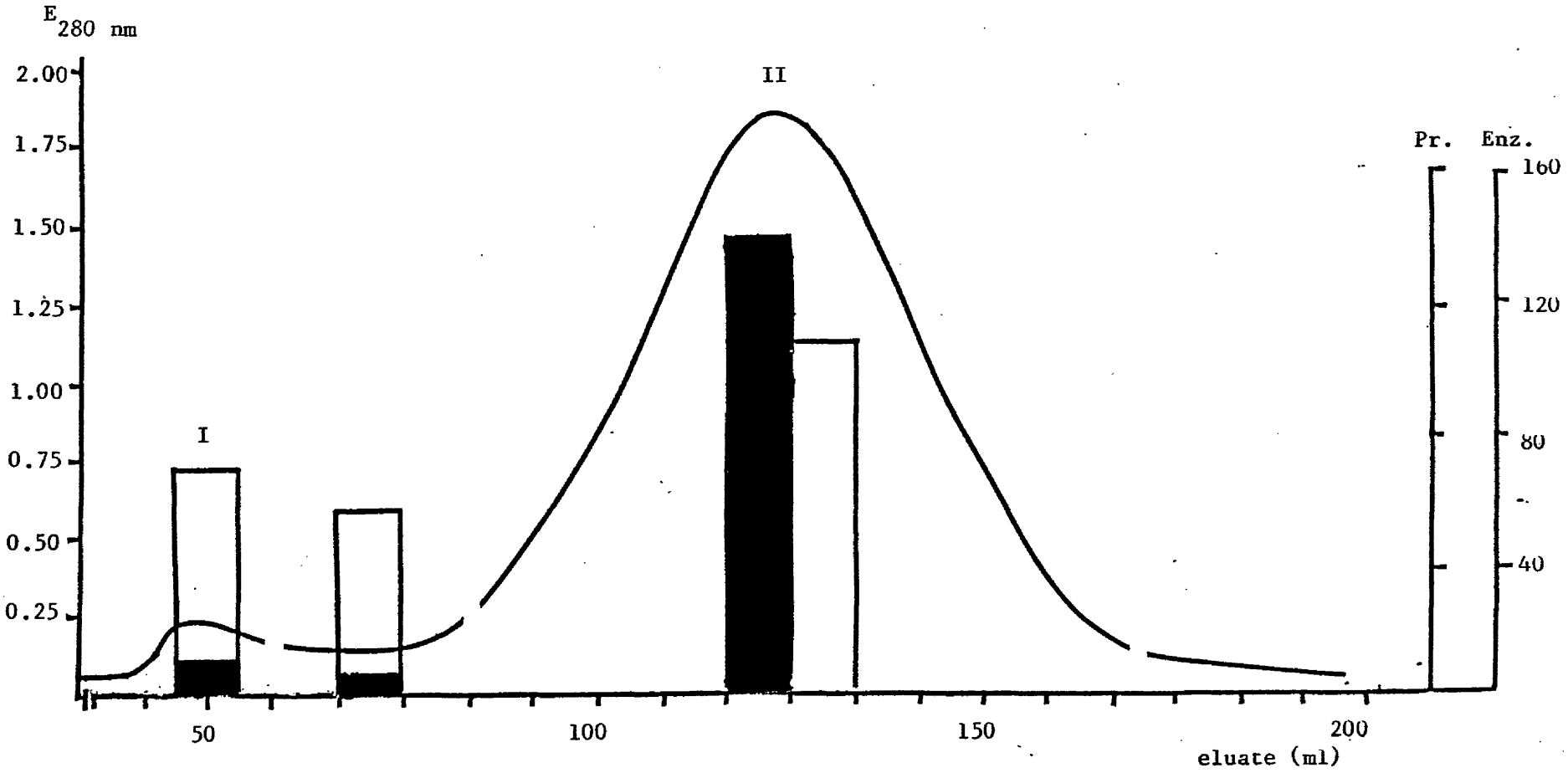


Figure 9 : Sephadex G 100 filtration of crude neuraminidase.

Column dimensions : 1.6 x 81 cm; Flow rate 0.8 ml min^{-1}; eluate 0.05 M NaAc buffer, pH 5.5

Legend: ● : Total protein value of peak in mg (based upon $E_{280\ nm}$ readings).

○ : Total neuraminidase activity value in ImU (based upon T.B.A. Assay)

major peak of neuraminidase was located between the first and second protein peaks. Much higher protein levels were present than found in the first peak. Protein estimation by the Lowry method showed that the protein value in peak 2 was twice that of peak 1.

In view of the finding that the peak of neuraminidase activity did not coincide with the protein peak in all semi-purification steps of the crude neuraminidase, the column fractions were examined by various methods. A typical result is shown in Figure 10. It will be noticed that the neuraminidase was eluted from the column between peaks I and II detected at $E_{280\ nm}$. However, the enzyme peak correlated reasonably with the protein peak obtained by the Lowry method. Consequently, the fractions between 78 and 110 ml of eluate containing the maximum neuraminidase activity were pooled. Such neuraminidase preparations obtained at different times were designated as N_1-N_4.

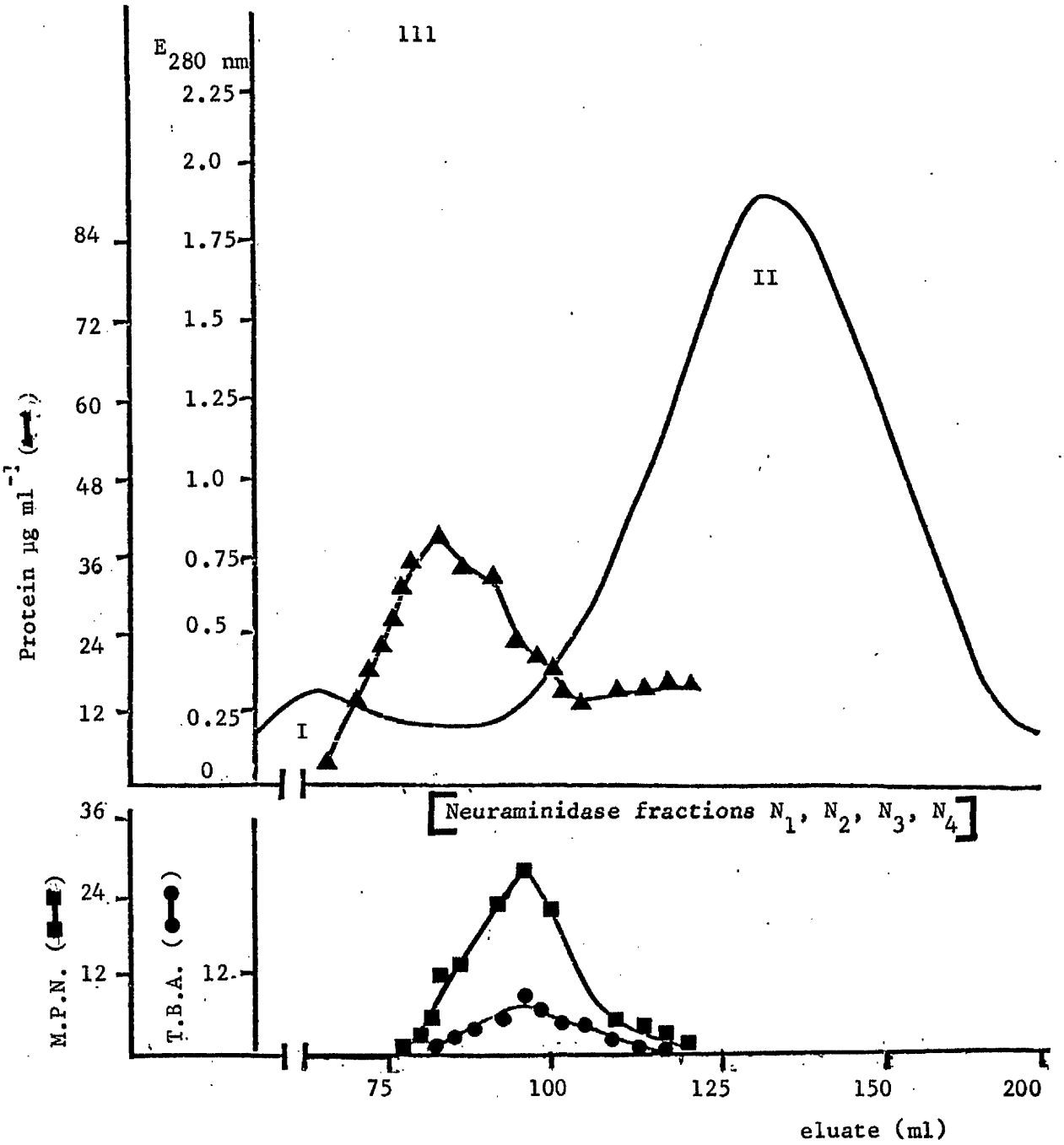


Figure 10: Locating neuraminidase activity (G100 column eluates).

Column dimensions: 1.5 x 81 cm; Flow rate: 0.8 ml min^{-1}
Sample volume: 3.0 ml; Eluate: 0.05M NaAc buffer pH 5.5

Legend:

a) $E_{280\ nm}$ elution profile (——)

b) Lowry protein estimation (µg protein as B.S.A.) (▲—▲)

c) Neuraminidase activity measured by the determination of:
   i) µg NANA liberated in the T.B.A. assay (●—●)
   ii) nmoles of methoxyphenol liberated in the M.P.N. assay (■—■).

3. CM-cellulose chromatography

Ion exchange chromatography on CM-cellulose gave the typical elution profile shown in Figure 11. The first $E_{280\ nm}$ peak showed a neuraminidase content of 6.8 ImU and the second one of 1.38 ImU by the T.B.A. assay. It was not possible to separate the enzyme from the brown pigment with the cationic exchanger, therefore it was not utilised further in the purification of crude enzyme preparations.

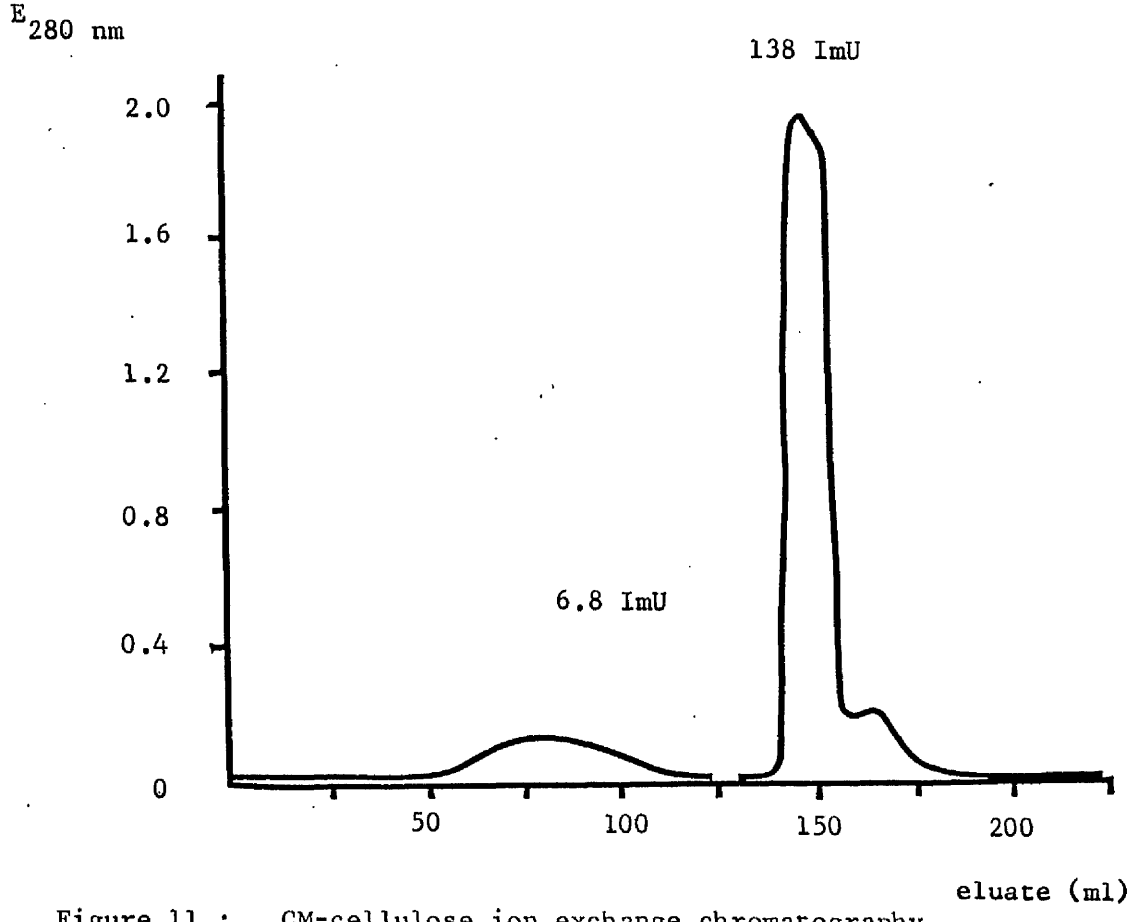


Figure 11 : CM-cellulose ion exchange chromatography of crude neuraminidase

Legend : The total neuraminidase activity contained in the pooled peak fractions is shown.

4. DEAE cellulose chromatography

Ion exchange chromatography on DEAE-cellulose produced the typical elution profile, Figure 12, in which seven $E_{280\ nm}$ peaks were separated with a stepwise gradient of NaAc buffer pH 4.4. Peaks VI and VII were the only peaks with neuraminidase activity when assayed by the T.B.A. assay. In addition, Peak VI was pigmented and Peak VII was non-pigmented. The activity level of Peak VI was 5.6 ImU mg^{-1}, and that of Peak VII was 3.5 ImU mg^{-1}.

Peak VI was re-chromatographed on DEAE, and three peaks were obtained after elution from the column with 1.0 M NaCl, Figure 13. Peaks were non-pigmented while Peak III was pigmented. Peaks I-III had neuraminidase activity levels of 1.7 ImU, 0.57 ImU and 1.0 ImU respectively.

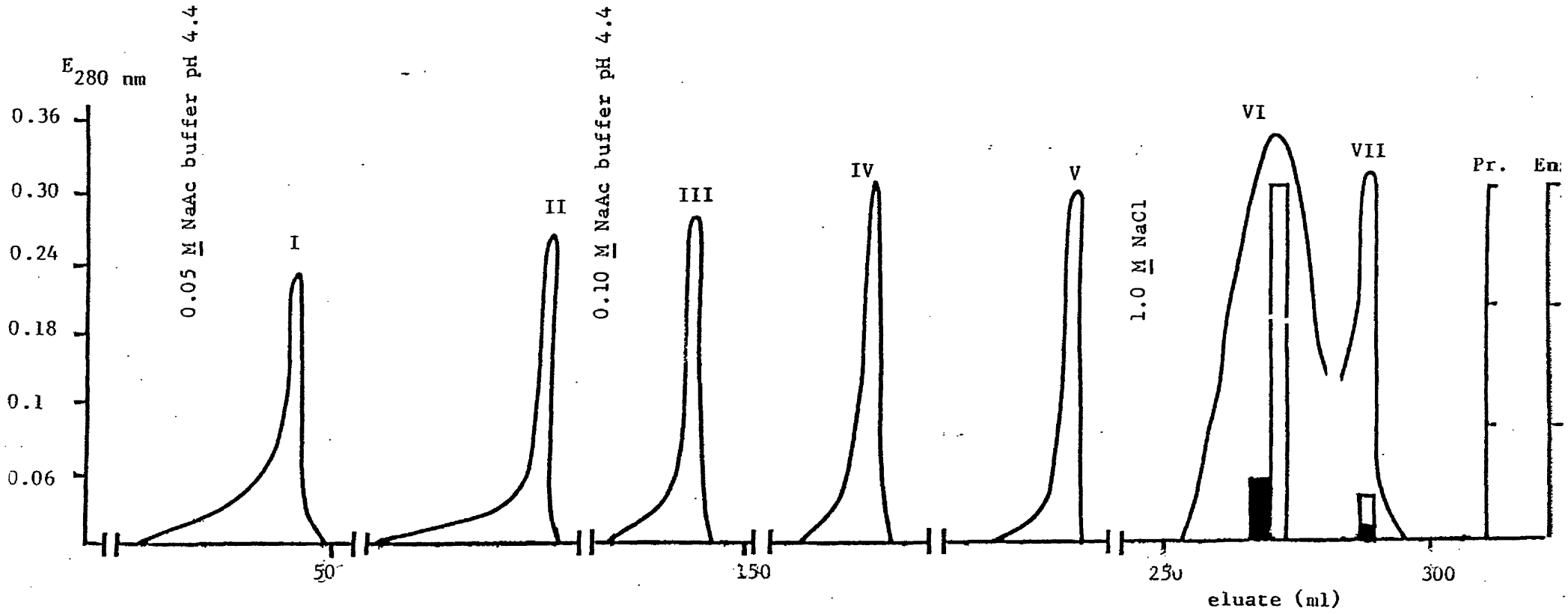


Figure 12 : DEAE-cellulose ion exchange chromatography of crude neuraminidase.

Legend :

● Total protein of peak in mg (based upon $E_{280\ nm}$ readings)

○ Total neuraminidase activity value in ImU (based upon T.B.A. assay).

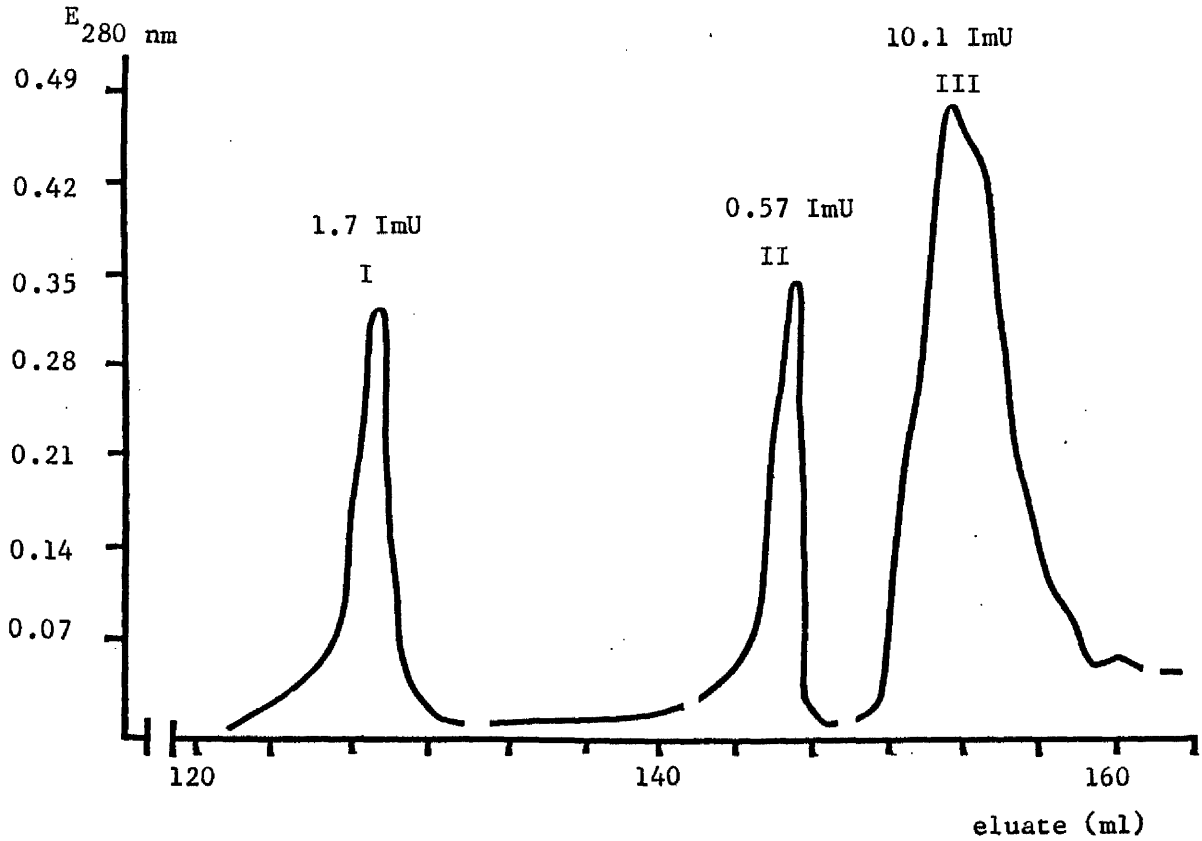


Figure 13 : Re-chromatographing of DEAE active peaks VI and VII

Peaks VI and VII from DEAE cellulose (see Figure 12) were pooled and rechromatographed with 1.0 M NaCl as eluate.

5. Protein estimation

A standard curve was obtained in the Lowry method by plotting μg protein (B.S.A.) against the $E_{750\ nm}$ values (Figure 14). The constant k obtained from the standard curve was 407. The protein content of each of the semi-purified Sephadex G 100 neuraminidase preparations (N_1-N_4) was expressed as μg of protein ml^{-1} as B.S.A. (Table 10).

A standard curve for the Micro-biuret method was obtained by plotting μg of protein (ovalbumin), against $E_{555\ nm}$ values (Figure 15). The constant k obtained from the standard curve was 4687.5. The protein content of each of the semi-purified Sephadex G 100 neuraminidase preparations (N_1-N_4) was expressed as μg of protein ml^{-1} as ovalbumin (Table 10). The protein content of the semi-purified Sephadex G 100 neuraminidase preparations (N_1-N_4) were compared by using both Lowry and Micro-biuret methods (Table 10).

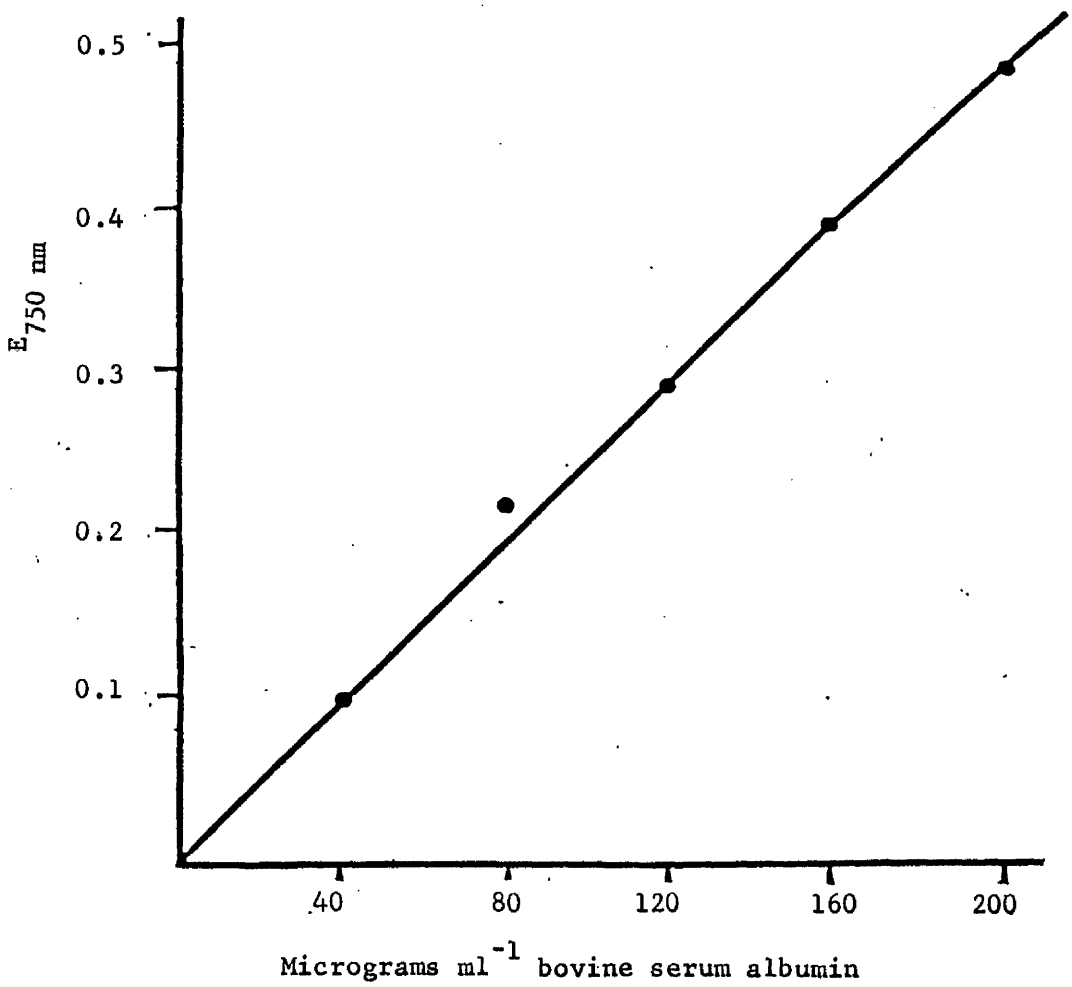


Figure 14 : Standard curve for protein estimation (Lowry method).

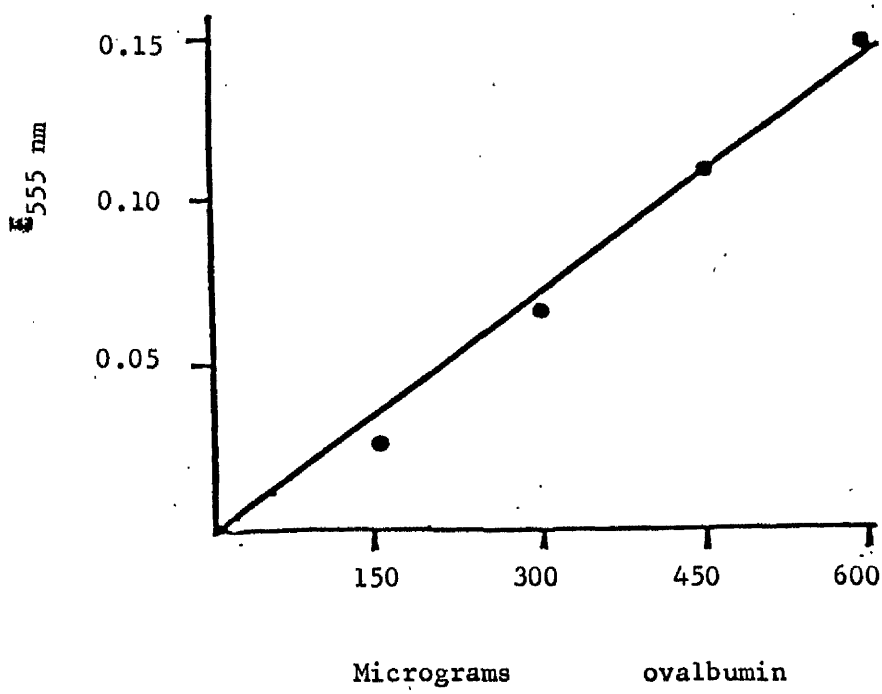


Figure 15 : Standard curve for Micro-biuret protein estimation.

Semi-purified G 100 Neuraminidase Preparation	Protein ($\mu g\ ml^{-1}$) as measured by	
	Lowry (B.S.A. Std.)	Micro-Biuret (Oval. Std.)
N_1	562.0	319.0
N_2	134.0	108.0
N_3	37.0	32.8
N_4	167.0	136.0

Table 10 : Protein estimation of neuraminidase active preparations.

Lowry Bovine serum albumin k value = 407

$\therefore E_{750\ nm} \times 407 \times 10 = \mu g$ protein ml^{-1}.

Microbiuret ovalbumin k value = 4688

$\therefore E_{555\ nm} \times 4688 = \mu g$ protein ml^{-1}.

II. ENZYME ASSAYS

1. T.B.A. procedure for neuraminidase

A standard curve was obtained by plotting the concentration of NANA (μmoles of N-acetylneuraminic acid) against the values at $E_{549\ nm}$ (Figure 16). Two different standard curves were produced, namely, one for N-butanol and one for cyclohexanone as the organic phase. The NANA constant, k, was 40.92 for N butanol and 32.92 for cyclohexanone.

It will be clear from Table 11 that in the 30 min T.B.A. assay neither the Koch-Light nor the Sigma neuraminidase preparations showed the level of enzyme activity as cited by the manufacturers' data of T.B.A. assay 690 and 290 ImU respectively. The levels of neuraminidase activity in the semi-purified preparations from the culture fluid of V. cholerae grown in bovine colostrum:proteose peptone medium (Table 11) varied between 30-55 ImU $(mg\ protein)^{-1}$.

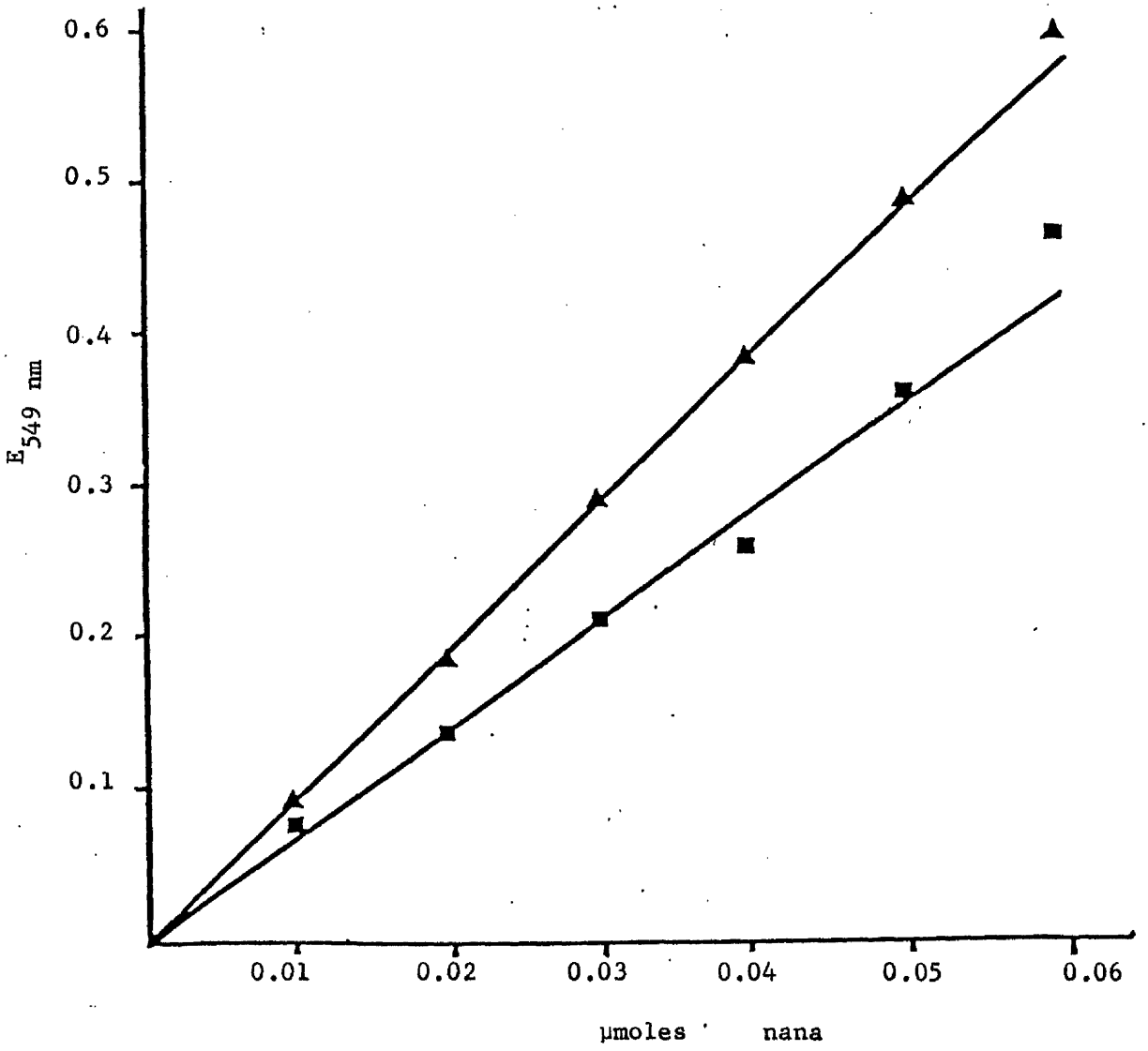


Figure 16 : Standard curve for measurement of NANA in T.B.A. procedure.

▲ = Standard NANA curve with cyclohexanone (distilled) as the organic phase

■ = Standard NANA curve with N-butanol as the organic phase.

Neuraminidase	Equivalent µg NANA released	µg NANA released ml^{-1}	µg NANA released $(mg\ protein)^{-1}$	Specific Activity ImU $(mg\ protein)^{-1}$
Koch-Light (V. cholerae)	4.7	47	N.D.	N.D.
(4.0 ImU ml^{-1})	6.1	61	N.D.	N.D.
Sigma (Cl. perfringens)	10.1	101	N.D.	N.D.
(3.4 ImU ml^{-1})	11.9	119	N.D.	N.D.

Pooled G 100 Preparations

Code	Total volume (ml)	Total protein (mg microbiuret)				
N_1	8.0	2.6	9.2	92	283.0	30.5
N_2	31.0	3.35	5.5	55	509.0	55.0
N_3	14.0	0.46	1.52	15.2	462.0	50.0
N_4	20.0	2.72	3.23	32.3	237.5	25.6

Table 11 : Neuraminidase activity by T.B.A. procedure.

Legend: $\frac{\mu g\ NANA\ ml^{-1} \times \text{total volume}}{\text{total microbiuret protein}} = \mu g\ NANA\ released\ (mg\ protein)^{-1}$ in sample = A;

$$\text{Specific Activity} = \frac{A}{9279} \times 1000 = ImU\ (mg\ protein)^{-1}.$$

2. M.P.N. procedure for neuraminidase

A standard curve was obtained by plotting nanomoles (nmoles) of 3-methoxyphenol (M.P.) against $E_{580\ nm}$ values (Figure 17). The M.P. constant, k, was 105.26. The nmoles of 3-methoxyphenol released was a reflection of the level of neuraminidase activity. The level of neuraminidase activity could also be expressed as μg of NANA released by multiplying nm of 3-methoxyphenol by 0.4 (the conversion factor from the ratio of the molecular weight of 3-methoxyphenol 124.14 : the molecular weight of NANA 309.3). An elution profile of neuraminidase active peaks eluted from a G 100 Sephadex column expressed as μg of NANA liberated was plotted (Figure 18).

The M.P.N. assay revealed that preparations N_1-N_4 had levels of specific activity ranging from 81-741 ImU $(mg\ protein)^{-1}$ (Table 12).

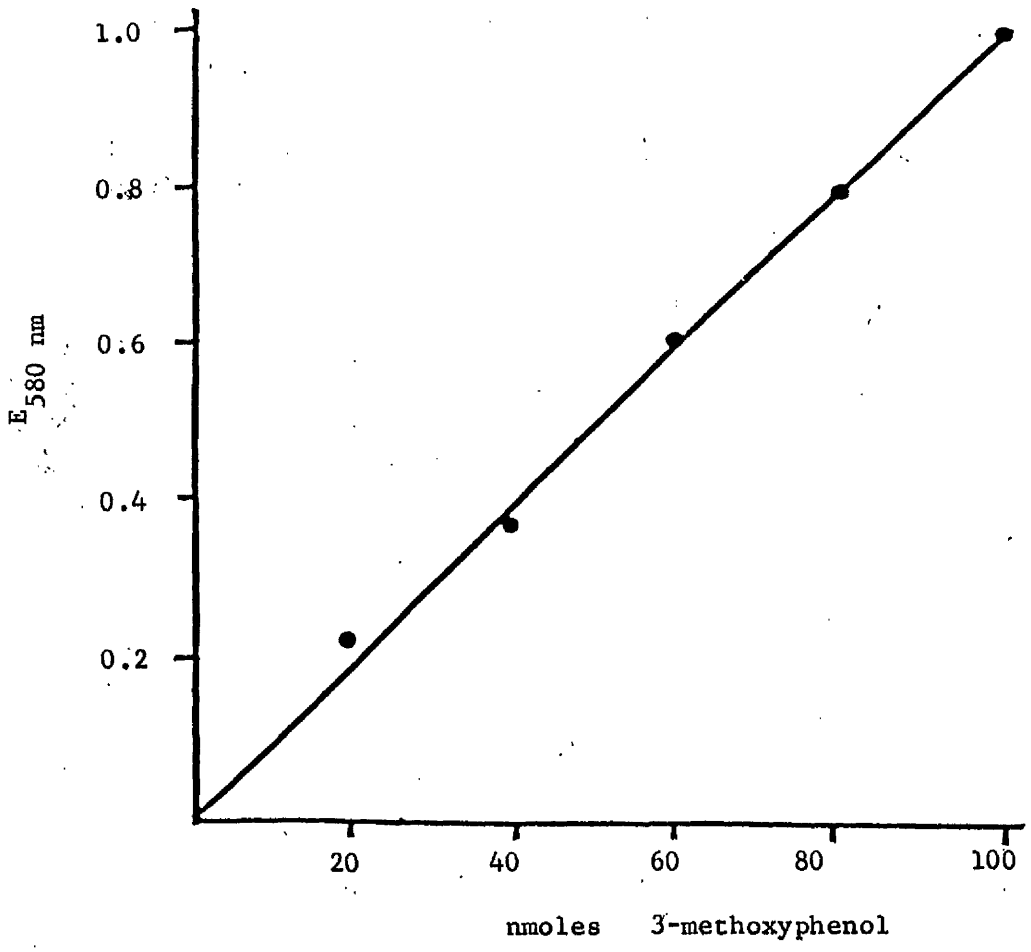


Figure 17 : Standard curve for measurement of M.P. in M.P.N. procedure.

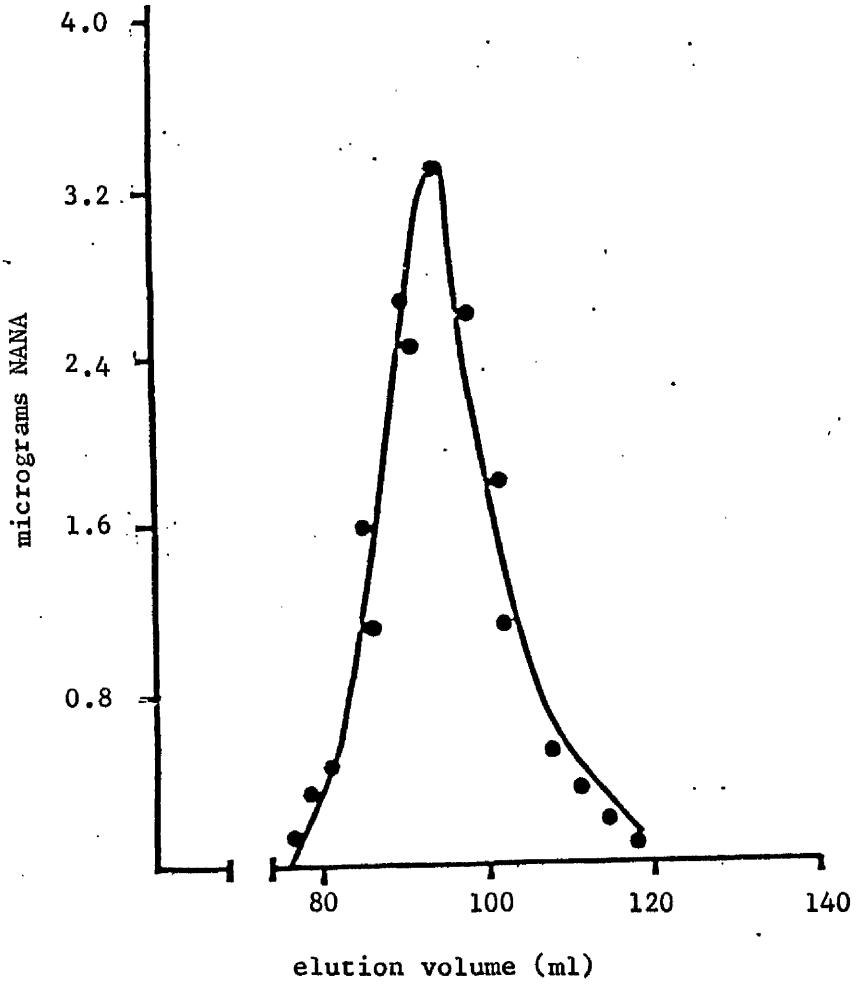


Figure 18 : Elution profile of neuraminidase active peak from G 100 column (M.P.N. procedure).

Neuraminidase	µg M.P. released	Equivalent µg NANA released	µg NANA released ml^{-1}	µg NANA released $(mg\ protein)^{-1}$	Specific activity ImU $(mg\ protein)^{-1}$
Koch-Light (*V. cholerae*) (4.0 ImU ml^{-1})	0.836	0.335	3.35	N.D.	N.D.
Sigma (*Cl.Perfringens*) (3.4 ImU ml^{-1})	3.59	1.44	14.4	N.D.	N.D.
Pooled G 100 Preparations					
Code — Total volume (ml) — Total protein (mg microbiuret)					
N_1 — 8.0 — 2.6	9.54	3.83	38.25	117.7	81
N_2 — 31.0 — 3.35	13.59	5.45	54.50	504.3	346
N_3 — 14.0 — 0.46	8.83	3.54	35.42	1078.0	740.72
N_4 — 20.0 — 2.72	32.67	13.10	131.00	963.2	661

Table 12 : Neuraminidase activity by M.P.N. procedure

Legend:

$$\frac{\mu g\ NANA\ ml^{-1} \times \text{total volume}}{\text{total microbiuret protein}} = \mu g\ NANA\ released\ (mg\ protein)^{-1}\ in\ sample = A;$$

$$\text{Specific Activity} = \frac{A}{1457.3} \times 1000 = ImU\ (mg\ protein)^{-1}.$$

3. Proteinase activity

A standard curve was obtained by plotting μg of proteinase (as trypsin) against the $E_{280\ nm}$ values (Figure 19). The proteinase constant, k, obtained from the standard curve was 142.85. The enzyme content in each of the semi-purified preparations was expressed as μg of proteinase ((mg micro-biuret protein)$^{-1}$).

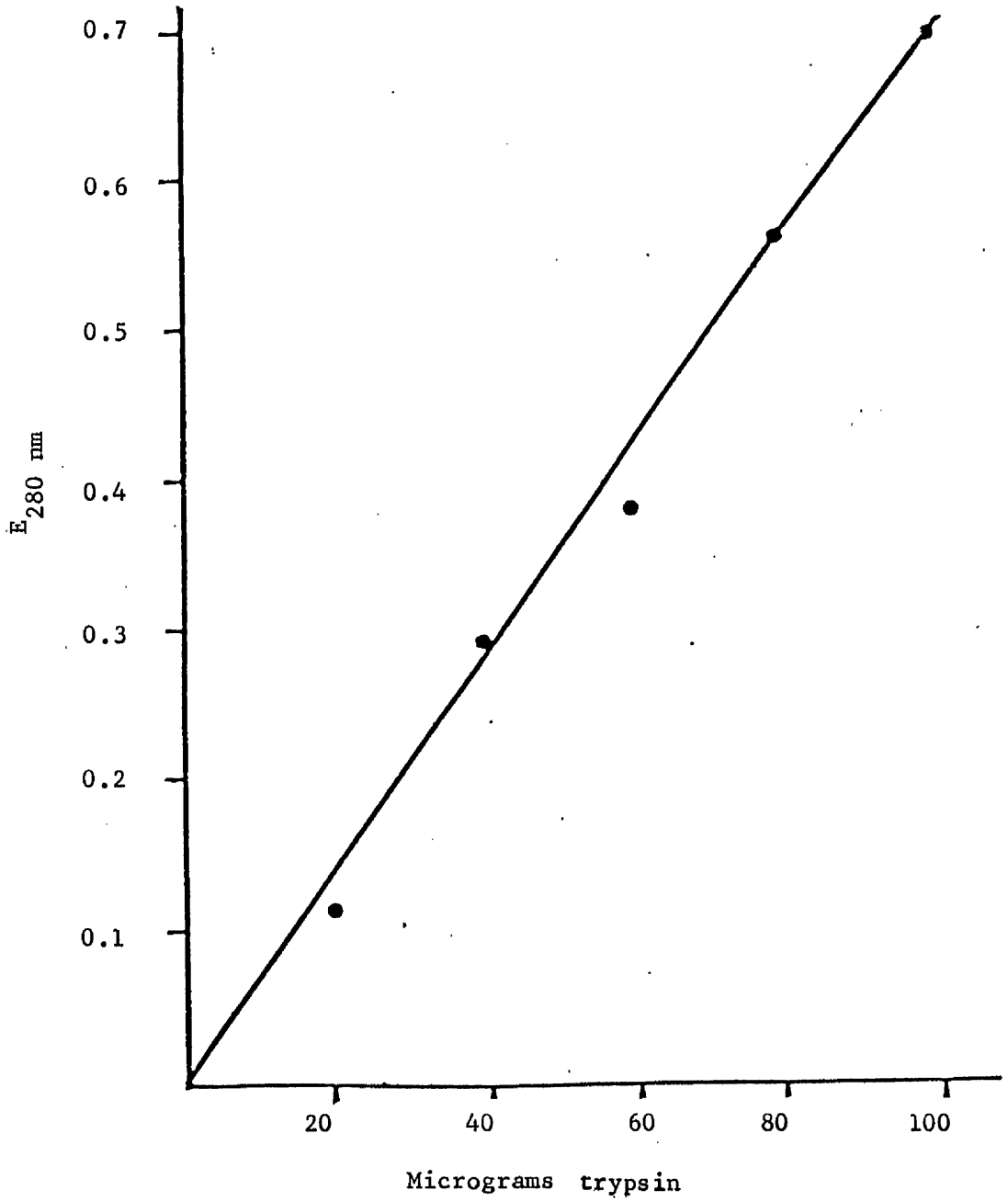


Figure 19 : Standard curve for proteinase activity.

Sample	Volume	Total Protein (mg micro-biuret)	ml^{-1}	Proteinase (µg) (as trypsin) (mg micro-biuret protein)$^{-1}$)
Pooled G 100 Fractions				
N_1	8.0	2.6	16.71	51.42
N_2	31.8	3.35	4.00	37.01
N_3	14.0	0.46	1.86	56.51
N_4	20.0	2.72	0	-

Table 13 : Proteinase activity.

Legend : µg proteinase ml^{-1} = A;

$$\frac{A \times \text{total volume}}{\text{total protein}} = \text{µg proteinase (as trypsin) (mg micro-biuret protein)}^{-1}.$$

4. Phospholipase-C

A standard curve was obtained by plotting ngrams of phospholipase-C against the difference in $E_{340\ nm}$ readings ($\triangle E = E_1 - E_2$, where E_1 = reading prior to the addition of glycerol kinase and E_2 = reading subsequent to the addition of glycerol kinase (Figure 20). The phospholipase-C constant, k, obtained from the standard curve was 559. The levels of phospholipase-C activity are shown in Table 14.

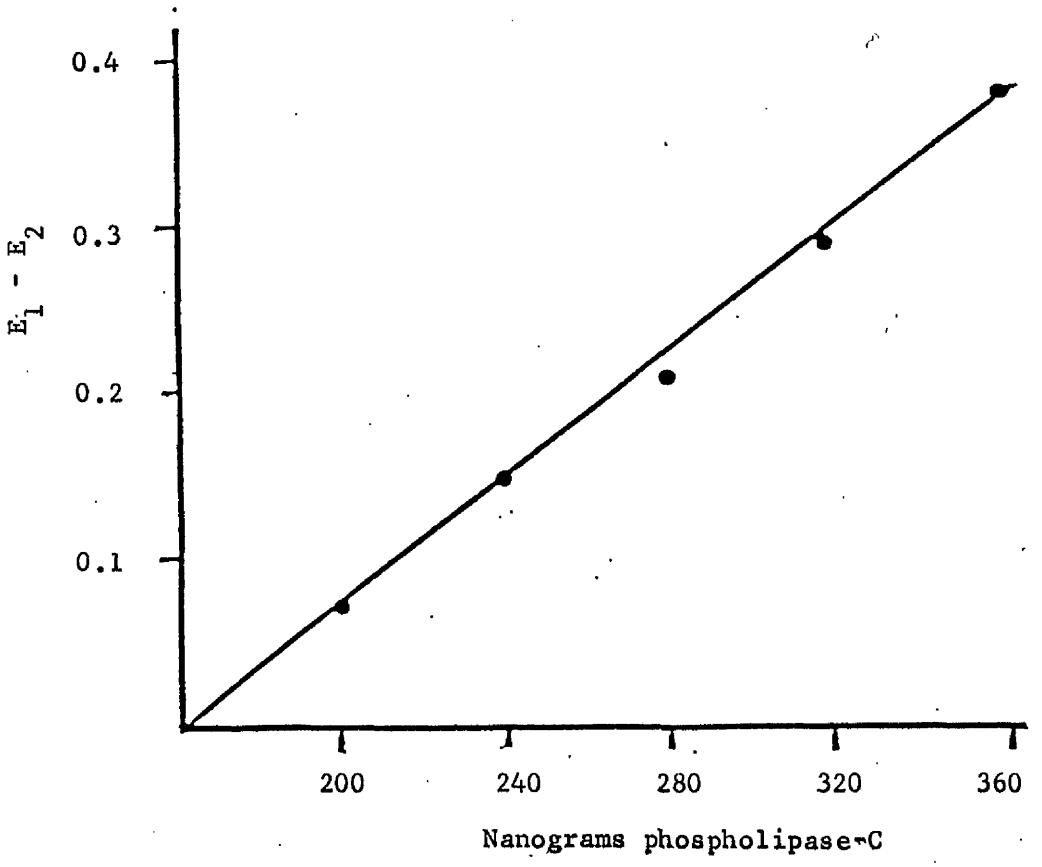


Figure 20 : Standard curve for phospholipase-C activity.

Legend: $E_1 = E_{340\ nm}$ prior to glycerol kinase treatment.

$E_2 = E_{340\ nm}$ after glycerol kinase treatment.

	Total vol (ml)	Total Protein (mg micro-biuret)	△E	µg lecithin hydrolysed	µg hydrolysed ml^{-1}	Phospholipase-C ImU $(mg\ protein)^{-1}$
Control (240 ng phospholipase-C: Boehringer (B. cereus) + T.C.A.)			0	0	0	-
240 ng phospholipase-C			0.142	0.079	3.95	N.D.
Pooled G100 Preparations						
N_1	8.0	2.6	0.190	0.106	5.31	1.35
N_2	31.0	3.35	0.170	0.095	4.75	3.64
N_3	14.0	0.46	0.202	0.113	5.65	14.24
N_4	20.0	2.72	0.207	0.116	5.79	3.53

Table 14 : Phospholipase-C activity.

Legend: $\frac{\mu g\ \text{lecithin hydrolysed}\ ml^{-1}}{\text{total protein}} \times \text{total volume} =$

$\mu g\ \text{lecithin hydrolysed}\ (mg\ protein)^{-1} = A;$

Specific activity = $\frac{A}{12063} \times 1000 = \text{ImU}\ (mg\ protein)^{-1}.$

5. Endoglycosidase

A standard curve was obtained by plotting the number of μg of neutral sugar (glucose) against $E_{490\ nm}$ (Figure 21). The phenol-H_2SO_4 constant, k, obtained from the curve was 121.95. The endoglycosidase activity of the N_1-N_4 preparations was obtained by measuring the release of neutral sugar (glucose) from an ovalbumin substrate, and comparing it against the level of neutral sugar (glucose) released from ovalbumin substrate by commercial endoglycosidase of known activity (Table 15).

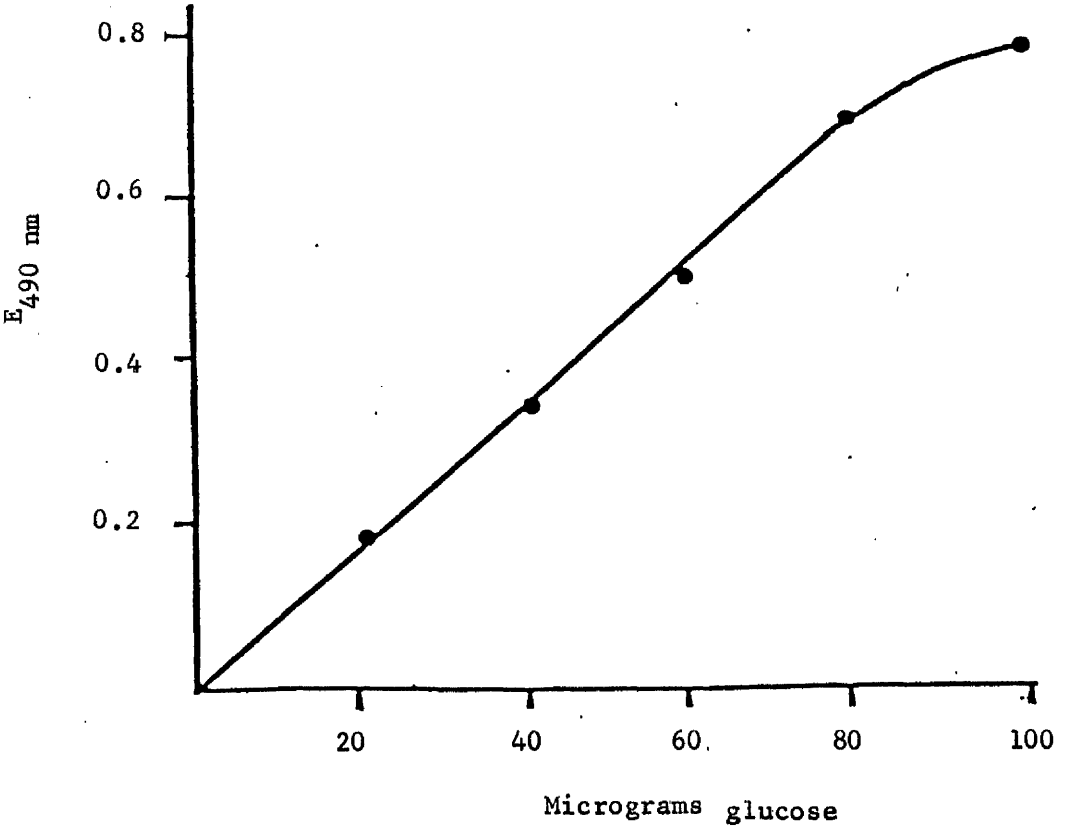


Figure 21 : Standard curve for neutral sugar (as glucose).

			µg glucose released	m.u. ml^{-1} relative to standard	Endoglycosidase m.u. $(mg\ protein)^{-1}$
Commercial Endoglycosidase H 6.0m.u.(20 µl)			9.145	-	0.1
Pooled G 100 Preparations (20 µl)					
	Total vol. (ml)	Total protein (mg micro-biuret)			
N_1	8.0	2.6	6.1	200	0.615
N_2	31.0	3.35	5.73	188	1.74
N_3	14.0	0.46	4.39	144	4.36
N_4	20.0	2.72	45.12	1480	10.88

Table 15 : Endoglycosidase activity.

Legend: µg glucose released by preparation = A

$$\frac{A \times 6.0\text{m.u. of commercial enzyme}}{9.145} \times 50 =$$

m.u. ml^{-1} of preparation;

$$\frac{\text{m.u.ml}^{-1}\text{ of preparation}}{\text{total protein}} \times \text{total value} \times 0.001^{*} =$$

Enzyme units $(mg\ protein)^{-1}$.

*Conversion factor to express as units (mg protein).

6. Aldolase

A standard curve was obtained by plotting μg of aldolase against the mean values of $\triangle E_{340\ nm} \times 10^{-2}\ min^{-1}$ (Figure 22). There were no changes in the $E_{340\ nm}$ values at 1 min intervals during the 5 min test period for the N_1-N_4 preparations. Therefore it was concluded that the N_1-N_4 preparations did not possess aldolase activity. The aldolase standard, however, gave variations in the $E_{340\ nm}$ reading during the same 5 min period.

7. Summary of enzyme activities in preparations N_1-N_4

The various enzyme activities associated with the Sephadex G 100 preparations (N_1-N_4) are shown in Table 16.

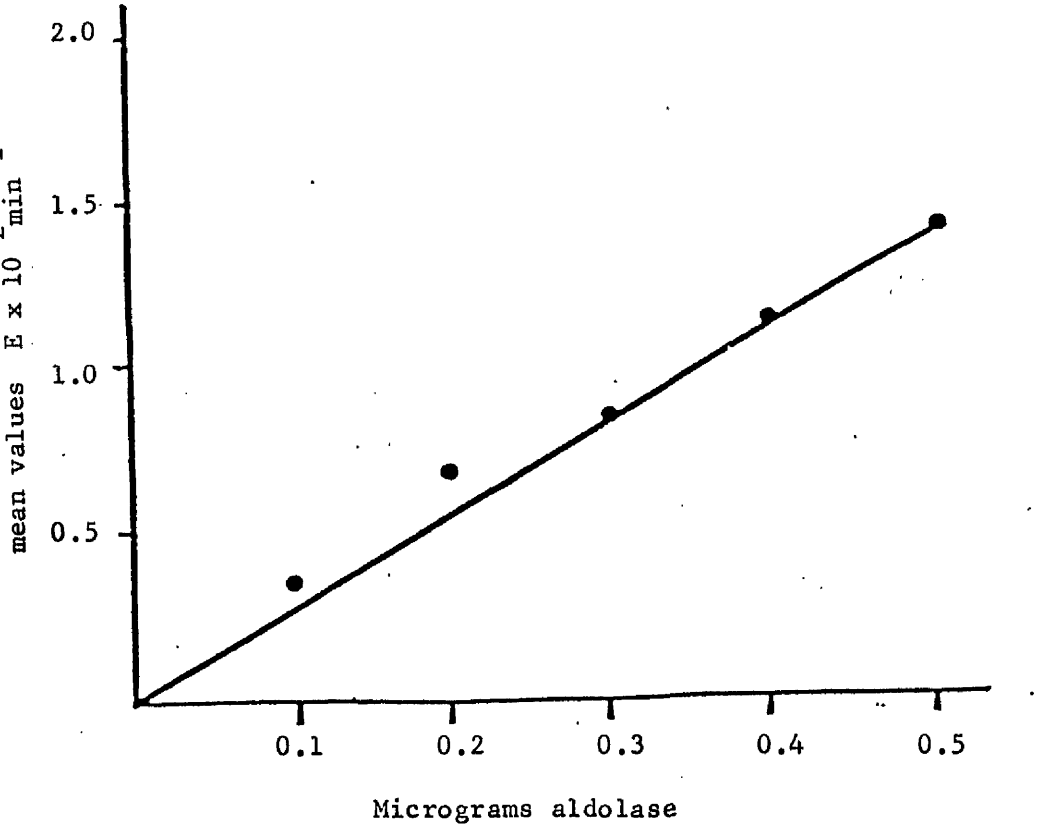


Figure 22 : Standard curve for aldolase concentration.

Legend: In this procedure the reaction is read at $E_{340\ nm}$ at 1 minute intervals (for a period of 5 minutes) and $\triangle E$ extinction was calculated at each 1 minute interval. From mean values of $\triangle E_{340\ nm}$ min^{-1} (obtained from the $\triangle E_{340\ nm}$ values), the concentration of aldolase (µg) could be read from the curve on the x-axis.

Pooled G100 Preparation		Lowry protein as B.S.A. mg ml^{-1}	Micro-biuret as oval-bumin mg ml^{-1}	Total protein mg (micro-biuret)	Per milligram microbiuret protein				
Sample	Total volume (ml)				Neuraminidase ImU T.B.A	Neuraminidase ImU M.P.N.	Proteinase (as trypsin) µg	Phospholipase-C ImU	Endoglycosidase* units
N_1	8.0	0.562	.319	2.6	30.0	81	51.42	1.35	0.615
N_2	31.0	0.134	.108	3.35	55.0	346	37.0	3.64	1.74
N_3	14.0	0.037	.033	0.46	50	740	56.5	14.24	4.36
N_4	20.0	0.167	.136	2.72	25.6	661	0	3.53	10.88

Table 16 : Summary of enzyme activities in neuraminidase preparation

* Calculated relative to release of glucose by commercial endoglycosidase.

III PREPARATION OF IgG IMMUNOGLOBULIN (BY DEAE)

Ion exchange chromatography on D.E.A.E.-cellulose gave an elution profile with two $E_{280\ nm}$ peaks. The first peak eluted with 0.01 M PO_4 buffer pH 7.5 contained the IgG, Figure 23.

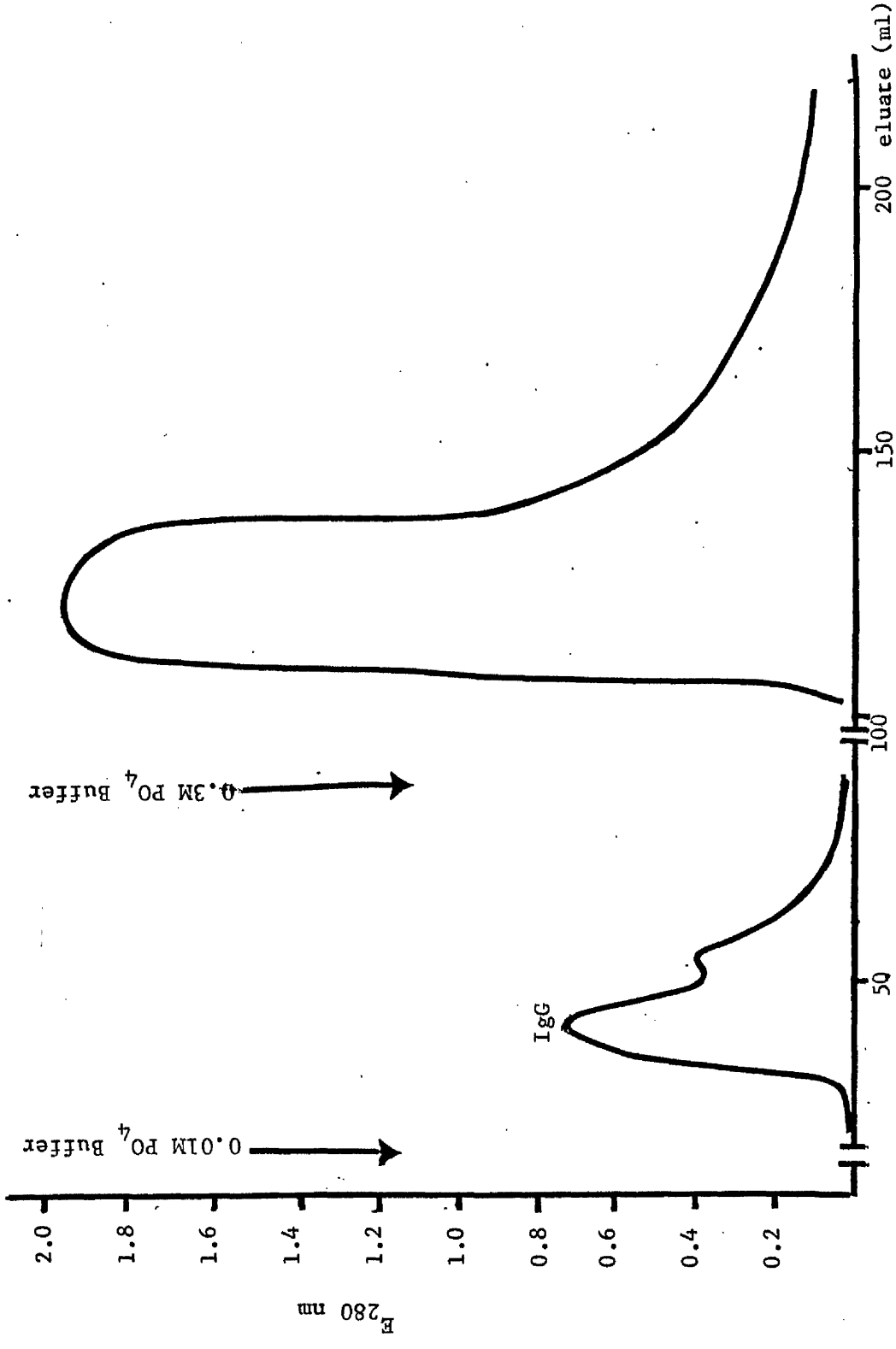


Figure 23 : Preparation of IgG immunoglobulin (by DEAE).

Column dimensions: 1.5 x 29 cm, Flow rate: 0.7 ml min^{-1}.

IV PURIFIED CHOLERA ENTEROTOXIN

Previous work in the Microbiology Department showed that cholera enterotoxin could be purified from crude culture filtrate by Sephadex G 100 filtration. The purified toxin was eluted from the column immediately after the void volume with borate gelatin buffer as the eluate, Figure 24. The peak fractions were concentrated to 5.0 ml and neuraminidase activity was measured by the T.B.A. and M.P.N. assays. No detectable neuraminidase activity was found in the toxin preparation.

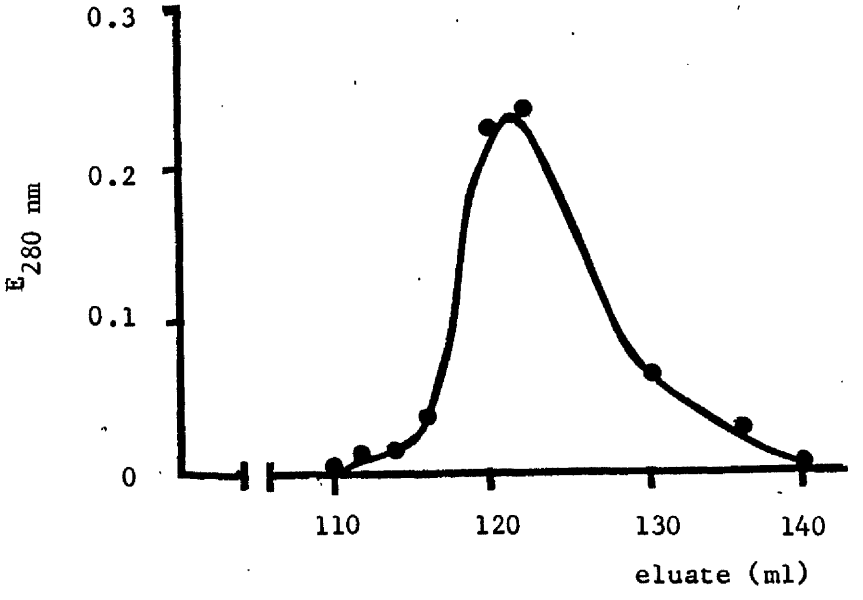


Figure 24 : Elution profile of V. cholerae enterotoxin on Sephadex G100.

Column dimensions: 2.6 x 81 cm, flow rate 0.8 ml min^{-1}.

Sample: crude N.I.A.I.D. V. cholerae enterotoxin 3 g $(5.0\ ml)^{-1}$ borate gelatin buffer.

V EFFECTS OF NEURAMINIDASE AND OTHER CHEMICAL TREATMENTS ON GOBLET CELL HISTOCHEMISTRY

Treatment with neuraminidase changed the number of cells stained by either Alcian Blue or PAS (Plates 2 and 4). The counts have been displayed in a bar graph (Figure 25). Differences in cell counts were analysed statistically by use of the t-test in its double tailed form (pp 232-233, Appendix VIII).

It will be noticed from Figure 25 that the number of goblet cells stained by Alcian Blue for the presence of NANA (sialic acid, N-acetylneuraminic acid) was reduced from 978 $(17.68\ mm^2)^{-1}$ to 499 after treatment with 136 ImU of neuraminidase for 24 h at $37^{\circ}C$. At the same time the number of goblet cells in the neuraminidase treated rat ileal sections stained by PAS increased to 342 $(17.68\ mm^2)^{-1}$ compared to the control value 211.

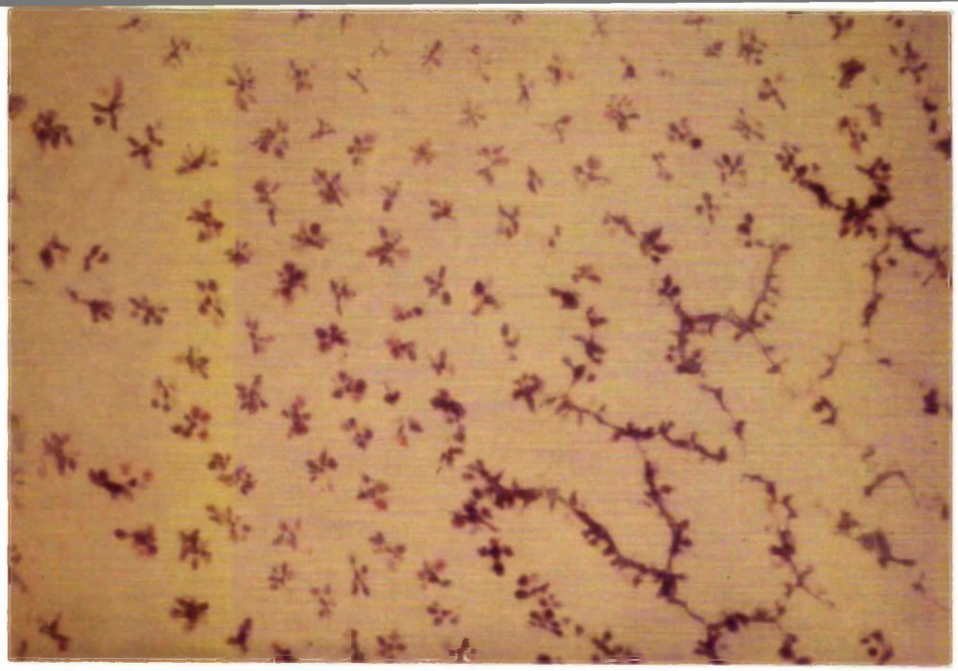
Statistical analysis indicated that the counts in the neuraminidase treated ileal sections stained by Alcian Blue or PAS were significantly different, at the $p < 0.0002$ and $p < 0.001$ levels respectively. There was no significant difference in the Alcian Blue stained sections between untreated sections and those treated with distilled water or 0.05 M NaAc buffer, pH 5.5.

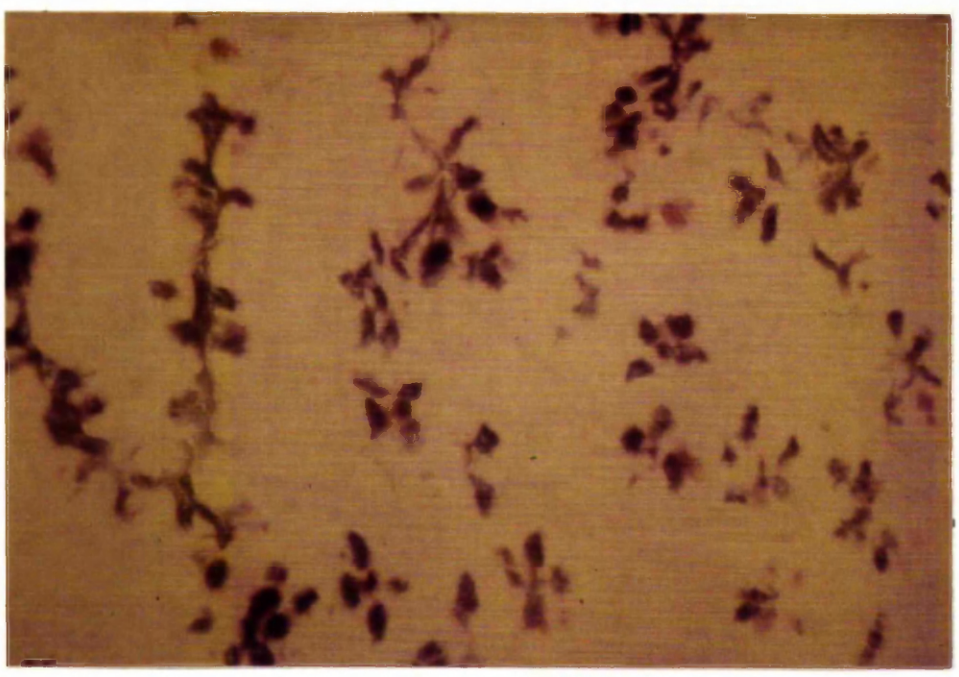
A Rat ileum, cross section, vapour fixed, stained with Alcian blue/PAS pH 2.5 (X 164).

B As above, except stained with Alcian blue/PAS at pH 2.5 and higher magnification (X 656).

C As B above

Plate 2 : Histological sections of rat ileum.



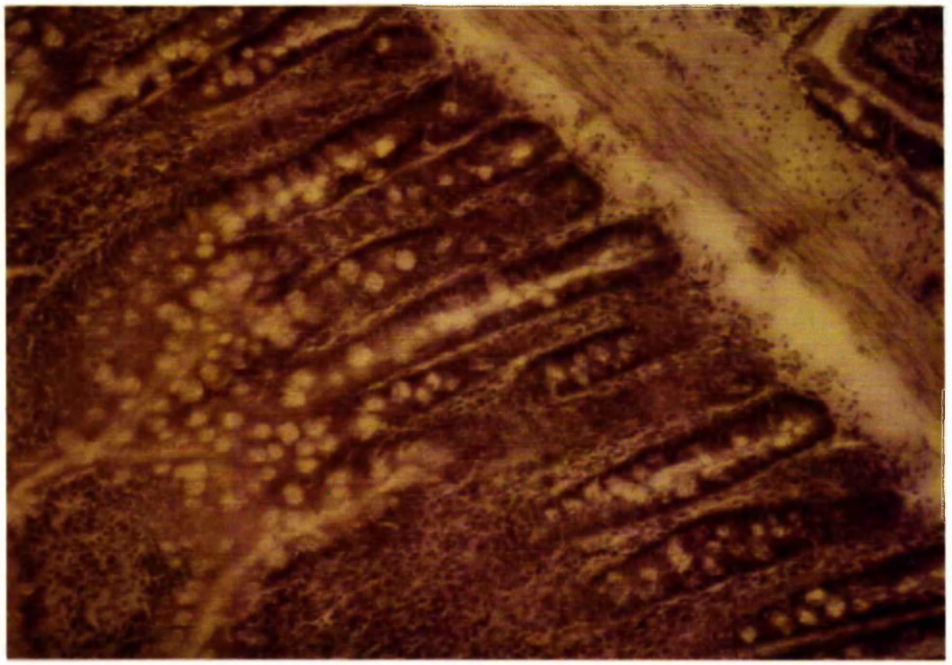


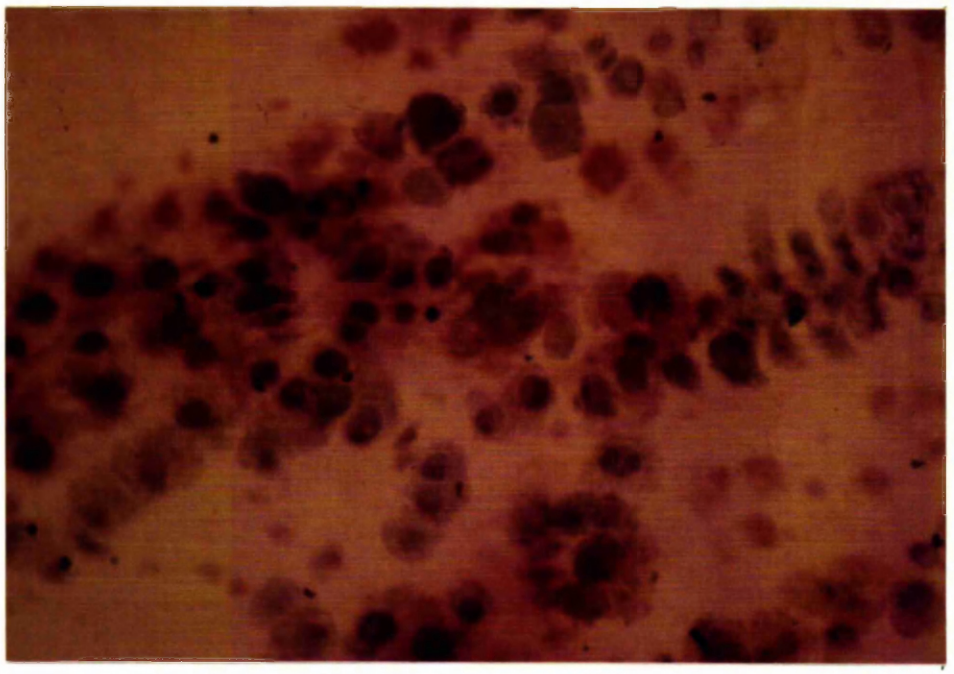
A Rat ileum, cross section, vapour fixed, untreated, and stained with Haemotoxylin and Eosin (X 164).

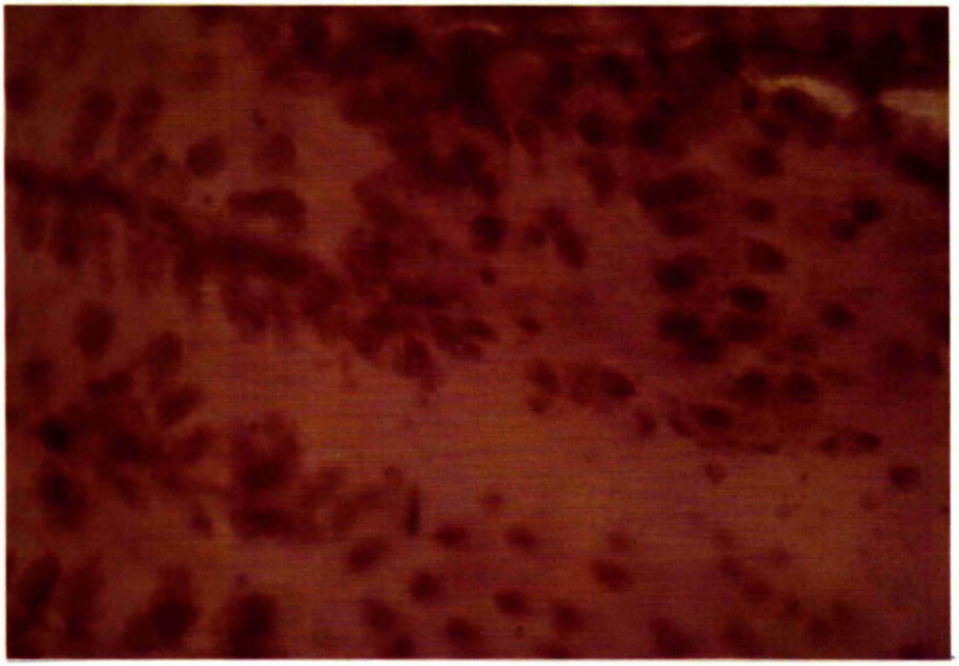
B Rat ileum, longitudinal section, vapour fixed, untreated, and stained with Alcian blue/PAS (X 656).

C As B above, except treated with Cl.perfringens neuraminidase (136 ImU Worthington) for 24 h at 37°C before staining.

Plate 3 : Histological sections of rat ileum.





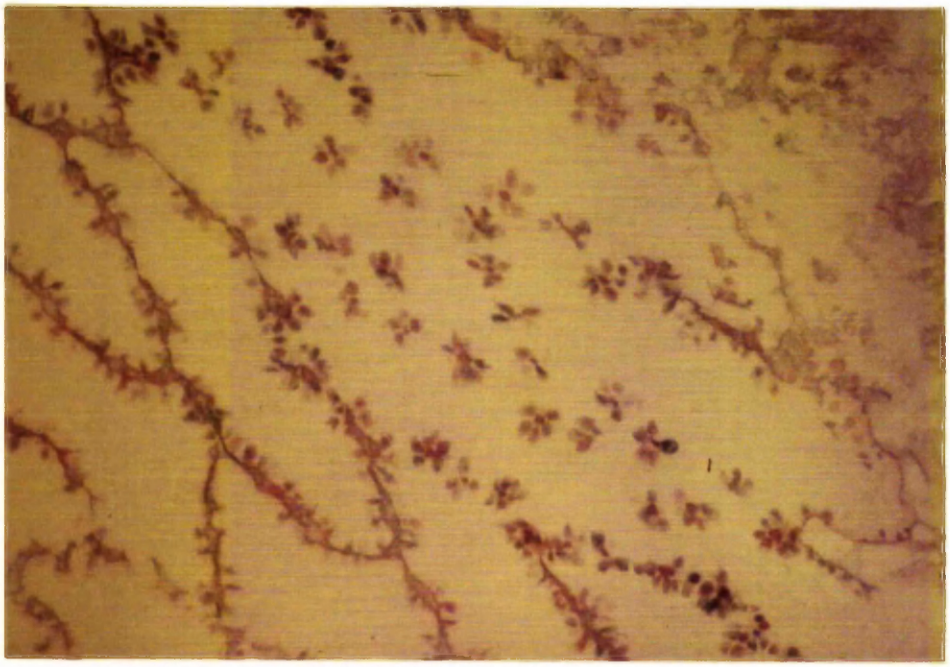


A Rat ileum, longitudinal section, vapour fixed, treated with Cl. perfringens neuraminidase (136 ImU Worthington) for 24 h at 37°C, and stained with Alcian blue/PAS at pH 2.5 (X 164).

B As above, except cross section and at higher magnification (X 656).

C As A above, except buffer control (no enzyme) and higher magnification (X 656).

Plate 4 : Histological sections of rat ileum.



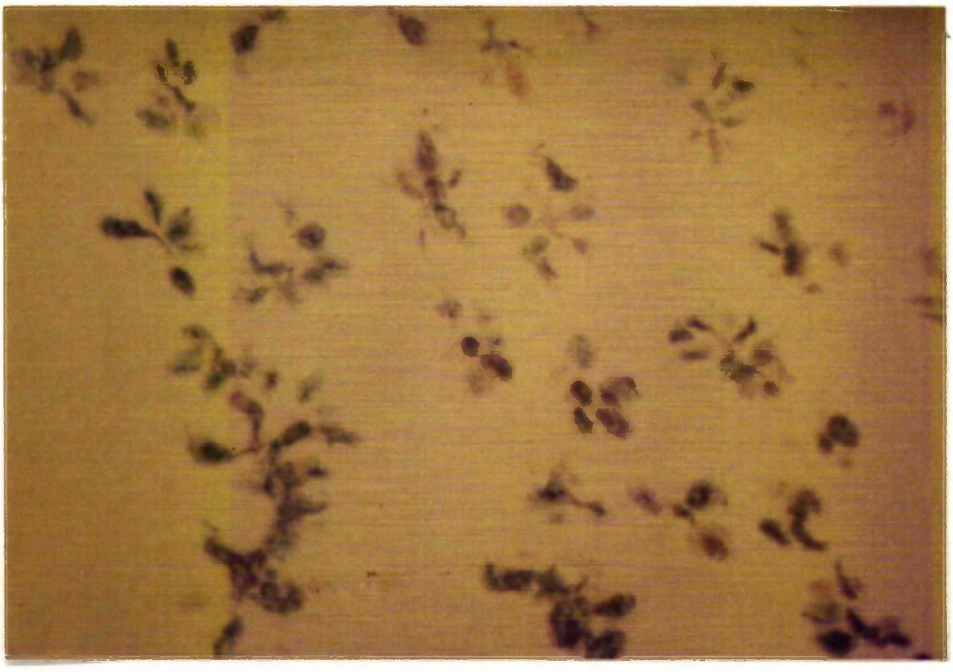


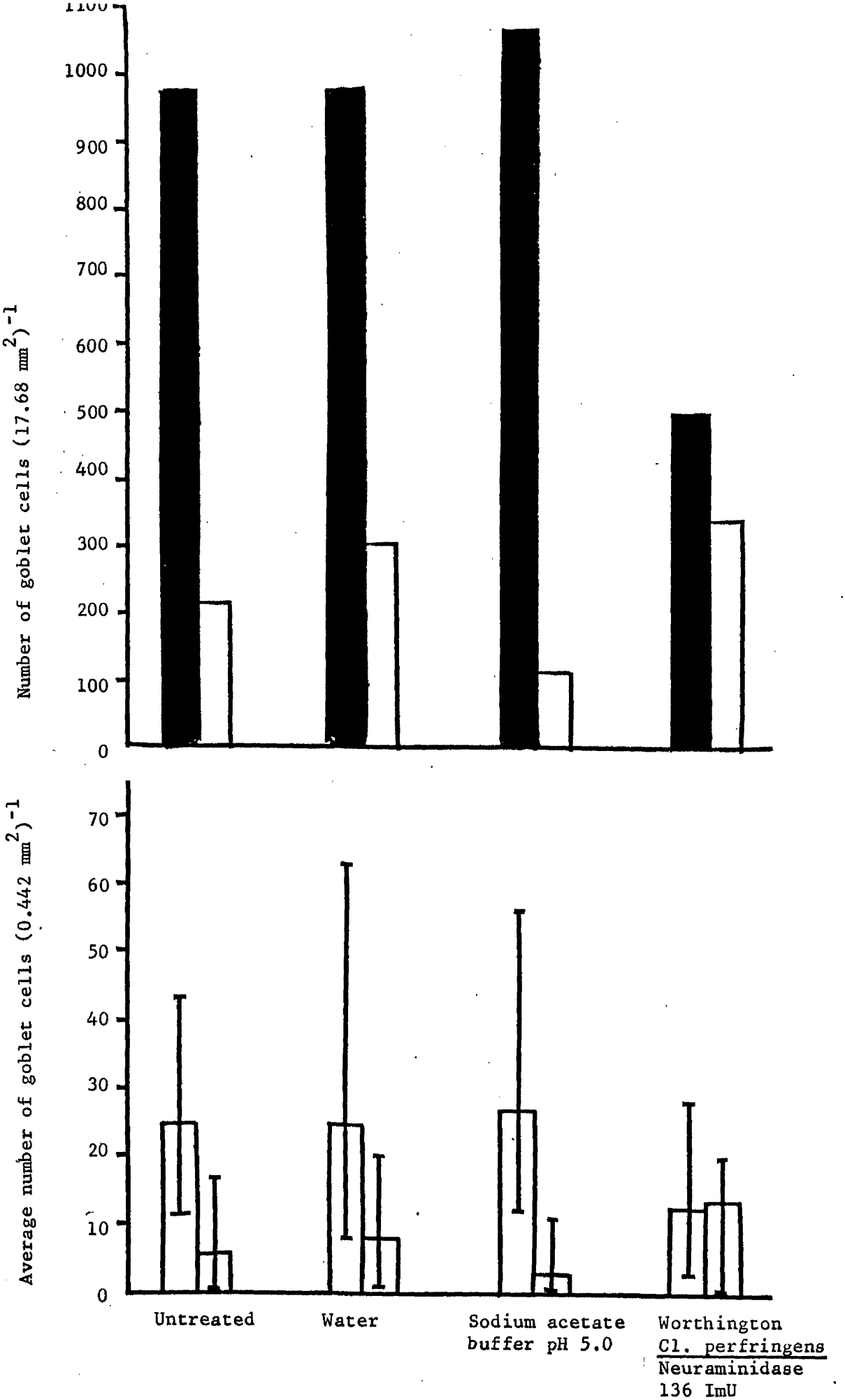
Figure 25 : Effect of commercial neuraminidase (Worthington Cl. perfringens)

Legend:

*Note: No. ImU cited is total enzymic activity in preparation.

■, Goblet cells stained by Alcian Blue to show the presence of neuraminic acid, and

□, Goblet cells stained by PAS to show mucopolysaccharide.


Number of goblet cells (17.68 mm^2)$^{-1}$
1100
1000
900
800
700
600
500
400
300
200
100
0
Average number of goblet cells (0.442 mm^2)$^{-1}$
70
60
50
40
30
20
10
0
Untreated
Water
Sodium acetate buffer pH 5.0
Worthington Cl. perfringens Neuraminidase 136 ImU
controls

I. Effects of commercial neuraminidase

In view of these results it was decided to compare the neuraminidase activity of a number of commercial enzyme preparations against the V. cholerae Sephadex G 100 preparation N_2 (see Plates 2-4; Figure 26). The most active commercial preparation with respect to loss of Alcian Blue staining in tissue sections when compared with untreated sections was the Sigma Cl. perfringens neuraminidase (Plate 2). In addition, the Cl. perfringens neuraminidase treated sections showed the highest PAS count $(17.68\ mm^2)^{-1}$ when compared to untreated sections. It will be noticed with both preparations of Cl. perfringens neuraminidase that there was a change from Alcian Blue to PAS staining. However, with the V. cholerae preparations not only was there a change in the content of NANA-containing sialomucin but, in addition, many of the goblet cells seemed to lose PAS stainable mucosubstance.

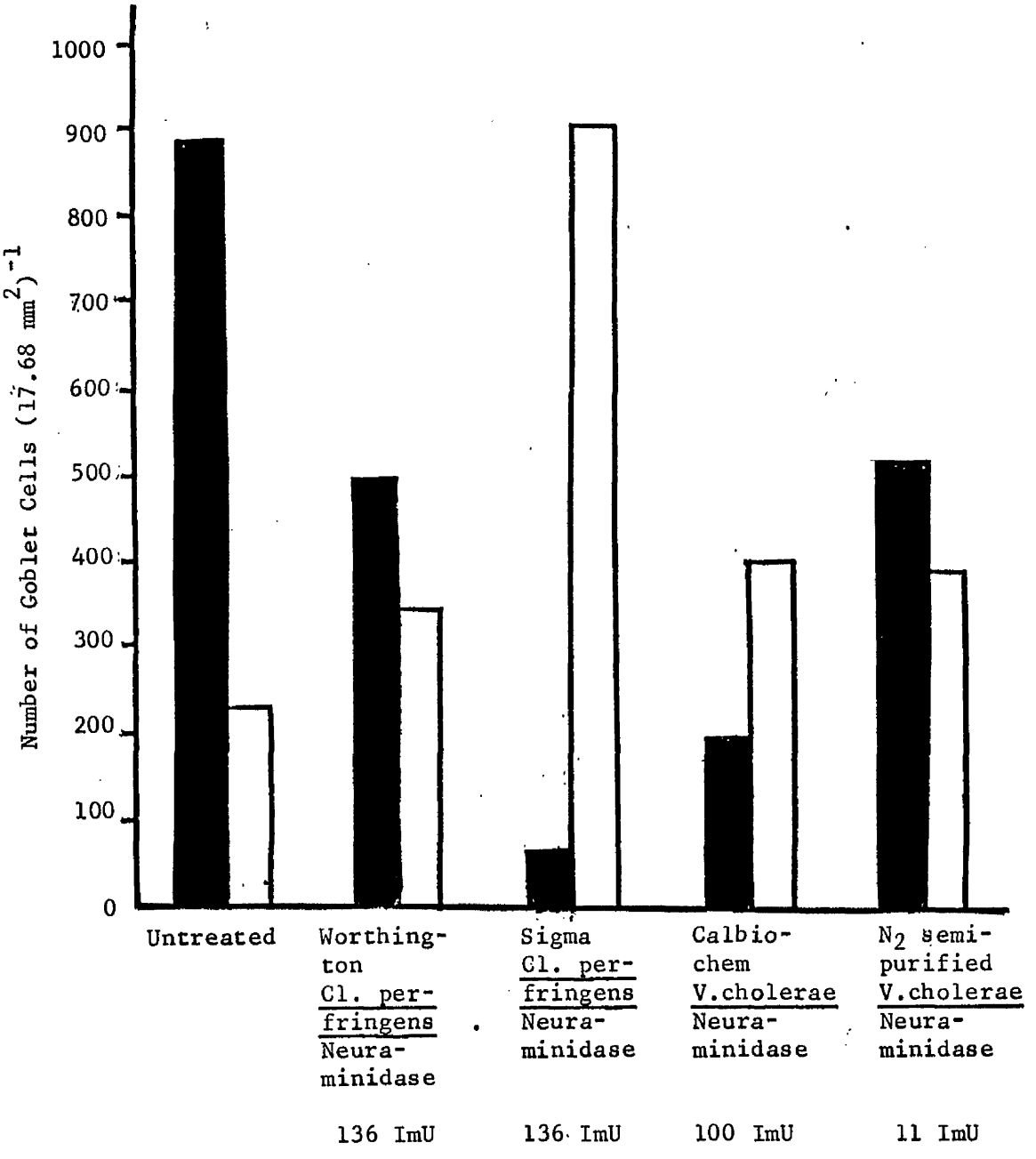


Figure 26 : Effect of several neuraminidase preparations.

Legend: Comparison of the total number of goblet cells (17.68 mm^2)$^{-1}$.

■, Alcian Blue staining goblet cells,

□, PAS staining goblet cells.

2. Effect of cholera enterotoxin

The counts of sections treated with Sephadex G 100 purified enterotoxin showed no significant difference from those of the controls treated with borate gelatin buffer pH 7.5. However, some difference was noted between the comparative PAS counts ($p < 0.0002$ Figure 27).

On the other hand, sections treated with neuraminidase showed that the staining in the goblet cells switched from a neuraminic acid to a positive mucopolysaccharide reaction.

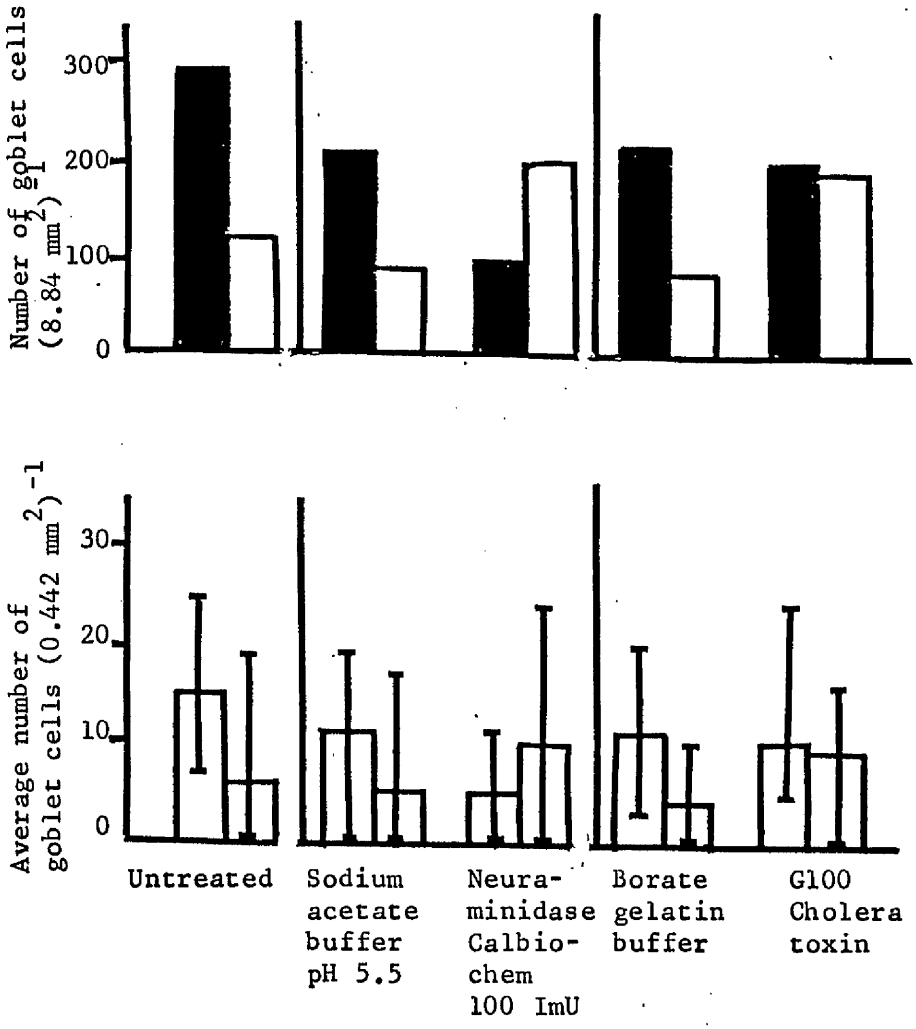


Figure 27 : Effect of cholera enterotoxin.

Legend: Comparison of the total number of Goblet Cells $(8.84\ mm^2)^{-1}$.

■, Alcian Blue staining Goblet Cells, □, PAS staining Goblet Cells. The lower part of the figure shows the average number of goblet cells $(0.442\ mm^2)^{-1}$, and the range of counts.

3. Effect of trypsin inhibitor and trypsin inhibitor + neuraminidase

The trypsin inhibitor and trypsin inhibitor plus neuraminidase was tested to see if the small amount of proteinase present in the N_2 semi-purified preparation could perhaps have been due to the proteinase trypsin. Pretreatment of tissue sections alone with trypsin inhibitor did not significantly alter the count $(8.84 \text{ sq mm})^{-1}$ with respect to the level of Alcian blue stain when compared to untreated sections. By contrast, the presence of trypsin inhibitor significantly increased ($p < 0.02$) the level of PAS staining when compared to untreated sections. When trypsin inhibitor was added with N_2 semi-purified *V. cholerae* neuraminidase, it caused the PAS count to be significantly increased with respect to untreated sections, but the level was not significantly different with respect to the Alcian blue staining (pp 236-237, Appendix VIII).

4. Effect of specific anti-neuraminidase serum (DST R77)

The counts of Alcian Blue and PAS reactions in goblet cells of untreated sections were compared with those of NaAc buffer neuraminidase (N_2) and neuraminidase:anti-neuraminidase antiserum treated ileal sections (Figures 28, 29). Treatment of the ileal sections with NaAc buffer did not cause a significant alteration in the Alcian Blue or PAS counts when compared with those of untreated sections. Because the N_2 preparation of V. cholerae neuraminidase, and anti-neuraminidase antiserum (DST-R77) were both suspended in NaAc buffer, the counts of the other treatments were compared with those of the NaAc treatment.

When sections were treated with diluted neuraminidase the Alcian Blue count was significantly lower ($p < 0.0002$ than that of the sections which were treated with NaAc buffer, while the PAS count was significantly higher ($p < 0.0002$) than that of the buffer treated sections (pp 238-242, Appendix VIII). Sections which were treated with antineuraminidase serum (0.1 ml, DST-R77) plus N_2 V. cholerae neuraminidase (0.2 ml) showed that the alteration from Alcian Blue to PAS reaction of the goblet cells could be prevented, in part, by the addition of specific antiserum (Figure 28). When the volume of the anti-neuraminidase serum to the N_2 preparation was increased it was apparent that the initial neutralisation was overtaken by a decrease in the Alcian Blue count (Figure 29). Statistically, the difference in the counts for neuraminidase plus 0.1 ml antiserum and 0.4 ml antiserum was significant at the $p < 0.0002$ level. It will be noticed that the Alcian Blue count in the antiserum control was significantly lower than the count for untreated sections ($p < 0.0002$) and the PAS reaction was significantly higher ($p < 0.0002$).

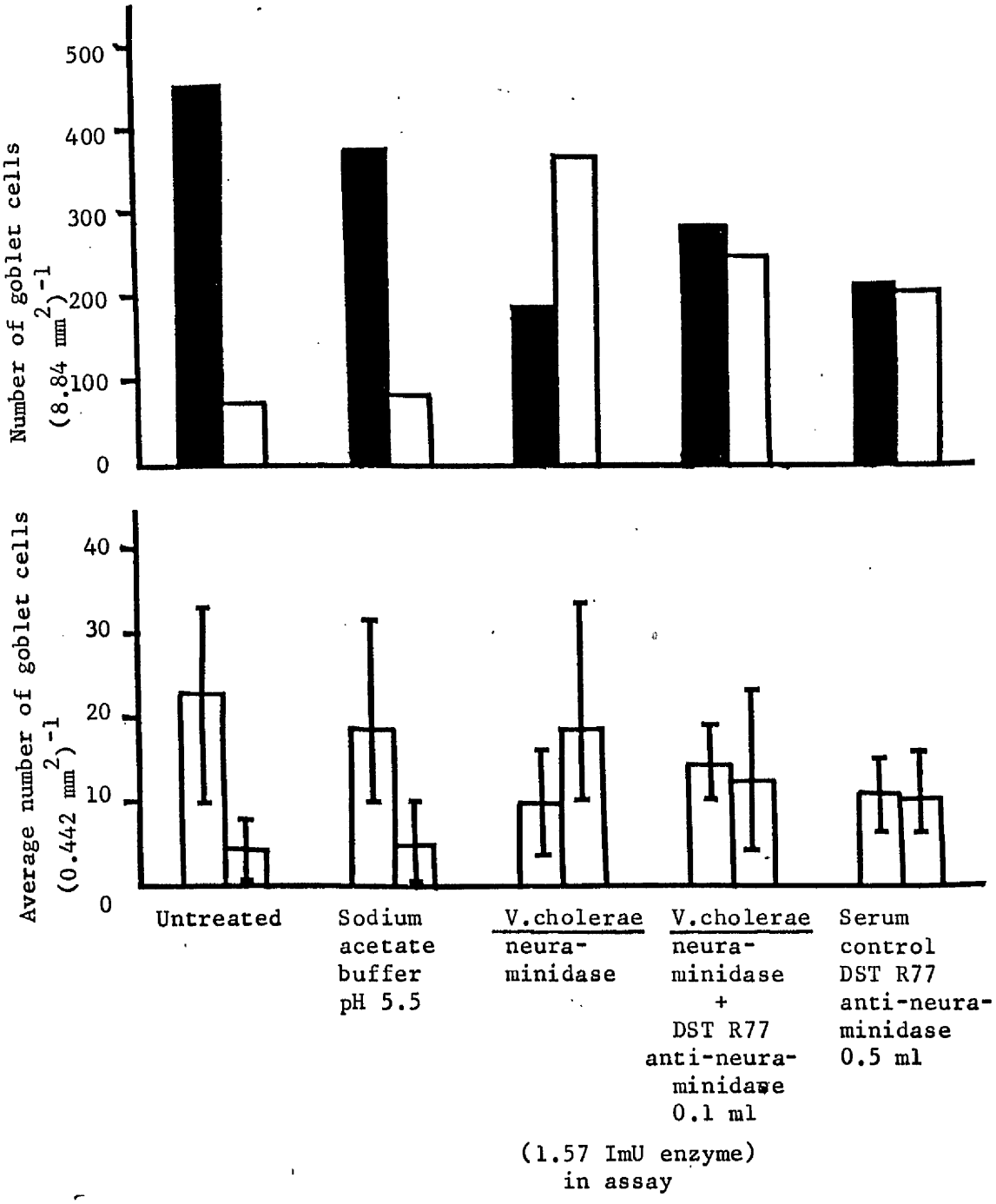


Figure 28: Effect of specific anti-neuraminidase serum (DST R77)

Legend: Comparison of the total number of goblet cells

■, Alcian Blue staining goblet cells;
□, PAS staining goblet cells.

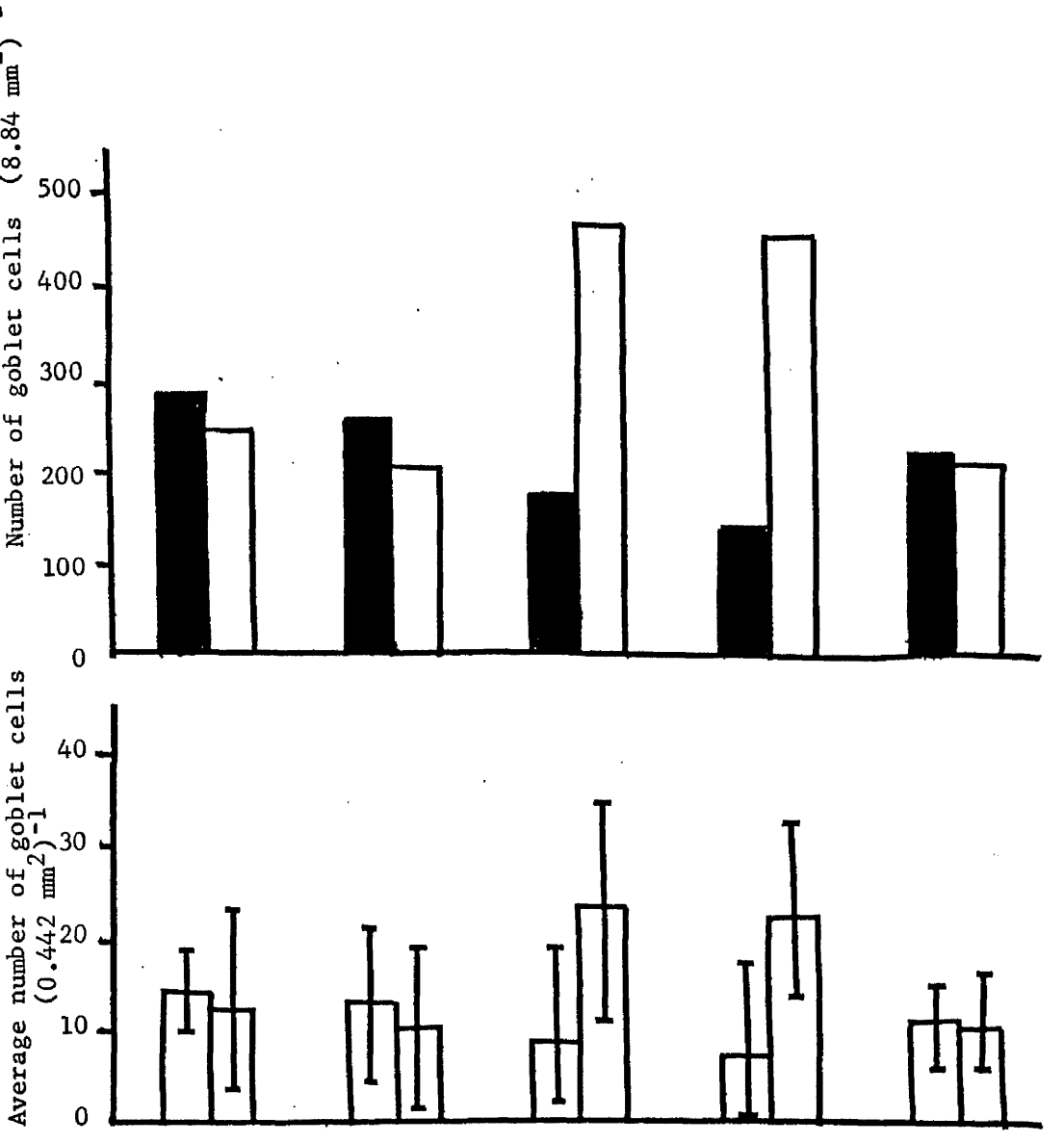


Figure 29 : Effect of specific anti-neuraminidase serum (DST R77)

Legend: Comparison of the total number of goblet cells;

■, Alcian Blue staining goblet cells;

□, PAS staining goblet cells.

5. Effect of anti-neuraminidase serum (DST R78) on non-cholera neuraminidase

It will be noticed from Figure 30 that the Cl. perfringens neuraminidase effected a similar alteration in the staining reaction of fixed rat ileal sections to that previously reported in Figure 28. There was a significant difference in the number of goblet cells stained with Alcian Blue ($p < 0.0002$) or PAS ($p < 0.0002$) in untreated rat ileal sections or those treated with Cl. perfringens neuraminidase (pp 243-248, Appendix VIII).

However, the addition of increasing volumes of anti-V. cholerae neuraminidase serum (DST R78) neutralised the alteration in change of staining from Alcian Blue to PAS. In the presence of 0.3 ml anti-V. cholerae neuraminidase serum the Alcian Blue counts were similar to those sections left untreated ($p < 0.10$) as shown in Figures 31 and 30.

The rabbit anti-neuraminidase serum DST R77, produced against the G75 preparation, did not appear to neutralise V. cholerae neuraminidase and DST R78, produced against Koch-Light V. cholerae neuraminidase, appeared to neutralise Cl. perfringens neuraminidase. Consequently, it was decided to examine the effect of DST-R78 whole antiserum or the IgG fraction on the action of V. cholerae neuraminidase. As shown in Figure 32 the whole antiserum increased the activity of the N_2 V. cholerae neuraminidase preparation. The change of staining from Alcian Blue to PAS was significantly greater with either enzyme plus 0.25 ml DST-R78 ($p < 0.0002$), or with 0.25 ml

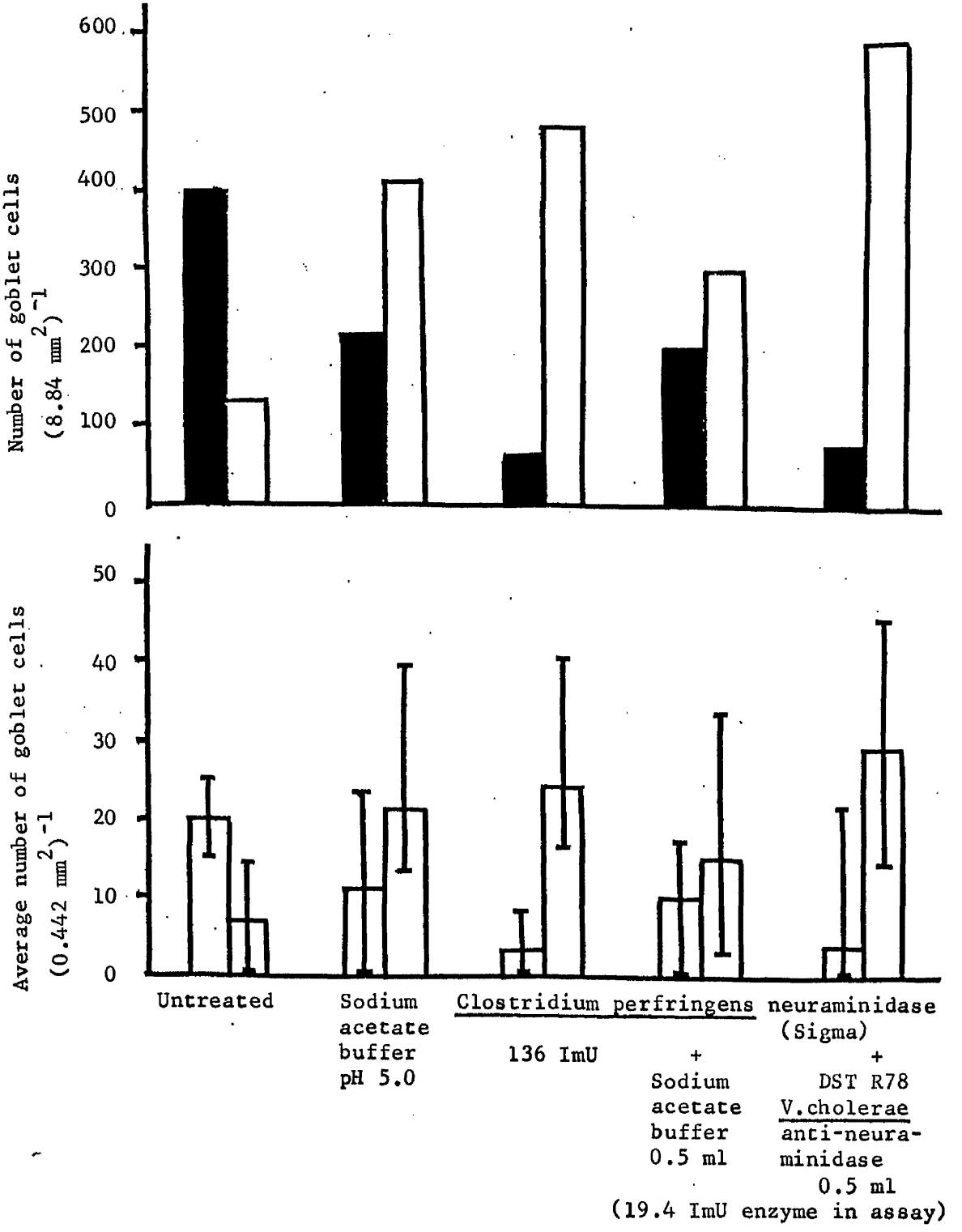


Figure 30 : Effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase.

Legend: ■, Alcian Blue staining goblet cells;
□, PAS staining goblet cells.

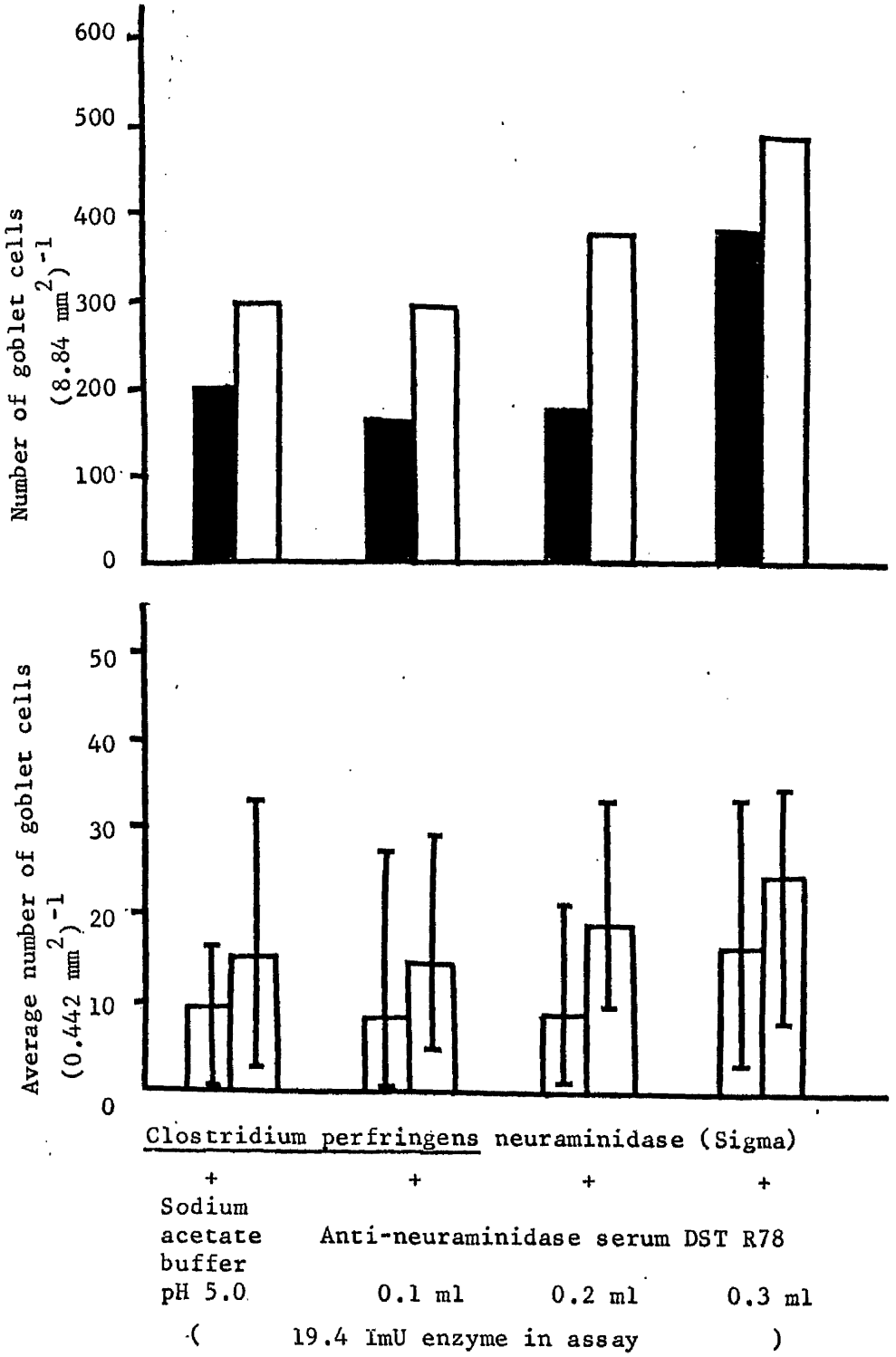


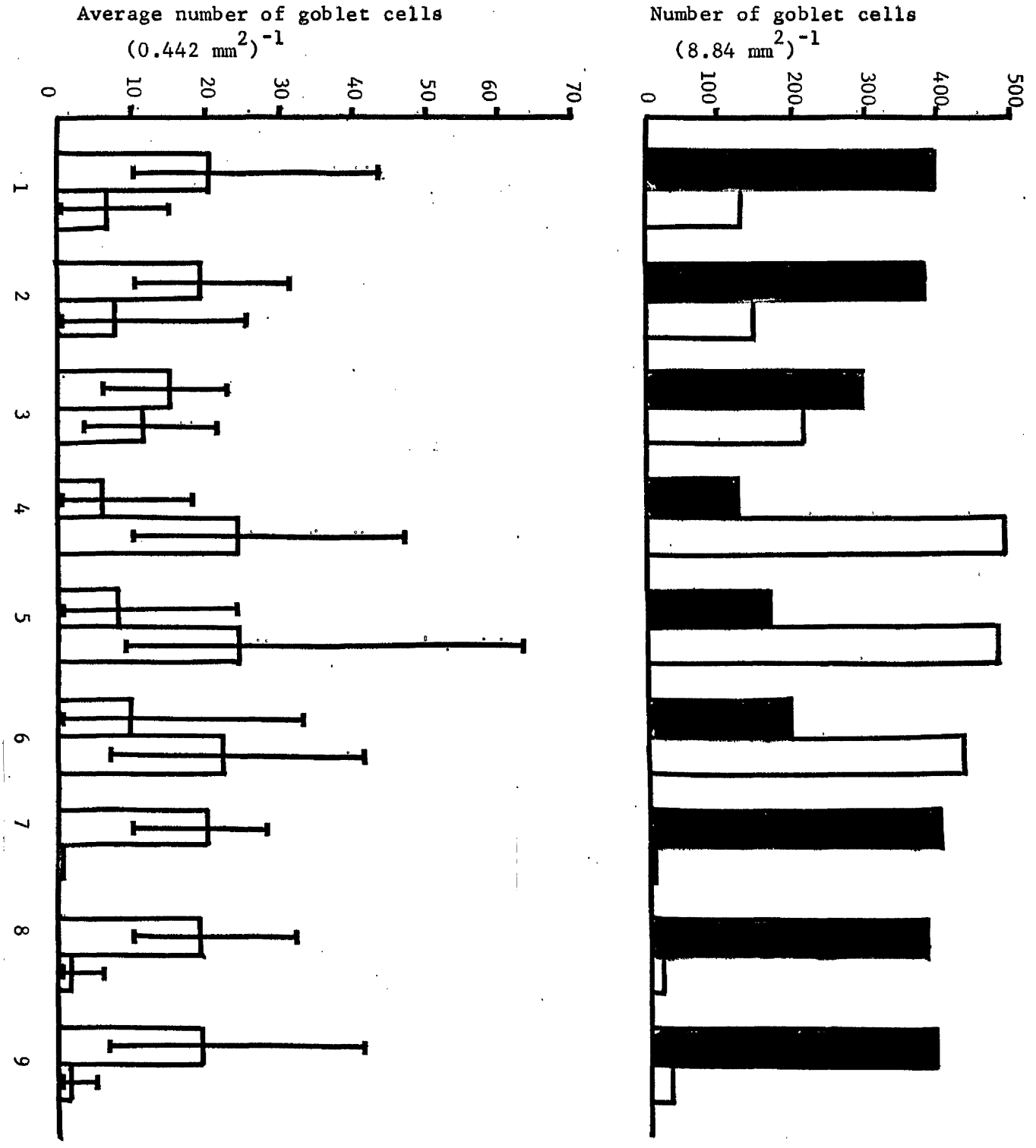
Figure 31: Effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase.

Legend: ■, Alcian Blue staining goblet cells;
□, PAS staining goblet cells.

Figure 32: Effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78).

Legend : ■, Alcian Blue staining goblet cells; □, PAS staining goblet cells.

1. Untreated
2. Sodium acetate buffer pH 5.0.
3. N_2 *V. cholerae* neuraminidase (1.57 ImU in assay)
4. N_2 (1.57 ImU in assay) + DST R78 anti-neuraminidase, 0.05 ml.
5. N_2 (1.57 ImU in assay) + DST R78 anti-neuraminidase, 0.25 ml.
6. Serum control DST R78 0.25 ml
7. N_2 (1.57 ImU in assay) + IgG anti-neuraminidase (DST R78), 0.05 ml.
8. N_2 (1.57 ImU in assay) + IgG anti-neuraminidase (DST R78), 0.25 ml.
9. Serum control IgG anti-neuraminidase (DST R78), 0.25 ml.


Average number of goblet cells
(0.442 mm^2)$^{-1}$
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
Number of goblet cells
(8.84 mm^2)$^{-1}$
0
100
200
300
400
500

DST-R78 alone ($p < 0.001$) when compared with untreated rat ileal sections (pp 249-256, Appendix VIII).

However, when the rat ileal sections were treated with 0.25 ml of the IgG fraction of DST-R78 and N_2 there was complete neutralisation of the N_2 neuraminidase activity (pp 249-256, Appendix VIII). In addition, there was no effect produced by 0.25 ml of the IgG fraction of DST-R78 alone. There was an absence of PAS staining with sections treated with 0.1 ml neuraminidase and 0.05 ml IgG fraction. In an earlier experiment it was found that the combination of NaAc and PO_4 buffers caused a reduction in PAS staining.

6. Effect of non-immune serum

The Alcian Blue and PAS counts $(8.84\ mm^2)^{-1}$ of untreated sections were compared with those of either neuraminidase treated or neuraminidase plus increasing volumes of non-immune whole rabbit serum (Figure 33). There was no significant difference between the number of goblet cells stained with Alcian Blue in rat ileal sections treated with the N_2 preparation alone or in combination with 0.1 ml normal rabbit serum (pp 257-263 Appendix VIII). However, if the amount of normal rabbit serum was increased to 0.4 ml there was a significant difference ($p < 0.0002$). There was also a significant difference in the goblet cells stained by PAS after the addition of normal rabbit serum to the reaction mixture ($p < 0.0002$).

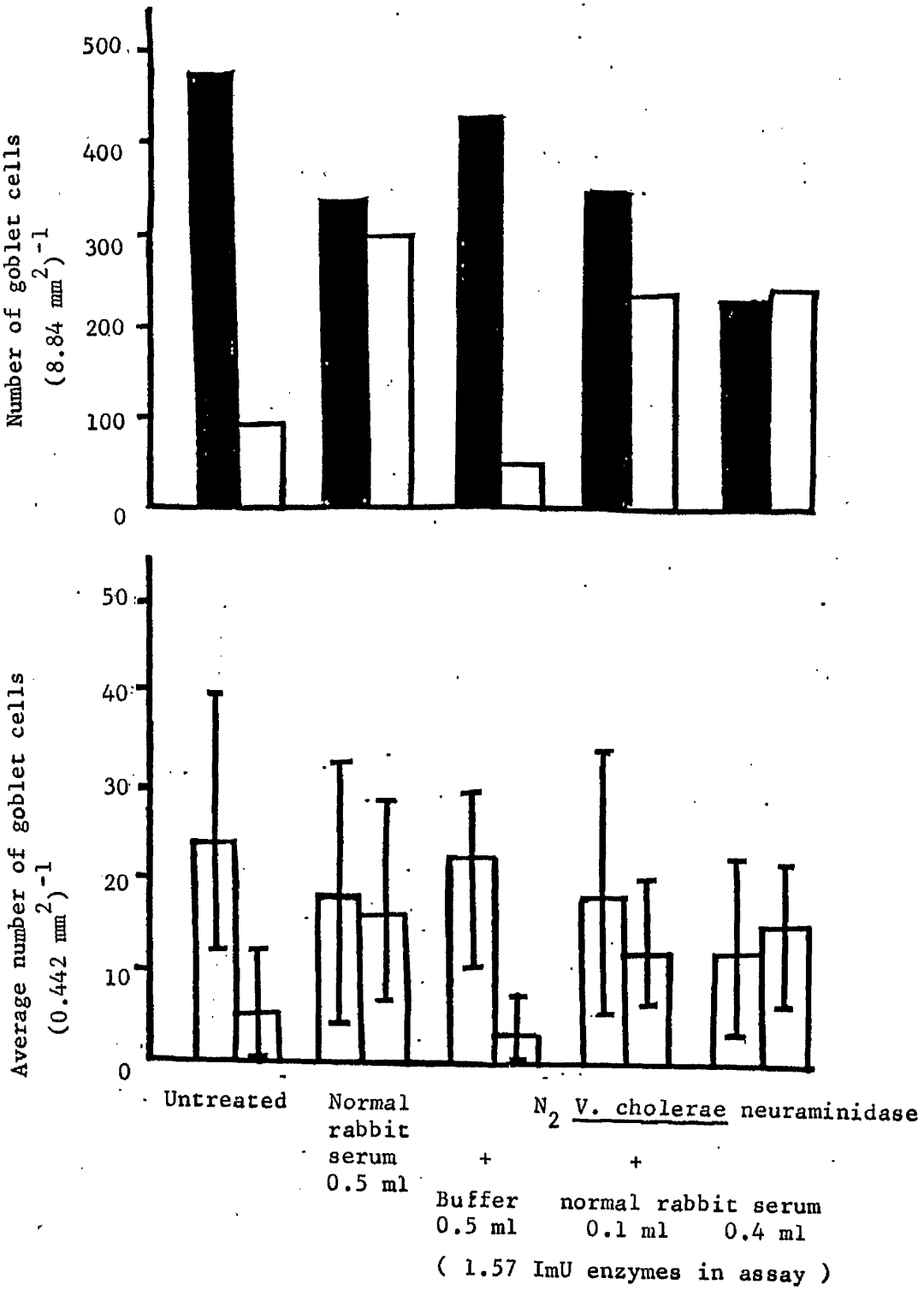


Figure 33 : Effect of non-immune serum.

Legend: ■, Alcian Blue staining goblet cells; □, PAS staining goblet cells.

VI EFFECT OF SPECIFIC ANTISERUM ON NEURAMINIDASE ACTIVITY

a) Effect on T.B.A. assay

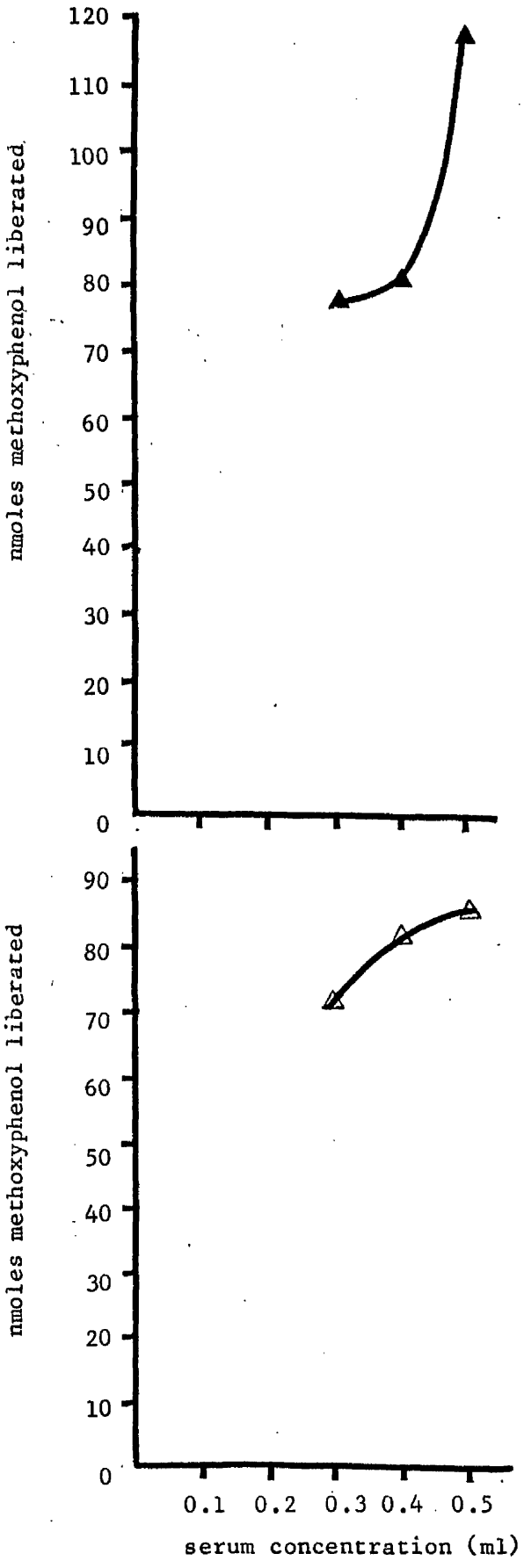
The absorption of neuraminidase preparations with the rabbit anti-*V. cholerae* neuraminidase serum (DST R77) for 1 h at 37°C effectively reduced the amount of NANA released from mucin (p 264, Appendix IX). However, in the course of the experiment it was found that 0.5 ml of serum released 0.5 µg NANA from mucin as measured by the T.B.A. assay. In addition the neuraminidase preparations released T.B.A. reactive material, possibly NANA, from the serum itself; 1.25 µg by N_2, 0.5 µg by N_3, 1.85 µg by N_4 preparations and 3.5 µg by the Sigma *Cl. perfringens* neuraminidase.

b) Effect on M.P.N. assay

Since it was apparent that the addition of excess amounts of the rabbit anti-neuraminidase serum caused an increase in the amount of T.B.A. reactive material released from a standard quantity of mucin, it was decided to repeat the experiment with the specific substrate 2-(3'-Methoxyphenyl-N-acetyl-α-neuraminic acid for neuraminidase activity. When increasing volumes of anti-neuraminidase serum were added to a constant amount of either N_2 or N_3 semi-purified *V. cholerae* neuraminidase, a corresponding increase in the levels of methoxyphenol liberated occurred (Figure 34).

Figure 34 : Absorption of neuraminidase activity
(M.P.N. procedure)

Legend:
▲, N_2 neuraminidase fraction (1.57 ImU in assay); △, N_3 neuraminidase fraction (1.43 ImU in assay).


nmoles methoxyphenol liberated
120
110
100
90
80
70
60
50
40
30
20
10
0
nmoles methoxyphenol liberated
90
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
serum concentration (ml)

c) Effect of specific IgG fractions and sera (T.B.A. assay)

When the IgG fraction of DST-R78 anti-neuraminidase and the whole serum were compared, it was found that the former absorbed neuraminidase activity. The addition of whole anti-neuraminidase serum appeared to increase the enzymic activity as increasing volumes of it were added to a fixed volume of enzyme (Figure 35A).

When the IgG fraction of non-immune rabbit serum was compared with non-immune whole rabbit serum, it was found that the former possessed no neutralising activity (Figure 35B). As in the case of whole anti-neuraminidase anti-serum the whole non-immune serum appeared to increase enzymic activity as increasing volumes of it were added to a fixed volume of enzyme (Figures 35A, 35B).

d) Effect of specific IgG fractions and sera (M.P.N. assay)

When the IgG fraction of the rabbit anti-neuraminidase serum (DST R78) was compared with the whole anti-neuraminidase serum, the former absorbed the neuraminidase activity as the volume was increased (Figure 36A). In the case of the whole anti-neuraminidase serum an increase in the volume of anti-serum caused an increase rather than a decrease in neuraminidase activity (Figure 36A).

When the IgG fraction of non-immune whole rabbit serum was compared with non-immune whole rabbit serum, no absorption of neuraminidase activity occurred (Figure 36B).

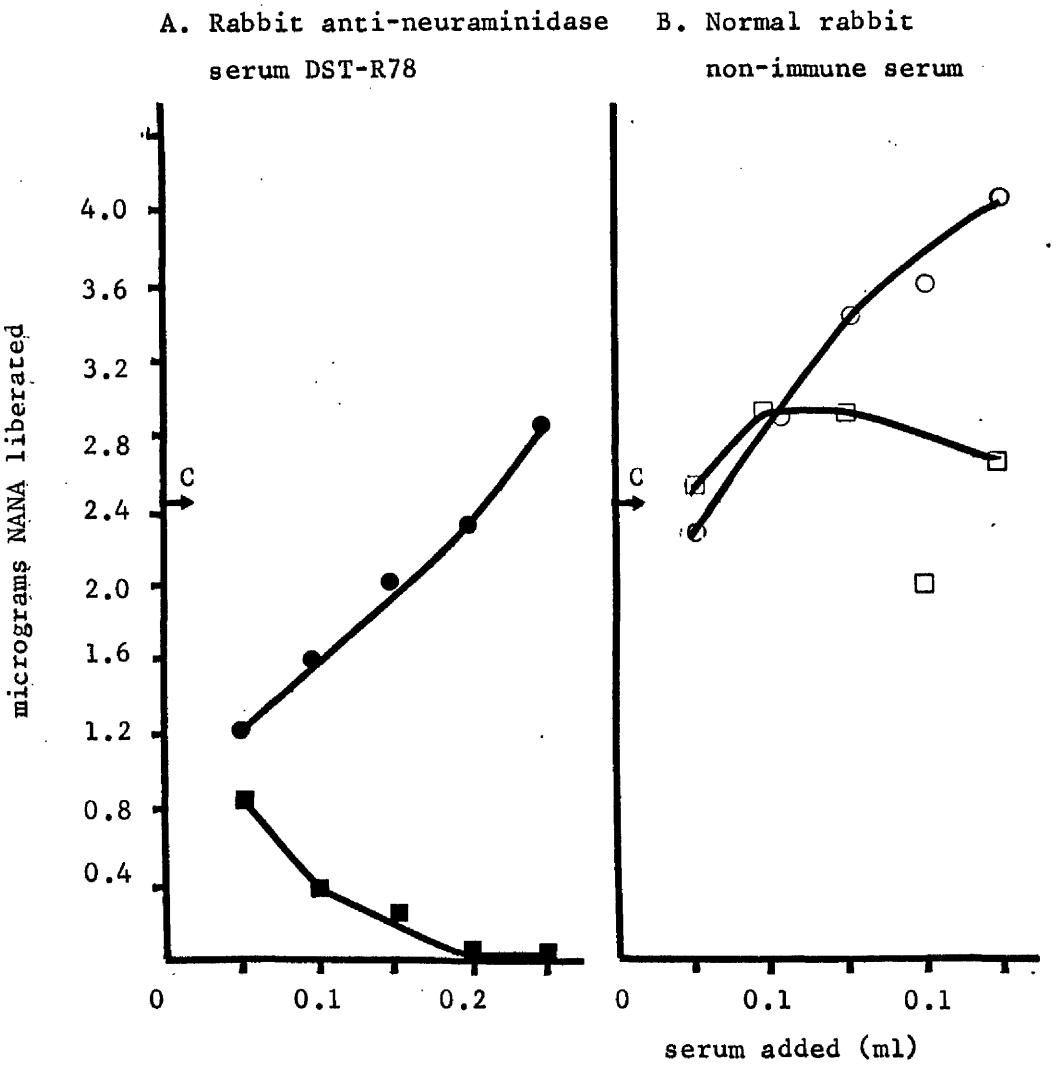


Figure 35 : Absorption of neuraminidase activity (T.B.A. procedure).

Legends: ■,□, IgG fraction
●,○, Whole serum
C, Enzyme control

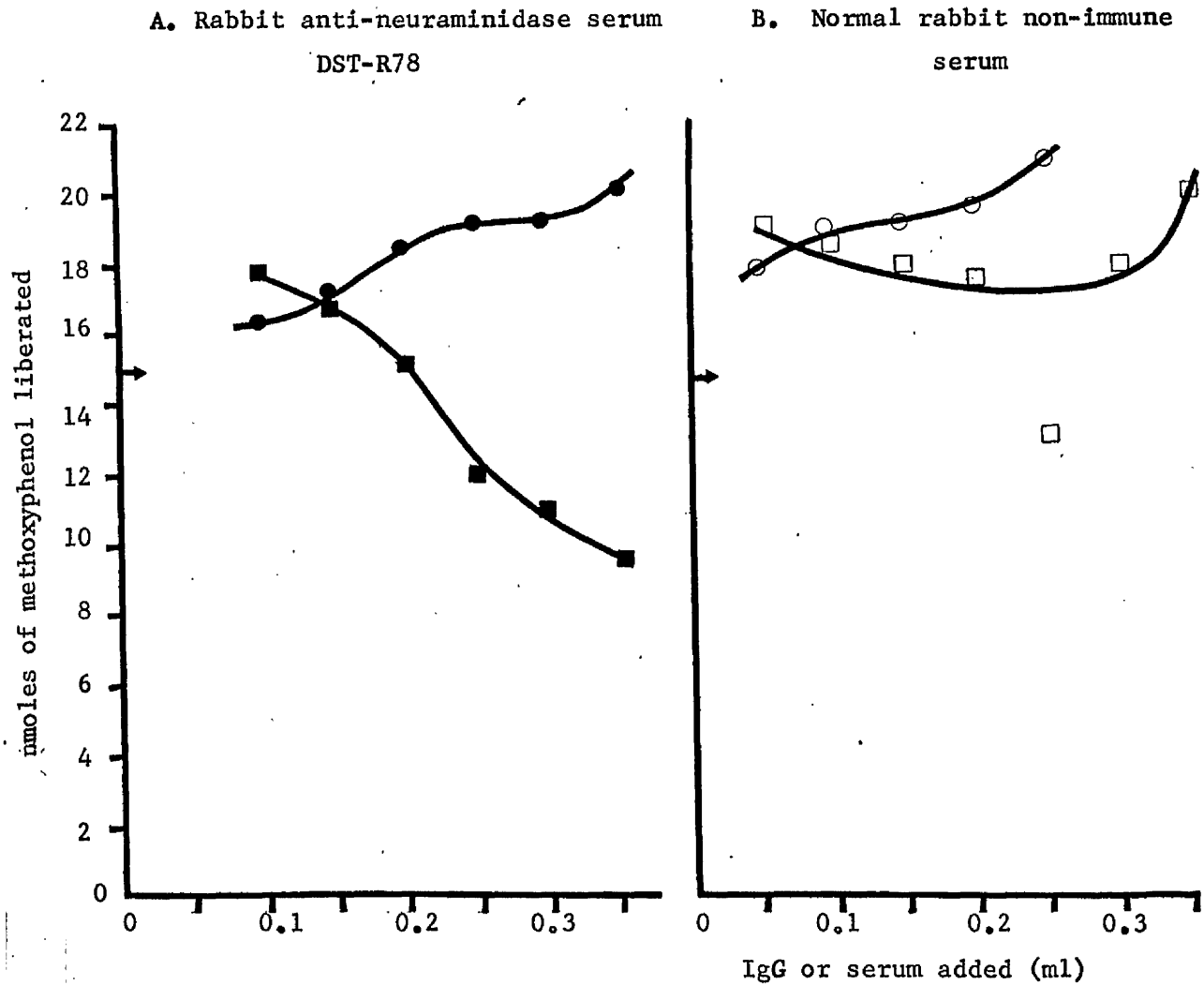


Figure 36: Effect of IgG fraction and serum on neuraminidase activity (M.P.N.)

Legends: ■,□, IgG fraction; ●,○, whole serum; C, Enzyme control.

VII DILUENT RELATED SIALOMUCIN LOSS AND NEURAMINIDASE INDUCED NANA LIBERATION

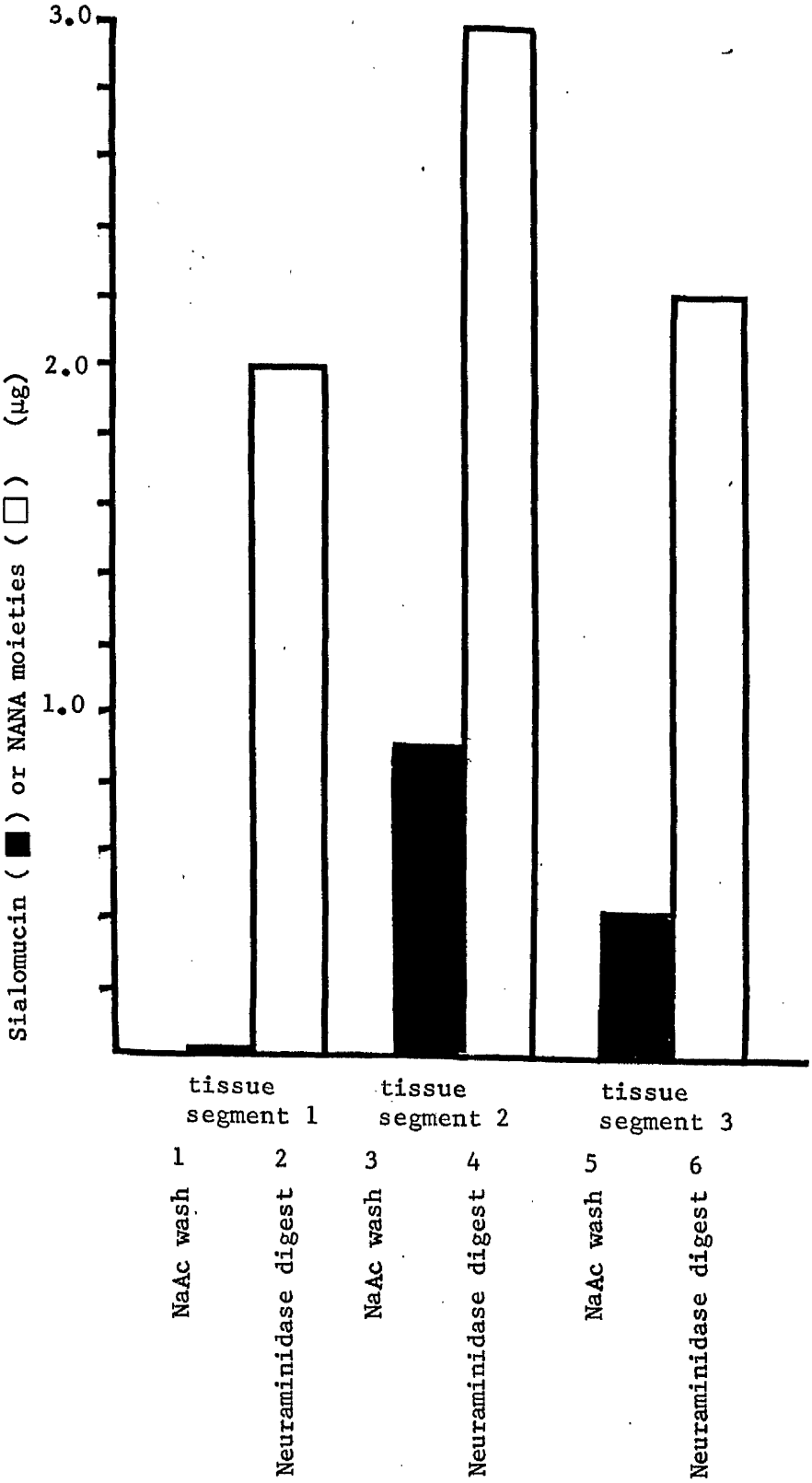
Initially, experiments were done with unfixed rat ileal segments but it was apparent that treatment of these with aqueous solutions (i.e. P.B.S. or C.A.Y.E. medium) removed the mucin layer completely. For this reason it was necessary to do the experiments with fixed tissue segments.

The loss of sialomucin or NANA in the washings from 3 pairs of tissue segments was determined by the T.B.A. assay. The greatest loss of sialomucin occurred when the tissue was exposed to C.A.Y.E. broth after a wash in NaAc buffer (Figure 37). It was noticed that even with fixed tissue segments there was some loss of sialomucin with sodium acetate buffer or C.A.Y.E. medium. This imposed limitations on the quantitative determination of the release of NANA from tissue segments by V. cholerae neuraminidase. It was necessary to differentiate between the mucin lost by aqueous solubility or by enzymic activity. Consequently, the values for sialomucin and NANA were relative to each other.

Figure 37 : Diluent related sialomucin loss and neuraminidase induced NANA release.

Legend: (■) Sialomucin released non-enzymatically;

(□) NANA (N-acetylneuraminic acid).


3.0
2.0
1.0
Sialomucin (■) or NANA moieties (□) (µg)
tissue segment 1
tissue segment 2
tissue segment 3
1
NaAc wash
2
Neuraminidase digest
3
NaAc wash
4
Neuraminidase digest
5
NaAc wash
6
Neuraminidase digest

VIII EFFECT OF NEURAMINIDASE ON ADHESION OF *V. CHOLERAE* TO RAT ILEUM

The adhesion of *V. cholerae* to fixed rat ileal tissue segments after pretreatment with either NaAc buffer or *V. cholerae* neuraminidase, was compared to determine whether there was an increase in adherent organisms after exposure to neuraminidase. In three experiments, B, F and H there was a slight increase in the number of adherent organisms. In experiments A, D, E and G there was a decrease in the number of adherent organisms (Table 17). However, when the results were examined statistically it was apparent that there was no significant difference between the number of organisms adhering to neuraminidase-treated and untreated ileal tissue ($t = 0.745$ with 7 degrees of freedom). The solutions exposed to the tissue segments were also tested by the T.B.A. assay to determine: (a) loss of sialomucin (non-enzymatically released substances) and (b) N-acetylneuraminic acid moieties released by *V. cholerae* neuraminidase.

These data were analysed as shown in Table 18. The recovery of *V. cholerae* was expressed as the ratio of *V. cholerae* adhering to neuraminidase-treated : untreated tissue and tabulated in the rank order of increase in binding. When this order was compared with the rank order of sialomucin release by buffer or neuraminic acid release by neuraminidase/neuraminidase + *V. cholerae* organisms/*V. cholerae* organisms alone no particular relationship between the rank orders and binding was apparent.

Table 17 : Effect of neuraminidase pretreatment on the binding of V. cholerae organisms to fixed rat ileal tissue

V. cholerae organisms were suspended in phosphate buffered saline.

Neuraminidase utilised in these experiments was Galbiochem V. cholerae neuraminidase 1.0 I.U. ml^{-1}.

Legend: *reaction to non-enzymatically released substances in NaAc buffer wash.

Table 17 :

Step	Treatment of rat ileal segment 1.0 sq.cm.	Recovery of *V. cholerae* $(sq.cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.cm.)^{-1}$ of tissue	µg NANA
Experiment A				
I	NaAc buffer wash	-	0.4*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		4.9
	or			
	B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (5.0×10^9 CFU) after neuraminidase pretreatment	98,000		1.8
	or			
	B) Exposure to *V. cholerae* organisms (5.0×10^9 CFU) after NaAc buffer pretreatment	115,000		0.25
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Step		Treatment of rat ileal segment 1.0 sq.cm.	Recovery of *V. cholerae* $(sq.cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.cm.)^{-1}$ of tissue	µg NANA
ExperimentB					
	I	NaAc buffer wash	-	0*	-
	II	Either:			
		A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		0.66
		or			
		B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
	III	Either:			
		A) Exposure to *V. cholerae* organisms (2.45×10^9 CFU) after neuraminidase pretreatment)	750,000		0.30
		or			
		B) Exposure to *V. cholerae* organisms (2.45×10^9 CFU) after NaAc buffer pretreatment)	415,000		0
		or			
		C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Table 17 : (continued)

Step	Treatment of rat ileal segment 1.0 sq.cm.	Recovery of *V. cholerae* $(sq.cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.cm.)^{-1}$ of tissue	µg NANA
Experiment C				
I	NaAc buffer wash	-	0.3*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		4.17
	or			
	B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (5.3×10^9 CFU) after neuraminidase pretreatment	46,500		0.64
	or			
	B) Exposure to *V. cholerae* organisms (5.3×10^9 CFU) after NaAc buffer pregreatment	45,000		0.23
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Table 17: (continued)

Step	Treatment of rat ileal segment 1.0 sq.cm.	Recovery of *V. cholerae* $(sq.cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.cm.)^{-1}$ of tissue	µg NANA
Experiment D				
I	NaAc buffer wash	-	0.2*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		1.3
	or			
	B) NaAc pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (2.2×10^{-10}) after neuraminidase pretreatment	97,500		0.2
	or			
	B) Exposure to *V. cholerae* organisms (2.2×10^{-10}) after NaAc buffer pretreatment	250,000		0
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Table 17: (continued)

Step	Treatment of rat ileal segment 1.0 sq.cm.	Recovery of *V. cholerae* $(sq.cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.cm.)^{-1}$ of tissue	µg NANA
Experiment E				
I	NaAc buffer wash	-	1.38*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		2.54
	or			
	B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (2.0×10^{10} CFU) after neuraminidase pretreatment	100,000		0.737
	or			
	B) Exposure to *V. cholerae* organisms (2.0×10^{10} CFU) after NaAc buffer pretreatment	110,000		0.355
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Table 17: (continued)

Step	Treatment of rat ileal segment 1.0 sq.cm.	Recovery of *V. cholerae* $(sq.\ cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.\ cm.)^{-1}$ of tissue	µg NANA
Experiment F				
I	NaAc buffer wash	-	1.16*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		2.72
	or			
	B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (2.0×10^{10} CFU) after neuraminidase pretreatment	150,000		0
	or			
	B) Exposure to *V. cholerae* organisms (2.0×10^{10} CFU) after NaAc buffer pretreatment	100,000		0.041
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Table 17: (continued)

Step	Treatment of rat ileal segment 1.0 sq. cm.	Recovery of *V. cholerae* $(sq.\ cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq.\ cm.)^{-1}$ of tissue	µg NANA
Experiment G				
I	NaAc buffer wash	-	0.86*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		2.24
	or			
	B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (1.0×10^{10} CFU) after neuraminidase pre-treatment	238,000		0.90
	or			
	B) Exposure to *V. cholerae* organisms (1.0×10^{10} CFU) after NaAc buffer pre-treatment	330,000		0.11
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment.	-	0*	-

Table 17 : (continued)

Step	Treatment of rat ileal segment 1.0 sq. cm.	Recovery of *V. cholerae* $(sq. cm.)^{-1}$ ileal tissue C.F.U.	µg sialomucin released into solution $(sq. cm.)^{-1}$ of tissue	µg NANA
Experiment H				
I	NaAc buffer wash	-	0.437*	-
II	Either:			
	A) Neuraminidase pretreatment (before the addition of *V. cholerae* organisms)	-		1.31
	or			
	B) NaAc buffer pretreatment (before the addition of *V. cholerae* organisms)	-	0*	-
III	Either:			
	A) Exposure to *V. cholerae* organisms (1.0×10^{10} CFU) after neuraminidase pretreatment	665,000		0.33
	or			
	B) Exposure to *V. cholerae* organisms (1.0×10^{10} CFU) after NaAc buffer pretreatment	134,000		0
	or			
	C) Exposure to C.A.Y.E. medium alone after NaAc buffer pretreatment	-	0*	-

Table 17: (continued)

Experiment	Recovery of V. cholerae (treated tissue untreated tissue)	NANA % released (rank order) (a) Sialomucin	(b) Enzyme alone	Enzyme + V.cholerae	V. cholerae alone
D	0.39	0.14(2)*	27 (3)	15 (2)	0 (1)
G	0.72	0.62(6)	46 (4)	40 (7)	5 (5)
A	0.85	0.29(4)	100 (8)	37 (6)	5 (5)
E	0.91	1.00(8)	52 (5)	29 (5)	14 (8)
C	1.03	0.22(3)	85 (7)	15 (2)	6 (7)
F	1.5	0.84(7)	56 (6)	0 (1)	2 (4)
B	1.8	0 (1)	13 (1)	45 (8)	0 (1)
H	4.96	0.32(5)	23 (2)	25 (4)	0 (1)

Table 18 : Analysis of V. cholerae binding to fixed rat ileal tissue

Legend: a) : maximum level of released NANA (%)

b) : $\frac{\mu g \text{ sialomucin released into solution}}{\text{maximal level of released sialomucin } (\mu g) \text{ achieved in the experimental series.}}$

* rank order.

IX EFFECT OF NEURAMINIDASE ON ADSORPTION OF CHOLERA ENTEROTOXIN TO RAT ILEUM

The stock solution of N.I.A.I.D. cholera enterotoxin (3.0 mg (ml BGB pH 7.5)$^{-1}$) contained 600 skin blueing doses. The enterotoxin solution after exposure to the tissue segments, was diluted to give solutions containing theoretically, 60, 30 and 15 BD. Rabbits were injected i.d. with 0.1 ml of each of these dilutions. The presence of active toxin was revealed 24 h later by the injection of Pontamine Sky Blue dye.

The effect of V. cholerae neuraminidase on the adsorption of cholera enterotoxin to ileal tissue is shown in Table 19. It was apparent that the treatment of rat ileal segments significantly increased the capacity of the tissue to adsorb V. cholerae enterotoxin. Whereas in untreated tissue there was only slight, if any, binding of the enterotoxin after treatment with neuraminidase for 1 h, more than 400 blueing doses of enterotoxin were adsorbed to the tissue.

In a further experiment the effect of adding the cholera enterotoxin at the same time as the neuraminidase solution was compared. Although there was evidence that neuraminidase combination with cholera enterotoxin stimulated adsorption to the rat ileal tissue, this effect was greater if the enzyme treatment preceded the addition of enterotoxin by one hour (Table 20).

Treatment of Tissue	Area of blueing reaction mm^2 Number of blueing doses injected 6.0	3.0	1.5	Number of blueing doses adsorbed by ileal tissue segment
NaAc buffer	259	185	139	< 50
Calbiochem. Neuraminidase solution in NaAc buffer (1.0 I.U. ml^{-1})	231	35	25	500<and > 400

Table 19 : Effect of V. cholerae neuraminidase on the adsorption of cholera enterotoxin to fixed rat ileal tissue segments as measured by residual blueing activity in the supernate.

Treatment of Tissue	Area of blueing dose (mm^2) Number of blueing doses injected 6.0	3.0	1.5	0.75	Number of blueing doses adsorbed by ileal tissue
a) Toxin added after treatment with either: i) NaAc buffer	160	134	104	N.D.	< 50
ii) Neuraminidase solution (Calbiochem, *V. cholerae* 1.0 $I.U.ml^{-1}$)	156	66	36	N.D.	400
b) Toxin added together with either: i) NaAc buffer	N.D.	226	175	150	<25
ii) Neuraminidase solution (Calbiochem, *V. cholerae* 1.0 $I.U.ml^{-1}$)	N.D.	180	111	51	<150 and ≥75

Table 20 : Effect of *V. cholerae* neuraminidase on the adsorption of cholera enterotoxin to fixed rat ileal tissue segments.

DISCUSSION

I ISOLATION AND PURIFICATION

Growth of V. cholerae in proteose peptone bovine colostrum medium stimulated the production of good yields of neuraminidase of the order of 12.42 IU litre^{-1} in the crude preparation. After Sephadex G 100 semi-purification the yield increased to 43.6 IU litre^{-1}. The yield compared favourably to that obtained by Ada, French and Lind (1961) who utilised a more complicated procedure for purification (see page 32). The $E_{280\ nm}$ peak and the Lowry protein peak did not coincide with the main peak of neuraminidase activity when individual column fractions were analysed which might indicate closeness of peaks of impurity. The latter peak was detected both by the T.B.A. and by the M.P.N. assays. Consequently, a direct comparison of activity in terms of IU (mg protein)$^{-1}$ between these results and those of Ada, French and Lind (1961) was not meaningful.

It was noticeable with DEAE-cellulose chromatography that at least five protein peaks which were separated from the methanolic precipitate possessed no enzymic activity. Two peaks eluted from the column with 1.0 M NaCl were associated with enzymic activity. These peaks were incompletely separated so the effect noted with possible peaks of protein impurity with Sephadex G 100 was not observed. In some of the chromatographic procedures it was not possible to separate the enzyme from the brown pigment present in the culture fluid. This pigment

interfered with the $E_{280\ nm}$ readings. For these reasons the neuraminidase preparations were semi-purified by Sephadex G 100 filtration and detection of enzyme peaks was achieved with the T.B.A. or M.P.N. assays (Figure 10). However, none of these methods allowed the separation of a highly purified neuraminidase preparation. Significant levels of proteinase and endoglycosidase activity were found together with slight phospholipase-C activity.

The assay for N-acetylneuraminic acid aldolase was included because the earlier investigation of Ada, French and Lind (1961) revealed that N-acetylneuraminic acid aldolase, if present, would interfere with any assay in which neuraminidase activity was measured by the liberation of N-acetylneuraminic acid moieties (e.g. thiobarbituric acid assay; Warren, 1959) since the aldolase would degrade NANA into N-acetylmannosamine. Although one did not expect aldolase activity, this was checked because Schick and Zilg (1978) had also included the determination in their study; no activity was found in any preparations obtained in the present work. The V. cholerae neuraminidase preparation of these workers purified by chromatography twice on a DEAE column did not possess any endoglycosidase, proteinase, phospholipase-C or aldolase activity. Although they achieved a 424-fold increase in the purification of enzyme this was accomplished by an 85% loss in yield. Similarly Ada, French and Lind (1961) used a complicated scheme of purification but a 79.0% loss in yield. The crystalline preparation (Ada, French and Lind, 1961) lacked proteinase and aldolase activity. Though my Sephadex G 100 V. cholerae neuraminidase preparation did not achieve the degree of purity of either Ada, French and Lind (1961) or Schick and Zilg (1978), it was a much simplified rapid procedure, and produced a semi-purified preparation that could be utilised in parallel experiments with a more highly purified commercial preparations

of V. cholerae neuraminidase. Gel filtration allowed a 3.88 fold concentration in specific enzyme activity.

The T.B.A. assay was not the most specific method to determine neuraminidase activity levels. Non-enzymatically released substances from mucin as well as enzymatically released N-acetylneuraminic acid moieties reacted in the assay. This weakness, however, was put to a practical use to measure the loss of sialomucin, in supernates of, unfixed, washed and unwashed rat ileal segments (see page 170).

The M.P.N. assay which measured neuraminidase activity by the liberation of 3-methoxyphenol moieties from an artificial substrate (2-3' methoxyphenol-N-acetyl-D-neuraminic acid), proved to be highly specific. In general it was noticed that the quantity of neuraminidase was greater when measured by this method.

The semi-purified neuraminidase preparations N_1-N_4 contained up to 0.5% (w/w) proteinase. Although the amount of proteinase was small it was not discounted that it might interact with the neuraminidase in the other biological experiments. In this respect it is interesting that Chien et al (1975) found that commercial preparations of neuraminidase were contaminated with endo-β-N-acetylglucosaminidase and proteinase. The latter hydrolysed Azocoll, haemoglobin, casein, ovalbumin, human serum albumin and α_1-acid glycoprotein. In this study an endo-β-N-acetylhexosaminidase was present in considerable quantities in the neuraminidase preparation. This enzyme might attack the β-glycosidic ester link in the bovine submaxillary mucin (see Figure 4a), between N-acetylglucosamine linked to the β-carboxyl group of aspartic or the γ-carboxyl group of glutamic acid. Ovine and bovine mucin contain N-acetylneuraminic acid and N-acetylgalactosamine and the latter was used in these investigations. However, it should be remembered that

human mucin contains N-acetylneuraminic acid, N-acetylgalactosamine plus fucose and galactose. The oligosaccharide groups of the mucin are linked to either serine or threonine in the polypeptide chain (White et al, 1978). It is also possible to propose that the crude N-acetyl-hexosaminidase might attack the α-glycosidic link between N-acetylgalacto-samine and D-galactose in the monosialoganglioside G_{M_1} (the enterotoxin receptor on the cell membrane).

It was apparent that the purification procedures used in the present study did not allow the separation of these enzymes by gel filtration nor by ion-exchange chromatography.

II NEURAMINIDASE ACTIVITY AND NEUTRALISATION

Gad (1969) found that pretreatment of human alimentary tract histological sections with V. cholerae neuraminidase caused a loss in Alcian blue staining. This was attributed to the loss of sialic acid (N-acetylneuraminic acid) moieties, a fact which was exploited in this study where it was observed that there was a characteristic whorl of goblet cells in cross-sections of villi. In addition, loss of Alcian blue staining was accompanied by a positive PAS staining reaction. Therefore, to quantitate the neuraminidase activity histochemically random counts were made in tissue sections and the number of Alcian blue : PAS stained cells was determined - the new goblet cell assay.

When sections treated with NaAc buffer were compared with sections treated with Worthington Cl. perfringens neuraminidase a significantly lower number of goblet cells stained Alcian blue was observed after enzyme treatment (Plates 2-4). This assay proved to be quite useful as a histochemical reflection of the removal of N-acetyl-

neuraminic acid moieties by V. cholerae enzyme. The assay, however, could not be used to measure secretion of sialomucin caused by cholera enterotoxin (Forstner et al, 1981) as a function of loss in Alcian blue staining of fixed rat ileum sections. Treatment of fixed rat ileum sections with cholera enterotoxin did not significantly alter the level of Alcian blue staining when compared with control sections.

It was observed in some instances that V. cholerae neuraminidase N_2 and the Calbiochem commercial sample actually reduced the total number of stained goblet cells. This was noted to be due in one experiment to the complete evagination of the goblet cells. This effect was not so readily noticeable with the Sigma or Worthington Cl. perfringens enzyme preparations. The goblet cell assay was also used to determine the effect of a specific V. cholerae anti-neuraminidase serum. Initially, problems were encountered because the whole rabbit antisera (DST R77 and DST R78) activated the change from Alcian Blue to PAS. This was attributed to the presence of a serum sialidase. However, after a double purification of the serum IgG fraction on DEAE this activity was removed. In addition the IgG fraction of the anti-neuraminidase serum absorbed the enzyme and neutralised activity prior to treatment of the rat ileal tissue section (Figure 32). It was also noticed that occasionally this neutralisation tended to affect the intensity of the PAS stain. The buffer used with the IgG might be responsible for this effect.

These results were confirmed biochemically by the T.B.A. and M.P.N. assays. In the T.B.A. assay both the neuraminidase and endo-glycosidase activities would provide a positive reaction but the M.P.N. assay only measures the specific activity of neuraminidase. In both assays there was an increase in the measurable levels of free neuraminic

acid in the presence of increasing quantities of rabbit anti-neuraminidase (Figures 35A and 36A). With normal rabbit serum a similar effect was observed (Figures 35B and 36B) and this confirmed that a serum sialidase was present.

On the other hand, the twice purified IgG fraction of rabbit anti-neuraminidase serum caused a significant decrease in the enzyme activity with increasing amounts of IgG when assayed by the M.P.N. assay. This effect was not found with the IgG of normal rabbit serum.

These neutralisation experiments contradict the statement by Palese, Bucher and Kilbourne (1973) that neuraminidase is not neutralised by specific antiserum when M.P.N. is used as substrate. They postulated that antibody molecules failed to block the active site of the enzyme by steric hindrance due in part to the small molecular size of methoxyphenol-neuraminic acid (423 dal.). However, it is more likely that their results were confused by the presence of the sialidase in the antiserum.

III MEASUREMENT OF THE LOSS OF N-ACETYLNEURAMINIC ACID AND SIALOMUCIN IN FIXED RAT ILEAL SEGMENTS TREATED WITH NEURAMINIDASE

Freter et al (1981) stated that the vast majority of the vibrios associated with the mucus gel rather than the mucosal epithelium when rabbit ileal slices were exposed to V. cholerae organisms. If the neuraminidase is involved in the adhesion of V. cholerae to the gut surface by exposing receptor sites this would presuppose that a continuous mucus layer must be available over the ileal surfaces. However, as shown in Plate 1 this may not be the usual case in experimental animals of all ages. Rozee et al (1982) noted that in

mice a continuous mucin layer was observed after 24 days. In experiments described in this thesis an attempt was made to retain the mucin layer in a state which would not preclude enzymic attack. In addition, aqueous solutions were shown to release mucin and this would affect the neuraminidase study. This difficulty had been encountered by Pearse (1968c) and Schmitz-Moormann (1969). The problem was controlled by a fixation procedure cited by Culling (1974). It must be stated that some non-enzymatic loss of mucin did occur after fixation but this was considerably less than that found with fresh tissue. Recently, this point has been re-emphasised. Ramphal and Pyle (1983a) advocated the retention of the mucin layer in assessing the adhesion of bacteria to tissue. They urged investigators to utilise an immobilisation procedure to prevent the unavoidable loss of mucin during the obligatory rinsing steps which are necessary in adhesion experiments.

IV EFFECT OF NEURAMINIDASE ON THE ADHESION OF V. CHOLERAE TO RAT ILEUM

The investigation differed from the earlier investigations of Freter (1969), Bhattacharjee and Srivastava (1978) and Bhattacharjee and Srivastava (1979) who used extirpated animal ileal segments to measure V. cholerae adsorption, in that: (a) rat ileal tissue was used rather than rabbit ileal tissue, (b) rat ileal segments were fixed in an attempt to preserve the sialomucin layer and (c) loss of sialomucin and N-acetylneuraminic acid as well as adhesion of V. cholerae to the extirpated tissue segment were measured.

In 8 experiments in which one group of tissue segments was pretreated with V. cholerae neuraminidase, and another group was

pretreated with NaAc buffer before exposure to V. cholerae organisms, a significant increase in the number of adherent organisms did not occur after enzyme pretreatment (Tables 17 and 18). In an earlier investigation using Kanagawa-positive Vibrio parahaemolyticus and foetal intestinal cell monolayer which had been pretreated with Cl. perfringens neuraminidase, Carruthers (1977) did not find any significant difference in the number of adherent organisms when compared with the controls. Arbuthnott and Smyth (1979) pointed out that numerous limitations exist in a single in vitro adsorption system, and advised the use of extreme caution when drawing conclusions for an in vivo situation.

Perhaps the greatest limitation in the use of the tissue segment in an in vitro assay is the inability, as mentioned above, to prevent non-enzymatic loss of mucin. Therefore, without the preservation and maintenance of such a layer, firm conclusions about the effects of various agents on V. cholerae adsorption in vivo cannot be drawn. It would be profitable to compare this in vitro system with an in vivo system where the effects of neuraminidase on the adsorption of V. cholerae to the sialomucin layer could be monitored, for example, by scanning electron microscopy. Consequently, it must not be ruled out that these initial adsorption studies signify that the neuraminidase is not involved in adherence in vivo although a positive effect was observed with the binding of enterotoxin to the ileal surface.

In addition, it is interesting that Attridge and Rowley (1983) found that V. cholerae organisms bound equally to both mucosal and serosal surfaces of mouse intestinal tissue and that even when the tissue was boiled, the V. cholerae organisms still showed undiminished binding. Consequently, although the V. cholerae organisms bound to the paraformaldehyde fixed rat ileal segments it should be remembered that

the numbers of adherent organisms represent the combined effect of binding to both mucosal and serosal surfaces. Since the neuraminidase preparation did not cause an increase in binding the results of Attridge and Rowley (1983) were not thought to alter the conclusions drawn in this study.

V EFFECT OF *VIBRIO CHOLERAE* NEURAMINIDASE ON ADSORPTION OF CHOLERA ENTEROTOXIN TO RAT ILEUM

Treatment of rat ileal segments with *V. cholerae* neuraminidase did affect the binding of the cholera enterotoxin to the rat ileal segments. There was a significant difference in the amount of enterotoxin remaining in the test fluid between the neuraminidase-treated and untreated tests. It was necessary to measure the toxin adsorption by a reverse assay, namely, measurement of the free enterotoxin left in solution because the homogenate of tissue with bound enterotoxin failed to produce the typical skin blueing reaction. A direct assay could be done with a radio-labelled sample of enterotoxin.

These results strengthen the claims of Gascoyne and van Heyningen (1979) and Ackerman, Wolken and Gelder (1980) published during the period of experimental work.

When rabbit intestinal homogenates were incubated in the presence of *V. cholerae* neuraminidase, Gascoyne and van Heyningen (1979) found that their capacity to bind cholera enterotoxin was increased four to five-fold. This increase in binding was thought to be due to the conversion of neuraminidase-labile to neuraminidase-stable gangliosides. The neuraminidase-labile gangliosides were converted to the stable cholera enterotoxin-binding G_{M_1} ganglioside. Essentially, similar

results were obtained by Ackerman, Wolken and Gelder (1980) who found an increase in the binding of cholera enterotoxin to the cell surface of human neutrophils after treatment with V. cholerae neuraminidase. This investigation, however, contradicted the finding of Holmgren (1981) who stated that V. cholerae neuraminidase treatment of rabbit intestinal epithelium failed to create new receptors for the binding of cholera enterotoxin. Holmgren (1981), however, did not state whether the sialomucin layer, a region rich in glycoprotein, a possible source of gangliosides for cholera enterotoxin binding, and an important component of the intestinal mucosa was preserved. If treatment with exogeneous neuraminidase does create and unmask receptors in the sialomucin layer, perhaps this could account for the discrepancy between these two sets of observations. In epithelial tissue devoid of a sialomucin layer, there would be a fixed number of receptors of cholera enterotoxin, and neuraminidase treatment would not increase this number.

The observation that neuraminidase activity increases the binding of the enterotoxin to the mucin layer deserves further investigation. If such a mechanism occurred in the course of a cholera infection it would seem that enterotoxin molecules bound to the mucin layer might be sloughed off and would be unable to exert their effect on the epithelial cells lining the small intestine.

It should also be considered that Schneider and Parker (1982) proposed that the neuraminidase activated the enterotoxin molecule. Therefore, one must not ignore the possibility that the results described in this thesis might also be due to the action of the neuraminidase on the toxin molecule itself and not on the exposure of additional receptor sites.

VI GENERAL DISCUSSION

The procedures used in this investigation for the production and purification of V. cholerae neuraminidase were chosen to provide sufficient quantities of active enzyme for experimental work. In unison with other research groups who have become interested in the exoenzymes of V. cholerae since this investigation began (Schick and Zilg, 1978; Schneider and Parker, 1978, 1982), it was apparent that a high degree of purification had to be sacrificed in order to maintain high yields and levels of enzymic activity. The enzyme preparation contained neuraminidase, endo-N-acetylhexosaminidase and proteinase. The specific activity of the neuraminidase was measured by the action on the specific substrate methoxyphenol : neuraminic acid. However, it was realised that in the thiobarbituric acid assay the moles of neuraminic acid released could be due to the concerted action of both the neuraminidase and the endoglycosidase. The small level of proteinase would not be expected to interfere with these assays.

Ramphal and Pyle (1983b) recently found that i) the particular origin of the neuraminidase utilised in mucin experiments and ii) the chemical nature of the host mucin does make a difference. Both Cl. perfringens and V. cholerae neuraminidases cleaved NANA from a bovine submaxillary mucin but only V. cholerae neuraminidase released NANA and reduced adhesion of Pseudomonas aeruginosa organisms to mouse tracheal tissue. From this finding it would appear that neuraminidase from a particular organism is specific for certain sialic-acid glycoproteins. Whether these limitations upon the use of a neuraminidase derived from a specific organism are due to differences in the steric configuration of the intended target sialic-acid glycoprotein or to enzyme

specificities of the neuraminidase has still to be determined. However, in the context of this study it would seem possible to suggest that additional enzyme activities associated with the "semi-purified" neuraminidase may play a part in this phenomenon. It seems relevant to retain the use of the term "mucinase complex" until such time as we have highly purified, individual enzymes.

It is interesting that other workers (Burnet and Stone, 1947a; Kusama and Craig, 1970; Schneider and Parker, 1978, 1982) have also observed that V. cholerae produce an array of exo-enzymes that may attack the mucin layer. Because of the reported difficulties in separating these enzymes there has been a tendency to group the activity under the broad term of "mucinase" (Burnet and Stone, 1947a; Burnet, 1948, 1949; Jensen, 1953; Singh and Ahuja, 1953; Freter, 1955; Kusama and Craig, 1970; Schneider and Parker, 1982). In the natural environment it seems reasonable to propose that the numerous exo-enzymes do interact in the breakdown of mucus leading to the adhesion of V. cholerae organisms to the mucus itself (Schrank and Verwey, 1976; Reed and Williams, 1978; Freter, O'Brien and Halstead, 1978; Freter et al, 1981) and finally to the epithelial surface. It is important to examine the biological activities of the individual exo-enzymes in order to elucidate the precise mechanism of these interactions. At present it would still seem to be relevant to retain the term "mucinases".

Recently, Schneider and Parker (1982) discussed V. cholerae exo-cellular enzymes and stated that there are several possible mechanisms which are not mutually exclusive by which the mucinase could affect the process of V. cholerae pathogenesis, namely, mucinase (1) may suppress the normal flora, thus permitting the V. cholerae organisms to initiate infection, (2) could proteolytically activate cholera entero-

toxin, enhancing uptake by intestinal cells, (3) could bring about the degradation of the intestinal mucin thus facilitating penetration of the mucus barrier to allow adherence to the intestinal epithelium, and (4) could degrade the mucus to provide nutrition for the vibrios. In this respect the results produced in this thesis provide additional support for the ideas set out in (2) and (3) above.

There seems to be little doubt that the neuraminidase action is part of a complicated process whereby V. cholerae is able to colonise the gut. Although as has been shown in the introduction V. cholerae neuraminidase has been characterised (Gottschalk, 1957; Pye and Curtain, 1961; Neurath, Hartzell and Rubin, 1970), assayed for its activity (Warren, 1959; Palese, Bucher and Kilbourne, 1973), purified (Ada, French and Line, 1961; Cuatrecasas and Illiano, 1971; Schick and Zilg, 1978), utilised for removing influenza virus receptors from erythrocytes (Burnet and Stone, 1947b), utilised for a variety of histological studies (Quintarelli et al, 1961; Kent, 1963; Mowry, 1961; Gad, 1969; Yamada and Shimazu, 1979), utilised to increase the binding of cholera enterotoxin to a variety of mammalian tissues (Gascoyne and van Heyningen, 1979; Ackerman, Wolken and Gelder, 1980) and utilised for a variety of other biological and biochemical applications (Finne, 1978a,b; Corfield, Beau and Schauer, 1978; Knight, Nahrwald and Rosenberg, 1978), a clear idea of its true role in V. cholerae pathology is still unclear.

The work in this thesis has extended the knowledge about "mucinase" and it would be interesting to continue the study examining further interactions with other exo-enzymes and also to determine whether antibodies against these enzymes would provide protection for the mammalian host against V. cholerae.

REFERENCES

ACKERMAN, A.G., WOLKEN, K.W. AND GELDER, F.B. (1980) Surface distribution of monosialogangliosides G_{M_1} on human blood cells and the effect of exogenous G_{M_1} and neuraminidase on cholera toxin labelling. A quantitative immunocytochemical study. J.Histochem.Cytochem., 28, 1100-1112.

ADA, G.L., FRENCH, E.L. AND LIND, P.E. (1961) Purification and properties of neuraminidase from V. cholerae. J.Gen.Microbiol. 24, 409-421.

ALLEN, A. (1983) Mucus - a protective secretion of complexity. Trends Biochem.Sci., 8, 169-173.

ALLWEISS, B., DOSTAL, J., CAREY, K.E., EDWARDS, T.F. AND FRETER, R. (1977) The role of chemotaxis in the ecology of bacterial pathogens. Nature, 266, 448-450.

AMINOFF, D. (1961) Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem.J., 81, 384-392.

ARBUTHNOTT, J.P. AND SMYTH, C.J. (1979) Bacterial adhesion in host/pathogen interactions in animals. pp 165-198. In: Ellwood, D.C., Melling, J. and Rutter, P. (eds). Adhesion of Microorganisms to Surfaces. Academic Press, London.

ATTRIDGE, S.R. AND ROWLEY, D. (1983) The specificity of V. cholerae adherence and the significance of the slime agglutinin as a second mediator of in vitro attachment. J.Infect.Dis., 147, 873-881.

BASU, M., BASU, S., SHANABRUCH, W.G., MOSKAL, J.R. AND EVANS, C.H. (1976) Lectin and cholera toxin binding to guinea pig tumor (10481) cell surfaces before and after glycosphingolipid incorporation. Biochem.Biophys.Res.Commun., 71, 385-392.

BEISENHERZ, G. VON, BOLTZE, H.J., BÜCHER, TH., CZOK, R., GARBADE, K.H., MEYER-ARENDT, E. AND PFLEIDERER, G. (1953) Diphosphofructose-aldolase, dehydrogenase und pyruvat-kinase aus kaninchemuskulatur in einem arbeitsgang. Zeit. Naturforsch., 8b, 555-577.

BENNETT, V., O'KEEFE, E. AND CUATRECASAS, P. (1975) Mechanism of action of cholera toxin and the mobile theory of hormone receptor-adenylate cyclic interactions. Proc.Acad.Sci.U.S.A., 72, 33-37.

BENYAJATI, C. (1966) Experimental cholera in humans. Brit.Med.J., 1, 140-142.

BHATTACHARJEE, J.W. AND SRIVASTAVA, B.S. (1978) Mannose-sensitive haemagglutinins in adherence of V. cholerae eltor to intestine. J.Gen.Microbiol., 107, 407-410.

BHATTACHARJEE, J.W. AND SRIVASTAVA, B.S. (1979) Adherence of wild-type and mutant strains of V. cholerae to non-immune and immune intestinal tissue. Bull.World Health Organization, 57, 123-128.

BIENENSTOCK, J. (1979) The physiology of the local immune response. pp 3-13. In: Asquith, P. (ed.) Immunology of the Gastrointestinal Tract. Churchill Livingstone, Edinburgh.

BINDER, H.J., FILBURN, B. AND FLOCH, M. (1975) Bile acid inhibition of intestinal anaerobic organisms. Amer.J.Clin.Nutr. 28, 119-125.

BIZZINI, B. (1977) Tetanus toxin structure as a basis for elucidating its immunological and neuropharmacological activities. pp 177-212. In: Cuatrecasas, P. (ed). The Specificity and Action of Animal, Bacterial and Plant Toxins. Vol. 1. Chapman and Hall, London.

BOEHM, L. (1838) Die kranke Darmscheimhaut in de asiatischen cholera. A. Duncker, Berlin.

BOEHRINGER MANNHEIM GmbH (1983) Enzymatic phospholipid analysis. p 17. In: Biochemica, Boehringer Mannheim, Mannheim.

BOURNE, H.R., LEHRER, R.I., LICHTENSTEIN, L.M., WEISSMANN, G. AND ZURIER, R. (1973) Effects of cholera enterotoxin on adenosine 3', 5' monophosphate and neutrophil function. Comparison with other compounds which stimulate leukocyte adenyl cyclase. J.Clin.Invest., 52, 698-708.

BRANDTZAEG, P. (1974) Mucosal and glandular distribution of immunoglobulin components; differential localization of free and bound SC in secretory epithelial cells. J.Immunol., 112, 1553-1559.

BURNET, F.M. (1948) The mucinase of V. cholerae. Aust.J.Exp.Biol.Med. Sci., 26, 71-80.

BURNET, F.M. (1949) Ovomucin as a substrate for the mucinolytic enzymes of V. cholerae filtrates. Aust.J.Exp.Biol.Med.Sci., 27, 245-252.

BURNET, F.M. AND STONE, J.D. (1947a) Desquamation of intestinal epithelium in vitro by V. cholerae filtrates: characterization of mucinase and tissue disintegrating enzymes. Aust.J.Exp.Biol.Med. Sci., 25, 219-226.

BURNET, F.M. AND STONE, J.D. (1947b) The receptor destroying enzyme of V. cholerae. Aust.J.Exp.Biol.Med.Sci., 25, 227-233.

CARRUTHERS, M.M. (1977) In vitro adherence of Kanagawa-Positive V. parahaemolyticus. J.Infect.Dis., 136, 588-592.

CARRUTHERS, M.M. AND ANDERSON, B. (1979) Inhibition of adherence by Kanagawa-Positive V. parahaemolyticus: A physicochemical effect. J.Infect.Dis., 140, 119-122.

CHIEN, S., YEVICK, S.J., LI, S. AND LI, Y. (1975) Presence of endo-β-N-acetylglucosaminidase and protease activities in the commercial neuraminidase preparations isolated from Cl. perfringens. Biochem.Biophys.Res.Commun., 65, 683-690.

CHITNIS, D.S., SHARMA, K.D. AND KAMAT, R.S. (1982) Role of bacterial adhesion in the pathogenesis of cholera. J.Med.Microbiol., 15, 43-51.

COHNHEIM, J.F. (1882) Vorlesungen über allgemeine pathologie. A. Hirschwald, Berlin.

COOPER, G.N. AND JACKSON, G.D.F. (1981) Immune responses of rats to live V. cholerae: antibodies in serum intestinal secretions. Parasit. Immunol., 3, 57-68.

CORFIELD, A.P., BEAU, J.M. AND SCHAUER, R. (1978) Desialylation of glycoconjugates using immobilized V. cholerae neuraminidase preparation, properties and use of the bound enzyme. Hoppe Seyler's Z.Physiol., 356S, 1335-1342.

CRAIG, J.P. (1965) A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (London), 207, 614-616.

CRAIG, S.W. AND CUATRECASAS, P. (1975) Mobility of cholera toxin receptors on rat lymphocyte membranes. Proc.Nat.Acad.Sci.U.S.A., 72, 3844-3848.

CUATRECASAS, P. (1973a) Cholera toxin-fat cell interaction and the mechanism of activation of the lipolytic response. Biochem., 12, 3567-3577.

CUATRECASAS, P. (1973b) Interaction of wheat germ agglutinin and concanavalin A with isolated fat cells. Biochem., 12, 1312-1327.

CUATRECASAS, P. (1973c) Interaction of V. cholerae enterotoxin with cell membranes. Biochem., 12, 3547-3558.

CUATRECASAS, P. (1973d) Gangliosides and membrane receptors for cholera toxin. Biochem., 12, 3558-3566.

CUATRECASAS, P. AND ILLIANO, G. (1971) Purification of neuraminidase from V. cholerae, Cl. perfringens and influenza virus by affinity chromatography. Biochem.Biophys.Res.Commun., 44, 178-184.

CUATRECASAS, P., PARIKH, I. AND HOLLENBERG, M.D. (1973) Affinity chromatography and structural analysis of V. cholerae enterotoxin-ganglioside agarose and the biological effects of ganglioside-containing soluble polymers. Biochem., 12, 4253.

CULLING, C.F.A. (1974) Handbook of Histopathological and Histochemical Techniques, 3rd edition, p 54. Butterworths, London.

DAHLE, H.K. AND SANDVIK, O. (1971) Comparative electrophoretic and serological analyses of V. comma and Aer. liquefaciens proteinases. Acta Path.Microbiol.Scand., Sec. B, 79, 686-690.

DE, S.N. (1959) Enterotoxicity of bacteria-free culture filtrate of V. cholerae. Nature (London), 183, 1533-1534.

DRASAR, B.S. (1974) Some factors associated with geographical variations in the intestinal microflora. pp 187-196. In: Skinner, F.A. and Carr, J.G. (eds). The Normal Microbial Flora of Man. Academic Press, London.

DUBOIS, M., GILLES, K.A., HAMILTON, J.E., REBERS, P.A. AND SMITH, F. (1956) Colorimetric method for determination of sugars and related substances. Anal.Chem., 28, 350-356.

DUTTA, N.K., PANSE, M.V. AND KULKARNI, D.R. (1959) Role of cholera toxin in experimental cholera. J.Bact., 78, 594-595.

ELLIOTT, L.H., CARPENTER, C.J., SACK, R.B. AND YARDLEY, J.H. (1970) Small bowel morphology in experimental canine cholera. A light and electron microscopic study. Lab.Invest., 22, 112-120.

ERLANDSEN, S.L. AND CHASE, D.G. (1974) Morphological alterations in the microvillous border of villous epithelial cells produced by micro-organisms. Amer.J.Clin.Nutr., 27, 1277-1286.

ERLANDSON, S.L. AND CHASE, D.B. (1972) Paneth cell function: phago-cytosis and intracellular digestion of intestinal microorganisms. I. Hexamita muris. J.Ultrastruct.Res., 41, 296-318.

FELSENFELD, O. (1963) Some observations on the cholera (eltor) epidemic in 1961-62. Bull.Wld.Hlth.Org., 28, 289-296.

FELSENFELD, O. (1966) A review of recent trends in cholera research and control. Bull.Wld.Hlth.Org., 34, 161-195.

FINKELSTEIN, R.A. (1973) Cholera. CRC Crit.Rev.Microbiol., 2, 553-623.

FINKELSTEIN, R.A. (1975) Immunology of cholera. Curr.Top.Microbiol. Immunol., 69, 137-195.

FINKELSTEIN, R.A., FUJITA, K. AND LO SPALLUTO, J.J. (1971) Procholera-genoid: an aggregated intermediate in the formation of choleragenoid. J.Immunol., 107, 1043-1051.

FINKELSTEIN, R.A., LA RUE, M.K. AND LO SPALLUTO, J.J. (1972) Properties of the cholera exo-enterotoxin : effects of dispersing agents and reducing agents in gel filtration and electrophoresis. Infect.Immun. 6, 934-944.

FINKELSTEIN, R.A. AND LO SPALLUTO, J.J. (1969) Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. J.Exp.Med., 130, 185-202.

FINKELSTEIN, R.A., BOESMAN, M., NEOH, S.H., LA RUE, M.K. AND DELANEY, R. (1974) Dissociation and recombination of the cholera enterotoxin (Choleragen). J.Immunol., 113, 145-150.

FINKLER, D. AND PRIOR, J. (1884) Untersuchungen über Cholera nostras. Dtsch.Med.Wschr., 10, 579.

FINNÉ, E. (1978a) Specificity of action of neuraminidase according to its bacteriological origin. Endokrinologie, 72, 3, S. 363-364.

FINNÉ, E. (1978b) Inactivation of endogenous FSH by neuraminidase from V. cholerae. Endokrinologie, 72, 3, S. 365-366.

FISHMAN, P.H., MOSS, J. AND OSBORNE, J.G. JR. (1978) Interaction of choleragen with the oligosaccharide of ganglioside G_{M_1}: Evidence for multiple oligosaccharide binding sites. Biochem., 17, 711-716.

FLOREY, H.W. (1933) Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa. J.Pathol.Bacteriol., 37, 283-289.

FORSTNER, J.F., JABBAL, I. AND FORSTNER, G.G. (1973) Goblet cell mucin of rat small intestine. Chemical and physical characterization. Can.J.Biochem., 51, 1154-1166.

FORSTNER, J.F., ROOMI, N.W., FAHIM, R.E. AND FORSTNER, G.G. (1981) Cholera toxin stimulates secretion of immunoreactive intestinal mucin. Amer.J.Physiol., 240, G 10-16.

FRANKLIN, M.A. AND SKORYNA, S.C. (1971) Studies on natural gastric flora: Survival of bacteria in fasting human subjects. Can.Med. Assoc.J., 105, 380-386.

FRESH, J.W., VERSAGE, P.M. AND REYES, V. (1964) Intestinal morphology in human and experimental cholera. Arch.Path. (Chicago), 77, 529-537.

FRETER, R. (1955) The serologic character of cholera vibrio mucinase. J.Infect.Dis., 97, 238-245.

FRETER, R. (1956) Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. J.Exp.Med., 104, 411-418.

FRETER, R. (1969) Studies of the mechanism of action of intestinal antibody in experimental cholera. Texas Reports on Biol. and Med., 27, Suppl. 1, 299-316.

FRETER, R. (1970) Mechanism of action of intestinal antibody in experimental cholera. II. Antibody mediated antibacterial reaction at the mucosal surface. Infect.Immun., 2, 556-562.

FRETER, R. (1972) Parameters affecting the association of vibrios with the intestinal surface in experimental cholera. Infect.Immun., 6, 134-141.

FRETER, R. (1974) Gut associated immunity to cholera. pp 315-326. In: Barua, D. and Burrows, W. (eds). Cholera. W.B. Saunders, Philadelphia.

FRETER, R. AND JONES, G.W. (1976) Adhesive properties of V. cholerae: Nature of interaction with intact mucosal surfaces. Infect.Immun. 14, 246-256.

FRETER, R., O'BRIEN, P.C.M. AND HALSTEAD, S.A. (1978) Adhesion and chemotaxis as determinants of bacterial association with mucosal surfaces. pp 429-437. In: McGhee, J.R., Mestecky, J. and Babb, J.L. (eds). Advances in Experimental Medicine and Biology. Plenum Press, New York.

FRETER, R., O'BRIEN, P.C.M. AND MACSAI, M.S. (1979) Effect of chemotaxis on the interaction of cholera vibrios with intestinal mucosa. Amer.J.Clin.Nutr., 32, 128-132.

FRETER, R. AND O'BRIEN, P.C.M. (1981a) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: Chemotactic responses of V. cholerae and description of motile nonchemotactic mutants. Infect.Immun., 34, 215-221.

FRETER, R. AND O'BRIEN, P.C.M. (1981b) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: Fitness and virulence of nonchemotactic V. cholerae mutants in infant mice. Infect.Immun., 34, 222-233.

FRETER, R. O'BRIEN, P.C.M. AND MACSAI, M.S. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: In vivo studies. Infect.Immun., 34, 234-240.

FRETER, R., ALLWEISS, B., O'BRIEN, P.C.M., HALSTEAD, S.A. AND MACSAI, M.S. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: In vitro studies. Infect.Immun., 34, 241-249.

FUBARA, E.S. AND FRETER, R. (1972) Availability of locally synthesized and systemic antibodies in the intestine. Infect.Immun., 6, 956-981.

FUBARA, E.S. AND FRETER, R. (1973) Protection against enteric bacterial infection by secretory IgA antibodies. J.Immunol., 111, 395-403.

GAD, A. (1969) A histochemical study of human alimentary tract mucosubstance in health and disease. I. Normal and Tumours. Brit.J. Cancer, 23, 52-63.

GALLAGHER, J.T. AND CORFIELD, A.P. (1978) Mucin-type glycoproteins - new perspectives on their structure and synthesis. Trends Biochem. Sci., 3, 38-41.

GANGAROSA, E.J., BEISEL, W.R., BENYAJATA, C. AND SPRING, H. (1960) The nature of the gastrointestinal lesion in asiatic cholera and its relationship to pathogenesis: a biopsy study. Amer.J.Trop.Med., 9, 112-135.

GASCOYNE, N. AND VAN HEYNINGEN, W.E. (1975) Binding of cholera toxin by various tissues. Infect.Immun., 12, 466-469.

GASCOYNE, N. AND VAN HEYNINGEN, W.E. (1979) Unmasking of actual and potential receptor sites for cholera toxin in intestinal mucosal homogenates. J.Infect.Dis., 139, 235-236.

GIBBONS, R.J. AND VAN HOUTE, J. (1971) Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect.Immun., 3, 567-573.

GILL, D.M. AND KING, C.A. (1975) The mechanism of action of cholera toxin in pigeon erythrocyte lysates. J.Biol.Chem., 250, 6424.

GOODPASTURE, E.W. (1923) Histopathology of intestine in cholera. Philippine J.Sci.(Sect.B), 22, 413-423.

GOTSCHLICH, F. (1905) Vibrions cholériques isolés au campement de Tor. Retour du pèlerinage de l'année 1905. Rapport adressé au président du Conseil quarantenaire d'Egypte, Alexandria. Bull.Inst.Pasteur, 3, 726.

GOTTSCHALK, A. (1956) Neuraminic acid: the functional group of some biologically active mucoproteins. Yale J.Biol.Med., 28, 525.

GOTTSCHALK, A. (1957) Neuraminidase: the specific enzyme of influenza virus and V. cholerae. Biochim.Biophys.Acta., 23, 645-646.

GUENTZEL, M.N. AND BERRY, L.J. (1975) Motility as a virulence factor for V. cholerae. Infect.Immun., 11, 890-897.

GUENTZEL, M.N., FIELD, L.H., EUBANKS, E.R. AND BERRY, L.J. (1977) Use of fluorescent antibody in studies of immunity to cholera in infant mice. Infect.Immun., 539-548.

HAKSAR, A., MAUDSLEY, D.V. AND PERSON, F. (1974) Neuraminidase treatment of adrenal cells increases their response to cholera enterotoxin. Nature, 15, 539-548.

HASSALL, A.H. (1855) In : Report of the committee for scientific inquiries in relation to the cholera epidemic of 1854. Eyre and Spottiswoode, London.

HEIMER, R. AND MEYER, K. (1956) Studies on sialic acid of submaxillary mucoid. Proc.Nat.Acad.Sci.U.S.A., 42, 728-735.

HOLDEMAN, L.V., GOOD, I.J. AND MOORE, W.E. (1976) Human fecal flora: variation in bacterial composition within individuals and possible effect of emotional stress. Appl.Environ.Microbiol., 31, 359-375.

HOLLENBERG, M.D., FISHMAN, P.H., BENNETT, V. AND CUATRECASAS, P. (1974) Cholera toxin and cell growth: role of membrane gangliosides. Proc.Nat.Acad.Sci.U.S.A., 71, 4224-4228.

HOLMGREN, J. (1973) Comparison of the tissue receptors for V. cholerae and E. coli enterotoxins by means of gangliosides and natural cholera toxoid. Infect.Immun. 8, 851-859.

HOLMGREN, J. (1981) Actions of cholera toxin and the prevention and treatment of cholera. Nature, 292, 413-417.

HOLMGREN, J. AND LÖNNROTH, L. (1975) Oligomeric structure of cholera toxin: characteristics of H and L subunits. J.Gen.Microbiol., 86, 49.

HOLMGREN, J., LÖNNROTH, I. AND SVENNERHOLM, L. (1973a) Fixation and inactivation of cholera toxin by G_{M_1} ganglioside. Scand.J.Infect. Dis., 5, 77-78.

HOLMGREN, J., LÖNNROTH, I. AND SVENNERHOLM, L. (1973b) Tissue receptor for cholera exotoxin: postulated structure from studies with G_{M_1} ganglioside and related glycolipids. Infect.Immun. 8, 208-214.

HOLMGREN, J., MÅNSSON, J.E. AND SVENNERHOLM, L. (1974) Tissue receptor for cholera exotoxin: structural requirements of G 11 ganglioside in toxin binding and inactivation. Med.Biol., 52, 229-233.

HOLMGREN, J., SVENNERHOLM, A.M. (1977) Mechanism of disease and immunity in cholera: a review. J.Infect.Dis., 136, Suppl., S105-112.

HOLMGREN, J., SVENNERHOLM, A.M. AND ÅHRÉN, C. (1981) Nonimmunoglobulin fraction of human milk inhibits bacterial adhesion (hemagglutination and enterotoxin binding of E. coli and V. cholerae. Infect.Immun., 33, 136-141.

HOLMGREN, J., SVENNERHOLM, A.M. AND LINDBLAD, M. (1983) Receptor-like glycocompounds in human milk that inhibit classical and eltor V. cholerae cell adherence (hemagglutination). Infect.Immun., 39, 147-154.

HUMASON, G.L. (1967) Animal Tissue Techniques. 2nd edition, pp 144, 495, W.H. Freeman and Company, San Francisco.

HUGH, R. (1964) The proposed conservation of the genetic name Vibrio pacini 1854 and designation of the neotype strain of Vibrio cholerae pacini 1854. Int.Bull.Bacteriol.Nomencl.Taxon., 14, 87-101.

HUTCHINSON, D.W. AND KABAYO, J.P. (1977) Review, current thoughts on neuraminidase. Trends Biochem.Sci., 2, 1-3.

JENSEN, K.E. (1953) Immunologic characterization on mucinolytic enzyme of V. cholerae. J.Infect.Dis., 93, 107-110.

JONES, G.W. AND FRETER, R. (1976a) Adhesive properties of V. cholerae: adhesion to isolated rabbit brush border membranes and haemagglutinating activity. Infect.Immun., 14, 232-239.

JONES, G.W. AND FRETER, R. (1976b) Adhesive properties of V. cholerae: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect.Immun., 14, 240-245.

KAMAL, A.M. (1974) The seventh pendemic of cholera. pp 1-7. In: Barua, D. and Burrows, W. (eds). Cholera. W.B. Saunders Company, Philadelphia.

KENT, S.P. (1963) A study of mucins in tissue sections by the fluorescent antibody technique. III. The specificity of antibody to salivary gland mucins and the effects of chemical alterations of mucins on the specificity of the antibody. Ann.N.Y.Acad.Sci., 106, 389-401.

KING, C.A. AND VAN HEYNINGEN, W.E. (1973) Deactivation of cholera toxin by a sialidase-resistant monosialoganglioside. J.Infect.Dis., 127, 639-647.

KING, C.A. AND VAN HEYNINGEN, W.E. (1975) Evidence for the complex nature of the ganglioside receptor for cholera toxin. J.Infect.Dis., 131, 643-648.

KLOB, J.M. (1867) Pathologisch-antomische studien über das wesen des cholera-processes. Duncker and Humblot, Leipzig.

KNIGHT, P.R., NAHRWALD, M.L. AND ROSENBERG, A. (1978) Sialic acid release by V. cholerae sialidase (neuraminidase) from the outer surface of neural cells is greatly raised by halothane. J. Neurochem., 30, 1645-1647.

KNOP, J. (1980a) Effect of V. cholerae neuraminidase on the mitogen response of T lymphocytes. I. Enhancement of macrophage T-lymphocyte cooperation in concanavalin-A-induced lymphocyte activation. Immunobiol., 157, 474-485.

KNOP, J. (1980b) Effect of V. cholerae neuraminidase on the mitogen response of T-lymphocytes. II. Modulation of the lymphocyte response to macrophage released factors by neuraminidase. Immunobiol., 157, 486-498.

KOCH, R. (1883) Der sweite bericht der deutschen cholera commission. Dtsch.Med.Waschr., 9, 743.

KOCH, R. (1884a) In : Conferenz zur erörterung der cholerafrage. Berl.Klin.Wschr., 21, 477, 493, 509.

KOCH, R. (1884b) In: Die conferenz zur erörterung der cholerafrage. Dtsch.Med.Wschr., 10, 499-519.

KOLLE, W. AND PRIGGE, R. (1928) Cholera asiatica. p 1. In: Kolle, W., Kraus, R. and Uhlenhuth, P. Handbuch der pathogenen mikroorganismer. 3rd edition, Vol. IV, G. Fischer, Jena.

KRAEMER, P.M. (1968) Cytotoxic, hemolytic and phospholipase contaminants of commercial neuraminidase. Biochim.Biophys.Acta., 167, 205-208.

KRAUS, R. (1903) Zur differenzierung des choleravibrio von artverwandten vibrionen. Wien Klin.Wschr., 16, 1382.

KRAUS, R. AND PŘIBRAM, E. (1905) Zur frage der toxinbildung des choleravibrio. Wien Klin.Wschr., 18, 999.

KRAUS, R. (1922) Über die verschiedenheit der eltor - von den choleravibrionen. Münch.Med.Wschr., 69, 499.

KUNITZ, M. (1947) Crystalline trypsin inhibitor. II. General properties. J.Gen.Physiol., 30, 291-310.

KUSAMA, H. AND CRAIG, J.P. (1970) Production of biologically active substances by two strains of V. cholerae. Infect.Immun., 1, 80-87.

LA BREC, E.H., SCHNEIDER, H., MAGNANI, T.J. AND FORMAL, S.B. (1964) Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J.Bact., 88, 1503-1518.

LEVEN, R. AND BRAUER, R.W. (1951) The biuret reaction for the determination of proteins - an improved reagent and its application. J.Lab.Clin.Med., 38, 474-480.

LO SPALLUTO, J.J. AND FINKELSTEIN, R.A. (1972) Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid). Biochem.Biophys. Acta.,257, 158-166.

LONGMATH, N. (1966) King cholera, the biography of a disease. pp 1-10. Hamish Hamilton, London.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J. (1951) Protein measurement with the folin phenol reagent. J.Biol.Chem., 193, 265-275.

MACNAMARA, C. (1876) A history of asiatic cholera. Macmillan and Company, London.

MALLORY, F.B. (1944) Pathological Technique. W.B. Saunders Company, Philadelphia.

MILES, A.A. AND MISRA, S.S. (1938) The estimation of the bactericidal power of the blood. J.Hyg.(Lond.), 38, 732-748.

MOSS, J., MANGANIELLO, V.C. AND FISHMAN, P.H. (1977) Oxidation of gangliosides in cultured cells: effects of choleragen. Biochem., 16, 1977.

MOWRY, R.W. (1963) The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with the periodic acid-schiff reaction. Ann.N.Y.Acad.Sci., 106, 402-423.

MYEROWITZ, R.L., GORDON, R.E. AND ROBBINS, J.B. (1973) Polysaccharides of the genus Bacillus cross-reactive with the capsular polysaccharides of D. pneumoniae type III, H. influenzae type b, and N. meningitidis Group A. Infect.Immun., 8, 896-900.

MYEROWITZ, R.L. AND NORDEN, C.W. (1977) Effect of neonatal gastro-intestinal colonization with cross-reacting E. coli or anticapsular antibody production and bacteremia in experimental H. influenza type b disease of rats. Infect.Immun., 17, 83-90.

McCLELLAND, D.B.L. (1979) Bacterial and viral infections of the gastro-intestinal tract. pp 214-245. In: Asquith, P. (ed). Immunology of the gastrointestinal tract. Churchill Livingstone, Edinburgh.

NAGY, B., MOON, H.W. AND ISAACSON, R.E. (1977) Colonization of porcine intestine by enterotoxigenic E. coli: Selection of piliated forms in vivo, adhesion of piliated forms to epithelial cells in vitro, and incidence of a pilus antigen among porcine enteropathogenic E. coli. Infect.Immun., 16, 344-352.

NEALE, A. (1831) Researches to establish the truth of the Linnaean doctrine of animate contagions. Longmans, London.

NELSON, E.T., CLEMENTS, J.D. AND FINKELSTEIN, R.A. (1976) V. cholerae adherence and colonization in experimental cholerae: Electron microscopic studies. Infect.Immun., 14, 527-547.

NEURATH, A.R., HARTZELL, R.W. AND RUBIN, B.A. (1970) Isoelectric focusing of neuraminidase. Experientia, 26, 1210-1211.

ONIONS, C.T. (ed). (1973) The shorter Oxford English Dictionary On Historical Principles. 3rd edition, p 329. Clarendon Press, Cambridge.

O'KEEFE, E. AND CUATRECASAS, P. (1978) Cholera toxin and membrane gangliosides in normal and transformed cells. J.Membrane Biol., 42, 61-79.

PACINI, F. (1854) Osservazioni microscopiche e deduzioni patologiche sul cholera asiatico. F. Bencini, Firenze.

PALESE, P., BUCHER, D. AND KILBOURNE, E.D. (1973) Applications of a synthetic neuraminidase substrate. Appl. Microbiol., 25, 195-201.

PANGBORN, M.C. (1951) A simplified purification of lecithin. J.Biol.Chem., 188, 471.

PEARSE, E.A.B. (1968a) Histochemistry : Theoretical and Applied. 3rd edition, Vol. 1, pp 305-307. Williams and Wilkins Company, Baltimore.

PEARSE, E.A.B. (1968b) Histochemistry : Theoretical and Applied. 3rd edition, Vol. 1, pp 298, 302. Williams and Wilkins Company, Baltimore.

PEARSE, E.A.B. (1968c) Histochemistry : Theoretical and Applied. 3rd edition, Vol. 1, pp 55-56. Williams and Wilkins Company, Baltimore.

PEARSON, E.S. AND HARTLEY, H.O. (eds). (1966) Biometricka tables for statisticians. 3rd edition, Vol. I, pp 21, 146. Cambridge University Press, Cambridge.

PETERSON, J.W. (1974) Tissue binding properties of the cholera toxin. Infect.Immun., 10, 157-166.

PETTENKOFER, M.J. VON (1855) Untersuchungen und beobachtungen über die verbreitungsort der cholera nebst betrachtungen über maszregeln, derselben einhalt zu thun. Cotta, München.

PIERCE, N.F. (1973) Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of V. cholerae and E. coli. J.Exp.Med., 137, 1009-1023.

POGER, M.E. AND LAMM, M.E. (1974) Localization of free and bound secretory component in human intestinal epithelial cells - a model for the assembly of secretory IgA. J.Exp.Med., 139, 629-642.

POLLITZER, R. (1959) Cholera. pp 1-50. World Health Organization, Geneva.

PYE, J. AND CURTAIN, C.C. (1961) Electrophoretic sedimentation and diffusion behavior of crystalline neuraminidase from V. cholerae. J.Gen.Microbiol., 24, 423-425.

QUINTARELLI, G., TSUIKI, S., HASHIMOTO, Y. AND PIGMAN, W. (1961) Studies of sialic acid-containing mucins in bovine submaxillary and rat sublingual glands. J.Histochem.Cytochem., 9, 176-183.

RADASZKIEWICZ, T., WEIRICH, E. AND DENK, H. (1979) Erythrocyte adherence to the marginal zone of mouse spleen follicle mediated by receptor)s) for neuraminic acid. Z. Immuno-Forsch., 155, 319-329.

RAMPHAL, R. AND PYLE, M. (1983a) Adherence of mucoid and nonmucoid P. aeruginosa to acid-injured tracheal epithelium. Infect.Immun., 41, 345-351.

RAMPHAL, R. AND PYLE, M. (1983b) Evidence for mucins and sialic acid as receptors for P. aeruginosa in the lower respiratory tract. Infect. Immun., 41, 339-344.

REED, W.P. AND WILLIAMS, R.C. JR. (1978) Bacterial adherence: First step in pathogenesis of certain infections. J.Chron.Dis., 31, 67-72.

RÉVÉSZ, T. AND GREAVES, M. (1975) Ligand-induced redistribution of lymphocyte membrane ganglioside G_{M_1}. Nature, 257, 103-106.

ROSENBERG, C.E. (1962) The Cholera Years. pp 1-9. University of Chicago Press, Chicago.

ROZEE, K.R., COOPER, D., LAM, K. AND COSTERTON, J.W. (1982) Microbial flora of the mouse ileum mucous layer and epithelial surface. Appl. Environ.Microbiol., 43, 1451-1463.

SAHYOUN, N. AND CUATRECASAS, P. (1975) Mechanism of activation of adenylate cyclase by cholera toxin. Proc.Nat.Acad.Sci.U.S.A. 72, 3438.

SAVAGE, D.C. (1972) Survival on mucosal epithelia, epithelial penetration and growth in tissues of pathogenic bacteria. p 25. In: Smith, H. and Pearce, J.H. Microbial Pathogenicity in Man and Animals. Cambridge University Press, Cambridge.

SAVAGE, D.C. (1977) Microbial ecology of the gastrointestinal tract. Annu.Rev.Microbiol., 31, 107-133.

SAVAGE, D.C. (1980) Colonization by and survival of pathogenic bacteria on intestinal mucosal surfaces. pp 177-206. In: Bitton, G. and Marshall, K.C. (eds). Adsorption of Microorganisms to Surfaces. John Wiley and Sons, New York.

SCHICK, H.J. AND ZILG, H. (1978) Production and quality control of therapeutically applicable V. cholerae neuraminidase. pp 81-85. In: Griffith, A.H. and Regamey, R.H. (eds). International Symposium on Biological Preparations in the treatment of Cancer: 1977 London. Vol. 33, S. Karger, Basel.

SCHMITZ-MOORMANN, P. (1969) Zur histochemie der neuraminsaüre. Histochemie, 20, 78-86.

SCHNEIDER, D.R. AND PARKER, C.D. (1978) Isolation and characterization of protease-deficient mutants of V. cholerae. J.Infect.Dis., 138, 143-151.

SCHNEIDER, D.R., SIGEL, S.P. AND PARKER, C.A. (1981) Characterization of V. cholerae protease activities with peptide analysis. J.Clin. Microbiol., 13, 80-84.

SCHNEIDER, D.R. AND PARKER, C.D. (1982) Purification and characterization of the mucinase of V. cholerae. J.Infect.Dis., 145, 474-482.

SCHRANK, G.D. AND VERWEY, W.F. (1976) Distribution of cholera organisms in experimental V. cholerae infections : Proposed mechanisms of pathogenesis and antibacterial immunity. Infect.Immun., 13, 195-203.

SEDLACEK, H.H., STARK, J., SEILER, F.R., ZIEGLER, W. AND WIEDANDT, H. (1976) Cholera toxin induced redistribution of sialoglycolipid receptor at the lymphocyte membrane. FEBS Letters, 61, 272-276.

SEDMAK, J.J. AND GROSSBERG, S.E. (1973) Comparative enzyme kinetics of influenza neuraminidases with the synthetic substrate methoxyphenylneuraminic acid. Virology, 56, 658-661.

SHORE, E.G., DEAN, A.G., HOLIK, K.J., DAVIS, B.R. (1974) Enterotoxin-producing E. coli and diarrheal disease in adult travelers : a prospective study. J.Infect.Dis., 129, 577-582.

SINGH, G. AND AHUJA, M.L. (1953) Observations on the intestinal epithelium desquamating enzyme of vibrios isolated from cholera and non-cholera sources. Ind.J.Med.Res., 41, 285-296.

SMITH, H. (1977) Microbial surfaces in relation to pathogenicity. Bacteriol.Rev., 41, 475-500.

SNOW, J. (1849) On the mode of communication of cholera. John Churchill, London.

SOLOVIEV, V.D., KOBRINSKY, G.D., GALTSEVA, G.V. AND SAYAMOV, R.M. (1975) The role of neuraminidase of cholera vibrios in the pathogenesis of experimental cholera. Zh.Mikrobiol.Epidemiol.Immunobiol.(Moscow), 5, 94-99.

SPICER, S.S. AND WARREN, L. (1960) The histochemistry of sialic acid containing mucoproteins. J.Histochem.Cytochem., 8, 135-137.

SRIVASTAVA, R., SINHA, V.B. AND SRIVASTAVA, B.S. (1980) Events in the pathogenesis of experimental cholera : Role of bacterial adherence and multiplication. J.Med.Microbiol., 13, 1-9.

SRIVASTAVA, R. AND SRIVASTAVA, B.S. (1980) Short Communication - Isolation of a non-adhesive mutant of V. cholerae and chromosomal localization of the gene controlling mannose-sensitive adherence. J.Gen.Microbiol., 117, 275-278.

STAERK, J., RONNEBERGER, H.J., WIEGANDT, H. AND ZIEGLER, W. (1974) Interaction of ganglioside $G_{G\ tet}1$ and its derivatives with choleragen. Eur.J.Biochem., 48, 103-110.

STEPHEN, J. AND PIETROWSKI, R.A. (1981a) Bacterial Toxins. p 6. Thomas Nelson and Sons Ltd., Surrey.

STEPHEN, J. AND PIETROWSKI, R.A. (1981b) Bacterial Toxins. pp 19, 20. Thomas Nelson and Sons Ltd., Surrey.

STICKER, G. (1912) Die Cholera. In: Abhandlungen aus der seuchengeschichte und seuchenlehre, Band II, iv, p 592. A Tüpelmann, Giessen.

TANAMAL, S.T.W. (1959) Notes on paracholera in Sulawesi (Celebes). Amer.J.Trop.Med., 8, 72-78.

TANNOCK, G.W. AND SAVAGE, D.C. (1974) Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect.Immun., 9, 591-598.

THOMAS, H.C. AND JEWELL, D.P. (eds). Clinical Gastrointestinal Immunology. p 33. Blackwell Scientific Publications, Oxford.

TOMASI, T.B. AND GREY, H.M. (1973) Structure and function of immunoglobulin A. Progress in Allergy, 16, 81-213.

VAN HEYNINGEN, S. (1974) Cholera toxin : interaction of subunits with ganglioside G_{M_1}. Science (Wash. D.C.), 183, 656-657.

VAN HEYNINGEN, S. (1977) Cholera toxin. Biol.Rev., 52, 509.

VAN HEYNINGEN, S. AND KING, C.A. (1975) Short Communication - Subunit A from cholera toxin is an activator of adenylate cyclase in pigeon erythrocytes. Biochem.J., 146, 269-271.

VAN HEYNINGEN, W.E., CARPENTER, C.C., PIERCE, N.F. AND GREENOUGH, W.B. (1971) Deactivation of cholera toxin by ganglioside. J.Infect.Dis., 124, 415-418.

VAN HEYNINGEN, W.E. AND MELLANBY, J. (1973) A note on the specific fixation, specific deactivation and non-specific inactivation of bacterial toxins by gangliosides. Naunyn Schmiedebergs Arch. Pharmacol., 276, 297-302.

VAN LOGHEM, J.J. (1938) Un vibrion "El Tor" pathogène isolé aux Indes Néerlandaises. Bull.Off.Int.Hyg.Publ., 30, 1520.

VERTIEV, Y.V., SHAGINIYAN, I.A. AND EZEPCHUK, Y.V. (1981) Immunoserological characteristics of homogeneous choleragen and neuraminidase preparations. Zbl.Bakt.Hyg., I Abt. Orig. A, 249, 235-241.

VIRCHOW, R.L.K. (1879) Gesammelte Abhandlungen aus dem Gebiete offenlichen Medicin und der Seuchenlehre von Rudolf Virchow. Band I, p 151. A. Hirschwald, Berlin.

WARREN, L. (1959) The Thiobarbituric Acid Assay of sialic acids. J.Biol.Chem., 234, 1971-1975.

WHITE, A., HANDLER, P. AND SMITH, E.L. (1968) Principles of Biochemistry. 4th edition, p 881. McGraw-Hill, New York.

WHITE, A., HANDLER, P., SMITH, E.L., HILL, R.L. AND LEHMAN, I.R. (1978) Principles of Biochemistry. 6th edition (International Student Edition), pp 1047-1048, McGraw-Hill Kogakusha Ltd., Tokyo.

WILLIAMS, R.C. AND GIBBONS, R.J. (1972) Inhibition of bacterial adherence by secretory immunoglobulin A: A mechanism of antigen disposal. Science, 177, pp 697-699.

WORLD HEALTH ORGANIZATION (1965) The new threat of cholera. Wld.Hlth.Org.Chron., 19, 227-232.

YAMADA, K. AND SHIMIZU, S. (1979) The use of peroxidase-labelled Limulus polyphemus agglutinin for the histochemistry of sialic acid-containing glycoproteins in light microscopy. Histochem.J., 4, 457-471.

YANCEY, R.J., WILLES, D.L. AND BERRY, L.J. (1978) Role of motility in experimental cholera in adult rabbits. Infect.Immun., 22, 387-392.

YOUNG, D.B. AND BROADBENT, D.A. (1982) Biochemical characterization of extracellular proteases from V. cholerae. Infect.Immun., 37, 875-883.

APPENDICES

Appendix I

BUFFERS AND DILUENTS

Appendix I

BUFFERS AND DILUENTS

Ammonium sulphate for protein precipitation

a) 100% saturated ammonium sulphate solution:

Ammonium sulphate (B.D.H.)	69.7 g
Distilled water	100 ml

b) 50% saturated ammonium sulphate solution:

Ammonium sulphate	29.7 g
Distilled water	100 ml

Borate gelatin buffer (Dr. Stewart-Tull, personal communication)

Boric acid	3.1 g
NaCl	4.0 g
NaOH	80 mg
Gelatine 6% (w/v)	5.0 ml

Adjust the volume to 1 litre with distilled water. The pH of the buffer is 7.5.

Phosphate buffered saline (P.B.S.)

a) Solution A

$Na_2HPO_4 \cdot H_2O$	3.58 g

Adjust the volume to 1 litre with distilled water.

b) Solution B

$NaH_2PO_4 \cdot 2H_2O$	1.56 g

Adjust the volume to 1 litre with distilled water.

c) Buffer:

Solution A	720 ml
Solution B	280 ml
NaCl	17.53 g
Distilled water	1.0 litre

The pH of the buffer is adjusted to 7.2.

Sodium acetate buffer

a) Solution A (0.3M acetic acid)

Glacial acetic acid	18.0 ml

Adjust the volume to 1 litre with distilled water.

Appendix I

b) Solution B (0.3M sodium acetate):

sodium acetate	24.6 g

Adjust the volume to 1 litre with distilled water.

c) Buffer:

Solution C (0.05M acetic acid)	48 ml
Solution D (0.05M sodium acetate)	452 ml
Distilled water	500 ml

Adjust the pH of the buffer to 5.0.

f) Buffer for use with V. cholerae neuraminidase:

Solution C	48 ml
Solution D	452 ml
Distilled water	500 ml
$CaCl_2$	1.0 g
NaCl	10.0 g

Adjust the pH of the buffer to 5.5.

Sodium Phosphate Buffer

a) Solution A (0.3M sodium phosphate dibasic):

$Na_2HPO_4 \cdot H_2O$	42.58 g

Adjust the volume to 1 litre with distilled water.

b) Solution B (0.3M sodium phosphate monobasic):

$NaH_2PO_4 \cdot 2H_2O$	44.77 g

Adjust the volume to 1 litre with distilled water.

c) Buffer:

Solution A	160 ml
Solution B	840 ml

Adjust the pH to 7.5.

d) Buffer for use in M.P.N. assay for neuraminidase activity:

Solution C (0.1M sodium phosphate monobasic)	877 ml
Solution D (0.1M sodium phosphate dibasic)	123 ml
$CaCl_2$	222 mg

The volume was brought up to 1 litre with distilled water. The pH was adjusted to 5.9.

Appendix I

Triethanolamine Buffer (Boehringer Mannheim, 1983)

Triethanolamine hydrochloride (0.1M) 1.86 g

The volume was brought up to 100 ml with distilled water. The pH was adjusted to 7.6 with 1.0N NaOH.

Triethanolamine Buffer + Calcium Chloride (Boehringer Mannheim, 1983)

Triethanolamine hydrochloride (0.1M) 1.86g

$CaCl_2.2H_2O$ 22 mg

The volume was brought up to 100 ml with distilled water. The pH was adjusted to 7.6 with 1.0N NaOH.

Triethanolamine Buffer + Magnesium Sulphate (Boehringer Mannheim, 1983)

Triethanolamine hydrochloride (0.3M) 6.0 g

$MgSO_4.7H_2O$ 100 mg

The volume was brought up to 100 ml with distilled water. The pH was adjusted to 7.6 with 1.0N NaOH.

Tris (hydroxymethyl) aminomethane Buffer

a) Solution A (0.2M Tris):

Tris 24.2 g

The volume was adjusted to 1 litre with distilled water.

b) Solution B (0.2M HCl):

Concentrated HCl 1.0 ml

The volume was adjusted to 60 ml with distilled water.

c) Buffer:

Solution C (0.05M Tris) 250 ml

Solution D (0.05M HCl) 25 ml

The volume was brought to 1 litre with distilled water. The pH was adjusted to 9.0.

Appendix II

MEDIA

Appendix II

MEDIA

Casamino acid-yeast extract broth (C.A.Y.E.) (Dr. J.P. Craig, personal communication)

Casamino acid (vitramin free - Difco, U.S.A.)	30.0 g
Yeast extract (Difco)	3.0 g
K_2HPO_4	500 mg

Bring to volume of 1 litre with distilled water. The pH was adjusted to 7.0 with 1.0N NaOH. The broth was dispensed into 16 x 125 mm tubes in 10 ml amounts. The media was autoclaved for 15 min at 120°C.

Gel diffusion media (for Ouchterlony plates)

Ion agar No. 2 (Oxoid)	15.0 g
NaCl	16.0 g
Methyl orange (Kodak Ltd)	120 mg
10% Phenol (w/v) saline solution	50 ml

Bring to volume of 1 litre and steam for 45 min. After steaming the methyl orange and phenol saline solution were added and the gel media was filtered through Whatman Grade 11 filter paper. It was stored as 25 ml amounts.

2% (w/v) proteose peptone broth

Meat peptone (Gibco-Europe, Uxbridge, U.K.)	20.0 g

The volume was adjusted to 1 litre with distilled water and autoclaved for 15 min at 120°C.

Proteose peptone-Colostrum for neuraminidase production

(Ada, French and Lind, 1961)

Meat peptone (Gibco)	150.0 g
Colostrum dialysate (Univ. of Glasgow, Cochno Farm)	7.0 litres
Glycerol (B.D.H.)	112.5 ml
Distilled water	8.0 litres

a) Proteose peptone component:

Meat peptone	150.0 g

The volume was adjusted to 7.0 litres with distilled water. The peptone broth was poured into a 15 litre fermenter vessel and autoclaved for 60 min at 120°C.

Appendix II

b) Colostrum component:

Bovine Colostrum	1.5 litres
Distilled water	7.0 litres

The colostrum was placed into dialysis tubing (Visking, 7.0 cm diam) and placed in a tank which contained distilled water saturated with chloroform (antibacterial agent), for 8-14 days. The dialysate in the tank was aerated for 2 h with air filtered through a 0.45 μm filter membrane (Oxoid) to expel any trace of chloroform which remained. The colostrum was transferred to a 20 litre millipore pressure vessel (Millipore Co., Bedford, Mass. U.S.A.), and filtered through a 0.45 μm membrane filter which was held in a 142 mm millipore filter holder under nitrogen pressure into the 15 litre fermenter vessel. It was necessary to replace the 0.45 μm filter membrane several times with presterilised filter membranes. These filter membrane changes were carried out under sterile technique.

c) Glycerol component:

Glycerol (B.D.H.)	112.0 ml

The glycerol was poured into an erlenmeyer flask and autoclaved for 30 min at 120°C. The glycerol was aseptically added to the fermenter vessel.

Thiosulphate-citrate bile salt sucrose agar (T.C.B.S.)

T.C.B.S. powdered agar (BBL-Cockeysville, Maryland, U.S.A.)	86 g

The powdered agar was suspended in 1 litre of distilled water, heated with frequent agitation (to dissolve the powder) and boiled for 1 min. The T.C.B.S. agar was cooled to 45-50°C and dispensed into petri-dishes.

T_1N_1 broth (Dr. J.P. Craig, personal communication)

Bacto-tryptone (Difco)	8.0 g
NaCl	8.0 g

The volume was brought to 1.0 litre with distilled water and the pH was adjusted to 7.2 with 1.0N NaOH. The broth was dispensed into 16 x 125 mm tubes in 5 ml amounts. The broth was autoclaved for 15 min at 120°C.

Appendix III

REAGENTS

Appendix III

REAGENTS

Aldolase assay

a) β-Nicotinamide-adenine dinucleotide, reduced:

β-NADH-Na_2.$2H_2O$ (Grade I, disodium salt, 99%, Boehringer Mannheim) 10 mg

0.1M Triethanolamine buffer, pH 7.6 1.0 ml

b) Fructose-1.6-diphosphate:

$C_6H_{10}O_{12}P_2Na_3$.($C_6H_{14}N_4$).$10H_2O$ (crystallised tetracyclohexyl-ammonium salt, Boehringer Mannheim) 30 mg

0.1M Triethanolamine buffer, pH 7.6 1.0 ml

c) Glycerol-3-phosphate dehydrogenase/Triosephosphate isomerase:

GDH/TIM (crystalline suspension in ammonium sulphate suspension, Boehringer Mannheim)

d) Aldolase:

Aldolase (ex rabbit muscle; crystalline suspension in ammonium sulphate solution, 10 mg ml^{-1}, Boehringer Mannheim)

The working solution was obtained by diluting the manufacturer's solution 100-fold in 0.1M triethanolamine buffer pH 7.6.

Endoglycosidase assay - phenol sulphuric acid assay for carbohydrates

a) Ovalbumin:

Ovalbumin (Grade V, Sigma) 2.0 mg

0.05 M Sodium acetate buffer, 0.5% SDS (w/v) pH 5.5 0.2 ml

b) Trichloroacetic acid:

12.5% (w/v) Trichloroacetic acid 12.5 g

Adjust the volume to 100 ml with distilled water

c) Phenol:

80% (w/v) Phenol (Reagent Grade, B.D.H.) 8.0 g

Adjust the volume to 10 ml with distilled water

d) Glucose (stock solution):

Glucose (Analar) 100 µg

Bring the volume to 1.0 ml with distilled water.

Appendix III

e) Endoglycosidase:

Endoglycosidase H (ex Streptomyces griseus, Miles Labs U.K., 0.1 unit vial)	0.1 unit
Distilled water	100 µl

The working solution was obtained by diluting 2 µl of enzyme solution in 100 µl of a 0.1M NaCl, 0.1% (w/v) bovine serum albumin solution.

Lowry assay

a) Folin A reagent:

2% (w/v) Na_2CO_3	2.0 g

Bring to a volume of 100 ml with 0.1N NaOH.

b) Folin B reagent:

Freshly prepare in equal proportions 1% (w/v) $CuSO_4.5H_2O$ and 2% (w/v) sodium tartrate.

c) Folin C reagent:

Add 50 ml of Folin A reagent to 1.0 ml of Folin B reagent.

d) Folin D reagent:

Folin-Ciocalteau's reagent (Sigma, 2N).

Folin-Ciocalteau reagent is diluted 2-fold with distilled water to obtain the 1.0N Folin D reagent.

e) Protein standard:

Bovine serum albumin (Grade V, Sigma)	1.2 mg
Distilled water	1.0 ml

Micro-biuret assay

a) Copper sulphate:

2.5% (w/v) $CuSO_4.5H_2O$	2.5 g

Bring to a volume of 100 ml with distilled water.

b) Sodium hydroxide (3.0N):

NaOH	120 g

Bring to a volume of 1 litre with distilled water.

c) Protein standard:

Ovalbumin (Grade V, Sigma)	1.5 mg
Distilled water	1.0 ml

Appendix III

M.P.N. assay

a) Diazonium salt of 4 amino-2,5 di methoxy-4' nitroazobenzene:

DS-ASNB (Koch-Light)	6.0 mg
0.4M Sodium phosphate buffer, pH 7.0	1.0 ml

This solution must be prepared on the day used and filtered through Whatman filter paper.

b) Di-sodium E.D.T.A. (0.1M):

Di-sodium E.D.T.A.	4.12 g
0.1 M Sodium phosphate buffer, pH 7.0	10.0 ml

c) Methoxyphenol:

Methoxyphenol (1.14 g ml^{-1}, B.D.H.)	1.0 ml
0.1 M Sodium phosphate buffer, 2 mM $CaCl_2$, pH 5.9	9.18 ml

The solution of methoxyphenol is diluted 1000 fold in 0.1 M Sodium phosphate buffer, 2 mM $CaCl_2$ pH 5.9 to give a final concentration of 100 nmoles $(0.1\ ml)^{-1}$.

d) Sodium hydroxide (0.5 N):

NaOH	20 g

The volume is adjusted to 1 litre with distilled water.

e) 2-(3' methoxyphenyl)-N-acetyl-α-neuraminic acid (10 mM):

M.P.N. (Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A.)	50.0 g
0.1 M Sodium phosphate buffer pH 5.9	11.82 ml

Phospholipase-C assay

a) Lipase:

Lipase (ex Rhizopus arrhizus; suspension in ammonium sulphate solution, 10 mg ml^{-1} Boehringer Mannheim)

b) Trichloroacetic acid (1.5 M)

$CaCl_3COOH$	24.5 g
Distilled water	100 ml

Appendix III

c) ATP/NADH/PEP solution:

$ATP\text{-}Na_2H_2.3H_2O$ (crystallised disodium salt, Boehringer, Mannheim)	40mg
$NADH\text{-}Na_2$ (disodium salt grade II Boehringer Mannheim)	10 mg
PEP-Na (crystallised mono-sodium salt, Boehringer Mannheim)	20 mg
$NaHCO_3$	100 mg
0.3M Triethanolamine buffer, pH 7.6	2.0 ml

d) Lactate dehydrogenase:

LDH (ex rabbit muscle, crystalline suspension in ammonium sulphate solution, Boehringer Mannheim)

e) Pyruvate kinase:

PK (ex rabbit muscle, crystalline suspension in ammonium sulphate solution, Boehringer Mannheim)

f) Glycerolkinase:

Glycerolkinase (ex Candida mycoderma, suspension in ammonium sulphate, Boehringer Mannheim)

g) Phospholipase-C:

Phospholipase-C (ex Bacillus cereus, grade II, suspension in ammonium sulphate, Boehringer Mannheim)

h) Lecithin:

Lecithin (ex egg yolk, prepared according to method of Pangborn, 1951)	200 mg
Redistilled water	10.0 ml

The lecithin suspension is exposed to ultrasonic treatment for 5 min. The ultrasonic treated lecithin serves as the substrate for the phospholipase-C assay.

Proteinase

a) Trichloroacetic acid:

CCl_3COOH	5.0 g
Distilled water	100 ml

Appendix III

b) Trypsin:

Trypsin (Sigma)	20 mg.
0.05M Tris buffer, pH 9.0	10 ml

c) Casein:

Casein (Hammarsten grade, B.D.H.)	1.0 g
0.1 M Sodium phosphate buffer, pH 7.6	100 ml

Thiobarbituric acid assay

a) N-acetylneuraminic acid:

NANA (Sigma)	6.18 mg
0.05 M Sodium acetate buffer, pH 5.5	10 ml

b) Phosphotungstic acid:

Phosphotungstic acid (B.D.H.)	5.0 g
2.5 N HCl	100 ml

c) Periodate solution:

$NaIO_4$ (Sigma)	4.27 g
Distilled water	38.3 ml
Concentrated Phosphoric acid (Analar)	61.7 ml

d) Arsenite solution:

Na_2SO_4	17.75 g
Distilled water	250 00 ml
5.0 N H_2SO_4	5.00 ml
Sodium arsenite (B.D.H.)	25.00 g

e) Thiobarbituric acid:

Thiobarbituric acid (B.D.H., 2X recrystallised)	4.5 g
0.5 M Na_2SO_4	750.00 ml

f) N-butanol

g) Cyclohexanone:

Cyclohexanone (re-distilled)

h) Bovine submaxillary mucin:

Bovine submaxillary mucin (Sigma)	20 mg
0.05 M Sodium acetate buffer, (1.0 g) $CaCl_2$ (10 g) NaCl, pH 5.5.	5.0 ml

Appendix III

Commercial neuraminidase preparations:

1) *V. cholerae* neuraminidase (Koch-Light; 200 ImU ml^{-1}):
The working solution of this enzyme is obtained by making a 5-fold dilution of the enzyme in 0.05 M Sodium acetate buffer, $CaCl_2$ (1.0 g) NaCl (10 g), pH 5.5. The activity of the working solution is 1.0 ImU in the assay.

2) *V. cholerae* neuraminidase (Calbiochem; 1000 ImU ml^{-1} or 100 ImU in assay).

3) *Cl. perfringens* neuraminidase (Sigma; 170 ImU mg^{-1}):
Cl. perfringens neuraminidase 1.0 mg
0.05 M Sodium acetate buffer, pH 5.0 5.0 ml
The activity of the working solution is 3.4 ImU in the assay.

4) *Cl. perfringens* neuraminidase (Worthington; 1360 ImU ml^{-1} or 136 ImU in assay).

Appendix IV

FERMENTER DIAGRAMS

BATCH CULTURE FERMENTER

A (L.H. ENGINEERING, LTD., STOKE POGES, BUCKS, ENGLAND)

Legend:

A = Sampling port

B = Filling port

C = Water in

D = Air in

E = Air out

F = Anti-Foam Probe

G = pH Probe

H = Water out

I = Water flow connection carrying heater water to "water in" connection

J = Water flow connection carrying water from main "Flow out" opening

K = Thermometer opening

L = Condenser

M = Temperature control gauge

N = Water flow from fermenter vessel

O = Water flow into fermenter vessel

P = Air flow gauge

Q = Air flow opening connected to air filter

R = Impellar

S = connection between impellar rotor blade and driving motor

T = Anti-foam mixture (1% Silcolapse)

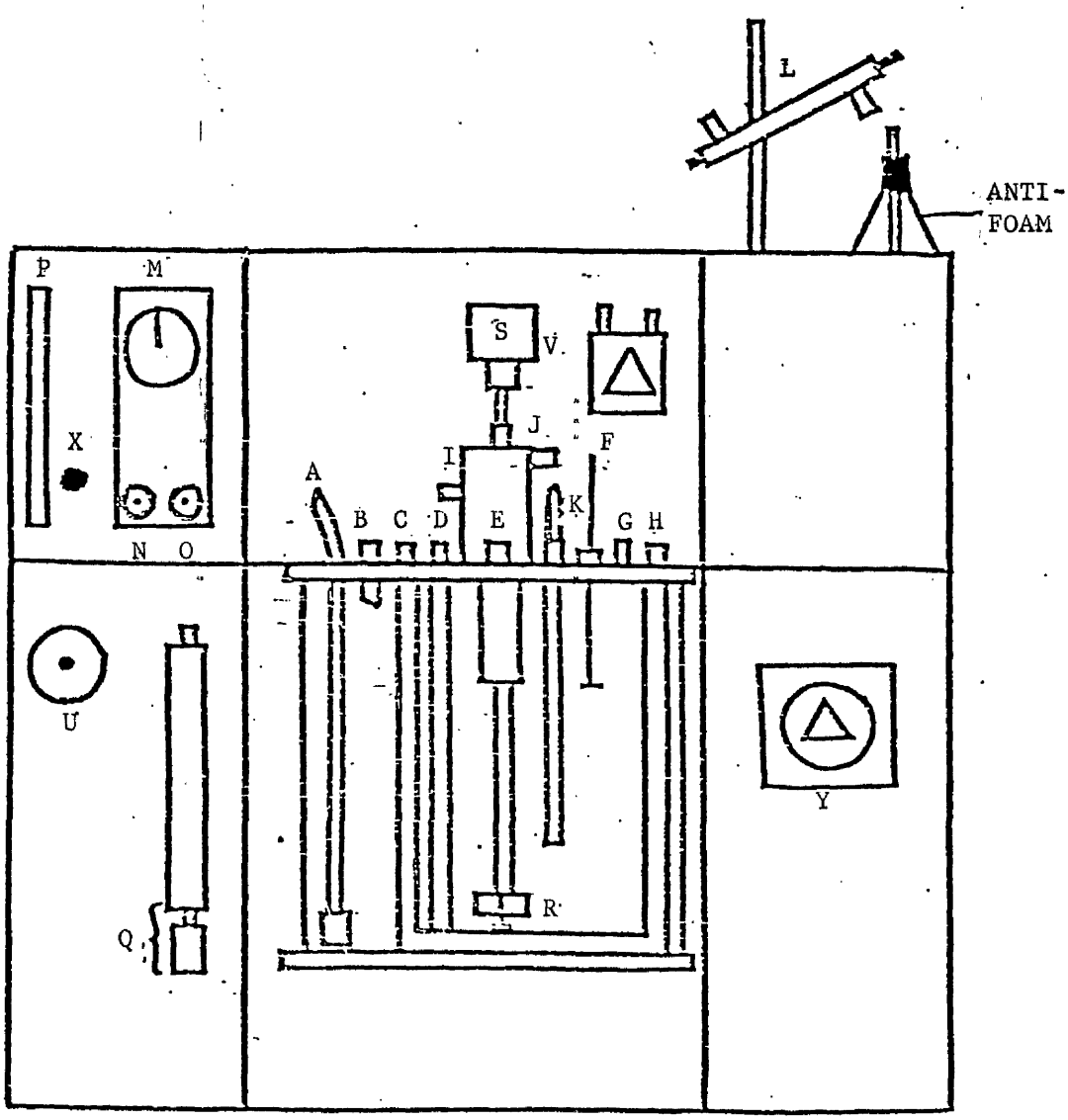
U = Speed control for impellar

V = Anti=foam pump

X = Air control dial

Y = Anti=foam dose control

Z = Sampling bottle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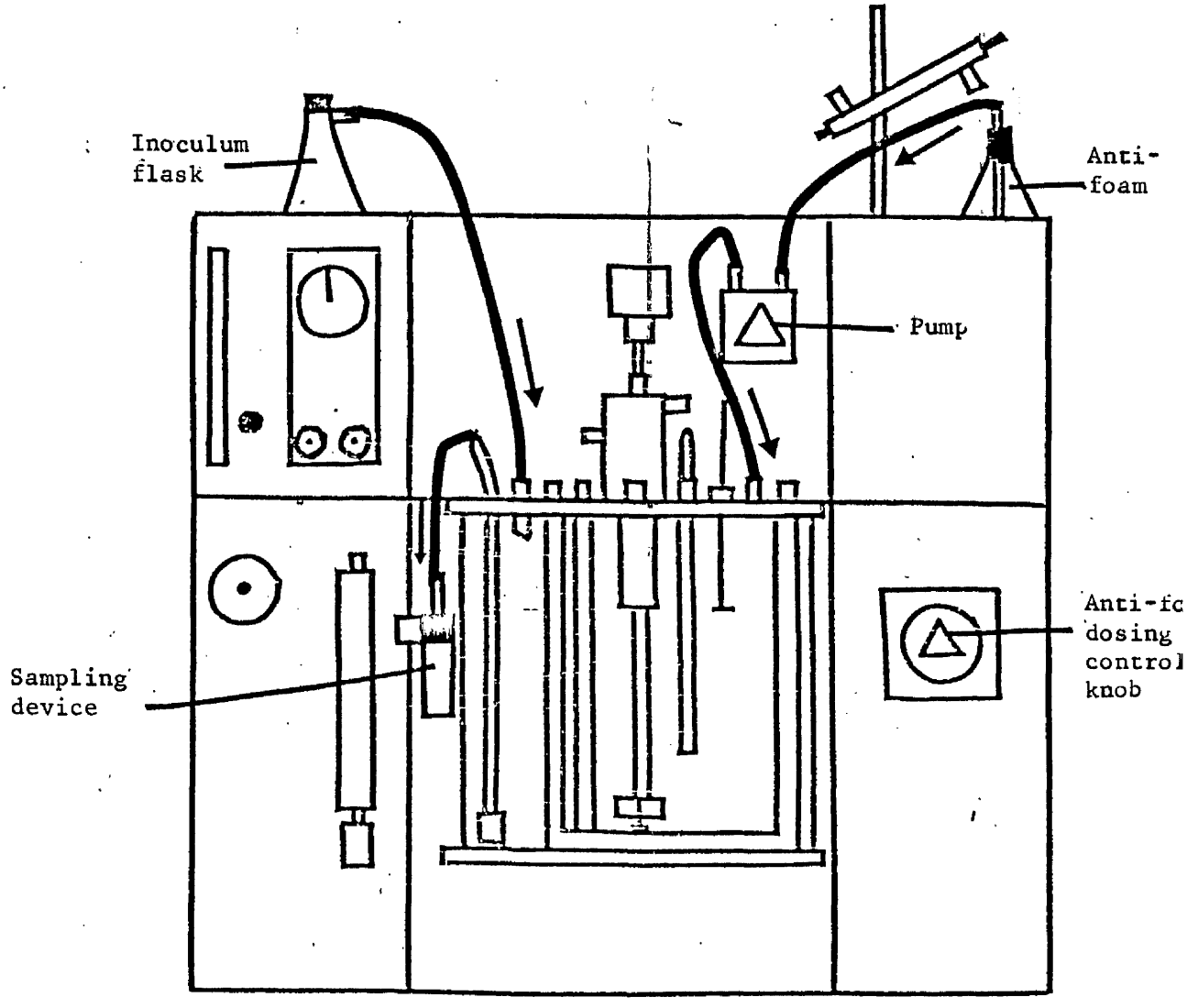


BATCH CULTURE FERMENTER

B (Anti-foam system, sampling system and addition of inoculum via filling port.)

BATCH CULTURE FERMENTER

C (Temperature control gauge, water heating system)

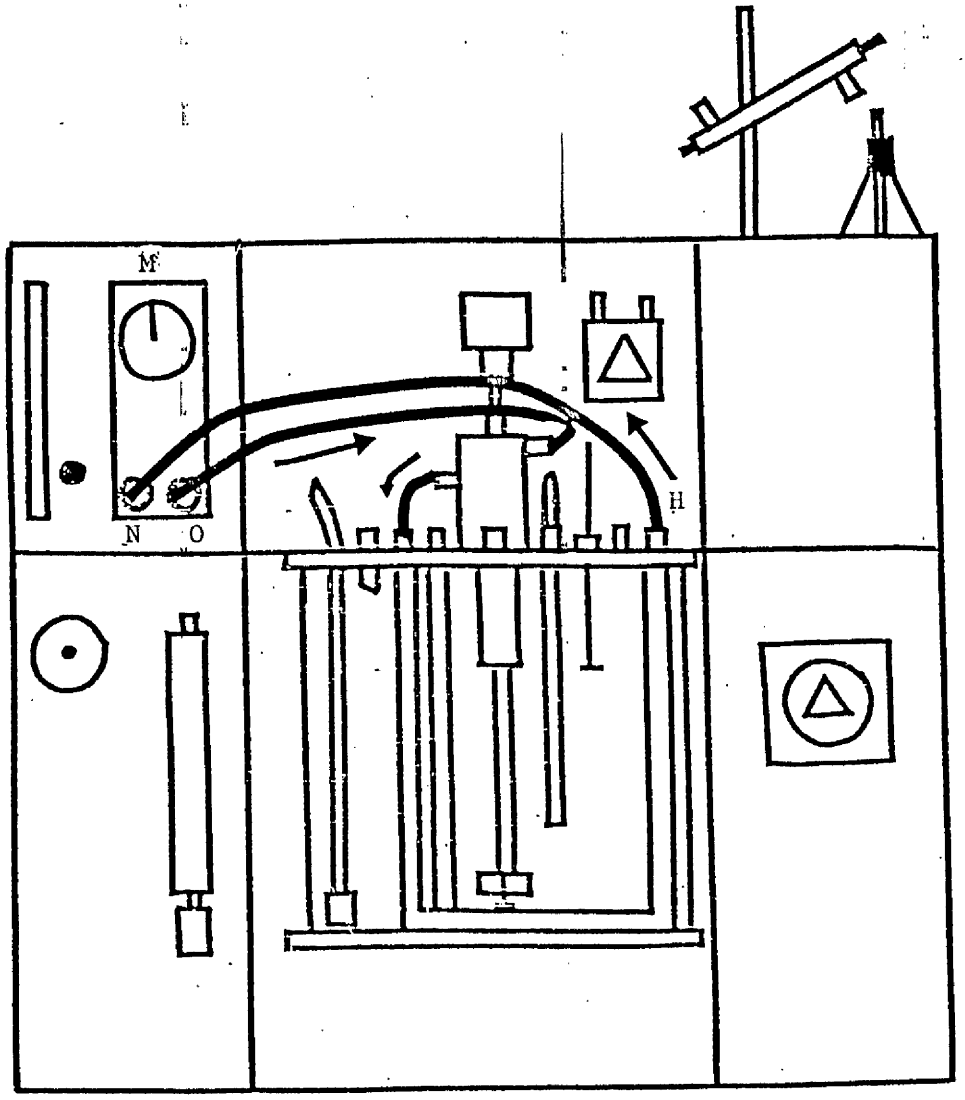
Legend: The arrow indicates the direction of flow of the cooling water

H = Water out opening

M = Temperature control gauge of fermenter vessel

N = Water flow from fermenter vessel

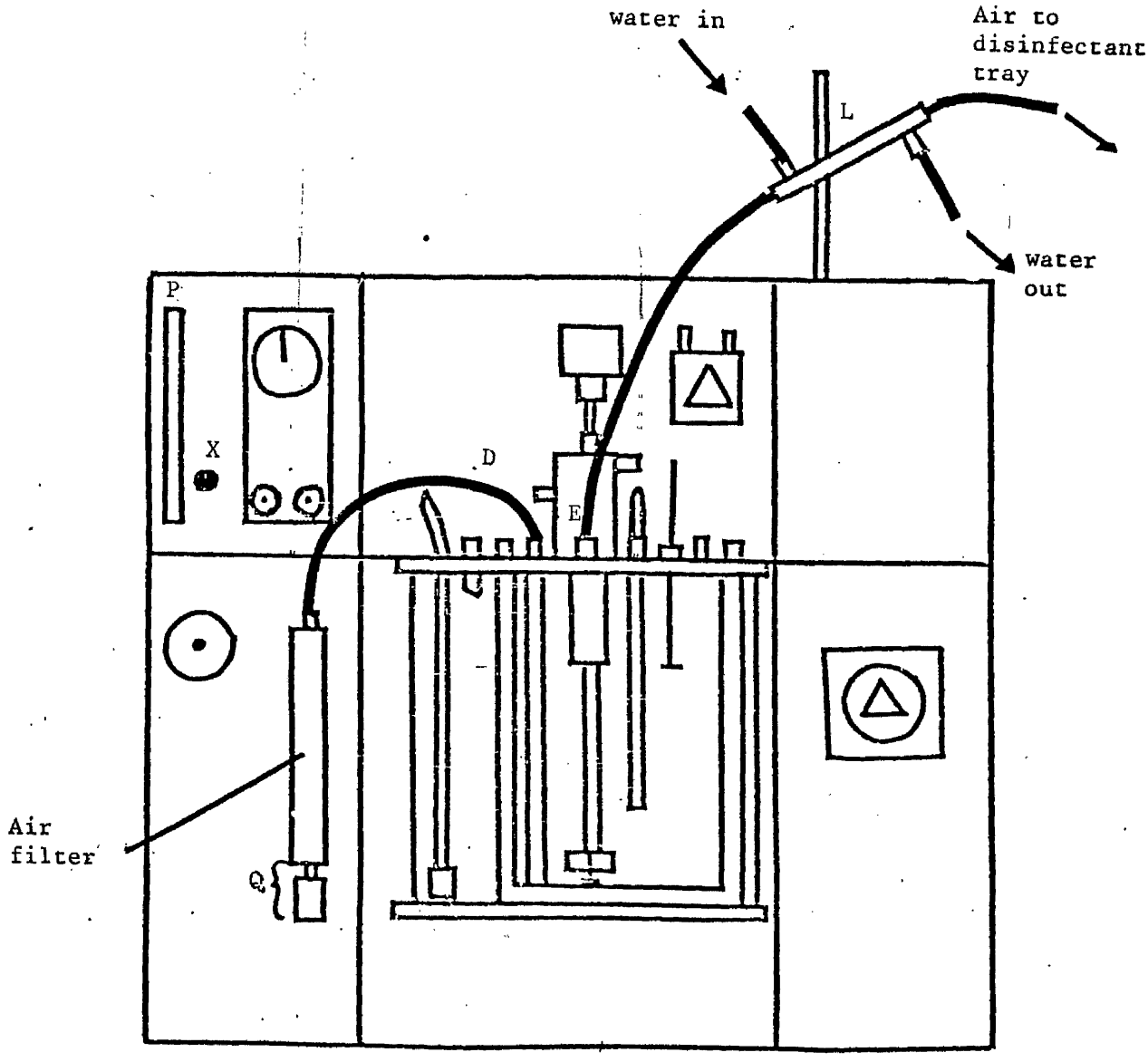
O = Water flow into fermenter vessel



BATCH CULTURE FERMENTER

D (Air flow system)

Legend:
- D = Air in connection
- E = Air out connection
- L = Condenser
- P = Air flow gauge
- Q = Air flow opening connected to air filter
- X = Air control dial



Appendix V

STAINS

Appendix V

STAINS

Alcian Blue/PAS, pH 2.5 (Mowry, 1963)

a) Alcian blue:

Alcian blue (Raymond Lamb, London)	100 g
Concentrated acetic acid	300 ml

The volume is adjusted to 1 litre with distilled water.

b) Schiff reagent:

Pararosaniline Chloride (Kodak Ltd.)	2.0 g

The volume is adjusted to 200 ml with distilled water. The pararosaniline chloride is decolourised by bubbling sulphur dioxide through the solution until it took on a straw yellow appearance.

c) Periodate solution:

1% (v/v) HIO_4	1.0 ml
Distilled water	99.0 ml

d) Sodium meta-bisulphite:

$Na_2S_2O_5$ (B.D.H.)	9.5 g

The volume is adjusted to 100 ml with distilled water.

Haematoxylin and Eosin (Humason, 1967)

a) Harris Haematoxylin - without acetic acid (Mallory, 1944):

Haematoxylin	1.0 g
Absolute alcohol	10.0 ml
Ammonium alum	20.0 g
Distilled water	200.00 ml

Note: The Harris Haematoxylin was obtained ready prepared from the supplier, Raymond Lamb, London.

b) Eosin yellow:

Eosin yellow (Kodak Ltd.)	2.0 g

The volume is adjusted to 200 ml with distilled water.

c) Scott's solution (Humason, 1967)

Sodium bicarbonate	2.0 g
$MgSO_4$	20.0 g

The volume is adjusted to 1 litre with distilled water.

APPENDIX VI

STAINING PROCEDURES

Appendix VI

STAINING PROCEDURES

Alcian Blue/PAS, pH 2.5 (Mowry, 1963)

a) Stain in Alcian blue for 30 min.
b) Wash in running water.
c) Oxidise for 10 min in 1% (v/v) aqueous HIO_4.
d) Wash in running water.
e) Treat with Schiff's reagent
f) Rinse 3 times in sodium metabisulphite for a period of 2 min at each time interval.
g) Rinse in running water.
h) Dehydrate, clear, and mount in D.P.X.

Haematoxylin and Eosin (Progressive method, Humason, 1967)

a) Stain in Harris Haematoxylin for 2-5 min.
b) Rinse in running water 3-5 min.
c) Immerse in Scott's solution for 3 min.
d) Rinse in running water 3-5 min.
e) Immerse in eosin yellow counterstain 1-3 min.
f) Rinse in running water.
g) Dehydrate, clear, and mount in D.P.X.

Appendix VII

NEURAMINIDASE PLUS SERA

Tube No.	Millilitres added 1	2	3	4	5	6	7
V. cholerae neuraminidase (N_2, 55 ImU ml^{-1})	0.2	0.2	0.2	0.2	0.2	.	.
DST R77 rabbit anti-neuraminidase serum	0.1	0.2	0.3	0.4	.	0.5	.
0.05 M NaAc buffer pH 5.5	0.4	0.3	0.2	0.1	0.5	0.2	0.7

Tube No.	Millilitres added 1	2	3	4	5	6
Cl. perfringens neuraminidase (680 ImU ml^{-1})	0.2	0.2	0.2	0.2	0.2	.
DST R78 rabbit anti-neuraminidase serum	0.1	0.2	0.3	0.5	.	.
0.05 M NaAc buffer pH 5.0	0.4	0.3	0.2	.	0.5	0.7

Tube No.	Millilitres added 1	2	3	4	5	6	7	8
V. cholerae neuraminidase (N_2, 55 ImU ml^{-1})	.	0.1	0.1	0.1	.	0.1	0.1	.
DST R78 rabbit anti-neuraminidase serum.	.	0.05	0.25	0.25	.	.	.	
IgG (R78) fraction of rabbit anti-neuraminidase serum	.	.	.	.	.	0.05	0.25	0.25
0.05 M NaAc buffer pH 5.5	0.35	0.25	0.20	.	0.1	0.20	.	0.10

Tube No.	Millilitres added 1	2	3	4	5
V. cholerae neuraminidase (N_2, 55 ImU ml^{-1})	.	.	0.2	0.2	0.2
Normal rabbit serum	.	0.5	.	0.1	0.4
0.05 M NaAc buffer pH 5.5	0.7	0.2	0.5	0.4	0.1

Tube No.	Millilitres added 1	2	3	4	5	6	7	8	9	10	11	12
V.cholerae neuraminidase (N_2, 55 ImU ml^{-1})	.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
DST R78 rabbit anti-neuraminidase serum	.	.	.	.	.	.	.	0.05	0.1	0.15	0.20	0.25
IgG (DST R78 fraction of rabbit anti-neuraminidase serum	.	.	0.05	0.10	0.15	0.20	0.25	.	.	.	.	.
0.05 M NaAc buffer pH 5.5	0.35	0.25	0.20	0.15	0.10	0.05	.	0.20	0.15	0.10	0.05	.

Serum Control Tube No.	Millilitres added 1	2	3	4	5	6	7	8	9	10
DST R78 rabbit anti-neuraminidase serum	.	.	.	.	.	0.05	0.10	0.15	0.20	0.25
IgG (DST R78) fraction of rabbit anti-neuraminidase serum	0.05	0.10	0.15	0.20	0.25	.	.	.	.	.
0.05 M NaAc buffer pH 5.5	0.30	0.25	0.20	0.15	0.10	0.30	0.25	0.20	0.15	0.10

**The above experiment was repeated with normal rabbit sera and the IgG fraction of normal rabbit serum

Tube No.	Millilitres Added 1	2	Test 3	4	5	Controls 6	7	8	9
V. cholerae neuraminidase *(N_2-N_4)	0.2	0.2	0.2	0.2	.	.	.	.	.
DST R77 rabbit anti-neuraminidase serum	.	0.3	0.4	0.5	0.5	.	0.3	0.4	0.5
Sigma *Cl.perfringens* neuraminidase (30 ImU ml^{-1})	.	.	.	.	0.2	.	.	.	.
0.05 M NaAc buffer pH 5.5	0.5	0.2	0.1	.	.	0.7	0.4	0.3	0.2

*N_2 = 0.05 I.U. ml^{-1}, N_3 = 0.01 I.U.ml^{-1}, *N_4 = 0.03 I.U.ml^{-1}.

**The neuraminidase was diluted 1:5 in 0.05 M NaAc buffer pH 5.5 for M.P.N. and T.B.A. assays.

***Tubes 4-6 were also assayed without the addition of bovine submaxillary by the T.B.A. assay in order to check for the possible addition of sialic acid from serum.

Appendix VIII

EFFECTS OF NEURAMINIDASE AND OTHER CHEMICAL TREATMENTS ON GOBLET CELL HISTOCHEMISTRY

Appendix VIII

The effect of commercial neuraminidase (Worthington Cl. perfringens)

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	Mean *Geom
Alcian Blue Untreated (x.s.)	1	18	18	23	21	17	20	20	14	19	19			
		17	27	21	28	26	16	27	27	23	20	421	21	20.7
PAS		6	5	4	1	1	0	0	3	2	0			
		16	9	8	8	7	4	3	6	5	6	94	4.7	0
Alcian Blue Water (x.s.)	2	9	8	10	9	13	16	17	19	18	28			
		11	14	14	17	16	14	11	12	22	18	296	14.8	14.1
PAS		5	6	3	3	1	3	5	6	0	7			
		12	9	8	7	5	6	7	5	2	11	111	5.6	0
Alcian Blue (x.s.) 0.05M NaAc buffer pH 5.0	3	14	23	25	30	13	21	24	20	18	15			
		15	12	20	16	27	16	18	19	21	21	388	19.4	18.8
PAS		5	4	3	3	0	0	0	0	0	0			
		10	11	7	8	0	0	0	2	7	8	68	3.4	0
Alcian Blue (x.s.) Neuraminidase (136 ImU)	4	3	7	1	4	5	7	3	3	2	5			
		13	16	19	21	22	13	15	12	18	15	204	10.2	7.5
PAS		8	6	4	2	6	16	6	2	3	0			
		20	17	14	14	10	11	12	9	16	8	184	9.2	0
Alcian Blue Untreated (1.s.)	5	43	17	39	27	27	31	15	41	31	17			
		22	23	30	45	38	28	24	24	24	11	557	27.9	26.2
PAS		7	8	9	4	7	3	4	5	8	3			
		3	5	8	4	9	8	3	9	7	3	117	5.9	5.4
Alcian Blue Water (1.s.)	6	11	13	13	12	10	10	15	19	17	9			
		63	57	48	56	48	53	62	53	59	50	678	33.9	26.2
PAS		6	5	5	3	3	5	3	6	4	3			
		13	15	20	15	15	15	13	18	13	13	193	9.7	7.8
Alcian Blue (1.s.) 0.05M NaAc buffer pH 5.0	7	23	18	16	18	16	24	21	20	16	14			
		45	56	61	50	45	44	45	56	44	50	682	34.1	30.1
PAS		0	0	0	1	2	0	0	1	0	2			
		5	2	3	5	3	4	2	3	5	6	44	2.2	0
Alcian Blue (1.s.) Neuraminidase (136 ImU)	8	6	11	8	8	4	10	4	11	12	5			
		15	20	13	22	21	25	23	22	28	27	295	14.8	12.4
PAS		12	5	5	5	3	3	8	10	2	2			
		14	9	7	9	13	11	13	14	6	7	158	7.9	6.7

*Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots \times \ldots X_n}$

Appendix VIII

The effect of commercial neuraminidase (Worthington Cl. perfringens)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 78 d.f.
Alcian Blue				
Untreated	1+5	978	6.78	<0.0002
Neuraminidase (136 ImU)	4+8	499		
PAS				
Untreated	1+5	211	-3.53	<0.001
Neuraminidase (136 ImU)	4+8	342		
Alcian Blue				
Untreated	1+5	978	-0.892	<0.50
0.05M NaAc buffer pH 5.0	3+6	1070		
PAS				
Untreated	1+5	211	3.52	<0.001
0.05M NaAc buffer pH 5.0	3+6	112		
Alcian Blue				
Untreated	1+5	978	0.0314	>0.8
Water	2+6	974		
PAS				
Untreated	1+5	211	-2.46	>0.02
Water	2+6	304		

Appendix VIII

The effect of cholera enterotoxin

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	Mean *Geom.
Alcian Blue	1													
Untreated (x.s.)		13	12	18	9	16	18	19	9	15	14			
		19	18	13	12	12	13	24	11	11	15	292	14.6	14.1
PAS		6	9	6	5	5	7	6	9	4	0			
		10	8	10	9	6	0	9	8	3	0	120	6.0	0
Alcian Blue														
0.05M NaAc buffer	2													
pH 5.5		20	29	12	25	14	9	8	0	11	14			
		9	10	3	2	0	7	14	18	9	6	210	10.5	0
PAS		10	0	0	0	10	0	0	0	0	10			
		0	10	0	10	0	0	12	9	11	8	90	4.5	0
Alcian Blue														
Neuraminidase (100 ImU)	3													
Calbiochem V.cholerae		7	6	4	2	2	4	0	0	0	0			
		9	7	7	6	4	9	10	7	3	11	98	4.9	0
PAS		12	0	9	16	9	16	0	15	0	16			
		12	15	6	7	5	0	15	16	14	17	200	10	0
Alcian Blue														
Borate Gelatin buffer	4													
pH 7.3 (x.s.)		6	5	10	8	10	5	12	3	10	3			
		12	15	9	9	13	12	20	17	19	18	216	10.8	9.5
PAS		3	8	3	5	3	0	0	0	0	0			
		5	1	6	7	6	10	7	9	6	5	84	4.2	0
Alcian Blue	5													
Cholera enterotoxin														
G 100 purified (x.s.)		24	14	14	11	10	9	9	7	8	7			
		5	8	9	9	11	11	11	7	8	7	199	10	9.4
PAS		8	9	10	13	10	16	9	15	5	0			
		6	10	13	7	12	10	8	10	7	9	187	9.4	0

* Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots \times \ldots X_n}$

Appendix VIII

The effect of cholera enterotoxin

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Cholera enterotoxin G100 purified	1 5	292 199	3.32	< 0.01
PAS				
Untreated	1	120		
Cholera enterotoxin G100 purified	5	187	-2.14	<0.05
Alcian Blue				
Borate Gelatin buffer pH7.3	4	216		
Cholera enterotoxin G100 purified	5	212	0.12	>0.80
PAS				
Borate Gelatin buffer pH7.3	4	84		
Cholera enterotoxin G100 purified	5	187	-4.76	<0.0002
Alcian Blue				
Untreated	1	292		
Borate Gelatin buffer pH7.3	4	216	2.42	<0.05
PAS				
Untreated	1	120		
Borate Gelatin buffer pH7.3	4	84	1.18	<0.50
Alcian Blue				
Untreated	1	292		
Neuraminidase (100 ImU) Calbiochem V. cholerae	3	98	7.47	<0.0002
PAS				
Untreated	1	120		
Neuraminidase (100 ImU) Calbiochem V. cholerae	3	200	-2.02	<0.10
Alcian Blue				
0.05M NaAc buffer pH 5.5	2	210		
Neuraminidase (100 ImU) Calbiochem V. cholerae	3	98	3.34	<0.01
PAS				
0.05M NaAc buffer pH 5.5	2	90		
Neuraminidase (100 ImU) Calbiochem V. cholerae	3	200	-2.96	<0.01
Alcian Blue				
Untreated	1	292		
0.05M NaAc buffer pH 5.5	2	210	2.22	< 0.05
PAS				
Untreated	1	120		
0.05M NaAc buffer pH 5.5	2	90	0.85	< 0.50

Appendix VIII

Effect of Trypsin Inhibitor and Trypsin Inhibitor + Neuraminidase

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	Mean *Geom.
Alcian Blue	1													
Untreated (x.s.)		9	9	10	5	6	9	6	3	6	5			
		6	7	0	3	5	0	4	5	0	0	98	4.9	0
PAS		13	7	9	10	8	11	0	5	9	2			
		7	6	0	6	5	0	0	0	0	6	104	5.2	0
Alcian Blue	2													
Water (x.s.)		13	9	7	0	0	3	13	9	6	6			
		5	5	2	9	4	0	0	0	0	0	91	4.6	0
PAS		12	13	9	13	7	9	8	0	5	14			
		5	8	4	6	9	6	0	0	0	0	128	6.4	0
Alcian Blue														
0.05M NaAc buffer	3													
pH 5.5 (x.s.)		9	5	4	6	6	8	6	5	9	4			
		6	3	0	5	4	5	4	0	0	0	89	4.5	0
PAS		11	9	8	10	9	11	4	10	14	18			
		9	7	8	0	5	0	10	0	6	8	157	7.9	0
Alcian Blue	4													
Semi-purified														
V. cholerae														
neuraminidase														
(5.5 ImU) (x.s.)		7	2	2	0	3	0	0	0	0	0			
		0	4	0	5	0	4	3	5	0	5	40	2	0
PAS		5	10	6	14	8	10	8	14	28	30			
		0	7	10	8	5	4	0	9	0	0	176	8.8	0
Alcian Blue (x.s.)	5													
Trypsin Inhibitor		7	6	8	5	2	5	7	7	3	2			
		0	5	3	2	3	3	0	4	4	4	80	4	0
PAS		9	13	12	8	14	5	10	12	25	7			
		8	0	10	9	10	8	3	3	6	5	177	8.9	0
Alcian Blue (x.s.)	6													
Semi-purified														
V. cholerae														
neuraminidase														
(5.5 ImU) +														
Trypsin Inhibitor		7	4	4	12	2	4	2	6	4	6			
		0	0	0	0	0	1	0	3	0	0	55	2.8	0
PAS		26	27	13	17	19	16	25	18	19	24			
		0	0	7	7	6	5	0	4	0	0	233	11.7	0

* Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots \times \ldots X_n}$

Appendix VIII

Effect of trypsin inhibitor and trypsin inhibitor + neuraminidase

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significanc (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	98	1.03	<0.50
Trypsin Inhibitor	5	80		
PAS				
Untreated	1	104	-2.44	<0.02
Trypsin Inhibitor	5	177		
Alcian Blue				
Untreated	1	98	2.13	<0.05
Semi-purified V.cholerae neuraminidase (5.5ImU) + Trypsin Inhibitor	6	55		
PAS				
Untreated	1	104	-2.70	<0.02
Semi-purified V.cholerae neuraminidase (5.5 ImU) + Trypsin Inhibitor	6	233		
Alcian Blue				
Untreated	1	98	3.3	<0.010
Semi-purified V.cholerae neuraminidase (5.5 ImU)	4	40		
PAS				
Untreated	1	104	-1.76	<0.1
Semi-purified V.cholerae neuraminidase (5.5 ImU)	4	176		
Alcian Blue				
Untreated	1	98	0.478	<0.80
0.05M NaAc buffer pH 5.5	3	89		
PAS				
Untreated	1	104	-1.92	<0.10
0.05M NaAc buffer pH 5.5	3	157		
Alcian Blue				
Untreated	1	98	0.289	<0.80
Water	2	91		
PAS				
Untreated	1	104	-0.853	<0.50
Water	2	128		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R77)

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	*Geom.
Alcian Blue	1													
Untreated (x.s.)		27	20	30	25	32	25	30	33	27	31			
		18	15	21	18	20	17	24	11	17	10	451	22.6	21.4
PAS		7	7	2	0	0	0	0	6	4	7			
		3	6	5	4	2	0	2	8	5	6	74	3.7	0
Alcian Blue														
0.05M NaAc buffer	2													
pH 5.5 (x.s.)		26	29	22	31	20	28	21	20	27	19			
		15	13	11	10	16	12	11	18	12	13	376	18.8	17.7
PAS		5	7	5	0	1	0	1	0	0	6			
		8	10	6	9	7	3	7	4	3	4	86	4.3	0
Alcian Blue	3													
Neuraminidase (V.cholerae)														
N_2 (11 ImU) + NaAc buffer														
0.2 ml + 0.5 ml (x.s.)		5	9	6	12	4	5	12	9	11	6			
		6	5	10	9	11	15	16	13	12	10	186	9.3	8.6
PAS		27	22	33	19	18	16	21	23	17	24			
		17	19	11	21	13	10	11	15	10	18	365	18.3	17.3
Alcian Blue														
Neuraminidase + Anti-ser.														
N_2 (11 ImU) + R77	4													
0.2 ml + 0.1 ml (x.s.)		13	17	16	16	13	15	10	14	13	11			
		11	12	13	15	17	12	15	19	17	14	283	14.2	14
PAS		6	5	4	12	15	10	12	21	6	12			
		16	14	11	21	12	16	11	23	9	8	244	12.2	11
Alcian Blue														
Neuraminidase + Anti-ser.														
N_2 (11 ImU) + R77	5													
0.2 ml + 0.2 ml (x.s.)		21	11	2	12	13	15	7	5	9	12			
		18	9	10	14	19	21	16	15	13	16	258	12.9	11.6
PAS		11	13	16	8	5	10	3	2	0	1			
		17	12	15	19	12	18	13	11	10	8	204	10.2	0
Alcian Blue														
Neuraminidase + Anti-ser.														
N_2 (11 ImU) + R77	6													
0.2 ml + 0.3 ml (x.s.)		8	2	3	1	5	8	2	8	6	3			
		10	5	11	9	13	15	15	19	18	13	174	8.7	6.7
PAS		31	32	25	23	23	30	33	28	34	25			
		18	16	17	15	26	14	20	22	21	11	464	23.2	22.2
Alcian Blue														
Neuraminidase + Anti-ser.														
N_2 (11 ImU) + R77	7													
0.2 ml + 0.4 ml (x.s.)		5	3	0	3	0	0	11	2	4	0			
		12	13	10	7	12	6	17	13	12	7	137	6.9	0
PAS		19	17	20	23	27	17	14	17	15	16			
		32	29	26	28	21	26	25	26	25	22	445	22.3	21.7
Alcian Blue	8													
Anti-ser. + 0.05M NaAc														
0.5 ml + 0.2 ml (x.s.)		10	14	11	7	12	6	8	10	7	11			
		13	11	15	12	11	11	15	12	8	10	214	10.7	10.4
PAS		9	13	13	11	7	8	14	11	10	8			
		6	8	9	10	14	12	9	16	9	6	203	10.2	9.8

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R77)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	451	7.21	<0.0002
Anti-ser.+0.05M NaAc 0.5 ml + 0.2 ml	8	214		
PAS				
Untreated	1	74	-7.30	<0.0002
Anti-ser.+ 0.05M NaAc 0.5 ml + 0.2 ml	8	203		
Alcian Blue				
Untreated	1	451	8.07	<0.0002
Neuraminidase+Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.4 ml	7	157		
PAS				
Untreated	1	74	-14.05	<0.0002
Neuraminidase+Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.4 ml	7	445		
Alcian Blue				
Untreated	1	451	7.05	<0.0002
Neuraminidase+Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.3 ml	6	174		
PAS				
Untreated	1	74	-11.92	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.3 ml	6	464		
Alcian Blue				
Untreated	1	451	5.06	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.2 ml	5	258		
PAS				
Untreated	1	74	-4.06	<0.001
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.2 m	5	204		
Alcian Blue				
Untreated	1	451	5.15	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.1 ml	4	283		
PAS				
Untreated	1	74	-6.30	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.1 ml	4	244		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R77)

Treatment and Standing	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	451	7.66	<0.0002
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	186		
PAS				
Untreated	1	74	-9.93	<0.0002
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	365		
Alcian Blue				
Untreated	1	451	1.75	<0.10
0.05M NaAc buffer pH 5.5	2	376		
PAS				
Untreated	1	74	-0.63	<0.80
0.05M NaAc buffer pH 5.5	2	86		
Alcian Blue				
0.05 M NaAc buffer pH 5.5	2	376	5.65	<0.0002
N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	186		
PAS				
0.05M NaAc buffer pH 5.5	2	86	-9.25	<0.0002
N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	365		
Alcian Blue				
0.05M NaAc buffer pH 5.5	2	376	2.95	<0.01
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.1 ml	4	283		
PAS				
0.05M NaAc buffer pH 5.5	2	86	-5,66	< 0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.1 ml	4	244		
Alcian Blue				
0.05M NaAc buffer pH 5.5	2	376	3.16	<0.01
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.2 ml	5	258		
PAS				
0.05M NaAc buffer pH 5.5	2	86	-4.05	<0.01
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.2 ml	5	204		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R77)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
0.05M NaAc buffer pH 5.5	2	376	5.26	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.3 ml	6	174		
PAS				
0.5 M NaAc buffer pH 5.5	2	86	-11.29	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.3 ml	6	464		
Alcian Blue				
0.05M NaAc buffer pH 5.5	2	376	6.29	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.4 ml	7	137		
PAS				
0.05M NaAc buffer pH 5.5	2	86	-13.13	<0.0002
Neuraminidase + Anti-ser. N_2 (11 ImU) + R77 0.2 ml + 0.4 ml	7	445		
Alcian Blue				
0.05M NaAc buffer pH 5.5	2	376	4.85	<0.0002
Anti-ser. + 0.05M NaAc 0.5 ml + 0.2 ml	8	214		
PAS				
0.05M NaAc buffer pH 5.5	2	86	-6.14	<0.0002
Anti-ser. + 0.05M NaAc 0.5 ml + 0.2 ml	8	203		
Alcian Blue				
Neuraminidase (*V.cholerae*) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	186	-5.09	<0.0002
Neuraminidase + Anti-ser. 0.2 ml + 0.1 ml	4	283		
PAS				
Neuraminidase (*V.cholerae*) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	365	3.39	<0.01
Neuraminidase + Anti-ser. 0.2 ml + 0.1 ml	4	244		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R77)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	186	-2.62	<0.02
Neuraminidase + Anti-ser. 0.2 ml + 0.2 ml	5	258		
PAS				
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	365	4.39	<0.0002
Neuraminidase + Anti-ser. 0.2 ml + 0.2 ml	5	204		
Alcian Blue				
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	186	0.41	<0.80
Neuraminidase + Anti-ser. 0.2 ml + 0.3 ml	6	174		
PAS				
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	365	-2.46	<0.02
Neuraminidase + Anti-ser. 0.2 ml + 0.3 ml	6	464		
Alcian Blue				
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.5 ml	3	186	1.72	<0.10
Neuraminidase + Anti-ser. 0.2 ml + 0.4 ml	7	137		
PAS				
Neuraminidase (V.cholerae) N_2 (11 ImU) + NaAc buffer 0.2 ml + 0.2 ml	3	365	-2.27	<0.05
Neuraminidase + Anti-ser. 0.2 ml + 0.4 ml	7	445		

Appendix VIII

The effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	Mean *Geom.
Alcian Blue	1													
Untreated (x.s.)		20	22	23	18	16	19	24	23	18	20			
		18	16	19	25	20	23	22	15	21	17	299	20	19.8
PAS		6	13	6	4	0	0	4	2	1	7			
		9	11	14	13	12	2	10	6	1	10	151	6.6	0
Alcian Blue														
0.05M NaAc buffer	2	6	5	7	10	4	5	9	0	11	7			
pH 5.0 (x.s.)		11	18	23	13	12	13	17	21	17	12	221	11.1	0
PAS		23	33	14	19	13	14	13	14	15	24			
		21	39	27	24	15	19	16	25	29	19	416	20.8	19.7
Alcian Blue	3													
Neuraminidase (Cl.perfr.)		0	0	0	2	0	0	3	2	0	0			
(136 ImU) (x.s.)		0	6	5	7	6	8	7	6	3	5	60	3	0
PAS		28	34	28	23	17	25	40	37	23	21			
		25	21	19	18	19	16	18	22	21	26	481	24.1	23.3
Alcian Blue														
Neuraminidase + 0.05M	4	8	5	9	9	5	6	8	3	3	0			
NaAc (136 ImU)(x.s.)		14	16	11	15	14	16	13	16	17	10	198	9.9	0
		9	8	8	10	10	8	3	6	8	7			
PAS		25	27	22	33	24	20	15	21	19	16	299	15	12.6
Alcian Blue	5													
Neuraminidase + Anti-ser.														
(136 ImU) + R78		7	3	0	10	5	3	5	6	5	3			
0.2 ml + 0.1 ml (x.s.)		4	4	8	20	27	11	10	10	13	11	165	8.3	0
PAS		10	5	4	25	12	22	29	8	7	5			
Alcian Blue (x.s.)		11	17	19	21	18	20	17	14	14	17	295	14.8	12.9
Neuraminidase + Anti-ser.														
(136 ImU) + R78	6													
0.2 ml + 0.2 ml		4	5	6	3	8	3	5	1	3	5			
		13	21	14	12	16	12	11	10	8	13	173	8.7	6.9
PAS		19	23	26	15	12	20	14	13	13	10			
		27	26	32	17	18	15	12	21	33	16	382	19.1	18.0
Alcian Blue	7													
Neuraminidase + Anti-ser.														
(136 ImU) + R78		12	26	12	11	9	10	7	6	8	3			
0.2 ml + 0.3 ml		19	32	33	29	22	23	18	19	16	11	326	16.3	13.8
PAS		17	22	16	33	19	22	32	16	18	8			
		30	33	32	33	27	34	23	27	28	17	487	24.4	23
Alcian Blue (x.s.)	8													
Neuraminidase + Anti-ser.														
(136 ImU) + R78		7	5	4	0	0	0	0	0	0	0			
0.2 ml + 0.5 ml		17	21	19	0	0	0	0	0	2	1	76	3.8	0
PAS		32	35	30	32	30	45	30	34	29	14			
		29	40	25	25	29	34	20	26	22	30	591	29.6	28.7

*Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots \times \ldots X_n}$

Appendix VIII

The effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	399	9.70	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.5 ml	8	76		
PAS				
Untreated	1	131	-12.45	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.5 ml	8	591		
Alcian Blue				
Untreated	1	399	1.75	<0.10
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.3 ml	7	326		
PAS				
Untreated	1	131	-8.92	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.3 ml	7	487		
Alcian Blue				
Untreated	1	399	8.43	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.2 ml	6	173		
PAS				
Untreated	1	131	-6.81	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.2 ml	6	382		
Alcian Blue				
Untreated	1	399	7.52	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.1 ml	5	165		
PAS				
Untreated	1	131	-4.35	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.1 ml	5	295		

Appendix VIII

The effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test p value 38 d.f.
Alcian Blue				
Untreated	1	399	7.66	<0.0002
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	198		
PAS				
Untreated	1	131	-3.89	<0.001
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	299		
Alcian Blue				
Untreated	1	399	18.39	<0.0002
Neuraminidase (Cl.perfrin.) (136 ImU)	3	60		
PAS				
Untreated	1	131	-9.64	<0.0002
Neuraminidase (Cl.perfrin.) (136 ImU)	3	481		
Alcian Blue				
Untreated	1	399	6.00	<0.0002
0.05M NaAc buffer pH 5.0	2	221		
PAS				
Untreated	1	131	-7.40	<0.0002
0.05M NaAc buffer pH 5.0	2	416		
Alcian Blue				
0.05M NaAc buffer pH 5.0	2	221	5.40	<0.0002
Neuraminidase (Cl.perfrin.) (136 ImU)	3	60		
PAS				
0.05M NaAc buffer pH 5.0	2	416	-1.48	<0.20
Neuraminidase (Cl.perfrin.) (136 ImU)	3	481		
Alcian Blue				
0.05M NaAc buffer pH 5.0	2	221	0.65	<0.80
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	198		
PAS				
0.05M NaAc buffer pH 5.0	2	416	2.36	<0.050
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	299		

Appendix VIII

The effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
0.05M NaAc buffer pH 5.0	2	221	1.44	<0.20
Neuraminidase + Anti-ser.	5	165		
(136 ImU) + R78				
0.2 ml + 0.1 ml				
PAS				
0.05M NaAc buffer pH 5.0	2	416	2.69	<0.02
Neuraminidase + Anti-ser.	5	295		
(136 ImU) + R78				
0.2 ml + 0.1 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.0	2	221	1.35	<0.20
Neuraminidase + Anti-ser.	6	173		
(136 ImU) + R78				
0.2 ml + 0.2 ml				
PAS				
0.05M NaAc buffer pH 5.0	2	416	0.77	<0.50
Neuraminidase + Anti-ser.	6	382		
(136 ImU) + R78				
0.2 ml + 0.2 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.0	2	221	-2.19	<0.05
Neuraminidase + Anti-ser.	7	326		
(136 ImU) + R78				
0.2 ml + 0.3 ml				
PAS				
0.05M NaAc buffer pH 5.0	2	416	-1.52	<0.20
Neuraminidase + Anti-ser.	7	487		
(136 ImU) + R78				
0.2 ml + 0,3 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.0	2	221	3.56	<0.01
Neuraminidase + Anti-ser.	8	76		
(136 ImU) + R78				
0.2 ml + 0.5 ml				
PAS				
0.5M NaAc buffer pH 5.0	2	416	-3.95	<0.001
Neuraminidase + Anti-ser.	8	591		
(136 ImU) + R78				
0.2 ml + 0.5 ml				

Appendix VIII

The effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	198	0.91	<0.50
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.1 ml	5	165		
PAS				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	299	0.082	>0.80
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.1 ml	5	295		
Alcian Blue				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	198	0.76	<0.50
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.2 ml	6	173		
PAS				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	299	-1.72	<0.10
Neuraminidase + Anti-ser. (136 ImU) 0.2 ml + 0.2 ml	6	382		
Alcian Blue				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	198	-2.79	<0.01
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.3 ml	7	326		
PAS				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	299	-3.71	<0.001
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.3 ml	7	487		

Appendix VIII

The effect of anti-neuraminidase serum (DST R78) against non-cholera neuraminidase

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	198	3.18	<0.01
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.5 ml	8	76		
PAS				
Neuraminidase + 0.05M NaAc (136 ImU) 0.2 ml + 0.5 ml	4	299	-6.04	<0.0002
Neuraminidase + Anti-ser. (136 ImU) + R78 0.2 ml + 0.5 ml	8	591		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Individual counts field^{-1}	Total count	Mean Arith.	Mean *Geom.
Alcian Blue Untreated (x.s.)	1	25 20 23 16 25 18 44 11 32 26			
		22 16 17 10 13 13 12 14 18 19	394	19.7	18.4
PAS		7 13 4 3 2 11 5 10 0 0			
		9 15 0 6 7 9 7 12 3 5	128	6.4	0
Alcian Blue Water (x.s.)	2	36 32 20 29 15 25 16 15 8 20			
		11 16 13 18 12 22 18 14 12 13	365	18.3	17.0
PAS		4 6 8 5 4 9 13 9 10 0			
		6 3 0 8 5 8 6 11 7 0	122	6.1	0
Alcian Blue 0.05M NaAc buffer pH 5.5 (x.s.)	3	10 22 31 16 17 19 21 18 10 25			
		24 26 20 14 20 12 14 15 24 22	380	19	18.2
PAS		14 12 2 0 3 1 3 6 8 0			
		25 16 8 3 0 0 6 16 14 5	142	7.1	0
Alcian Blue (x.s.) Neuraminidase (V.cholerae)(5.5 ImU)	4	18 13 19 7 9 11 5 7 11 9			
		11 5 18 10 16 21 8 13 4 17	232	11.6	10.5
PAS		22 17 21 18 23 16 25 18 12 21			
		10 11 9 8 13 12 11 9 8 15	299	15	14
Alcian Blue (x.s.) Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	20 18 6 11 23 12 13 18 11 22			
		13 11 17 7 14 10 19 15 17 19	296	14.8	14
PAS		16 19 12 9 2 21 17 13 8 4			
		12 8 9 14 8 9 11 9 7 3	211	10.6	9.2
Alcian Blue (x.s.) Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.05 ml	6	16 20 19 14 23 25 24 23 26 20			
		22 15 17 10 26 28 19 21 14 11	393	19.7	18.9
PAS		0 0 0 0 0 0 0 0 0 0			
		0 0 0 0 0 0 0 0 0 0	0	0	0
Alcian Blue (x.s.) Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	18 16 15 10 19 12 18 17 10 25			
		19 18 32 23 26 24 15 24 21 16	378	18.9	18.1
PAS		0 0 0 0 0 0 0 0 0 0			
		1 2 0 0 0 0 6 1 3 0	13	1	0
Alcian Blue (x.s.) IgG fract. + 0.05M NaAc 0.25 ml + 0.1 ml	8	41 24 29 30 18 40 12 15 18 12			
		17 10 18 32 30 16 7 14 8 9	390	20	17.6
PAS		3 1 0 0 0 0 0 3 0 0			
		0 1 1 5 0 1 0 4 2 1	22	1.1	0

*Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots x \ldots X_n}$

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	*Geom.
Alcian Blue (x.s.)	9													
Neuraminidase + Anti-ser.														
N_2 (5.5 ImU) + R78		8	0	10	3	2	4	0	0	5	6			
0.1 ml + 0.05 ml		10	9	7	9	18	11	2	3	11	4	122	6.1	0
PAS		46	47	41	30	38	26	25	24	47	20			
		11	19	17	10	14	18	13	16	13	10	485	24.3	21.3
Alcian Blue (x.s.)	10													
Neuraminidase + Anti-ser.														
N_2 (5.5 ImU) + R78		12	24	22	8	5	6	10	0	14	15			
0.1 ml + 0.2 ml		21	8	7	11	16	7	9	6	18	13	232	11.6	0
PAS		55	39	35	67	42	26	40	29	26	22			
		17	12	14	19	14	13	11	15	21	12	529	26.5	22.8
Alcian Blue (x.s.)	11													
Neuraminidase + Anti-ser.														
N_2 (5.5 ImU) + R78		6	8	9	0	0	0	5	7	0	4			
0.1 ml + 0.25 ml		9	16	24	13	3	13	16	13	6	10	162	8.1	0
PAS		40	63	28	41	14	24	36	37	30	24			
		13	21	15	21	10	12	9	10	12	15	475	23.8	20.4
Alcian Blue (x.s.)	12													
Anti-ser. + 0.05M NaAc		33	7	0	4	0	10	0	6	0	3			
0.25 ml + 0.1 ml		14	22	25	9	0	14	17	14	3	12	193	9.7	0
PAS		28	27	41	28	25	31	25	21	29	39			
		18	13	12	14	16	15	13	7	10	17	429	21.5	19.4

*Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots \times \ldots X_n}$

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical sitnificance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	394	3.64	<0.001
Anti-ser. + 0.05M NaAc 0.25 ml + 0.1 ml	12	193		
PAS				
Untreated	1	128	-6.43	<0.0002
Anti-ser. + 0.05M NaAc 0.25 ml + 0.1 ml	12	429		
Alcian Blue				
Untreated	1	394	5.02	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.25ml	11	162		
PAS				
Untreated	1	128	-5.28	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.25 ml	11	475		
Alcian Blue				
Untreated	1	394	3.54	<0.01
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.20 ml	10	232		
PAS				
Untreated	1	128	-5.55	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.20 ml	10	529		
Alcian Blue				
Untreated	1	394	6.50	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.05 ml	9	122		
PAS				
Untreated	1	128	-5.85	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.05 ml	9	485		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	394	0.068	>0.80
IgG fract. + 0.05 M NaAc 0.25 ml + 0.1 ml	8	390		
PAS				
Untreated	1	128	5.06	<0.0002
IgG fract. + 0.05 M NaAc 0.25 ml + 0.1 ml	8	22		
Alcian Blue				
Untreated	1	394	0.364	<0.80
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	378		
PAS				
Untreated	1	128	5.50	<0.0002
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	13		
Alcian Blue				
Untreated	1	394	0.023	>0.80
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.05 ml	6	393		
PAS				
Untreated	1	128	6.46	<0.0002
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.05 ml	6	0		
Alcian Blue				
Untreated	1	394	2.33	<0.05
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296		
PAS				
Untreated	1	128	-2.75	<0.01
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211		
Alcian Blue				
Untreated	1	394	3.78	<0.001
Neuraminidase (*V. cholerae*) (5.5 ImU)	4	232		
PAS				
Untreated	1	128	-5.47	<0.0002
Neuraminidase (*V. cholerae*) (5.5 ImU)	4	299		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	394	0.318	<0.80
0.05M NaAc buffer pH 5.5	3	380		
PAS				
Untreated	1	128	-0.379	<0.80
0.05M NaAc buffer pH 5.5	3	142		
Alcian Blue				
Untreated	1	394	0.593	<0.80
Water	2	365		
PAS				
Untreated	1	128	0.235	<0.80
Water	2	122		
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	4.37	<0.0002
Neuraminidase (V.cholerae) (5.5 ImU)	4	232		
PAS				
0.05M NaAc buffer pH 5.5	3	142	-3.98	<0.001
Neuraminidase (V.cholerae) (5.5 ImU)	4	299		
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	2.56	<0.02
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296		
PAS				
0.05M NaAc buffer pH 5.5	3	142	-1.79	<0.10
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211		
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	7.91	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.05 ml	9	122		
PAS				
0.05M NaAc buffer pH 5.5	3	142	-5.23	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.05 ml	9	485		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	3.94	<0.001
Neuraminidase + Anti-ser.	10	232		
N_2 (5.5 ImU) + R78				
0.1 ml + 0.20 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	142	-5.08	<0.0002
Neuraminidase + Anti-ser.	10	529		
N_2 (5.5 ImU) + R78				
0.1 ml + 0.20 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	5.73	<0.0002
Neuraminidase + Anti-ser.	11	162		
N_2 (5.5 ImU) + R78				
0.1 ml + 0.25 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	142	-4.76	<0.0002
Neuraminidase + Anti-ser.	11	475		
N_2 (5.5 ImU) + R78				
0.1 ml + 0.25 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	3.84	<0.001
Anti-ser. + 0.05M NaAc	12	193		
0.25 ml + 0.1 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	142	-5.45	<0.0002
Anti-ser. + 0.05M NaAc	12	429		
0.25 ml + 0.1 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	-0.38	<0.80
Neuraminidase + IgG fract.	6	393		
N_2 (5.5 ImU) + IgG (R78)				
0.1 ml + 0.05 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	142	4.56	<0.0002
Neuraminidase + IgG fract.	6	0		
N_2 (5.5 ImU) + IgG (R78)				
0.1 ml + 0.05 ml				

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	0.06	>0.80
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	378		
PAS				
0.05M NaAc buffer pH 5.5	3	142	4.05	<0.001
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	13		
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	380	-0.19	>0.80
IgG fract. + 0.05M NaAc 0.25 ml + 0.1 ml	8	390		
PAS				
0.05M NaAc buffer pH 5.5	3	142	3.76	<0.001
IgG fract. + 0.05M NaAc 0.25 ml + 0.1 ml	8	22		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296	5.83	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.05 ml	9	122		
PAS				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211	-4.42	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.05 ml	9	485		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296	1.82	<0.10
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.20 ml	10	232		
PAS				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211	-4.35	<0.0002
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.20 ml	10	529		

Appendix VIII

The effect of specific anti-neuraminidase serum (DST R78) and the IgG fraction (of DST R78)

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296	3.75	<0.001
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.25 ml	11	162		
PAS				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211	-3.96	<0.001
Neuraminidase + Anti-ser. N_2 (5.5 ImU) + R78 0.1 ml + 0.25 ml	11	475		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296	-3.09	<0.01
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.05 ml	6	393		
PAS				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211	9.30	<0.0002
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.05 ml	6	0		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	296	-2.50	<0.02
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	378		
PAS				
Neuraminidase + NaAc buffer N_2 (5.5 ImU) 0.1 ml + 0.25 ml	5	211	8.37	<0.0002
Neuraminidase + IgG fract. N_2 (5.5 ImU) + IgG (R78) 0.1 ml + 0.25 ml	7	13		

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	Mean *Geom.
Alcian Blue	1													
Untreated (x.s.)		28	38	27	31	36	35	25	17	25	17			
		40	20	14	18	17	20	18	12	17	22	477	22.9	22.5
PAS		0	5	0	0	1	2	5	4	6	7			
		8	4	12	6	3	7	8	3	7	10	98	4.9	0
Alcian Blue	2													
Water (x.s.)		16	13	22	25	12	14	18	11	26	20			
		12	14	19	11	9	12	10	12	18	16	310	15.5	14.8
PAS		1	0	9	12	1	2	3	1	4	0			
		4	6	5	6	5	4	0	7	5	5	80	4	0
Alcian Blue	3													
0.05M NaAc buffer pH 5.5		17	16	7	8	14	8	19	23	14	7			
		23	21	22	15	12	13	15	11	13	14	292	14.6	13.7
PAS		5	3	11	0	2	2	0	0	0	1			
		9	10	12	13	7	14	5	7	15	10	126	6.3	0
Alcian Blue (x.s.)	4													
Neuraminidase (V.cholerae)		11	17	9	10	9	13	9	15	13	11			
(11 ImU)		16	18	22	18	19	17	13	12	16	12	280	14	13.5
PAS		3	0	3	1	0	4	3	2	6	4			
		0	7	7	15	12	6	6	4	10	0	93	4.7	0
Alcian Blue (x.s.)	5													
Nueraminidase+NaAc buffer														
N_2 (11 ImU)		27	17	16	22	18	17	28	27	29	20			
0.2 ml + 0.5 ml		23	26	25	27	24	21	20	18	15	10	430	21.5	20.8
PAS		1	0	0	2	3	1	4	3	0	4			
		0	0	3	5	1	6	3	2	3	8	49	2.5	0
Alcian Blue (x.s.)	6													
Neuraminidase+Serum														
N_2 (11 ImU) + Normal ser.		27	25	34	17	30	24	17	12	21	16			
0.2 ml + 0.1 ml		19	12	18	5	14	11	19	14	6	12	353	17.7	16
PAS		12	15	10	9	7	15	20	6	8	13			
		13	18	14	10	12	13	11	10	9	7	232	11.6	11.1
Alcian Blue (x.s.)	7													
Neuraminidase+Serum														
N_2 (11 ImU) + Normal ser.		24	26	29	12	29	14	30	19	18	15			
0.2 ml + 0.2 ml		15	19	11	11	13	10	12	13	11	14	345	17.3	16.2
PAS		8	6	5	0	0	0	0	0	0	1			
		14	10	0	0	0	0	0	0	0	0	44	2.2	0
Alcian Blue (x.s.)	8													
Neuraminidase+Normal ser.														
0.2 ml + 0.3 ml		28	14	10	12	13	25	10	13	12	21			
		10	9	8	9	7	11	16	8	9	7	252	12.6	11.6
PAS		0	4	6	5	10	0	0	0	0	0			
		0	0	0	2	4	0	0	0	2	0	33	1.7	0

*Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots x \ldots X_n}$

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Individual counts field^{-1}										Total count	Mean Arith.	*Geom.
Alcian Blue (x.s.)	9													
Neuraminidase+Serum														
N_2 (11 ImU)+Normal ser.		14	5	9	3	14	18	22	14	20	6			
0.2 ml + 0.4 ml		12	7	5	8	13	12	10	17	10	11	230	11.5	10.3
PAS		17	21	19	17	9	6	9	8	12	10			
		11	9	12	10	7	19	14	18	9	7	244	12.2	11.4
Alcian Blue (x.s.)	10													
Normal ser. + 0.05M NaAc		32	29	15	18	11	26	29	19	26	25			
0.5 ml + 0.2 ml		12	13	8	10	4	13	12	7	16	15	340	17	15
PAS		16	23	12	28	16	11	19	10	19	21			
		14	9	6	17	9	10	15	13	10	22	300	15	14

*Geometric mean = $\sqrt[n]{X_1 \times X_2 \ldots x \ldots X_n}$

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	477	2.46	<0.02
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.1 ml	6	353		
PAS				
Untreated	1	98	-6.02	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.1 ml	6	232		
Alcian Blue				
Untreated	1	477	1.07	<0.50
Neuraminidase + 0.05M NaAc N_2 (11 ImU) 0.2 ml + 0.5 ml	5	430		
PAS				
Untreated	1	98	2.72	<0.01
Neuraminidase + 0.05M NaAc N_2 (11 ImU) 0.2 ml + 0.5 ml	5	49		
Alcian Blue				
Untreated	1	477	4.79	<0.0002
Neuraminidase (*V.cholerae*) (11 ImU)	4	280		
PAS				
Untreated	1	98	0.210	>0.80
Neuraminidase (*V.cholerae*) (11 ImU)	4	93		
Alcian Blue				
Untreated	1	477	4.21	<0.0002
0.05M NaAc buffer pH 5.5	3	292		
PAS				
Untreated	1	98	-1.01	<0.50
0.05M NaAc buffer pH 5.5	3	126		
Alcian Blue				
Untreated	1	477	3.83	<0.001
Water	2	310		
PAS				
Untreated	1	98	0.871	<0.50
Water	2	80		

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Untreated	1	477	2.61	<0.02
Normal ser. + 0.05M NaAc 0.5 ml + 0.2 ml	10	340		
PAS				
Untreated	1	98	-6.83	<0.0002
Normal ser. + 0.05M NaAc 0.5 ml + 0.2 ml	10	300		
Alcian Blue				
Untreated	1	477	5.60	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.4 ml	9	230		
PAS				
Untreated	1	98	-5.67	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.4 ml	9	244		
Alcian Blue				
Untreated	1	477	4.92	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.3 ml	8	252		
PAS				
Untreated	1	98	3.33	<0.01
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.3 ml	8	33		
Alcian Blue				
Untreated	1	477	2.74	<0.01
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.2 ml	7	345		
PAS				
Untreated	1	98	2.26	<0.05
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.2 ml	7	44		

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	0.42	<0.80
Neuraminidase (V.cholerae)	4	280		
(11 ImU)				
PAS				
0.05M NaAc buffer pH 5.5	3	126	1.12	<0.50
Neuraminidase (V.cholerae)	4	93		
(11 ImU)				
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	-4.26	<0.0002
Neuraminidase + NaAc buffer	5	430		
N_2 (11 ImU)				
0.2 ml + 0.5 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	126	3.09	<0.01
Neuraminidase + NaAc buffer	5	48		
N_2 (11 ImU)				
0.2 ml + 0.5 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	-1.50	<0.20
Neuraminidase + Serum	6	353		
N_2 (11 ImU) + Normal ser.				
0.2 ml + 0.1 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	126	-3.74	<0.001
Neuraminidase + Serum	6	232		
N_2 (11 ImU) + Normal ser.				
0.2 ml + 0.1 ml				
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	-1.40	<0.20
Neuraminidase + Serum	7	345		
N_2 (11 ImU) + Normal ser.				
0.2 ml + 0.2 ml				
PAS				
0.05M NaAc buffer pH 5.5	3	126	2.77	<0.010
Neuraminidase + Serum	7	44		
N_2 (11 ImU) + Normal ser.				
0.2 ml + 0.2 ml				

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	1.16	<0.50
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.3 ml	8	252		
PAS				
0.05M NaAc buffer pH 5.5	3	126	3.54	<0.01
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.3 ml	8	33		
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	1.91	<0.10
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.4 ml	9	230		
PAS				
0.05M NaAc buffer pH 5.5	3	126	-3.78	<0.001
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.4 ml	9	244		
Alcian Blue				
0.05M NaAc buffer pH 5.5	3	292	-1.11	<0.50
Normal ser. + 0.05M NaAc 0.5 ml + 0.2 ml	10	340		
PAS				
0.05M NaAc buffer pH 5.5	3	126	-5.06	<0.0002
Normal ser. + 0.05M NaAc 0.5 ml + 0.2 ml	10	300		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	430	1.88	<0.10
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.1 ml	6	353		
PAS				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	49	-9.56	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.1 ml	6	232		

Appendix VIII

The effect of non-immune serum

Treatment and Staining	Prep. No.	Total count	t-value	Statistical significance (two-tailed test) p value 38 d.f.
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5ml	5	430	2.24	<0.05
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.2 ml	7	345		
PAS				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	49	0.24	>0.80
Neuraminidase +Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.2 ml	7	44		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	430	5.12	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.3 ml	8	252		
PAS				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	49	1.01	<0.50
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.3 ml	8	33		
Alcian Blue				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	430	6.13	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Serum 0.2 ml + 0.4 ml	9	230		
PAS				
Neuraminidase + NaAc buffer N_2 (11 ImU) 0.2 ml + 0.5 ml	5	49	-8.43	<0.0002
Neuraminidase + Serum N_2 (11 ImU) + Normal ser. 0.2 ml + 0.4 ml	9	244		

Appendix IX

ABSORPTION OF NEURAMINIDASE ACTIVITY

(T.B.A. ASSAY)

Appendix IX

Neuraminidase preparation	Rabbit anti-neuraminidase DST R77 (ml)	Corrected values of NANA released* (µg)
N_2 1.57 ImU	0.3	0
N_2 1.57 ImU	0.4	1.50
N_2 1.57 ImU	0.5	1.50
N_2 1.57 ImU	0	3.60
N_3 1.43 ImU	0.3	0
N_3 1.43 ImU	0.4	0.25
N_3 1.43 ImU	0.5	0.50
N_3 1.43 ImU	0	0.25
N_4 1.0 ImU	0.3	1.25
N_4 1.0 ImU	0.4	1.50
N_4 1.0 ImU	0.5	2.50
N_4 1.0 ImU	0	3.00
Sigma 3.4 ImU	0.5	1.00
Sigma 3.4 ImU	0	3.3

*Mucin (1.6 mg) was added to each test. All values were corrected for the release of NANA i) from serum in the absence of mucin and ii) from mucin by serum in the absence of enzyme.
NANA was measured by the T.B.A. assay.