To My Mother, Father

and My Twin Brother

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TOXINS AND ENZYMES OF *CLOSTRIDIUM PERFRINGENS* TYPE A.

CYRIL J. SMYTH

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"As I aim at nothing but Truth, and, so far as in me lieth, to point out Mistakes that may have crept into certain Matters; I hope that in so doing those I chance to censure will not take it ill; and if they would expose any Errors in my own Discoveries, I'd esteem it a Service; all the more, because 'twould thereby give me Encouragement towards the Attaining of a nicer Accuracy."

A. van Leeuwenhoek (1700)

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"No scientist lives in isolation. What he is, is determined as much by his teachers and all the other influences of his cultural environment as by his innate individuality and his own efforts."

H.S.D. Garven.

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Object of the Research

The principal object of this investigation was to devise a convenient and rapid method for the purification of the α-toxin of *Clostridium perfringens* type A. Previous workers had experienced considerable difficulty in obtaining this factor free of the variety of extracellular toxins and enzymes produced by this organism. Despite the fact that it was the first toxin to have its mode of action defined in biochemical terms, many features remain in doubt in particular its physical characterisation. Moreover in light of the findings of Johnson and Bonventre (1967) with *Bacillus cereus* toxin, it was decided to re-investigate the possibility that the lecithinase, hot-cold haemolytic and lethal activities of this toxin were manifestations of a single moiety.

In 1967 and 1968 Vesterberg and Wadström published a series of studies on the use of a new electrophoretic technique termed isoelectric focusing, in the purification and characterisation of the extracellular products of *Staphylococcus aureus*. Not only was the resolving power of this technique clearly demonstrated for a complex mixture of factors, but several of the staphylococcal products were shown to exist in multiple forms. Using isoelectric focusing, investigations were undertaken into the reported occurrence of multiple forms of *Cl. perfringens* α-toxin and their possible role in the unitarian concept of the biological activities of α-toxin. Moreover, a study of α-toxin was merited on the basis of the increasing use of α-toxin from commercial sources as a biochemical probe of membrane structure.

As with many investigations avenues of research presented themselves as a by-product of these primary objectives. Indeed the simultaneous purification of collagenase, hyaluronidase and θ-toxin by isoelectric focusing allowed these factors to be characterised, and aspects of the cytolytic action of the θ-toxin of this organism as well as the α-toxin, to be investigated.

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LIST OF ABBREVIATIONS

Å	=	Ångstrom
ala	=	alanine
ATCC	=	American Type Culture Collection
AU	=	Azocoll digesting unit of collagenase activity
C	=	Centigrade
cm	=	centimetre
CM-	=	Carboxy methyl-
CN	=	Culture Number
p-CMB	=	Parachloromercuribenzoate
DEAE-	=	Diethylaminoethyl-
DICaB	=	Dimethylglutaric acid, isotonic saline, calcium acetate, bovine serum albumin buffer (α-toxin diluent, page 5A)
DNase	=	Deoxyribonuclease
DPFP	=	Di-isopropylfluorophosphate
E	=	Extinction
EDTA	=	Ethylene diamine tetra-acetate, disodium salt
ETU	=	Egg turbidity unit of α-toxin
g	=	gram or acceleration of free fall
gly	=	glycine
hr	=	hour
HU	=	Haemolytic unit of α-toxin or θ-toxin
IU	=	International unit of hyaluronidase
K_m	=	Michaelis constant - substrate concentration at half-maximal velocity
LD_{50}	=	Dose killing 50% of animals under test
leu	=	leucine
LPC	=	Lysophosphatidylcholine, lysolecithin

LU = Lethality Unit of α-toxin

LV = Lecithovitellin

Lyso PTC = Lysophosphatidylcholine

M = Molar

mamp = milliamp

mg = milligram

min = minute

ml = millilitre

MLD = Minimum Lethal Dose

mm = millimetre

mM = millimolar

MSE = Measuring and Scientific Equipment

M-I = Modified Medium of Adams et al. (see Appendix I)

M-II = Modified Medium of Murata (see Appendix I)

μ = micro

μg = microgram

μl = microlitre

NANA = N-acetyl neuraminic acid

NCIB = National Collection of Industrial Bacteria

NCTC = National Collection of Type Cultures

ND = Not determined

ng = nanogram

NH_2 = Amino

nm = Nanometer

NT = Not tested

OD = Optical density

-OH = -hydroxyl

OP = Orthophenanthroline

PBS = Phosphate buffered saline

PCU = Phospholipase C unit of α-toxin

pH = -Logarithm of the hydrogen ion concentration

pI = Isoelectric point

ppt = Precipitate

PTA = Phosphatidic acid

PTC = Phosphatidylcholine

PTE = Phosphatidylethanolamine

PTI = Phosphatidylinositol

PTS = Phosphatidylserine

PVP = Polyvinylpyrrolidone

rpm = revolutions per minute

SDS = Sodium dodecyl sulphate

sec = second

-SH = -sulphydryl

SLO = Streptolysin O

Sph = Sphingomyelin

SSES = Standardised erythrocyte suspension (sheep)

TCA = Trichloroacetic acid

TSH = Thyroid stimulating hormone

UV = Ultraviolet

v/v = volume for volume

V = Volt

W = Watt

WHO = World Health Organisation

w/v = weight for volume

Greek Letters and Symbols.

α	Alpha
β	Beta
γ	Gamma
δ	Delta
ϵ	Epsilon
ι	Iota
ζ	Zeta
η	Eta
θ	Theta
κ	Kappa
λ	Lambda
μ	Mu
ν	Nu
$>$	Greater than
$<$	Less than
"	Inch
$\rightarrow$	To

REVIEW OF THE LITERATURE

SECTION I : EMERGENCE OF CLOSTRIDIUM PERFRINGENS TYPE A

A. ANAEROBES - A HISTORICAL REVIEW

1. Discovery and Definition.

'Nous arrivons donc à cette double proposition:

1. Le ferment butyrique est un infusoire.
2. Cet infusoire vit sans gaz oxygène libre.

C'est, je crois, le premier exemple connu des ferments animaux et aussi d'animaux sans gaz oxygène libre.'

Pasteur, L. (1861a).

With these words just over a century ago, Pasteur heralded his discovery of anaerobiosis, 'la vie sans l'air', during his researches on butyric fermentation. The rod-shaped organism responsible for this type of fermentation was named Vibrion butyrique by Pasteur. This non-pathogenic spore-forming anaerobe is now known as Clostridium butyricum and is the type species of the genus. As a result of this study and subsequent studies on the fermentation of tartarate and alcohol (Pasteur, 1861b;1863), he coined the terms aerobe and anaerobe 'to denote the existence of two classes of lower beings, the ones unable to live without the presence of oxygen, and the others capable of multiplying ad infinitum out of contact of this gas'.

Nevertheless, although Pasteur may be regarded as the "Father of Anaerobiosis", he was not the first to describe organisms that could exist in the absence of air. Leeuwenhoek in his 32nd letter to the Royal Society in 1680 described an experiment in which he sealed off powdered pepper and fresh rain water in a glass tube (plate 1) (see Bulloch, 1938; Dobell, 1932). His 'animalcules' developed in this 'rarified' atmosphere. Furthermore, in 1776 Spallanzani (cited by Bulloch, 1938) showed that 'animalcules' developed under conditions of high vaccuum.

Plate 1: The pepper-tube of van Leeuwenhoek (1680)

The glass tube was filled to BK with pounded pepper and then to CI with clean rain water collected on May 26th 1680 in a clean china dish ("in which no victuals had been put for quite 10 years"). The tube was then sealed. The tube was left sealed for 5 days. The 'animalcules' he observed were different from those observed in a tube that was pulled out to a point (G) without the tube actually being sealed.

Dobell (1932)

A FIGURE FROM LETTER 32 (14 JUNE 1680)

Pasteur's ideas on anaerobiosis found ready opponents, since the dependence of life on oxygen was one of the central tenets in biology at that time. The principal criticisms were that it was difficult to purge apparatus free of oxygen and that yeasts lost their power of reproduction in the continued absence of air. However, in 1884 Lachowicz and Nencki vindicated Pasteur's experiments by demonstrating that putrefaction occurred under conditions in which no free oxygen was detected by the most sensitive tests available at that time.

2. Anaerobes as Causative Agents of Disease.

The association of anaerobes with disease processes was first clearly demonstrated by Feser (1876), who showed that Bacterium chauvoei, discovered in the previous year by Bollinger (1875), was the cause of blackquarter (blackleg) disease in cattle and sheep. Pasteur and Joubert (1877) described an anaerobic organism which they termed Vibrion septique, isolated from the blood of an infected guinea pig. This organism we now recognise as Clostridium septicum. Between 1875 and 1922 most of the pathogenic clostridia we recognise today were described and isolated in pure culture (Topley and Wilson, 1964).

The first description of Clostridium perfringens can be attributed to Achalme (1891), who isolated this bacterium from a case of acute rheumatic arthritis. Welch and Nuttal (1892) isolated the same organism from a cadaver and called it Bacillus aerogenes capsulatus. Fraenkel (1893) was the first to describe Cl. perfringens as the causative organism of gas gangrene. He designated his bacillus, Bacillus phlegmonis emphysematosae. Klein (1895) isolated a spore-forming anaerobe from the stools of 204 patients suffering from diarrhoea in a hospital. His organism, Bacillus enteritidis sporogenes, caused a syndrome of short duration characterised by severe abdominal pain

and a marked absence of vomiting. In 1899 Andrewes again isolated Klein's bacillus from cases of chronic diarrhoea associated with consumption of rice pudding. Veillon and Zuber (1898) named the organism Bacillus perfringens and Migula (1900) was responsible for the designation Bacillus welchii. Thus, as with many anaerobes, Achalme's bacillus had a number of synonyms by the beginning of this century.

3. The Genus Clostridium.

A comprehensive review of the arguments leading to the creation of the genus Clostridium is outwith the scope of this thesis (see Winslow et al., (1917; 1920)). Suffice it to say that Winslow et al., recommended the setting up of a family for gram positive, spore forming rod-shaped bacteria, the family Bacillaceae, comprising two genera. This proposal was accepted and incorporated into the first edition of Bergey's Manual of Determinative Bacteriology (1923). Although this scheme of subdivision has received wide acceptance, a somewhat more complex system has been developed by Prevot (1957). In his scheme the single genus Clostridium of Bergey's classification is divided into nine genera arranged in two orders and three families. However, it has received little support outside France.

In Great Britain it has been the practice to term Achalme's bacillus Clostridium welchii, in honour of W.H. Welch, who provided the first concise description of this organism. However, as the 7th Edition of Bergey's Manual (1957) has adopted the specific epithet 'perfringens' and this speciation has been used in most of the current literature on this organism and its extracellular products by researchers in Russia, Japan, Canada and the U.S.A., I shall adopt the binomial Clostridium perfringens throughout this thesis despite the common custom of British authors. Current synonyms of British, American and French origin are compared in Table 1,

Table 1 . Comparative Synonyms describing *Clostridium perfringens*.

Bergey's Classification (American)	British Synonym	Prevot's Classification (French)	Original Description
Clostridium perfringens type A	*Clostridium welchii* type A	*Welchia perfringens*	
Cl. perfringens type B	*Cl. welchii* type B	*W. agni*	*Bacillus agni*
Cl. perfringens type C	*Cl. welchii* type C	*W. agni var. paludis*	*Bacillus paludis*
Cl. perfringens type D	*Cl. welchii* type D	*W. agni var. wilsdoni*	*Bacillus ovitotoxicus.*
Cl. perfringens type E	*Cl. welchii* type E	*W. agni var. vitulitoxicus*	
Cl. perfringens type F	*Cl. welchii* type F	*W. perfringens var. hominitoxicus*	

together with terminologies coined for the original descriptions of types of Clostridium perfringens (vide infra).

B. CLOSTRIDIUM PERFRINGENS

This organism is defined as a gram positive rod, usually occurring singly or in pairs, producing central oval spores, and possessing a capsule. It is non-motile. Attempts to subdivide or type this species have been made using three methods:

1. biochemical criteria
2. cellular antigens
3. extracellular antigens.

The success of any of these methods depends on several factors :

a. the ecological sources of the strains tested

b. the stability of the characters chosen

c. the delineation of clear cut groups by the characters tested

d. the ease with which unknown strains can be assigned to a group designated by the characters chosen.

Attempts were made to type Cl. perfringens prior to 1916, but discussion of these attempts is not being undertaken here because of the doubtful purity of cultures (see Howard, 1928).

The emergence of Cl. perfringens as the most important single cause of gas gangrene during World War I and consequently the need for better isolation and identification techniques during this period led to the development of the McIntosh and Fildes anaerobic jar (1916). This apparatus brought to the study of anaerobes for the first time, the precision of pure culture that had escaped their study for the previous 30 years and dismissed the plethora of descriptions and names attributed to clostridial species. Of the above methods of typing, that employing extracellular

antigens has proved most successful. On account of its relevance to the later discussion of individual factors produced by Cl. perfringens type A, this aspect will be dealt with fully.

1. Typing of Cl. perfringens.

a. Biochemical Methods:

Simonds (1915) was able to subdivide the species into four types on the basis of fermentation of glycerol and inulin in a study of 20 strains. However, his groups could not be correlated with their ecological origin and his sources would indicate that all the strains tested were probably of type A (vide infra 'Toxinological Typing').

b. Serological Typing by Cellular Antigens:

Attempts at typing Cl. perfringens on the basis of either capsular or somatic antigens have been undertaken. Neither method has attained much recognition nor success, except in the case of typing of food-poisoning strains (Hobbs et al., 1953).

i. Capsular antigens: The most comprehensive study to date on the capsular polysaccharides of this organism was carried out by Svec and McCoy (1944). In a survey of some 60 strains, most of which were type A, the capsules proved to be immunologically similar by agglutination and precipitin tests. Chemical analyses of the capsular polysaccharides confirmed that they were chemically similar. However, polysaccharide production could not be correlated with the fermentation types of Simonds (1915) or the toxinological types of Wilsdon (1933).

ii. Somatic antigens: Various authors have met with little success in their efforts to type strains by this method (Hall, 1922; Howard, 1928; Henricksen, 1937; Henderson, 1940; Bychenko et al., 1959). Summarising

the findings of these authors I conclude that the main difficulty experienced has been the extreme heterogeneity of strains. However, comparing this method with toxinological groups, Rodwell (1941) and Bychenko et al., (1959) showed that bacterial cells of each toxinological type possessed different antigenic structures, but within each of these groups various serological groupings existed. Thus strain-specific types were created and confusion rather than order resulted. Moreover, antigenic types could not be correlated with the ability to ferment alcohols and sugars or with the production of toxic factors.

Gas gangrene type A strains did not cross react with food poisoning strains serologically. Whereas the former formed a very large heterogeneous group in which corresponding antisera were strain specific, the latter were serologically typable. Hobbs et al., (1953) were able to identify eight types. Since then the number of types has increased to 13. As an epidemiological tool in food poisoning outbreaks caused by Cl. perfringens type A, serological typing has proved extremely useful. It has provided the 'finger-printing' sensitivity necessary to relate isolates from patients' faeces to possible food vectors.

iii. Spore antigens: Because of the difficulties encountered in trying to obtain spores of this organism in vitro, their antigenicity has been little studied. Walker (1963), whilst investigating the spore antigens of clostridia, did not detect any cross reaction by agglutination tests between the spores of Cl. perfringens and those of Cl. bifermentans, Cl. sporogenes and Cl. sordellii. With the development of media specifically designed to induce sporulation (Ellner, 1956; Duncan and Strong, 1968), the promise of improved yields should favour a study of the antigenic mosaic of these structures. However, in the light of the experience of workers with capsular and somatic antigens, it would seem a forlorn hope that the serology of spores would define easily recognisable groups.

c. Toxinological Typing:

Although anaerobic infections were first recognised in animals (Feser, 1876; Pasteur and Joubert, 1877) it was not until the early 1920's that strains of Cl. perfringens became incriminated in cases of veterinary disease. Gaiger and Dalling (1923) and Dalling (1926) described cases of lamb dysentery due to an organism they named Bacillus agni. McEwen (1930) isolated strains of Cl. perfringens, designated Bacillus paludis, from cases of 'Struck' in adult lambs in Kent. Strains isolated from cases of enterotoxaemia in sheep in Australia and pulpy kidney disease in New Zealand were described by Bennetts (1932) and Gill (1932) and named Bacillus ovitotoxicus

Some light was shed on the relationship between these various strains of Cl. perfringens by Wilsdon (1931) in a study of some 50 strains. This study and subsequent investigations (Wilsdon, 1932-33) differentiated four varieties of Cl. perfringens on the basis of toxic components in culture filtrates of these organisms. Bull and Pritchett (1917a, 1917b) and Bull (1917) had earlier shown that broth culture filtrates (Wilsdon Type A) were lethal for experimental animals and that experimental animals could be protected against Cl. perfringens infection by immunisation with graded doses of such toxic filtrates. All the strains examined by Wilsdon were morphologically, biochemically and culturally identical. Nevertheless, cross neutralisation tests with antisera raised against culture filtrates of each strain delineated four groups.

Wilsdon recognised three different toxic moieties on the basis of these neutralisation tests, termed W, X and Z. He designated his types A, B, C and D as shown in Table 2. Two further types have since been recognised. Bosworth (1943) described an outbreak of enterotoxaemia in

cattle due to a strain that differed toxinologically from Wilsdon's types and which he termed type E. Type F was isolated by Zeissler and Rassfeld-Sternberg (1949) from cases of acute gastro-enteritis in Germany. However, more recently the taxonomic position of type F has been questioned by Sterne and Warrack (1964) (see page 13).

During the 1930's and 1940's various groups of workers investigated the toxinology of Cl. perfringens strains, and adopted a Greek letter system of nomenclature to identify the toxins they described. Unfortunately, this system suffered from confusion, and disputes over nomenclature arose with various groups assigning identical components different Greek letters or different components the same designation. The nomenclature in current use is that agreed to by a group of British workers (Nature, 1942).

Glenny et al., (1933) described five different toxic components produced by Wilsdon's types A, B, C and D which were named α, β, γ, δ and ε. They further showed that types B and C produced at least four toxins. Their system of nomenclature is equated with that of Wilsdon in Table 2. These factors were identified by cross-neutralisation experiments involving haemolysis, necrosis and lethality. Although they had controls for the possible existence of natural antibody, the lack of which had been the major criticism of Wilsdon's work, Glenny and his coworkers based their findings upon only one or two selected strains of each type. Despite the limited premise on which their results were based, the overall format of their conclusions has lasted.

It was not until 1937 that type A filtrates were shown to contain more than one factor. Prigge demonstrated two haemolysins which he named α and ζ, now recognised as equivalent to θ and α respectively. Macfarlane et al., (1941) designated the reversibly inactivated haemolysin found in

Table 2 . Toxinological Typing-Comparative Nomenclature.

Wilsdon's Type Classification	Wilsdon's Toxin Nomenclature	Toxin Nomenclature of Glenny et al.
A	W	α
B	W	α
	X	ε
	Z	β, γ, δ
C	W	α
	Z	β, γ, δ
D	W	α
	X	ε

type A filtrates as θ-toxin. A third component of such filtrates was identified by Ipsen and Davoli (1939) as produced by the Lechien strain, and termed η-toxin. Thus by 1942 seven serologically distinct toxins had been identified in culture filtrates of Cl. perfringens - α, β, γ, δ, ε, θ and η.

To this list ι-toxin was added by Bosworth in 1943, as the characteristic factor elaborated by his type E strains. Oakley et al., (1946) demonstrated that collagenase (k-toxin) produced by type A strains was serologically distinct from previously described toxins. Although a 'spreading factor' was first described in culture filtrates of type A, C (Cl. paludis) and D (Cl. ovitotoxicus) by McClean (1936) and shown to be unrelated to any of Glenny's toxins, it was not until 1951 that it was characterised by a Greek letter as μ-antigen by Oakley and Warrack. Oakley et al., (1948) found a proteinase which attacked azocoll powder and gelatin, but not collagen paper or muscle. It was not neutralisable by antikappa antiserum and was termed the λ-antigen. Butler (1942; 1943), whilst investigating post-abortal gas gangrene, noted the destruction of the nuclei of leucocytes in cervical smears. Similar findings were demonstrated by Robb-Smith (1945). He proposed that type A culture filtrates contained a deoxyribonuclease, a fact confirmed by Oakley and Warrack (1951). They called this enzyme ν-antigen.

Using the above twelve components, Oakley and Warrack (1953) typed some 500 strains of Cl. perfringens successfully. Re-assessing the criteria used in this type differentiation in a survey of 307 strains, Brooks et al., (1957) demonstrated the toxinological sub-division of the species on the basis of the four lethal antigens α, β, ε, and ι. These are usually referred to as the major lethal antigens, i.e. 'major' in the sense of

qualitative importance for typing because of their characteristic production by strains from widely different sources. Brooks et al., also added two more haemolysins to the list of components produced, designated "non α, θ, δ" haemolysins. Moreover, they demonstrated that differences existed in the distribution of soluble toxins and antigens within some of the accepted types (Table 3). These subtypes were ecologically significant, forming well defined homogeneous groups of strains differing from the classical pattern of antigen production. They were identified in types A, B and C. A further modification of this table occurred in 1964, when Sterne and Warrack relegated type F to a sub-type of type C, because strains of Cl. perfringens which had been isolated from cases of acute enteritis in Papua - New Guinea by Murrell and Roth (1963) were classical type C strains and differed only in being heat resistant from Zeissler and Rassfeld-Sternberg's classical type F strains.

Thus although this method of typing Cl. perfringens has satisfied the criteria outlined on page 6, exceptions exist. Various authors have reported that some of the toxins or antigens are not produced consistently or are lost in culture or through storage (Oakley and Warrack, 1953). Strains which lose their ability to produce a particular component are termed 'degraded' strains. For instance, Glenny et al., (1933) observed that type B strains lost the ability to produce ε-toxin, a finding also reported by Mason (1935). Taylor (1940) stated that a type C strain lost its ability to produce β-toxin and type D strains lost their ε-toxigenicity in Borthwick's study (1937).

It is generally accepted that in a population the natural frequency of mutation for any one character lies between 1×10^{-5} to 1×10^{-7}. This can be accelerated by exposure of the organism to chemical or physical

Table 3 : Distribution of the Major Lethal Toxins and Minor Lethal or Non-Lethal Factors Produced by the Various Types of Cl. perfringens

Type	Sub-Type	MAJOR LETHAL FACTORS				MINOR LETHAL OR NON-LETHAL FACTORS								Non α θ δ haemolysins	
		α	β	ε	ι	γ	δ	η	θ	κ	λ	μ	ν	Ox	Horse
A	(1) Classical Gas Gangrene	+++	0	0	0	0	0	+	++	+++	0	++	++	+	+
	(2) Food-Poisoning	+++	0	0	0	–	0	–	+	+++	0	+	+++	0	0
B	(1) Lamb dysentery	+++	+++	+++	0	++	0	–	+++	0	+++	+++	++	0	0
	(2) Enterotoxaemia sheep & goats (Iran)	+++	+++	+++	0	–	–	–	+++	+++	0	0	+	0	+ *
C	(1) Struck in sheep	+++	+++°	0	0	++	+++	–	+++	+++	0	0	++	0	0
	(2) Enterotoxaemia calves & lambs U.S.A.)	+++	+++	0	0	–	0	–	+++	+++	0	0	++	0	0
	(3) Enterotoxaemia piglets (Britain)	+++	+++	0	0	–	0	–	++	++	0	+	+++	+++	+++
	(4) Necrotic enteritis of man (Germany) Formerly type F	+++	+++	0	0	+++	0	–	0	0	0	0	+++	++	++
	(5) Necrotic enteritis of man (Papua – New Guinea) †	+++	+++	0	0	–	0	–	+++	++	0	+++	–	–	–
D	Enterotoxaemia of sheep, and pulpy kidney disease (Australia)	+++	0	+++	0	–	0	–	+++	+++	+	++	+++	+*	++*
E	Doubtful pathogen of sheep & cattle (Britain)	+++	0	0	+++	–	0	–	+++	+++	+++	+	++	++*	++*

After Brooks et al., (1957) and Sterne and Warrack (1964)

° Detected from the production of the appropriate anti-toxin by hyperimmunised horses.

* Haemolysis produced only in the presence of antisera.

+++ Produced by most strains (80% or above positive)
++ Produced by some strains (30–80% positive)
+ Produced by few strains (less than 30% positive)

} % calculated from data of Brooks et al., (except for type $C_{(5)}$) symbols modified where necessary to conform with this standardisation.

0 Not produced by any strains

– Not tested

† Based on data of Egerton and Walker (1964) and Murrell and Roth (1963)

agents. Paquette and Fredette (1967), for example, rendered toxigenic strains of type A avirulent by exposure to euflavine, this being correlated with loss of α-toxin or its decreased production. Thus typing difficulties can be caused by mutation.

Akama and Otani (1970) showed that the temperature of heating used in isolation of strains from faeces could affect the toxigenicity of isolates which were untypable with Hobb's sera (θ-toxin and collagenase). Atypical strains which were toxinologically type A, but differed biochemically from type A strains in that they fermented salicin and were strongly proteolytic, were isolated by Mansson and Olsson (1961) from the intestine of pigs.

Despite the above limitations this method of typing still remains in common usage as strains can be conveniently identified irrespective of their source or the disease they produce. A simple procedure for typing of isolates has been devised by Oakley and Warrack (1953) and Brooks et al., (1957).

The toxins and enzymes produced by Cl. perfringens type A are reviewed individually later in this thesis, together with other factors that have been described in culture filtrates or supernatants of type A. Their percentage distribution among type A strains is shown in Table 4.

Table 4 : Distribution of Extracellular Products among type A strains of Cl. perfringens

Extracellular Factor	Author	Year	No. of Strains Tested	No. of Strains Positive [o]
α-Toxin	Evans	1945a	30	30 (100%)
	Brooks et al.	1957	202	202 (100%)
θ-Toxin	Evans	1945a	30	20 (67%)
	Brooks et al.	1957	202	127 (63%)
Collagenase	Oakley et al.	1948	11	11 (100%)
	Brooks et al.	1957	202	178 (88%)
Hyaluronidase	Evans	1945a	30	7 (23%)
	Oakley et al.	1951	14	7 (50%)
	Brooks et al.	1957	202	105 (52%)
Deoxyribonuclease	Brooks et al.	1957	189	141 (75%)
Neuraminidase	Collee	1965b	9	8 (85%)
	Moss et al.	1967	12	0[a] (0%)
	Moss et al.	1967	20	17[b] (85%)
Non α, θ, δhaemolysins	Brooks et al.	1957	186	8[c] (4.3%)
	Brooks et al.	1957	200	7[d] (3.5%)
Fibrinolysin	Reed et al.	1941	See Table 22 of this thesis	
δ, ε, ι, β & λ Toxins *	Brooks et al.	1957	202	0 (0%)
Haemagglutinin	Collee	1965b	3	3 (100%)
η-Toxin	Ipsen and Davoli	1939	No data available	
Bursting Factor	Fredette et al.	1962	1	1
Histidine decarboxylase	Eggerth	1939	4	4 (100%)
	Gale	1941	7	7 (100%)
A enzyme	Schiff	1939	No data available	
H enzyme	Morgan	1946	No data available	
Enterotoxin	Duncan et al.	1968	29	14[e] (48%)
	Duncan et al.	1968	46	42[f] (91%)
	Duncan & Strong	1969	26	14[g] (54%)

[o] Figures in parenthesis = percentage of strains positive
a Food-poisoning strains of Hobbs type 1-13, except Hobbs type 4
b Classical type A strains from pathological specimens (Hobbs untypeable)
c Causing haemolysis on "Ox" blood agar plates, non neutralisable with anti α, θ or δ antisera
d Causing haemolysis on "horse" blood agar plates, non neutralisable with anti α, θ or δ antisera
* cf Ispolatovskaya (1971)
e Rabbit Ileal Loop positive strains from food poisoning outbreaks
f Type A strains tested giving a positive response by at least one of the methods used
g Strains causing diarrhoea

SECTION II : PRODUCTION AND SYNTHESIS OF CLOSTRIDIUM PERFRINGENS TYPE A TOXINS

A. TOXIN PRODUCTION BY CL. PERFRINGENS

1. Media and Strains.

Most authors have used one of two strains of Cl. perfringens type A for the production of extracellular toxins and enzymes, namely, strain S 107 (NCTC 8237) or strain BP6K (ATCC 10543). Over the past 15 years the factors affecting the biosynthesis of α-toxin in particular have been thoroughly investigated, mainly by Japanese and Russian workers. This aspect, which has not been studied in detail for other cytolytic toxins, has not been reviewed elsewhere and will thus be summarised below.

a. Complex Media:

Many media have been designed and used for the production of toxins by type A strains, mainly on an empirical basis. Peptone has been used as the principal nitrogenous ingredient by some authors, but van Heyningen (1941b) reported that commercial peptone did not give reproducibly good yields, perhaps due to the presence of excess iron (Logan et al., 1945; Murata et al., 1956; 1965). As a result of this, authors have prepared their own digests of casein, gelatin and horse muscle (van Heyningen, 1941b; Adams et al., 1947; Shemanova et al., 1965). Murata et al., (1956) criticised the media designed by authors to that date on the grounds that they were tedious to prepare and gave variable yields. They undertook a series of investigations to determine the conditions for optimal production of α-toxin of high titre. They designed a complex medium giving high yields of α-toxin (600-900 LD_{50}/ml)

which could be prepared readily. Factors shown to affect toxinogenesis were the initial pH of the medium, the carbohydrate source, the nitrogen source and inorganic mineral salts. However complex media are still used for reasons of convenience.

b. Chemically Defined Media:

A chemically defined medium is desirable for the study of factors that stimulate enzyme production. Boyd et al., (1948) were the first to grow Cl. perfringens in a defined medium consisting of amino acids, glucose, vitamins and inorganic salts. This medium did not support toxin production, although addition of the peptide glycyl-L-asparagine led to the production of small amounts of α-toxin (Jayko and Lichstein, 1959). Nakamura et al., (1968) studied the production of α-toxin by strain BP6K, and a food-poisoning strain, A 48, by making additions to a chemically defined tissue culture medium, which did not in itself support toxin formation. Of some 40 synthetic peptides tested, the addition of 21 of these, tested separately, stimulated α-toxin production in BP6K, whereas only 2 stimulated A 48. None of 6 free amino acids tested led to toxin formation by either strain. That the peptide content of the peptone added to the complex media stimulated toxinogenesis has been clearly demonstrated by the study of Tsukamoto et al., (1963).

In 1964 Murata and Yamamoto reported the preparation of a synthetic medium which could yield α-toxin of fairly high titre without the addition of peptides (200 LD_{50}/ml). It contained 19 amino acids, fructose, vitamins and various mineral salts. The effect on growth and toxin production of each component was tested by omission experiments or

varying its concentration (Murata et al., 1965). L-arginine was essential for toxin production, cysteine in concentrations of 100 µg/ml inhibited toxin formation (see page 42) and omission of adenine, biotin, calcium pantothenate and pyridoxamine-HCl reduced toxin production significantly. The presence of a carbohydrate was essential, the most effective being fructose. Divalent cations had profound effects on production: magnesium ions (Mg^{2+}) and ferrous ions (Fe^{2+}) were both necessary for growth and toxinogenesis, but concentrations of these ions in excess of the optimum concentration for each, 20 µg and 1-2 µg/ml respectively, decreased toxinogenesis; zinc ions (Zn^{2+}) and manganese ions (Mn^{2+}) were essential for toxinogenesis, the optimal concentrations being 6 µg/ml and 4 µg/ml respectively. Mn^{2+} further improved the yield of α-toxin significantly in the presence of Zn^{2+}. Although they showed that a phosphate concentration of 0.05M was optimal, later studies (Murata et al., 1968) revealed that it was the total concentration of sodium and potassium ions that played the promoting role in toxin production, rather than the phosphate concentration itself.

Although some insight into the requirements for toxin production has been gained from these studies, the role of individual components of the medium in toxin biosynthesis is not yet clear. Some substances may affect toxin biosynthesis, others toxin release, while some may affect both stages. For instance, the membranes of micro-organisms can undergo changes by alteration in the ionic strength or composition of the medium (Brown, 1964). It is quite conceivable that such changes may affect the release of the toxin or its biosynthesis, since ribosomes have been shown to be associated with membranes in bacteria (Kelley and Schaechter, 1968).

Furthermore the assessment of the role of trace elements in toxin biosynthesis is difficult because of the problems associated with obtaining pure samples of inorganic salts. An apparent requirement for a specific ion may be the result of an imbalance in the proportion of other ions in the medium or of the presence of toxic ions or chelating agents such as amino acids.

2. Growth and Biosynthesis.

Gale and van Heyningen (1942) clearly showed that α-toxin, θ-toxin and hyaluronidase were produced during the logarithmic phase of growth, a finding confirmed by Raynaud et al., (1955). Both groups showed that toxinogenesis ceased when growth ceased, and that α-toxin did not appear in the medium immediately after initiation of the logarithmic phase of growth, but after a lag equivalent to a doubling of cell numbers. Raynaud et al., (1955) argued that the lag was associated with de novo synthesis and not simply secretion of preformed toxin accumulated within the cells; they were unable to detect lecithinase activity (see page 28) in washed cells from this phase of the growth cycle using a variety of extraction procedures. Neither were they able to detect toxin inside cells during the log or stationary phases of the growth cycle. Three hypotheses are suggested by these data:

i. That the lag in α-toxin production at the commencement of logarithmic growth represents the period of induction or derepression of the structural genes associated with toxin biosynthesis, or

ii. That de novo synthesis of toxin only occurs at the time of cell division, or

iii. That toxin is continually synthesised, but is masked such as to render it indetectable, release of active toxin occurring at the time of cell division.

a. Enzyme Induction:

Adams et al., (1947) found that lecithin and glycerophosphoryl-choline stimulated lecithinase production in complex media. Also Nakamura et al., (1969) found that L-α-lecithin stimulated lecithinase production in chemically defined media in the presence of glycyl-glycine. However, α-toxin has been produced in the complete absence of added lecithin in chemically defined media by other authors (Murata et al., 1956; 1968; 1969; Tsukamoto et al., 1963; Nakamura et al., 1968). Moreover Turner et al., (1942) did not observe increased α-toxin production by adding lecithin to their medium. Thus whether α-toxin can be induced remains to be answered conclusively.

b. Intracellular Toxin:

Since the studies of Raynaud et al., (1955), other workers have confirmed the release of toxins and enzymes from type A strains during the logarithmic phase of growth. What appears to be more in conflict in the literature is the presence or absence of intracellular toxin at various stages in the growth cycle. Shemanova et al., (1967; 1968a; 1970) like Raynaud et al., (1955) and Rusinko et al., (1965) were unable to detect α-toxin by its lethal, enzymic or antigenic activities in disintegrated cell preparations. However, the extraction procedure of Shemanova et al., (1970), sonication for 30 hours at 30°C, was hardly conducive to the preservation of activity. Conversely, Meisel et al., (1959a; 1959b) and Ispolatovskaya et al., (1965) reported the detection of lecithinase in homogenates of mechanically disintegrated cells.

The work of Meisel et al., (1960; 1959b), who observed that an absence of glucose in the culture medium favoured the detection of intracellular toxin, demonstrated that medium composition could affect the level of α-toxin in cells. The investigation of Cross and Nakamura (1968) revealed a constant intracellular level of α-toxin in two strains of different origin indicating that perhaps extraction procedures rather than strain differences may have contributed to the disparity of results between various groups.

Other factors including the method of cell disintegration, the number of washes given to the cells prior to disintegration, the duration and temperature of disintegration, the stage in the growth cycle, the age of the cells, and the quantity of material examined may have influenced the results obtained.

Very little information is available regarding the presence or absence of other type A toxins within vegetative cells. Meisel et al., (1959c) and Ispolatovskaya et al., (1965) found collagenase in mechanically disrupted vegetative cells, whereas Shemanova et al.(1970) failed to detect this enzyme. Meisel et al., (1963) and Ispolatovskaya et al., (1965) detected intracellular hyaluronidase by mechanical disintegration of cells. By contrast Meyer et al., (1940a) were unable to detect hyaluronidase in autolysates of vegetative cells. Intracellular neuraminidase was detected by Moss et al., (1967) in gas gangrene strains of Cl. perfringens, but not in food poisoning strains. No data are available on the intracellular detection of θ-toxin and deoxyribonuclease. However, Shemanova et al., (1970) showed the accumulation of an alkaline proteinase intracellularly during growth.

c. Mechanism of Biosynthesis and Release of α-Toxin:

This aspect of toxinogenesis in type A cells has only recently been investigated. The literature on the subject is almost entirely restricted to that of Russian workers. Evaluation of their results is dependent on the availability and accuracy of translations and some reservations of the findings so far will be indicated.

i. Inactive precursors: Zai(n)chevskaya (1963) is cited by Shemanova et al., (1968a) as postulating the existence of lecithinase in the form of an inactive precursor or protoxin within Cl. perfringens vegetative cells. To test this hypothesis Shemanova and her coworkers labelled cellular proteins with ^{14}C by growing the organism in medium containing ^{14}C-glutamic acid. After harvesting and washing, organisms transferred to unlabelled medium continued to form α-toxin, but this was unlabelled as detected by immunoabsorption and immunoautoradiography. Identity of the α-toxin released was made by serological comparison with highly purified ^{14}C-labelled lecithinase and specific antibody raised to this lecithinase preparation in rabbits. Their experiments indicated that the α-toxin was synthesised de novo without the intracellular accumulation of a high molecular weight precursor.

ii. Masked toxin: In none of their experiments were Shemanova et al., (1968a) able to detect α-toxin in homogenates of disintegrated cells, either by its enzymic or by its lethal activity, even after concentration of homogenates to contain 20-30 mg protein/ml. Such homogenates had no antibody-combining capacity. However, immunisation of rabbits with the soluble protein fraction, free of ribosomes, raised antibody which neutralised purified lecithinase. As the sensitivity of their lecithinase assay was such as to detect 5 ng of highly purified toxin (van Heyningen

and Bidwell, 1948), they argued that quantities of protein less than this could not possibly elicit an immune response, particularly of the same magnitude as that occurring on administration of 175 μg of toxoided highly purified lecithinase. They thus proposed that cell sap contained a protein in which both the sites responsible for the catalytic and antigenic specificities of α-toxin were masked and that in the immunised rabbit antigenic determinants were unmasked leading to an antibody response.

Currently it is believed that an enzyme's activity is neutralised by its corresponding antibody by combination at a site separate from the active site of the enzyme (Arnon, 1971); this interaction of enzyme with antibody results in an alteration in the steric configuration of the enzyme's active site. Furthermore, Ispolatovskaya (1967) showed that α-toxin treated with chelating agents lost its enzymic activity, but retained its serological specificity. This inactivation was associated with the loss of a metal ion (see page 43). Thus lecithinase, like other enzymes, appears to have separate catalytic and neutralisation sites. Masking of biological activity could occur in the cell by the non-incorporation of the metal ion. Shemanova et al., (1968a) cited the findings of Semykina and Chernikova (1960) to support their hypothesis. However, I have been unable to obtain an English translation of this work.

It is interesting to note at this point that Montie and Ajl (1970) have shown that the murine toxin of Pasteurella pestis is membrane bound, and can be released by procedures which cause membrane disruption. Thus, the detection of cell associated toxin in Cl. perfringens may

depend on the method of disruption used. Since bacterial membranes have been shown to be sensitive to the active form of α-toxin, the existence of an inactive 'masked' form of α-toxin may be a protective mechanism within Cl. perfringens type A cells. Shemanova et al., (1968a)proposed that biosynthesis occurred in association with the bacterial membrane, with incorporation into or binding to the membrane of the α-toxin as an inactive form; release and activation of the toxin would then occur by stretching of the membrane through growth or at cell division.

iii. Site of synthesis and release: Electron-microscopic studies using ferritin labelled antibodies to stain ultrathin sections of cells of strain BP6K at various stages in the growth cycle during toxinogenesis failed to reveal the presence of toxin in association with the cytoplasmic membrane or mesosomes (Kushnarev et al., 1968; 1969; . Smirnova et al., 1971).

However, these investigations did not take account of the possibility that cell-associated toxin is immunologically masked. Indeed these negative findings could be interpreted as supporting Shemanova's hypothesis (vide supra).

Vlasenko et al., (1970) demonstrated that inhibition of cell division of Cl. perfringens type A by ampicillin and glycoglycine was accompanied by suppression of toxin production. Electron microscopy showed that ampicillin induced the formation of filamentous forms with impaired and incomplete cross walls. This finding favours the hypothesis of Raynaud et al., (1955) that toxin synthesis and/or release is related to cell division, a fact that might be verified by studying toxinogenesis in synchronous culture. Alterations in the cell wall which may allow release of α-toxin through this mechanical barrier were described by Kushnarev et al., (1968).

iv. Control of toxin synthesis: There is a paucity of genetic studies with anaerobic bacteria. There have been but two studies on the effect of mutagenic agents on type A cells. Paquette and Fredette (1967) reported that euflavine treatment of vegetative cells induced mutants which had lost the capacity to produce lecithinase as well as the ability to invade host animal tissue. It is generally accepted that acridine derivatives eliminate episomes, e.g. F factor from E. coli, K-12 (Hirota and Iijima, 1957). Because of the high frequency of mutation, up to 8% of the total viable bacteria at 3 μg euflavine/ml, Paquette and Fredette suggested that it was not improbable that an episome or bacteriophage might be involved. Indeed, Mahony and Kalz (1968) demonstrated that a temperate bacteriophage could be isolated from Cl. perfringens strain Lechien, one of the strains rendered non-toxinogenic by euflavine treatment.

Schulze et al., (1968) noted the wide variation in the lecithinase production of strains of type A (see also Robertson and Keppie, 1941; Keppie and Robertson, 1944; Evans, 1945a; Dolby and Macfarlane, 1956). These authors investigated the effect of nitroguanidine (N-methyl-N'nitro-N-nitroguanidine) on toxinogenesis. Mutants producing larger colony diameters than the wild type, also produced more α-toxin, whereas those mutants that possessed small colonies or colonies with irregular margins produced less lecithinase than the parent. Irregular margined colonies have been produced by UV-irradiation of Cl. perfringens cells. Moreover cells from these colonies were morphologically filamentous, (Shaikh, 1969). Therefore it would seem desirable that the studies of Schulze et al., be extended to determine whether decreased toxinogenesis is associated with morphological change of the cell type.

SECTION III : THE EXTRACELLULAR PRODUCTS OF CLOSTRIDIUM PERFRINGENS TYPE A

A. α-TOXIN

The designation of α-toxin for the principal toxic component of filtrates of Wilsdon type A cultures of Cl. perfringens is that of Glenny et al., (1933) and is equivalent to Wilsdon's 'X' factor. Although Glenny and his coworkers used this terminology in the belief that it was the sole toxin in such filtrates, this name now refers to one specific protein component of such filtrates.

In the 1930's increasing interest centred on the chemical nature and mode of action of bacterial toxins, as a result of the demonstration that toxic products elaborated by bacteria were responsible for the manifestations of certain diseases (Tetanus - von Behring and Kitasato, 1890; Diphtheria - Roux and Yersin, 1888; Botulism - van Ermengem, 1896; Gas gangrene - Bull and Pritchett, 1917a; 1917b). Two hypotheses were common - (a) that a toxin acted by causing a metabolic block in the host by competing with the normal substrate for the enzyme catalysing a specific reaction and (b) that the toxin was itself an enzyme attacking one or more normal components of the host's cells. Macfarlane and Knight (1941) pointed out 'that the basis of both of these hypotheses is that the initial lesion in the host cell is one of molecular dimensions and high chemical specificity'. Cl. perfringens α-toxin, historically, occupies a central position in the attention of toxinologists, being the first toxin to have its mode of action defined biochemically (vide infra). The demonstration of its enzymic nature has led workers on almost all exotoxins known to date to attempt to demonstrate some enzymic specificity in their preparations that might explain their actions in vivo.

Unfortunately, this approach has met with little success, as yet. Phospholipase C activity was established to be identical with specific components in culture filtrates of other clostridia, namely Cl. oedematiens β- and γ-toxins, Cl. haemolyticum α-toxin, and to be produced by Cl. bifermentans. Nevertheless, it was not until 1963 that Doery et al., demonstrated a clear association between enzymic activity and the toxin of another major group of pathogens, namely the β-toxin of Staphylococcus aureus. Curiously, this too proved to be a phospholipase C, but specific for sphingomyelin. To date Cl. perfringens α-toxin and staphylococcal β-toxin remain the only two major toxins to have their biochemical mode of action defined unequivocally. Equivocal results have indicated that staphylococcal δ-toxin may be associated with a phospholipase specific for phosphatidyl inositol (Doery et al., 1963; 1965; Wiseman and Caird, 1968), and intensive work on the mode of action of diphtheria toxin in recent years has indicated that this toxin may be an NADase (Pappenheimer, 1970).

1. The Enzymic Nature of α-Toxin.

Probably the most important single observation contributing to the development of knowledge of the mode of action of α-toxin was that of Nagler (1939) and Seiffert (1939) who both showed independently that when Cl. perfringens was grown in human serum, an opalescence developed in the medium and eventually a layer of fat rose to the surface. This phenomenon occurred with the culture filtrates of all types of Cl. perfringens identified at that time (types A, B, C and D). Furthermore the opalescence reaction could be inhibited by antitoxin. Nagler also demonstrated that the activity of his type A filtrates as quantitated by production of opalescence, paralleled the lethal action of these filtrates

and that antisera could be titrated in vitro using an end point assay for opalescence. That a similar reaction, the so-called lecithovitellin reaction or L.V. reaction, took place between toxin and a clear emulsion of egg-yolk, obtained by filtering a saline suspension of yolk, was shown by Macfarlane et al., (1941). This alternative substrate had the decided advantage that a more distinct opalescence was produced. By neutralisation techniques these authors showed that the reaction was due solely to α-toxin, the other then identified toxins β, γ and δ having no effect. They also demonstrated that both haemolysis produced by α-toxin and the lecithovitellin reaction were activated by Ca^{2+} and paralleled each other in filtrates, leading them to consider that 'both these activities might be manifestations of the same enzyme reaction'.

Macfarlane and Knight (1941) investigated the biochemical nature of the mode of action of α-toxin on egg-yolk emulsion. They showed that the toxin rendered almost all of the lipid phosphorus water-soluble and that formation of water-soluble phosphorus paralleled the development of turbidity in egg-yolk extract. Using partially purified lecithin from egg-yolk they were able to demonstrate its hydrolysis to phosphocholine and a diglyceride (see Figure 1).

Enzymes hydrolysing lecithins are classified in a broader group of enzymes called phospholipases, those capable of catalysing the breakdown of phospholipids, principally because such enzymes can be capable of attacking more than one phospholipid. There are four types of phospholipase which act on lecithin, described by their point of attack on the substrate molecule (Figure 1). Cl. perfringens lecithinase is a phospholipase C and is listed by the Enzyme Commission as EC.3.1.4.3., phosphatidylcholine cholinephosphohydrolase (Florkin and Stotz, 1964).

Figure 1 : Hydrolysis of Lecithin by Phospholipases

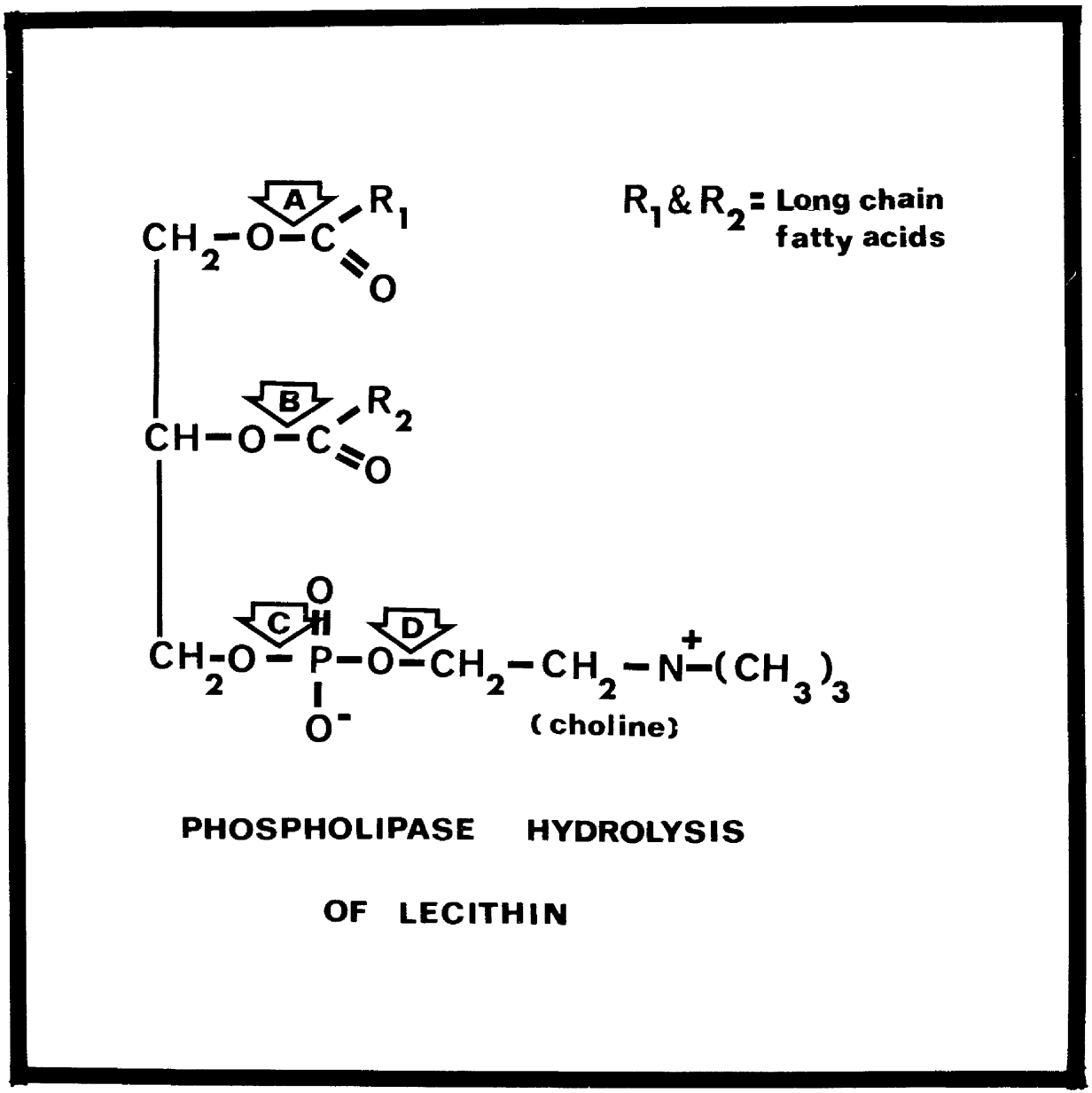
Phospholipase A - found in snake venoms and the poison of bees

Phospholipase B - found in extracts of Penicillium notatum

Phospholipase C - found in culture filtrates of bacteria

Phospholipase D - found in extracts of plant tissue

Points of attack by each phospholipase denoted by open arrow heads labelled with an appropriate index lette



PHOSPHOLIPASE HYDROLYSIS

OF LECITHIN

The nature of the opalescent material formed in the lecithovitellin reaction has been discussed by various authors. Macfarlane et al., (1941) considered that decomposition of an aqueous egg-yolk emulsion to form a cream of fat globules was due to the destruction of the lecithin causing a loss of stabilisation of the protein and fat emulsion. The cream or thick scum gave a free fat reaction. Crook (1942), however, showed that the curd contained all types of fat and some protein. The studies of Willis and Gowland (1962) have clarified the position somewhat. They demonstrated that much of the opalescent material was protein, as trypsin reduced the opalescence produced in egg-yolk agar plates by α-toxin and the opalescence produced in trypsinised egg-yolk agar plates was much less than that on control egg-yolk agar plates. They concluded that the opalescence was due to fat from free lecithin, to free fats in the emulsion coalescing after splitting of the emulsion, and to curding of water-insoluble proteins, vitellin and vitellenin, along with insoluble free fats.

2. Purification.

Table 5 lists those attempts at purification of α-toxin which have occurred since its mode of action on lecithin was elucidated. Prior to the late 1950's various precipitation techniques were employed, including salting out with ammonium sulphate, precipitation with methanol at acid pH and low temperature, acetone precipitation, acid precipitation and protamine precipitation. The rapid development of protein purification procedures in the late 1950's and 1960's led authors to include ion-exchange chromatography, gel filtration chromatography and various electrophoretic procedures in their purification protocols. Ion-exchange celluloses have been used as column fractionation steps as well as batch absorption steps

Table 5 Purification studies on Cl. perfringens α-toxin.

Authors	Year	Strain	Purification Procedure	Purity†	Comments
van Heyningen	1941b	S 107	1.Adsorption onto calcium phosphate in the presence of 40% Acetone; -10°C. 2.Elution of precipitate (ppt.) with 20% $(NH_4)_2SO_4$. 3.Repeat Stages (1) and (2).	-°	Purification = 38 fold relative to culture filtrate. Overall Yield = 54% Main drawback to method was the large volumes of Acetone involved.
van Heyningen and Bidwell	1948	S 107	1.90% $(NH_4)_2SO_4$ saturation ppt. 2.68% $(NH_4)_2SO_4$ saturation ppt. 3.Nucleic acid precipitation at pH=4.5 4.Protamine sulphate precipitation at pH=6.0. 5.Absorption of toxin with alumina cream (3.3%).	-°	Purification = 250 fold relative to Stage 1. Overall Yield = 20% α- and θ-toxins not separated. Stage 5 toxin also contained k- and μ-toxins.
Roth and Pillemer	1953	BP6K	1.Acid/Methanol (35%) precipitation pH=4.6; -5°C. 2.25% Methanol ppt.; -5°C; pH=5.7; protein concentration = 1%; ionic strength = 0.1. 3.50% Methanol ppt.; pH=9.0; ionic strength = 0.30; ppt. discarded. 4.Supernatant + cold distilled water 1:1; pH=4.65; -5°C; ppt.harvested.	N.T.	Purification = 1500 fold relative to culture filtrate. Overall Yield = 30%. Stage 4 toxin was not homogeneous, and contained 0.6% of original θ-toxin.
Meduski and Volkova	1957	SR12	1.Freeze dried culture filtrate. 2.60% $(NH_4)_2SO_4$ saturation ppt. 3.42% $(NH_4)_2SO_4$ saturation ppt. 4.45% $(NH_4)_2SO_4$ saturation ppt.	N.T.	
			Procedure 1.		
Habermann	1959	BP6K	1.35% Methanol ppt.; -5°C; pH=4.7-4.8. 2.20% Methanol ppt.; -5°C; pH=6.0. 3.50% Methanol ppt.; pH=8.0; ppt. discarded. 4.Supernatant + cold distilled water 1:1; pH=4.6; ppt. harvested. 5.Amberlite IRC 50 chromatography.	1	Purification = 71 fold relative to Stage 1. Overall Yield = 26%. Stage 5 toxin contained 0.05% θ-toxin, 0.2% k-toxin and 0.4% μ-toxin relative to Stage 1.
			Procedure 2.		
			1.Stage 1 as above. 2.Zone electrophoresis in starch columns. 3.Hydroxyapatite-celite chromatography.	1	Purification = 75 fold relative to Stage 1. Overall Yield = 40% θ-toxin content of Stage 3 α-toxin was 0.003% relative to Stage 1.
Stephen	1961	CN1491 Wellcome Research Laboratories.	1.Ultrfiltration through 8% collodion membranes. 2.Freeze dried. 3.Zone electrophoresis in ethanolysed cellulose columns; borate buffer pH=7.1. 4.Elution of column with borate-NaCl buffer.	1	Purification = 6 fold relative to Stage 1. Overall Yield = 80% Quantitative separation of α- from θ-, k- and μ-toxins.

Authors	Year	Strain	Purification Procedure	Purity†	Comments
Bangham and Dawson	1962	S 107	1.75% $(NH_4)_2SO_4$ saturation ppt. 2.40% $(NH_4)_2SO_4$ saturation ppt. 3.Acid precipitation at pH=4.9 4.Zone electrophoresis in glycerol density gradient.	1	Purification - no data available. Overall Yield - "low" - Bangham (1968) personal communication.
Ispolatovskaya and Levdikova	1962	BP6K-28	1.Absorption with DEAE-cellulose. 2.30% $(NH_4)_2SO_4$ saturation ppt. 3.36% Methanol ppt. 4.Repeat Stage 3. 5.50% Acetone ppt. 6.Repeat Stage 5. 7.CM-cellulose chromatography with gradient elution; Acetate buffer pH=5.6.	2	Purification - no data available. Overall Yield - no data available. Homogeneity not attained, as evidenced by ultra-centrifigation and immunodiffusion.
Ikezawa et al.	1964	BP6K	1.100% $(NH_4)_2SO_4$ saturation ppt. 2.Repeat Stage 1. 3.Sephadex G-100 chromatography.	N.T.	Purification = 4.4 fold relative to Stage 1. Overall Yield = 67%.
Shemanova et al.	1965	BP6K-28	1.Acid ppt. at pH=4.0 - 4.3 2.25% $(NH_4)_2SO_4$ saturation ppt. 3.Sephadex G-100 chromatography. 4.35% Methanol ppt.; -5°C. 5.Absorption with DEAE-cellulose; pH=5.6 6.Repeat Stage 5. 7.30% $(NH_4)_2SO_4$ saturation ppt. 8.Sephadex G-75 chromatography. 9.CM-cellulose chromatography; pH=5.6 gradient elution; acetate buffer. 10.30% $(NH_4)_2SO_4$ saturation ppt. 11.Sephadex G-75 chromatography. 12.Adsorption onto calcium phosphate in the presence of 60-70% Acetone at - 20°C. 13.Repeat Stage 11. 14.Lyophilisation.	1	Purification - no data available. Overall Yield - no data available. Stage 13 was homogeneous, but low molecular weight contaminants were observe by ultracentrifugation.
§Ito	1968	BP6K-N5	1.1% Zinc chloride ppt. 2.80% $(NH_4)_2SO_4$ saturation ppt. 3.Sephadex G-100 chromatography. 4.80% $(NH_4)_2SO_4$ saturation ppt. 5.Sephadex G-100 chromatography of pooled fractions.	1	Purification = 16 fold relative to Stage 2. Overall Yield = 31%. Stage 5 contained 2.3% of θ-toxin and 0.02% kappa relative to Stage 1 material.
§Shemanova et al	1968b	BP6K-28	1.100% $(NH_4)_2SO_4$ saturation ppt. 2.Fractional ppt. 18-25% $(NH_4)_2SO_4$ saturation. 3.Absorption with DEAE-cellulose,pH=4.5 4.Absorption with DEAE-cellulose,pH=5.6 5.35% Methanol ppt.; -5°C; pH=4.6-4.8 6.Sephadex G-100 chromatography. 7.60-65% Acetone ppt.; -20°C. 8.40% Acetone ppt. 9.Sephadex G-100 chromatography. 10.Lyophilisation.	1	Purification - no data available. Overall Yield = 0.05%. Weight of protein obtained = 12mg/80 litres of starting material.

§These purification studies were published after work on this thesis had commenced.
†Purity = No. of precipitin lines observed by immunodiffusion or immunoelectrophoresis against standard antiserum.
° Purification published prior to the development of gel double diffusion technique of Ouchterlony.
N.T.Not tested.

Ispolatovskaya (1962) used DEAE-cellulose in the latter manner to adsorb crude culture supernatants. Shemanova's procedures (1965; 1968b) seem unduly complex. It is difficult to justify the use of Sephadex G-75 on three occasions in a single purification protocol, particularly as G-100 was used at an earlier stage.

Preparative electrophoretic procedures used include column electrophoresis in potato starch (Habermann, 1959) and in ethanolysed cellulose columns (Stephen, 1961) and density gradient electrophoresis in glycerol (Bangham and Dawson, 1962). Problems such as low yields, the expense of equipment and the amount of material that could be processed have limited the use of electrophoretic methods.

Although a number of the purifications in recent years have resulted in highly purified toxin preparations as gauged by immunodiffusion or immunoelectrophoretic techniques, no one has as yet succeeded in crystallising α-toxin. No comparison of highly purified preparations from a number of different laboratories has been attempted as it has with staphylococcal delta toxin (Kreger et al., 1971) or Cl. botulinum type B toxin (Boroff et al., 1968). It is difficult to be categorical about the freedom of various preparations from other type A toxins and enzymes, as authors have been reluctant to provide this information or have not assayed for them. Shemanova et al., (1965) pointed out that although their preparation was homogeneous by immunoelectrophoresis it contained low molecular weight components of unspecified nature, as observed in the ultracentrifuge. With the exception of Habermann (1959) who used cellulose acetate electrophoresis to check the purity of his fractions, no use has been made of gel electrophoresis or membrane electrophoresis

by other authors to check for the presence of minor protein components not detected by immunodiffusion techniques.

A characteristic of most of these purification procedures was the use of large volumes of starting material, 50-200 litres being worked up at any one time, because of low yields at the end of a multi-stage process. The use of multiple step purification procedures increases the possibility that the toxin may undergo structural alteration, such as changes in conformation or aggregation. Such changes may or may not affect the biological properties of the resulting purified material. One of the objectives of this thesis was to develop a procedure whereby highly purified toxin could be prepared in conditions which would be unlikely to alter its native structure. Such a method would also obviate the need for specific precautions attendant on the preservation and storage of purified material prepared from a large volume of starting material by a complex and tedious series of stages.

3. Properties of α-Toxin.

a. Physical Properties:

i. Physical characteristics: Comparatively little is known of the physical characters of α-toxin, present knowledge having accumulated slowly over the past 10 years. This is surprising when one considers the importance attributed to this toxin for historical and theoretical reasons. The literature on molecular parameters for this toxin is summarised in Table 6.

It can readily be seen that there is a considerable discrepancy between the molecular weights determined by three groups, albeit that different methods were used in these determinations. The method used

Table 6 Physical Characteristics of Cl. perfringens α-Toxin.

Molecular Parameter	Value	Method of Determination.	Authors
Molecular Weight	106,000±3,000	Gamma ray inactivation	Meduski and Volkova (1957;1958)
	31,000	Gel filtration	Bernheimer and Grushoff (1967)
	51,200	Ultracentrifugation	Shemanova et al (1968 b)
§Sedimentation Coefficient (S)	7.9		Meduski and Volkova (1957;1958)
	3.8		Shemanova et al (1965)
	3.8-3.9		Shemanova et al (1968b)
Isoelectric Point (pI)	5.0	Measurement of electrophoretic mobility adsorbed to liquid paraffin emulsion	Bangham and Dawson (1962)
	5.8	Column electrophoresis at varying pH	Bangham and Dawson (1962)
	5.5		Dawson (1968)
Frictional Ratio (f/fo)	0.93	Method of Greenberg (1951)	Meduski and Volkova (1957;1958)
Molecular Diameter	69Å		Meduski and Volkova (1957)
†Diffusion Coefficient (D°_{20})	7.41	Method of Northrop and Anson (1929)	van Heyningen and Bidwell (1948)
	7.26	Method of Allison and Humphrey (1959)	Shemanova et al (1968b)
∫Electrophoretic Mobility	circa 0.0	Borate buffer pH=8.6 Conductance = 0.0020/ohm/cm	Uspenskaya and Meduski (1957)
	-1.2 to -1.6	Borate buffer pH=8.6 Conductance = 0.0035/ohm/cm	Ispolatovskaya and Larina (1959)
	-1.0	Borate buffer pH=8.6 Conductance = 0.0035/ohm/cm	Ispolatovskaya et al (1961)

§ Sedimentation Coefficient - given in Svedberg units.
† Diffusion Coefficient - given in Fick units (10^{-7}/cm^2/second)
∫ Electrophoretic Mobility - given for indicated pH values in units of 10^{-5} cm^2/volt/second.

by Meduski and Volkova is unique in the study of bacterial toxins, and depends on a linear relationship between -ln (initial enzyme activity-inactive enzyme) and the radiation dose. The test material was gamma-irradiated in the dry state.

The apparent arithmetic relationship of the molecular weights so far determined suggests that the toxin can aggregate or polymerize. For example, dehydration of proteins is known to cause the formation of cross-linking bonds. This could conceivably have contributed to the high molecular weight found by Meduski and Volkova.

Freshly prepared α-toxin was shown to be electrophoretically homogeneous at pH 8.6 by both Ispolatovskaya and Uspenskaya's groups, with a mobility close to zero. Bangham and Dawson determined the pI of α-toxin. They did not report observing heterogeneity in their highly purified preparation. These observations on the physical properties are pertinent to the discussion on 'Multiple Forms of α-Toxin' (See page 46).

Thus it can be seen that there is scope for intensive investigation on the physical properties of this protein. The information so gained may go some way to explaining some of its biological characters.

ii. Heat stability - the Arrhenius phenomenon: Early work established that α-toxin, like other proteins, is denatured by heat. Bull and Pritchett (1917a) found their lethal toxin (α-toxin) to be heat labile. Heating at 60°C reduced activity, whilst heating at 70°C totally inactivated the toxin. Macfarlane and Knight (1941), however, reported that their α-toxin was heat stable, 45% of the original activity remaining after heating at 100°C/10 minutes, a finding more recently corroborated by Weiss and Strong (1967), who reported that lecithinase in culture filtrates

retained more of its activity after heating at 100°C than at 75°C, and that the heat resistance of lecithinase was somewhat strain dependent.

Arrhenius (1907) stated that staphylococcal haemolysin was inactivated to a greater extent at 70°C than at 100°C, a phenomenon which he termed 'anomalous heat inactivation'. Furthermore toxin inactivated by heating at the lower temperature could be partially reactivated by brief heating at 100°C. This effect of heat reactivation has since been called 'The Arrhenius Phenomenon'. Guillamie et al., (1944) were the first to record this effect with Cl. perfringens α-toxin.

Some of the parameters affecting this inactivation/reactivation phenomenon with Cl. perfringens lecithinase, were investigated by Smith and Gardner (1950). Their significant observations were that lecithinase electrodialysed free of easily dissociable ions did not exhibit anomalous heat inactivation; that 'partially purified' lecithinase did not exhibit the phenomenon of anomalous heat inactivation; and that the greater the dilution of the preparation the greater the inactivation. Kushner (1957) has since verified that purified lecithinase was thermolabile while the activity of crude culture filtrates was heat resistant. Thus anomalous heat inactivation was due to the protective action of components of the culture medium. The reason for this is still obscure, but it may be that metal ions bind more strongly at the higher temperature making the protein less susceptible to heat denaturation.

Smith and Gardner also noted that a perceptible opalescence appeared in the lecithinase on heating and that inactivation paralleled its appearance. Heating of 'partially purified' lecithinase in the presence of Ca^{2+} resulted in the formation of opalescence and a sedimentable

precipitate, with loss of activity. When the precipitate was harvested, resuspended and briefly heated at 100°C, activity was recovered. They theorised that the 'Arrhenius phenomenon' could be explained in terms of the formation of reversible bonds between the lecithinase and Ca^{2+} or Mg^{2+} and that the complex so formed was disaggregated on heating at 100°C by the formation of an insoluble calcium or magnesium compound. Thus their findings provide at least one explanation of this curious effect and confirm the proposal of Landsteiner and von Rauchenbichler (1909) that inactivation was due to the formation of an inactive complex between toxin and a component of the culture filtrates.

A different mechanism to explain 'The Arrhenius Phenomenon' in relation to staphylococcal α-toxin has been proposed by Arbuthnott et al., (1967). They noted that 8M urea could disaggregate the insoluble precipitate obtained when staphylococcal α-toxin of high purity was heated at 60°C. These studies suggested that the precipitate was formed by the direct aggregation of the protein molecules of active α-toxin and that the brief heating at 100°C released active toxin by a reversal of this reaction. Indeed, the experiments of Smith and Gardner with Cl. perfringens α-toxin clearly merit reinvestigation with preparations of toxin that are relatively homogeneous.

iii. pH stability: Gale and van Heyningen (1942) studied the stability of crude bacterial filtrates. At pHs below 4 and above 9 rapid inactivation occurred at room temperature. These results are in good agreement with those of Nakamura and Cross (1968) who found that lecithinase activity was completely inactivated after one hour at pH 1-3, was unaffected at pH 6-9, and was inactivated at higher pH values. Thus extreme acid and alkaline conditions are inimical to α-toxin as with most other proteins.

b. Chemical Properties:

i. Analysis: The protein nature of purified α-toxin is established by its ultraviolet absorption spectrum and its reaction with specific reagents used for the detection of proteins or their quantitative estimation. Dalling and Ross (1938) are cited by Oakley (1943) as having destroyed the lethal activity of culture filtrates of type A strains with trypsin. No studies on the effect of specific enzymic hydrolysis by well characterised proteolytic enzymes have been undertaken with highly purified α-toxin, either as a means of sequence analysis or in an attempt to isolate toxic fragments of the toxin. No data are available on whether the toxin contains any carbohydrate moiety as an integral part of its structure. The question of association of metal ions will be dealt with later (see page 43). An amino acid analysis of highly purified toxin has been reported by Shemanova et al., (1968b).

ii. Effect of inhibitors and activators: Macfarlane and Knight (1941) clearly showed that the presence of Ca^{2+} greatly affected the lecithinase activity of α-toxin, activation being most marked with low concentrations of toxin, when any activating ions in the crude culture filtrate would have been diluted out. Macfarlane et al., (1941) had previously shown that Ca^{2+} enhanced the haemolytic activity of α-toxin. Bangham and Dawson (1962) demonstrated that highly purified α-toxin would only break down lecithin in the presence of Ca^{2+} or Mg^{2+}. Certain long chain amphipathic cations such as stearylamine could also act as activators for the breakdown of lecithin (see pages 45 and 51). By contrast, Saito and Mukoyama (1968) reported hydrolysis of lecithin in the absence of Ca^{2+} (cf. Pastan et al., 1968).

Ispolatovskaya and Klimacheva (1966) have investigated the effects of known enzyme inhibitors (see Table 7) on α-toxin. They used a preparation of α-toxin that was free of detectable collagenase, hyaluronidase, deoxyribonuclease, proteinase and θ-haemolysin. The partial inhibitory action of Cu^{2+} and Fe^{2+} could not be reversed by dialysis, Sephadex G-25 gel filtration, or the addition of ethylenediamine tetra-acetate (EDTA). Higher concentrations of these metal ions caused rapid loss of activity and precipitation of the protein with complete loss of activity. The comparative stability of the enzyme to the action of parachloromercuribenzoate (p-CMB), which combines specifically with sulphydryl groups (-SH groups) forming mercaptides, and to monoiodoacetate suggested that the heavy metals do not react by addition to -SH groups. Bubbling hydrogen sulphide from a Kipp's apparatus through solutions of the enzyme also resulted in rapid inactivation. Hydrogen sulphide is known to 'poison' metalloproteins or metalloenzymes by the formation of the sulphide of the particular metal ion involved. However, lecithinase activity was also inhibited rapidly by bubbling air or nitrogen through solutions of the enzyme (Macfarlane and Knight, 1941). These authors suggested that this was due to surface denaturation rather than oxidation as the enzyme was unaffected by a 1:100 dilution of '30 vol.' hydrogen peroxide, a finding confirmed by Ispolatovskaya and Klimacheva (1966). It is possible, therefore, that physical denaturation due to bubbling could account for the inhibitory effect of hydrogen sulphide.

Inactivation by the chelating agent, EDTA proved irreversible even after prolonged dialysis or the addition of divalent cations prior to or after the addition of EDTA. This supports the view that α-toxin is a metalloprotein. Di-isopropylfluorophosphate, specific for serine residues, caused a negligible drop in activity, indicating that it is

Table 7 Effects of Enzyme Inhibitors of Cl. perfringens α-toxin.

Test Chemical	Concentration	Time of Exposure (hours)	% Inhibition
Copper Sulphate	0.001M	12	90%
	0.02M	0	100%
Ferrous Sulphate	0.001M	12	90%
	0.02M	0	100%
p-CMB	0.001M	48	90%
Monoiodoacetate	0.002M	48	90%
Hydrogen Sulphide	-	0.25	100%
Hydrogen Peroxide	0.1M	24	100%
EDTA	0.002M	48	90%
	0.02M	0	100%
DFFP	1M	48	Negligible
	2M	48	Negligible
	4M	48	Negligible
Potassium Ferrocyanide	0.02M	2	100%
Cysteine	0.02M	2	100%
Thiogycollic Acid	0.02M	2	100%
Urea	8M	24	90%
Iodine	0.01M	144	90%
	0.1M	0	100%

After Ispolatovskaya and Klimachova (1966)

unlikely that serine is involved in the active site of the molecule. Of the other agents tested urea and low concentrations of iodine were without effect, whereas potassium ferrocyanide, cysteine, thioglycollic acid and higher concentrations of iodine were inhibitory.

c. The Metalloprotein Nature of α-Toxin:

From their experiments on the inhibition of the L.V. reaction by EDTA using highly purified toxin, Ispolatovskaya and Klimacheva (1966) concluded that inhibition of this enzyme was not solely due to chelation of activating ions by the EDTA, butto adirect effect on the lecithinase molecule itself. The following points support this view (a) the kinetics of action of EDTA on lecithinase, (b) inactivation of the enzyme by H_2S, (c) failure to restore enzyme activity by the addition of excess calcium ions before or after EDTA treatment, (d) failure to reverse the inhibition by dialysis or gel filtration with or without the subsequent addition of a metal ion activator, (e) the loss of toxic properties. Collectively these findings suggested that the inhibition was due to a structural change in the molecule associated with the loss or removal of a metal ion forming an integral part of it.

Ispolatovskaya (1967; 1970) using extensively purified α-toxin preparations has provided evidence that Zn^{2+} is the metal ion involved. Emission spectra of six preparations of lecithinase of differing purity and specific activity showed the presence of zinc from spectral lines at 334.5 nm and 330.2 nm. Also the enzyme was inactivated by o-phenathroline (OP), a chelating agent known to have a high affinity for Zn^{2+}; and restoration of activity could be achieved by the addition of Zn^{2+} to enzyme dialysed free of EDTA or OP. No reactivation was observed with Ca^{2+}, Mg^{2+} or Mn^{2+} but Co^{2+} caused reactivation to a lesser degree.

A similar conclusion had been reached by Moskowitz et al., (1956) and Moskowitz (1958) who, from their protection studies using chelating agents, suggested that a metal ion was intimately associated with the toxin. It was proposed that this might be Zn^{2+}. Moreover, evidence of a role for Zn^{2+} in α-toxin production has been furnished by Murata et al., (1956; 1965). Studies in complex media with a large number of cultures with varying nitrogen sources suggested that Zn^{2+} had a promoting effect on α-toxin production. In their chemically defined medium Zn^{2+} displayed a decisive effect on toxin production, although ineffective for growth (see page 19). Murata's studies did not, however, clarify whether the Zn^{2+} was essential for integration into the toxin molecule or a requisite cofactor for enzyme activity in biosynthesis of the toxin molecule. Also Sheldon (1960) showed that Zn^{2+} was a potent activator of α-toxin.

Evidence from research on other bacterial exoenzymes suggests that Zn^{2+} can be an integral part of their molecular structure. Ottolenghi (1965) showed that the phospholipase C of Bacillus cereus was a metalloenzyme containing Zn^{2+}. Also, through studies on the inhibition of Cl. histolyticum collagenase by cysteine, Seifter et al., (1959) and Harper and Seifter (1965) suggested that inactivation occurred through chelation of an intrinsic metal component and that this metal was probably Zn^{2+}. This was verified by growing Cl. histolyticum in ^{65}Zn-labelled medium and subsequently purifying ^{65}Zn-labelled collagenase (Harper, 1965, cited by Seifter et al., (1970)). Furthermore, these two bacteria belong to the same family as Cl. perfringens but it is too early to say whether these are chance findings or representative of a characteristic feature of metalloenzymes within this family.

i. The possible role of zinc and calcium in phospholipase activity: In association with protein molecules metal ions may function in one of several ways. The metal ion may be contained in the catalytic centre of the molecule and play some role in the action of the enzyme. It may act in binding the substrate to the enzyme, but have no direct function in the enzymic activity. It may be essential only for the maintenance of enzyme conformation. Finally the ion may act as a bridge between enzyme and substrate creating electrostatic conditions at the substrate-water interface suitable for enzyme-substrate interaction.

While it seems that Zn^{2+} is bound to the apoenzyme (Ispolatovskaya, 1967; 1970), Ca^{2+} activate α-toxin without binding irreversibly to the molecule. With the exception of the studies of Bangham and Dawson (1962) little is known of the mechanism of metal ion activation for this enzyme. These workers established that a positive zeta potential had to prevail at the lecithin-water interface before Cl. perfringens α-toxin would hydrolyse the lecithin substrate. This was produced by the addition of Ca^{2+}, maximum hydrolysis occurring when the mobility of the lecithin particles was about +0.5/sec/volt/cm. With increasing values of zeta potential, hydrolysis of lecithin declined. Moreover, enzymic activity was detected only above pH 5.5 i.e. when the protein had a net negative charge (Dawson, 1968).

Thus the roles of these two metal ions are clearly distinct. Yet, a number of questions remain unanswered. Nothing is known (i) of the nature of the enzyme-substrate bond and the function of Ca^{2+} in this, (ii) the molecular arrangements of the Ca^{2+} and Zn^{2+} during substrate-enzyme interaction (iii) the function of the Zn^{2+} in enzymic activity and

(iv) the stoichiometric ratio of Zn^{2+} to apoenzyme, or of Ca^{2+} to enzyme-substrate bond at maximal velocity of the reaction. These problems can only be satisfactorally tackled and answered with the provision of α-toxin of high specific activity.

d. Multiple Forms of α-Toxin:

Ispolatovskaya and Levdikova (1962) reported that when they fractionated their stage 3 toxin (see Table 5) on CM-cellulose using gradient elution with acetate buffer pH 5.6, the α-toxin was eluted as a single peak of lecithinase activity. However, when stage 6 toxin was chromatographed similarly i.e. toxin purified by a complex series of stages and lyophilised, the activity of the enzyme was eluted as two well defined peaks of activity. In reporting this observation, these authors were unable to determine whether the presence of two active components in the α-toxin preparation was due to the resolution of isoenzymic forms of the lecithinase or the result of slight changes in the structure of the enzyme caused by the purification methods used, which could have caused a modification of the charge on individual parts of the molecule.

In reinvestigating this aspect, (Ispolatovskaya, 1964), the lecithinase was purified as previously described (Ispolatovskaya and Levdikova, 1962) except that 'Stage 6 toxin' was subjected to a further purification on Sephadex G-75. Part of this material was concentrated with ammonium sulphate and passed through Sephadex G-75 again. All three purified preparations were lyophilised and then analysed by starch gel electrophoresis. Gels were sectioned and stained for protein. Transverse strips were eluted by a variety of procedures and assayed for lecithinase activity by phosphorus release from an egg-yolk emulsion.

A number of active components possessing lecithinase activity were revealed in each preparation, although the number of active components varied with the preparation. However, crude or purified material that had not been lyophilised was not analysed. Also Ispolatovskaya failed to establish whether the several components were produced by aggregation or polymerisation. Ispolatovskaya and Klimacheva (1966) reported that lecithinase was relatively stable to urea, but did not determine whether this treatment altered the electrophoretic pattern.

The technique of electrophoresis in starch or polyacrylamide gel has revealed heterogeneity in other bacterial toxins. Habeeb (1964; 1969) showed that Cl. perfringens ε-prototoxin purified to immunological homogeneity displayed electrophoretic heterogeneity in starch gel electrophoresis. The 4-5 components detected were serologically identical. Again this toxin was freeze-dried before analysis.

Here the observations of Uspenskaya and Meduski (1957) are worthy of mention, as they may provide a partial explanation of the multiple forms observed by Ispolatovskaya (1964). Both these authors and Ispolatovskaya and Larina (1959) reported that freshly prepared α-toxin was electrophoretically homogeneous. However, Uspenskaya and Meduski observed that when a seven-year old specimen of α-toxin, obtained from the Central Institute of Epidemiology and Bacteriology, Moscow, and therefore presumably freeze-dried, was subjected to electrophoresis, it occupied a broad zone of electrophoretic mobility. Their work clearly demonstrated that storage or ageing of the toxin caused certain changes in the toxin molecule. Ispolatovskaya and Larina investigated detoxification of crude α-toxin with formalin. Gradual modification of

α-toxin by formalin made the protein more acidic but the initial changes in electrophoretic mobility of α-toxin did not result in a loss of activity. These authors interpreted this as a gradual blocking or denaturation of the active centre of the enzyme molecule not involving the structural feature responsible for its antigenicity. They also suggested that the result of Uspenskaya and Meduski might have been due to spontaneous detoxification as a result of prolonged storage.

In the light of the foregoing discussion, some account of the effects of freeze-drying on proteins is necessary. The removal of water causes an increase in the concentration of salts present in the protein solution. Increased salt concentration may cause dissociation into sub-units or metal ions may bind to specific amino acid residues in the polypeptide chain impairing protein function or disrupting the tertiary structure. Changes in pH may occur with increasing hydrogen ion concentration as ice is removed, or alternatively with changes in salt concentration (Lea and Hawke, 1952). Protein solutions with an isoelectric point below pH 7 tend to become more acid with increasing salt concentration. Water molecules can be important for the structural integrity of some proteins e.g. β-lipoprotein of plasma (Oncley et al., 1950). The removal of water molecules necessary for structural integrity may cause denaturation or alternatively the dehydration effect may bring molecules into physical contact causing the formation of new cross-linking bonds. If these bonds are stronger than the normal hydrogen bonds and hydrophobic interactions maintaining protein conformation, alteration of the protein could occur.

Thus a number of important questions have to be answered:

1. Are the observed multiple forms of α-toxin native protein forms or artefacts caused by purification and freeze-drying?
2. Do these multiple forms, albeit if they are artefacts, differ in other respects apart from charge?
3. Assuming that α-toxin is a zinc metalloprotein, do these forms all possess zinc as an integral part of their structure?
4. Do they differ biologically or biochemically in any respects?

Work undertaken in this thesis goes some way to answering some of these questions. The technique of isoelectric focusing has been used to determine whether multiple forms exist in crude α-toxin. This method of electrophoresis allows the quantitative separation of isoenzymic forms of protein in pH gradients. It has revealed multiple forms in other bacterial toxins and enzymes e.g. staphylococcal hyaluronidase, deoxyribonuclease, α-toxin, δ-haemolysin, β-toxin and enterotoxin (Vesterberg et al., 1967; Wadström, 1967; Vesterberg, 1968a; Wadström, 1968; Kreger et al., 1971; Wadström and Möllby, 1971; Chang and Dickie, 1971). The method was also chosen with other points in mind. Firstly, it might prove a convenient and efficient method for the preparation of Cl. perfringens α-toxin in a highly purified state; secondly it has the intrinsic advantage of concentrating proteins as well as purifying them, a consideration of paramount importance in the case of bacterial toxins as quantitatively in crude material they represent but a small percentage of the total protein present; thirdly, this method might avoid the necessity of starting with large volumes of starting material and long tedious purification procedure with low yield; fourthly, the method might

also provide a means of obtaining a number of highly purified toxins of Cl. perfringens by a single step procedure. This would facilitate the study of the biological activity of individual toxic products.

4. Biological Characteristics of α-Toxin.

a. Substrate Specificity:

The literature on this aspect of α-toxin is extensive and contradictory. Several points have to be taken into account in an attempt to resolve these discrepancies. Firstly, not all authors have used highly purified lecithinase. Cl. perfringens α-toxin (phospholipase C) is readily available from several commercial sources as a freeze-dried culture filtrate or partially purified derivative, usually an ammonium sulphate precipitate. The value of studies on the hydrolysis of pure phospholipids under a variety of conditions is rendered questionable by the use of reagents of low purity. The only authors to avoid this criticism have been Bangham and Dawson (1962). Secondly, it has been assumed by many authors that the enzyme has the same avidity for pure phospholipids from various sources, without presenting evidence to substantiate this assumption. Thirdly, work carried out with pure phospholipids does not correlate with that carried out with mixtures of phospholipids, a fact that is probably explained by effects on the zeta potential by individual phospholipids in a mixture. Finally, the criteria of purity of phospholipids used by earlier workers are now known to be inadequate.

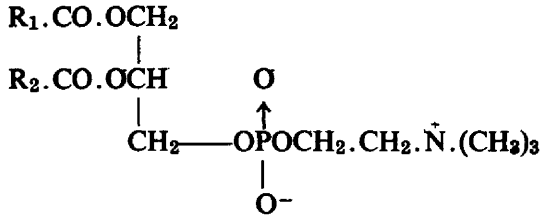
That lecithin was split to diglyceride and phosphocholine was first established by Macfarlane and Knight (1941) using crude lecithin. This finding has been confirmed many times since with pure substrates

(Zamecnik et al., 1947; Bangham and Dawson, 1962; Matsumoto, 1961 and others). Macfarlane and Knight's lecithin, phosphatidyl choline (PTC), contained other phospholipids (circa 30%) principally cephalins and sphingomyelin. The best characterised cephalins are phosphatidyl ethanolamine (PTE) and phosphatidyl serine (PTS). Unlike PTC, PTE and PTS, sphingomyelin does not contain a glycerol backbone. The structures of these phospholipids are shown in Figure 2 .

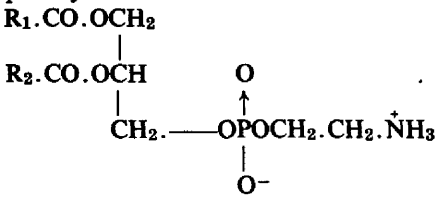
Since 1941, α-toxin has been shown to hydrolyse other phospholipids. These findings are summarised in Table 8 . Hydrolysis of sphingomyelin has been demonstrated in phospholipid mixtures or with the pure phospholipid by all authors except Zamecnik et al., (1947). Saito and Mukoyama (1968) demonstrated that two other phosphosphingolipids, ceramide aminoethylphosphonate and ceramide phosphorylethanolamine (sphingoethanolamine), were hydrolysed by α-toxin and that Ca^{2+} increased their rates of hydrolysis. The investigations carried out on PTE hydrolysis require qualification. In the studies of Zamecnik et al., (1947) and Macfarlane (1948) α-toxin neither attacked cephalin mixtures (PTE + PTS) nor the individual phospholipids. Hydrolysis of pure PTE in the presence of Ca^{2+} was demonstrated by Matsumoto (1961) and van Deenen et al., (1961) and, to a lesser extent, by Bangham and Dawson (1962), whereas Saito and Mukoyama (1968) reported its hydrolysis in the absence of activating ions, and Dyatlovitskaya et al., (1967a) found that only Zn^{2+} would potentiate its hydrolysis. Enzymatic hydrolysis of PTE in phospholipid mixtures was shown by de Gier et al., (1961), Matsumoto (1961) and Bangham and Dawson (1962), and in thromboplastin from brain by Kushner and Feldman (1958). Moreover, de Gier et al., and Bangham and Dawson observed a stimulation of PTE hydrolysis by the addition of PTC.

Figure 2 : Structure of Phospholipids commonly found in Membranes in comparison with Lecithin

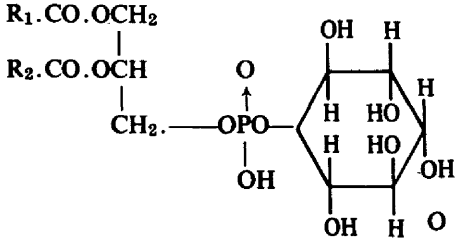
Lecithin: (Phosphatidyl choline)



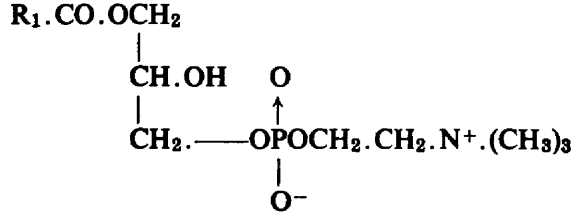
Phosphatidylethanolamine:



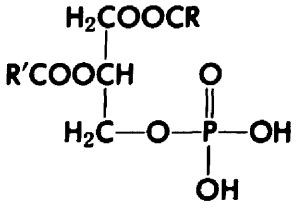
Phosphatidylinositol:



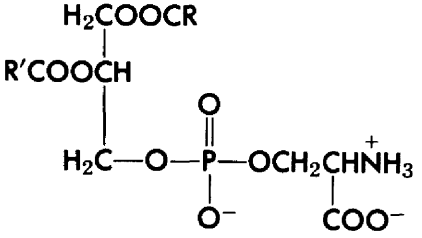
Lysolecithin:



L-α-Phosphatidic acid



Phosphatidyl serine



Sphingomyelin:

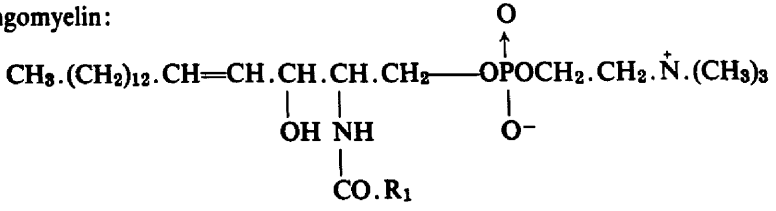


Table 8 : Hydrolysis of Phospholipids other than Lecithin by Cl. perfringens α-toxin.

Author	Year	Phospholipid Sph.	PTE	PTS	LysoPTC
Macfarlane	1942	+	ND	ND	ND
Zamecnik et al.	1947	–	–	–	–
Macfarlane	1948	+	–	–	ND
Kushner and Feldman	1958	ND	+	ND	ND
Sribney and Kennedy	1958	+	ND	ND	ND
Gowland and Willis	1961	ND	ND	ND	–
de Gier et al.	1961	+	+	+	ND
Matsumoto	1961	+	+	–	–
van Deenen et al.	1961	ND	+	ND	ND
Bangham and Dawson	1962	–	+	ND	ND
Dyatlovitskaya et al.	1967a	ND	+	ND	ND
Saito and Mukoyama	1968	+	+	ND	ND
Rosenberg and Condrea	1968	+	+	ND	ND

+ = hydrolysed

– = not hydrolysed

ND = Not Determined

Only de Gier et al., (1961) have reported that α-toxin hydrolysed PTS, although only to a minor extent in phospholipid mixtures from erythrocyte stroma. α-toxin also attacked choline plasmalogen -- a phosphatide with the fatty acid in the α' position replaced with an αβ-unsaturated ether (Kiyasu and Kennedy, 1960; Gottfried and Rapport, 1962; Norton et al., 1962; Ansell and Spanner, 1965) but not ethanolamine plasmalogen (Ansell and Spanner, 1965), or lysolecithin (Zamecnik et al., 1947; Matsumoto, 1961; Gowland and Willis, 1961) or lysocephalin (Matsumoto, 1961). In addition cerebroside, mono- and diphenylglycerophosphate, α-glycerophosphate and glycerylphosphorylcholine were not hydrolysed by α-toxin (Macfarlane and Knight, 1941; Macfarlane, 1942, 1948; Zamecnik et al., 1947).

Studies in recent years on the homogeneity of phospholipids from a single source have revealed that they exist as a heterogeneous mixture. For instance, lecithin from human red cells membranes appears to consist of some twenty different molecular species, differing in substituents on the α' and β carbons of the glycerol (van Golde et al., 1967). It has been calculated by van Deenen (1969) that the total number of molecular species of phospholipid in a human red cell membrane may be as high as 150-200. What effect this heterogeneity has on the avidity of enzyme for substrate is impossible to gauge at the moment.

b. The Effect of α-Toxin on Membrane Bound Structures in vitro:

The most important consideration in reviewing this aspect of studies carried out with α-toxin has been the nature and purity of the material used by various authors. The vast majority of work has been attempted with crude or ammonium sulphate precipitated toxin and with

commercially available phospholipase C derived from Cl. perfringens. Most authors have used these reagents without due consideration of the possible contributions of other factors to the effects observed. At best qualitative analysis for other toxins or enzymes was stated or controls using specified enzymes from other bacteria instituted, for example, by the use of Cl. histolyticum collagenase or Vibrio cholerae neuraminidase. The type of α-toxin preparation used is indicated in each of the following sections. These studies are reviewed in five sections (i) the erythrocyte, because it has been studied most extensively and because it presents the intriguing problem of the mechanism of hot-cold haemolysis; (ii) procaryotic cells, protoplasts and spheroplasts, because the lipid composition of their membranes differs significantly from that of mammalian cells; (iii) mammalian cells in suspension and monolayer; (iv) membrane bound organelles from mammalian cells and (v) membranes isolated from mammalian cells or cell-associated structures.

i. The erythrocyte: When susceptible erythrocytes are exposed to α-toxin they do not lyse immediately, but do so when the toxin-erythrocyte mixture is cooled to $0^{\circ}C - 4^{\circ}C$. This type of reaction was first described for staphylococcus β-toxin by Glenny and Stevens (1935) and is termed hot-cold haemolysis. It was van Heyningen (1941b) who first noted this phenomenon in relation to Cl. perfringens α-toxin. However, he also showed that high concentrations of α-toxin caused lysis at $37^{\circ}C$, and Macfarlane (1950) demonstrated that both hot and hot-cold haemolysis were the result of phospholipid hydrolysis in the erythrocyte membrane. Her studies showed that erythrocytes of different species varied in their susceptibility to hot-cold haemolysis, susceptibility decreasing in the sequence sheep>horse>rabbit>human. The rate at which haemoglobin leaked

out was not just a function of the rate of hydrolysis of phospholipid but of damage to the molecular architecture of the membrane. The level of critical damage was different in sheep and horse cells; horse erythrocytes tended to lyse completely when over 50% of the hydrolysable phospholipid had been attacked, whereas sheep erythrocytes could have up to 70-75% hydrolysed before complete haemolysis occurred. However, phospholipid extracted from sheep erythrocytes was more readily attacked by α-toxin than that from horse erythrocytes, leading Macfarlane to propose that differences in the rate of hydrolysis of phospholipid in intact cells might be due to differences in the relative proportions of sphingomyelin and PTC in the membranes. The susceptibility of erythrocytes of different species to α-toxin will be discussed in greater detail below (page number 57).

Detailed kinetic studies of haemolysis have been carried out by Burrows (1951), Ikezawa (1963) and Ikezawa and Murata (1964). Burrows used dialysed culture filtrate stated to give 'no haemolysis in isotonic phosphate buffer', and thus assumed to be free of θ-toxin. Using rabbit erythrocytes, he found a linear relationship between % hot-cold haemolysis and the logarithm of the α-toxin concentration over the range 20-70% haemolysis. The use of rabbit erythrocytes is interesting in light of Macfarlane's observations that these were insensitive to hot-cold haemolysis. Nevertheless, Burrow's studies clearly showed that the temperature of warm phase incubation, red cell concentration, buffer constitution and pH influenced the reproducibility of assays.

Ikezawa used partially purified toxin which appears from his rigorous controls to have been free of θ-toxin. He found that hot-cold

haemolysis by his α-toxin preparation obeyed first order kinetics with respect to erythrocyte concentration, and deduced from kinetic data that the amount of substrate was reduced to a critical level in the erythrocyte membrane (cf. Macfarlane, 1950, vide supra). The lag period to haemolysis consisted of two phases. The first, defined as latent period t_1, was independent of enzyme concentration and inhibited by antitoxin. The authors concluded that this represented the time taken for activation of the enzyme by the calcium-substrate complex. The second, designated latent period t_2, was inversely proportional to the enzyme concentration and addition of antitoxin caused only partial inhibition. This was thought to represent the interval between breakdown of the substrate in the erythrocytes and the onset of haemolysis.

No author has so far assessed the haemolytic spectrum of Cl. perfringens α-toxin by comparative doubling dilution titrations in the manner of Wiseman (1965) in his study of staphylococcal β-haemolysin. Burrows (1951) noted that the fragility of rabbit erythrocytes in the presence of α-toxin varied from animal to animal and from time to time in the same animal, but this variation 'was of a relatively low order'.

Ikezawa and Murata (1964) included an analysis of the kinetics of haemolysis of four species of mammalian erythrocytes. K_m values for Ca^{2+} for the substrate on each type of erythrocyte were obtained (Table 9). These figures demonstrate a 100 fold variation in the affinity of Ca^{2+} for the substrate on each cell, and reflect differences in the sensitivity of each type of cell to enzymic attack. The susceptibility of various species of erythrocytes to a haemolysin is usually examined under standardised conditions. In this case a constant concentration of Ca^{2+}

Table 9 . Kinetics of Haemolysis of Mammalian Erythrocytes by Cl. perfringens α-Toxin - K_m values of Calcium ions to Erythrocyte Substrates.

Species of Erythrocyte	K_m value for Calcium ions
Rabbit	1.25×10^{-4} M
Human (O)	2.63×10^{-4} M
Sheep	1.54×10^{-3} M
Horse	1.23×10^{-2} M

Compiled from the data of Ikezawa and Murata (196

will reflect in different rates of phospholipid hydrolysis according to erythrocyte species. Their findings can be interpreted in terms of Bangham and Dawson's model (1962) as being the concentrations of calcium necessary to create the zeta potential on the erythrocyte membrane required for K_m (i.e. $\frac{1}{2}$ maximal velocity of haemolysis). It must be pointed out that these data bear no strict relationship to susceptibility to cold shock. Indeed rabbit and human erythrocytes, although showing lower K_m values, were stable to cold shock whereas those of horse and sheep were not (Macfarlane, 1950).

In hot-cold haemolysis, lysis in the 'cold' phase was independent of the action of the toxin for van Heyningen (1941b) showed that antiserum added after incubation in the hot and prior to cooling, failed to prevent haemolysis. From their kinetic studies, Ikezawa and Murata argued that since cold lysis took place instantaneously, it was not rate limiting in the overall reaction sequence and therefore non-enzymic in nature; rather it involved cold shock to the cells. Addition of Ca^{2+} at this stage did not affect the haemolytic process. As is argued above, resistance of erythrocytes to cold shock must be an important factor in sensitivity differences between erythrocytes; but to invoke this theory could be an oversimplification as sheep erythrocytes do not undergo appreciable haemolysis after treatment with Bacillus cereus phospholipase C (Zwaal et al., 1971).

For the other hot-cold haemolysin, staphylococcus β-toxin, data are available relating susceptibility of erythrocytes to their phospholipid composition. Table 10 gives the phospholipid distribution in erythrocytes from various mammalian species and the spectrum of

Table 10 Relationship between Spectrum of Erythrocyte Sensitivity to Partially Purified Staphylococcal β-haemolysins and to the Phospholipid Distribution within the Erythrocyte Membranes.

Erythrocyte Species	Haemolytic Titres of β-haemolysins.†		Phospholipid Composition of Erythrocytes as percentage of the phospholipid present°°							
	Strain R-1	Strain 252-F	Sph	PTC	PTE	PTS	PTI	PTA	LPC	X
Sheep	2048	512	51.0	ND	26.2	14.1	2.9	0.3	ND	4.8
Ox	512	512	46.2	ND	29.1	19.3	3.7	0.3	ND	1.7
Man	256	128	26.9	28.9	27.2	13.0	1.3	2.2	-	-
Cat	64	64	26.1	30.5	22.2	13.2	7.4	0.8	0.3	-
Rabbit	64	32	19.0	33.9	31.9	12.2	1.6	1.6	0.3	-
Pig	32	32	26.5	23.3	29.7	17.8	1.8	0.3	0.9	-
*Fowl	32	32	21.4	42.0	23.5	4.8	2.0	1.0	2.6	2.7
Rat	4	4	12.8	47.5	21.5	10.8	3.5	0.3	3.8	-
Mouse	4	4								
Frog	4	4								
Dog	4	4	10.8	46.9	22.4	15.4	2.2	0.5	1.8	-
Guinea Pig	4	4	11.1	41.1	24.6	16.8	2.4	4.2	0.3	-
Horse	4	4	13.5	42.4	24.3	18.0	0.3	0.3	1.7	-

Sph - sphingomyelin.
PTC - phosphatidyl choline
PTE - phosphatidyl ethanolamine
PTS - phosphatidyl serine
PTI - phosphatidyl inositol
PTA - phosphatidic acid
LPC - lysophosphatidyl choline
X - unidentified phospholipids
ND - not detected.

†Data of Wiseman (1965)
*Data of Kates and James (1961)
°°After Rouser et al, 1968.

erythrocyte sensitivity to partially purified staphylococcus β-toxin from strains R-1 and 252-F. From this table it can be seen that susceptibility to hot-cold haemolysis can be correlated with the sphingomyelin content of the erythrocyte membranes (Wiseman and Caird, 1967). The staphylococcal enzymewas, however, without effect on pure PTC and PTE (Wiseman and Caird, 1967). Since de Gier et al., (1961), Matsumoto (1961) and Bangham and Dawson (1962) have shown that Cl. perfringens α-toxin can breakdown a variety of phospholipids in a mixture it is difficult to draw clear out correlations for Cl. perfringens α-toxin. Other factors including accessibility of phospholipid substrate and the relative importance of individual phospholipids in membrane structure and function should also be considered. Yet it is interesting that sheep erythrocytes, which are the most susceptible species to α-toxin, have the highest sphingomyelin content (see page 60).

Lucy (1964) proposed that phase changes between a globular structure and a continuous bimolecular leaflet organisation (lamellar) might be of physiological significance in allowing dynamic changes in the permeability of membranes. It is possible that α-toxin may cause the transformation of membrane lipids from the lamellar to a globular or micellar form, through hydrolysis of selected phospholipids in the membrane of the erythrocyte. Such a transformation would result in the formation of channels through which diffusion of solutes might be facilitated. Subsequent cold shock might exaggerage this effect. Thus susceptibility to lysis might depend on whether the phospholipids hydrolysed were involved in the maintenance of the lamellar state.

ii. Microbial cells, protoplasts and spheroplasts: Davie and Brock (1966), using the uptake of ^{14}C-glycine by the cells as a measure of

membrane damage, found that intact bacteria were resistant to α-toxin. Table 11 shows that protoplasts of Micrococcus lysodeikticus were more sensitive to lysis by α-toxin than the spheroplasts prepared from Streptococcus faecalis. Bernheimer et al., (1968b) found protoplasts of Strep. faecalis along with those of Sarcina lutea and spheroplasts of Escherichia coli to be completely resistant.

M. lysodeikticus membranes have been shown to contain little nitrogen-containing phospholipid, whereas Freimer (1963) showed that Group A streptococci had some choline containing phospholipid in their membranes. Yet the former membranes were more sensitive than Group D streptococci membranes. This observation cannot be explained, but the treatment used in the preparation of protoplasts or spheroplasts may be an important consideration.

It would be interesting to determine whether spheroplasts and protoplasts of clostridial species are sensitive to α-toxin (see page 25)

iii. Mammalian cells in suspension and monolayer: The application of tissue culture to the study of clostridial toxins was initiated by Barg (1932). In 1933 he showed that culture filtrates of Cl. perfringens were cytotoxic. Cl. perfringens filtrates were shown by Lasfargues and Delaunay (1946) to be toxic to guinea pig spleen explants. Chick embryo cells in tissue culture have been used as a titration system for the α-toxin of Cl. perfringens (Horvath, 1959; Zemskov, 1963; Shemanova et al., 1965). Shamraeva and Volkova (1966) investigated evidence in favour of the use of tissue cultures of chick fibroblasts for the determination of toxic activity and antitoxin combining power. It was asserted that toxicity can be detected more rapidly and inexpensively by

Table 11 Effect of *Cl. perfringens* α-toxin on Microbial Cells, Protoplasts and Spheroplasts.

Organism	Strain	Structure Tested	Preparation Technique	Source of α-toxin°	Dosage	Sensitivity
(1) *Micrococcus lysodeikticus*		Whole viable cells		Commercial (Worthington Biochemicals)	50µg	+ve
Streptococcus faecalis var zymogenes	X14S	As above		"	"	-ve
Strep. faecalis	X46S	As above		"	"	++ve
Strep. faecalis (mutant)	X46S-R1	As above		"	"	-ve
M. lysodeikticus		Protoplasts	Lysozyme	Commercial (Worthington Biochemicals)	50µg	++++
Strep. faecalis var zymogenes	X14S	Spheroplasts	Lysozyme + Trypsin	"	"	-
Strep. faecalis	X46S	Spheroplasts	Lysozyme + Trypsin	"	"	++
Strep. faecalis	X46S-R1	Spheroplasts	Lysozyme + Trypsin	"	"	+
(2) *Sarcina lutea*		Protoplasts	Lysozyme	Wellcome laboratories. Crude culture filtrate concentrate.	200µg	-
Strep. faecalis		Protoplasts	Lysozyme	"	"	-
Escherichia coli		Spheroplasts	Lysozyme + Penicillin	"	"	-

(1) Davie and Brock (1966)
(2) Bernheimer *et al* (1968b)

Symbols: Impairment of uptake of ^{14}C-glycine by whole cells:
-ve, resistant;
+ve, fairly resistant;
++ve, sensitive
Lysis:
- no lysis;
+ resistant to lysis;
++ moderately sensitive to lysis;
++++ sensitive to lysis

° Purity of α-toxin not recorded.

this method than by titration of the lethal effect in mice. Saltykov et al., (1961) demonstrated that the toxins of Cl. perfringens were toxic to chick embryo and Hela cells, but not to monkey fibroblast cells, HLS, Detroit 6 (human sternal bone marrow), and human embryo muscle cells. Although they do not specify the precise nature of the 'toxin' used the latter finding on muscle cells is interesting in the light of Grossman's work (1967) (see pages 65 and 66). These studies were, however, limited to descriptions of morphological damage to the cells.

Rodbell (1966) and Rodbell and Jones (1966) have studied the effects of α-toxin on free fat cells isolated from rat epididymal adipose tissue. Lysis of the fat cells occurred at high concentrations, whereas low concentrations of α-toxin stimulated glucose and amino acid utilisation. Their findings suggested that the effects of α-toxin on both glucose uptake and cellular integrity were manifestations of the amount of phospholipid hydrolysed by α-toxin (cf. section (i) 'The erythrocyte', page 55). Both fat cells in adipose tissue and muscle cells normally possess a basement membrane. Rodbell (1964; 1966) prepared free fat cells by treating adipose tissue with Cl. histolyticum collagenase and showed that this removed the basement membrane. Thus 'naked' fat cells were sensitive to α-toxin. The relevance of this finding to the observations of Saltykov et al., (1961) and to the possible penetration of α-toxin to the mitochondria of muscle cells requires investigation.

Platelets have been used as a model system by Bernheimer and Schwartz (1965) for studying the interaction of a bacterial toxin with membranes. A crude preparation of α-toxin, stated to contain no θ-toxin,

but not tested to prove or disprove this, destroyed rabbit blood platelets. These authors presented evidence to show that the effects that they had observed with the ε-prototoxin of Cl. perfringens type D were due to contaminating θ-toxin. Moreover streptolysin O (SLO), another oxygen labile haemolysin, destroyed platelets rapidly. Thus the morphological damage attributed to α-toxin remains in doubt.

It is clear that more detailed investigations of the interaction of α-toxin with mammalian cells, using homogeneous preparations, are required. This would provide results having immediate significance and would eliminate the need for negative controls using reagents of doubtful homogeneity e.g. Cl. histolyticum collagenase.

iv. Membrane-bound organelles from mammalian cells: Such structures from mammalian cells have been treated with α-toxin for two purposes: (a) as a means of determining a possible intracellular target site for toxic action that might account for cell death and (b) as a means of investigating the mechanism of action of other cytolytic agents by comparison with the effect of an agent of "known" substrate specificity.

The sensitivity of mitochondria and lysosomes to α-toxin has been tested. Macfarlane and Datta (1954) studied the action of crude α-toxin, a culture filtrate concentrate stated to contain other toxins and enzymes, on mitochondria isolated from the liver of various mammalian species; all preparations of mitochondria were sensitive. In contrast to the findings of Macfarlane (1950) with erythrocytes of different species, there was a complete absence of any marked species difference in the susceptibility of mitochondrial phospholipid to attack by α-toxin. Succinic oxidase activity was the most sensitive enzyme tested.

Cytochrome oxidase and succinic dehydrogenase activities were also impaired although to a lesser extent. Grossman et al., (1967) showed the same enzymic defects in mitochondria isolated from muscle cells treated in vitro with α-toxin and were able to correlate morphological damage of in vitro treated mitochondria with that observed in intoxicated muscle tissue. Curiously, they also observed that the oxidation of α-glycerophosphate associated with the inner mitochondrial membrane was more inhibited than the oxidation of pyruvate or malate associated with the outer mitochondrial membrane. It remains to be shown, nevertheless, whether α-toxin by itself is capable of penetrating intact muscle cells to impair mitochondrial respiration or whether other toxins and enzymes found in crude toxin contribute to this. It is possible that damage to muscle tissue caused by injury or inoculation could provide ready access, thereby bypassing any natural permeability barrier of the muscle cells.

Lysosomes from rabbit blood leucocytes were lysed by α-toxin as evidenced spectrophotometrically and by the release of β-glucuronidase, a lysosome-bound enzyme (Bernheimer and Schwartz, 1965). The toxin used was free of θ-toxin and hyaluronidase, but contained collagenase activity. No comparative studies on the resistance or sensitivity of lysosomes prepared from other tissues or from other species have as yet been carried out. Again the question of access of α-toxin to these structures remains unanswered. However, from the observations of Butler (1942; 1943) and Robb-Smith (1945) it is known that the nuclei of toxin-treated polymorphonuclear leucocytes underwent morphological damage. Whether this was due to the uptake of the α-toxin by the leucocytes, followed by lysis of the lysosomes and autodigestion by lysosomal enzymes, is an unresolved problem (see page 97).

v. Isolated membranes: In recent years increasing use has been made of specific enzymes in the investigation of the molecular architecture of membranes. Lenard and Singer (1968), treated human erythrocyte ghosts with a commercial preparation of α-toxin. Under the conditions of their experiments 68-74% of the total membrane phosphorus was released rapidly. Examination of the α-toxin treated membranes by circular dichroism revealed that they were indistinguishable from untreated controls. Such treatment did not, as judged by phase contrast microscopy, cause disruption of the ghosts or alteration in the overall conformation of the protein in the membrane. By contrast, Gordon et al., (1969) demonstrated slight changes both in the circular dichroism and optical rotatory dispersion spectra of α-toxin-treated erythrocyte membranes. These results were interpreted as indicating that hydrophobic interactions stabilise the membrane structure, whereas polar and ionic groupings on lipids in contact with the bulk aqueous phase are exposed to the action of the enzyme.

Finean and Martonosi (1965) treated muscle microsomes with α-toxin from Cl. perfringens. This membrane is derived from the endoplasmic reticulum. These membranes shrank to 45% of their original surface area after hydrolysis of 70% of the phospholipid in the membrane. These authors concluded that lipid occupies approximately 80% of the area of muscle microsomal membranes. It is of interest that phospholipid hydrolysis was accompanied by membrane shrinkage. As discussed earlier, this may to some extent contribute to the phenomenon of hot-cold haemolysis (see page 61).

Phospholipase C from Cl. perfringens has also been used to identify the chemical nature of features observed in the electron

microscope. Lessops (1967) used this enzyme to show that the lanthanum-staining material, observed in the presence of lanthanum ions as an electron opaque layer external to the cell membrane of embryonic chick cells, can be removed from the cell surface and might therefore be composed of phospholipid.

The Limitations of using crude α-toxin to treat membranous structures have been admirably exemplified by electron-microscopic studies of α-toxin treated erythrocyte ghosts, plasma membranes and membrane bound virus particles. The latter are considered under this section for two reasons :-

(1) the outer membrane coats of these virions were host-cell derived and therefore represent a form of isolated mammalian membrane

(2) the effects observed upon α-toxin treatment had features in common with the effects demonstrated on membranes isolated from mammalian cells per se.

With the exception of the studies of Habermann and Pohlmann (1959), Finean and Martonosi (1965) and Benedetti and Emmelot (1966) an ignorance of the Ca^{2+} dependence of α-toxin is evident, a fact that has certainly been a major cause of misinterpretation of results when coupled with a lack of appreciation of the crude nature of the commercial preparations of α-toxin employed. Unless otherwise stated all authors used Sigma type 1 phospholipase C from Cl. perfringens (α-toxin). Moreover with the above exceptions they used phosphate buffered saline (PBS) as the suspending fluid for treatment. The inhibitory effect of phosphate on α-toxin was demonstrated by Macfarlane and Knight (1941). Indeed this is the primary reason for using PBS as the suspending buffer for θ-toxin titration.

Following treatment with crude α-toxin, ring like structures were revealed by negative staining techniques on Rauscher leukaemia virus (Padgett and Levine, 1965; Kemp and Howatson, 1966), the WSN strain of influenza A virus (Simpson and Hauser, 1965), vesicular stomatitis virus (Simpson and Hauser, 1966a), human erythrocyte ghosts (Dourmashkin and Rosse, 1966) human, bovine, rabbit, sheep and goat erythrocyte ghosts (Kemp and Howatson, 1966), chick embryo fibroblast cell membranes (Simpson and Hauser, 1966a), rat liver plasma membranes (Benedetti and Emmelot, 1966; 1968). These had a diameter of 280-500Å and a border 60-70Å wide. In some cases they were only found in the plane of the membrane, in others dispersed as free rings or arcs which were arranged in ribbons, filaments or complex coiled aggregates. In all cases these structures had a globular substructure. Padgett and Levine (1965) and Simpson and Hauser (1965) initially suggested that these structures represented nucleoprotein elements released from the viral nucleoid by α-toxin, a conclusion later shown to be erroneous by the mammalian membrane studies.

The involvement of lipid components of viral and mammalian cell membranes in the formation of these rings was first demonstrated by Simpson and Hauser (1966b). Reconstruction experiments involving treatment of artificial mixtures of major lipid components of membranes established that cholesterol was essential (Simpson and Hauser, 1966b; Kemp and Howatson, 1966). Indeed, α-toxin treatment of cholesterol alone caused ring formation. Simpson and Hauser went on to demonstrate that the agent in crude α-toxin responsible for this phenomenon was not α-toxin *per se*, but a heat labile factor in the crude mixture.

By contrast Habermann and Pohlmann (1959) and Finean and Martonosi (1965) did not observe the formation of such structures on α-toxin treated human erythrocyte ghosts and rat and rabbit muscle microsomal membranes respectively. Instead both groups observed the appearance of dense droplets or globules in OsO_4 fixed specimens either in association with the treated membranes or free in the surrounding milieu. The fact that muscle microsomes contain very little cholesterol (Martonosi, 1968) in comparison with other membranes and the use of highly purified α-toxin by Habermann and Pohlmann may have contributed to these findings. Habermann and Pohlmann were unable to identify the nature of these globules by acetone or ethanol extraction or by treatment with lipase, whereas Finean and Coleman identified diglyceride as a component.

Although it must be borne in mind that α-toxin/substrate interaction could occur in the absence of Ca^{2+} and that the sphingomyelinase of Pastan et al., (1968) (see page 74) did not require Ca^{2+} for activity, the role of α-toxin, if any, in the formation of the ring-like structures is doubtful. Studies undertaken towards the end of work for this thesis sought to clarify the effect of highly purified α-toxin on membranes (see page 87).

c. Minimum Effective Doses in Biological Assays:

The quantities of protein of purified α-toxin preparations obtained by various authors giving rise to biological manifestations of α-toxin activity are summarised in Table 12. In most cases these values were calculated from the specific activities provided by the authors concerned.

Table 12 Biological Potency of Cl. perfringens α-Toxin*

Author	Year	Lethal Dose** LD_{50} (μg)	Lethal Dose** MLD (μg)	Haemolytic Units (μg x 10^{-2})	LV Units (μg)
van Heyningen	1941	ND	0.75	ND	ND
van Heyningen and Bidwell	1948	ND	0.63	ND	ND
Roth and Pillemer	1953	ND	0.14	1.67	ND
Habermann - A	1959	0.06	ND	1.89	ND
- B	1959	0.07	ND	2.21	ND
Stephen	1961	0.36	ND	62-124	17.5
Ispolatovskaya	1962	ND	ND	ND	0.06
Shemanova et al.	1965	ND	0.41-0.52	ND	0.04
Shemanova et al.	1968b	ND	0.25	ND	ND
Ito	1968	ND	ND	ND	2.3-3.2

* See Table 5 for Method of Purification

** All data refer to lethal dose for 20g mouse in 24 hours

ND = Not determined

5. Biological Activity-Unitarian Hypothesis.

Three biological activities have been associated with α-toxin - lethality, hot-cold haemolysis and lecithinase activity. The question of whether these three activities are manifestations of a single molecular species or due to three very closely related entities has intrigued investigators since the observations of Macfarlane and Knight (1941). Roth and Pillemer (1953) found that at all stages of their 130 fold purification, the lecithinase activity, lethal activity and hot-cold haemolytic activity remained proportional. However, since they did not obtain a homogeneous protein they added that 'conclusive proof that these three activities are functions of a single agent is lacking'. The purified preparations of Habermann (1959) obtained by two distinct purification procedures had the same ratios of haemolysin/lecithinase/ lethal toxin within the limits of their experimental error. Lethal and lecithinase activity were associated with the highly purified α-toxins of Shemanova et al., (1965) and Ispolatovskaya and Levdikova (1962), although these workers did not assess the ratios of these biological activities throughout the purification procedures. No information was available from Ispolatovskaya (1964) as to whether the multiple forms of lecithinase she observed possessed other activities (see page 46).

Activation and inhibition studies have also contributed to the unitarian view. Ispolatovskaya et al., (1961) showed that concentrations of EDTA greater than 2% (0.06M) irreversibly inactivated the lecithinase and hot-cold haemolytic activities of α-toxin. Also loss of lecithinase activity through toxoiding with formalin was associated with a gradual decrease in toxicity for mice (Ispolatovskaya and Larina, 1959). Nagler (1941) found that the inhibiting effect of

α-antitoxin on lecithinase activity paralleled its protective action against the lethal activity of the toxin. Both lecithinase activity and hot-cold haemolytic activity require Ca^{2+} for activation. Treatment of α-toxin with lecithin abolished its lethal and haemolytic effects (Zamecnik et al., 1945).

Conversely, Dolby and Macfarlane (1956) found that the enzymic activity of α-toxin towards aqueous lecithin was not directly related to its lethality for laboratory animals or its haemolytic titre with all strains of type A examined. The variance in the toxicity of α-toxin from different strains was attributed to conformational differences in the α-toxins elaborated. More recently, Lynch and Moskowitz (1968) found that two preparations of α-toxin of differing purity did not possess the same ratio of lecithinase to lethal activity. They, however, thought that this might be explained in terms of degraded lecithinase having the ability to combine with substrate but lacking toxicity in vivo. As the lecithinase was purified the amount of degraded toxin would decrease, and thus the toxic dose in vivo would apparently decrease.

Thus, although the concensus of opinion at the moment still supports the view elaborated by Macfarlane and Knight over 30 years ago, it still remains conceivable that the haemolytic, lethal and lecithinase activities are manifestations of separate groupings on the one molecule or are due to individual species of α-toxin, as yet unresolved by biochemical techniques. It is pertinent that the same arguments were advanced in the case of B. cereus toxin, that its haemolytic, phospholipase C and lethal activities were properties of a single molecule. Nevertheless, Johnson and Bonventre (1967) established by a variety of methods that three distinct extracellular products were involved.

Furthermore, the hydrolyses of lecithin and sphingomyelin have long been assumed to be a manifestation of a single enzyme produced by Cl. perfringens, viz. α-toxin, (Macfarlane, 1942, 1948; Matsumoto, 1961; Saito and Mukoyama, 1968).

Slein and Logan (1963; 1965) showed that Bacillus cereus culture filtrates contained a number of phospholipases. One of these split PTE and PTC, whereas others had a definite specificity for sphingomyelin and phosphatidyl inositol. An enzyme was also isolated from extracts of rat liver and brain that hydrolysed sphingomyelin, but not lecithin (Kanfer et al., 1966; Barnholz et al., 1966). The β-toxin of Staphylococcus aureus is also a hot-cold haemolysin, but does not hydrolyse lecithin, only sphingomyelin and lysolecithin (Doery et al., 1965; Maheswaren and Lindorfer, 1967; Wiseman and Caird, 1967). Thus there is evidence from other fields that phospholipases can hydrolyse sphingomyelin selectively.

In 1967 Macchia and Pastan, whilst investigating the effect of thyroid stimulating hormone on dog thyroid slices, sought to determine its mechanism and site of interaction on the thyroid by treating thyroid slices with specific enzymes prior to exposure to TSH. An enzyme in culture filtrates of Cl. perfringens strain BP6K was found to render the slices unresponsive to TSH, whilst itself increasing the oxidation of (1-^{14}C) glucose to $^{14}CO_2$ and stimulating the incorporation of ^{32}Pi into phospholipid. Initially they identified this component of culture filtrates as lecithinase C on the ground that the lecithinase and thyroid stimulating activities were inseparable over a 240 fold purification. However, in a subsequent paper, these same authors (Macchia et al., 1967) reported the separation of this thyroid factor from proteolytic

activity, θ-haemolysin, neuraminidase and lecithinase C. Thyroid factor was destroyed by pronase and trypsin and had an estimated molecular weight by gel filtration on Sephadex G-100 of 30,000. It was later identified as a sphingomyelinase by Pastan et al., (1968), which was not activated by Ca^{2+} or Mg^{2+}. Indeed Matsumoto (1961) proposed that α-toxin preparations might contain different enzymes for PTC, sphingomyelin and PTE.

The findings of Pastan et al., (1968) await confirmation by other authors and pose some interesting questions :-

(a) If sphingomyelinase is truly a unique molecular species in culture filtrates of Cl. perfringens, its activity, by implication and analogy with staphylococcus β-toxin, may explain the phenomenon of hot-cold haemolysis.

(b) This factor has the same molecular weight as that determined for lecithinase by Bernheimer and Grushoff (1967), again using Sephadex G-100 gel filtration. Thus is sphingomyelinase one of the multiple forms of α-toxin?

(c) Does this factor cross-react immunologically with lecithinase in gel diffusion and does it possess lethal activity?

As part of the isoelectric focusing studies of this thesis, an attempt has been made to provide some answers to these questions.

B. THETA TOXIN (θ-TOXIN)

The production of an oxygen-labile haemolysin by Cl. perfringens was first described by Wuth (1923) and Neill (1926). Filtrates exposed as thin layers to the air exhibited reduced haemolytic activity, but the

activity could be restored by treatment of the filtrates with sodium hydrosulphite, a reducing agent.

The clear definition of two haemolysins in type A filtrates, θ and α, was achieved through the work of Schnayerson and Samuels (1930), Prigge (1936), van Heyningen (1941b) and Gale and van Heyningen (1942). A review of this early work has been given by Oakley (1943) and the reader is referred to this valuable contribution for a detailed description of their findings.

That this haemolysin was serologically related to the oxygen-labile haemolysins produced by Streptococcus pneumoniae, Streptococcus pyogenes and Clostridium tetani, all of which had previously been shown to cross react serologically, was first demonstrated by Todd (1941; see also Todd 1934; 1938).

Investigations into the mode of action of this toxin, its chemical nature, and its purification have rarely been undertaken, probably because of the findings of Evans (1943a;1945a; 1945b) that virulence was not correlated with the production of θ-toxin and θ-antitoxin neither protected guinea pigs against type A infections nor increased the protective power of α-antitoxin. Furthermore, Neill (1926) had shown that normal serum inhibited the haemolytic activity of θ-toxin and Oakley and Warrack (1941) suggested that the occurrence of natural antibody neutralised this toxin in vivo.

1. Purification.

Prigge (1937) was the first to achieve a partial separation of the θ- and α-toxins by precipitation with ammonium and sodium sulphate.

In 1941 van Heyningen separated θ- from α-toxin, by adsorption onto erythrocytes, but unfortunately the θ-toxin was irreversibly bound to these cells. The only comprehensive attempt at purifying θ-toxin has been that of Roth and Pillemer (1955). Table 13 summarises their method along with those of other authors. The preparation of Roth and Pillemer was shown to contain two components by Tiselius electrophoresis, the θ-toxin accounting for 82% of the total protein. Habermann's preparation was immunologically homogeneous. Neither of these two authors estimated the molecular weight of their purified toxin. Stephen (1961) found that his preparation contained a mixture of antigens on immunoelectrophoresis. Although carried out with type D culture filtrates, Hauschild's purification has been included because of the separation and recovery achieved by his procedure.

2. Physical and Chemical Properties.

Most of our present information on θ-toxin is derived from the studies of Roth and Pillemer (1955) and Habermann (1959) and its properties are summarised in Table 14 .

Purified toxin was stable to lyophilisation and freezing at -35°C, but dilute solutions of toxin lost their activity quickly when frozen and the toxin was readily inactivated by boiling for 5 minutes. The toxin appears to be a protein as it reacts with protein stains, but no information is available on whether it contains carbohydrate or metal ions. Its sensitivity to proteinases has not been investigated.

3. Biological Characteristics.

a. Haemolysis:

Roth and Pillemer examined the degree of haemolysis produced

Table 13 Purification studies on Cl. perfringens θ-toxin.

Authors	Year	Strain	Purification Procedure	Purity†	Comments
Roth and Pillemer	1955	BP6K	1. 35% Methanol ppt. at pH=4.6 -5°C; redissolved at pH=6.0 in distilled water at 170 times the original protein concentration. 2. 20% Methanol ppt. pH=5.7; ionic strength = 2M NaCl. 3. 50% Methanol ppt.; ionic strength = 0.3; protein = 0.3mg/ml; pH = 8.95; -5°C. 4. pH=7 phosphate buffer added to protein =0.25mg/ml; pH=4.7; 0°C/2 hr; discard ppt; adjust pH=6.0; 20% Methanol ppt. -5°C; ppt. extracted with succinate buffer pH=6.0. 5. 10% Methanol ppt; pH=4.8; -5°C; discard ppt; adjust pH=5.25; increase methanol to 30%; extract ppt. with succinate buffer pH=6.0. 6. Preparative centrifugation at 750, 000g/ 1 hr.	N.T.	Purification = 4240 fold relative to culture filtrate. Overall Yield = 8-21%. Final product 82% pure. Free of hyaluronidase and collagenase. Contained less than 0.01% original lecithinase content of parent filtrate. Stage 1 of this purification gives a 265 fold purification relative to culture filtrate.
Habermann	1959	BP6K	A. 1. Stage (1) of Roth and Pillemer (1955) 2. Electrophoresis in starch column at pH=9.0. 3. DEAE-cellulose chromatography-elution with NaCl-phosphate buffer gradient pH=6.8	2	Purification = 22 fold relative to Stage 1 Overall Yield = 49% Fails to separate α-toxin from θ-toxin (contains 12% of α-toxin relative to Stage 1).
			B. 1. Stage (1) of Roth and Pillemer (1955). 2. 20% Methanol ppt.; -5°C; pH=6.0. 3. 50% Methanol ppt.; pH=8.0 4. Chromatography on hydroxylapatite; gradient elution with phosphate buffer.	1	Purification = 27 fold relative to Stage 1. Overall Yield = 144% Contained 0.03% α-toxin, 4% K-toxin and 0.07% hyaluronidase relative to Stage 1.
Stephen	1961	CN 1491	1. Concentration by ultrafiltration on 8% collodion membranes. 2. Zone electrophoresis in ethanolysed cellulose columns; borate buffer pH=7.1.	Complex of Antigens	Purification-insufficient da Overall Yield = 50-70%. Separated from α-toxin.
Hauschild	1965	D7	1. 60% $(NH_4)_2SO_4$ saturation of culture filtrates. 2. DEAE-cellulose chromatograph-stepwise elution (0.05M phosphate- pH=7.0 fraction.)	N.T.	θ-toxin contained -toxin Stage 2 Yield 94% relative to Stage 1.

† Symbol as in Table 5.

N.T. - not tested.

Table 14 Properties of Cl. perfringens θ-Toxin.

Molecular Weight	No data available from literature
†Sedimentation Coefficient	6.5 (a)
Optimum pH	6.75 - 6.8 (a)
Optimum Temperature	37°C (a)
Activators	Cysteine, sodium sulphite, sodium hydrosulphite (a)
Ineffective Activators	Ascorbic acid, potassium cyanide (a)
Inhibitors:	
(i) Oxidising agents	Hydrogen peroxide, potassium ferricyanide (a)
(ii) Thiol active agents	p-chloromercuribenzoate, iodoacetate.
Rate of Haemolysis Produced by θ-toxin	Linear with concentration of haemolysin (d)
Erythrocytes:	
(i) Adsorption at 0°C	+ve (b); -ve (a)
(ii) Addition of Stroma	Inactivates haemolytic activity (8.5ng/HU) (a)
Serological Characteristics	Neutralisable by anti-pneumolysin, anti-SLO, anti-tetanolysin, anti-cereolysin (c)
Biological Activities	Oxygen-labile haemolysin (b;c) Cardiotoxic (e) Lethal (a;b) Dermonecrotic (c)
Biological Potency	
(i) Haemolytic Unit (1 HU)	0.41ng (2.45×10^6 HU/mg) (a) 2.25ng (4.45×10^5 HU/mg) (f) (Method A of Table) 1.85ng (5.44×10^5 HU/mg) (f) (Method B of Table)
(ii) Lethal dose (20gm.mouse)	0.12µg (300 Activated haemolytic units) (a) 1.02µg (2,500 Unactivated haemolytic units) (a)

Authors:
(a) Roth and Pillemer, 1955.
(b) van Heyningen, 1941b.
(c) Todd, 1941.
(d) Bernheimer, 1947.
(e) Bernheimer and Cantoni, 1945.
(f) Habermann, 1959.

† Unitage as in Table 6

by θ-toxin as a function of time and found that haemolysis curves at different haemolysin concentrations all showed an initial linear phase. The rates of haemolysis were proportional to haemolysin concentration, confirming the finding of Bernheimer (1947) using crude culture supernatant. This finding suggested that θ-toxin may be enzymic in nature, but this has never been substantiated by the demonstration of a specific substrate, (see page 83). Bernheimer (1947) showed that an Arrhenius plot of the logarithm of the rate of haemolysis against the logarithm of the absolute temperature was linear for theta toxin between 0°C to 31°C, the critical thermal increment being 19,500. Similar studies with SLO and tetanolysin revealed that there were two phases of thermal increment, between 0-15°C and 20-30°C. An explanation of these findings is elusive. Bernheimer theorised that such changes may have been due to a shift in the rate-determining reaction in the series of reactions leading to lysis. This finding for θ-toxin again is not inconsistent with the view that it may be an enzyme. Table 14 gives data on the haemolytic potency of the preparations of θ-toxin to date. No haemolytic spectrum is available from the current literature.

b. Lethality and Dermonecrosis:

θ-toxin was lethal to mice on intravenous injection (Todd, 1941; Roth and Pillemer, 1955; Habermann, 1959). Lethal doses are shown in Table 14 . Death was sudden with no haemoglobinuria, as occurred with α-toxin. Bernheimer and Cantoni (1945) showed that θ-toxin was cardiotoxic, having a similar action to SLO on frog's heart. The rapid death of animals on intravenous administration of θ-toxin would suggest that this was the principal site of attack, although nothing is known of the mode of action of this toxin on the heart. Habermann (1960) also

showed that θ-toxin caused oedema in the lungs of rats injected intravenously. The finding of Todd (1941) that θ-toxin was dermonecrotic has not been further investigated.

c. Adsorption of θ-Haemolysin by Erythrocytes:

In 1941 van Heyningen reported complete adsorption of θ-haemolysin at 0°C from fresh culture filtrates and partially purified preparations. Yet, using aged culture filtrates only 70% of the haemolytic activity could be adsorbed onto erythrocytes after activation, even when the quantity of erythrocytes or the time of adsorption was increased. Neither toxoiding nor reversible inactivation was responsible for this observation (van Heyningen, 1941b) and no explanation of this phenomenon has as yet been offered. Moreover, Hauschild confirmed that θ-toxin was adsorbed onto sheep erythrocytes from culture filtrates of type D.

By comparison Roth and Pillemer reported that their highly purified θ-toxin was not at all adsorbed at 0°C, but in the presence of large amounts of haemolysin the erythrocytes were altered at 0°C and lysed when washed and resuspended in fresh buffer at 37°C. They concluded that if adsorption took place, their assay procedures were not sensitive enough to detect the loss of activity. However, occlusion of a lytic amount of toxin by the erythrocytes on centrifugation could have occurred. Their results could be interpreted as demonstrating that the lytic process did not involve physical adsorption of the toxin and was essentially enzymic in nature.

Like pneumolysin (Cohen et al., 1942) and SLO (Alouf and Raynaud, 1968c) θ-toxin is 'inactivated' by erythrocyte stroma (see Table 14). In their study Roth and Pillemer (1955) could not detect haemolytic activity in the/

supernatant of their erythrocyte stroma/θ-toxin mixture after incubation for one hour at 37°C; but these workers provided no evidence that the toxin was not adsorbed onto the stroma, and therefore assumed inactivation. 'Inactivation' was not observed with oxidised θ-toxin. Alouf and Raynaud (1968a) clearly showed that SLO binds to erythrocyte stroma. These authors also demonstrated that the adsorption of SLO onto rabbit erythrocytes which took place at 0°C, was rapid and not observed with the oxidised SLO. Furthermore the adsorption step was temperature independent, whereas lysis of the erythrocytes required a suitable temperature.

Similar studies with highly purified θ-toxin may clarify the above discrepancies.

d. Inhibition and Inactivation of θ-Toxin:

Thiol blocking agents inhibited θ-toxin, but this inhibition could be overcome by an excess of cysteine (Roth and Pillemer, 1955). These experiments were carried out in a system in which unactivated θ-toxin was first treated with inhibitor, reactivated with cysteine and then tested for haemolytic potency. By contrast Alouf and Raynaud (1968c) showed that activated SLO was inhibited by p-chloromercuribenzoate but the lytic activity of SLO was not inhibited by this agent when SLO was bound to erythrocytes. Similar experiments with θ-toxin would confirm whether it binds to erythrocytes or not.

θ-toxin was sensitive to oxidising agents (Table 14) as might be expected from its sensitivity to exposure to air. Gordon (1931) reported that θ-toxin was inhibited by congo red, but this inhibition was reversible, a fact that requires confirmation with highly purified θ-toxin. There has been but one report on the inhibition of θ-toxin by lecithin

(Gordon et al., 1954). These workers also found that when θ-toxin inhibited by lecithin was ultracentrifuged, haemolytic activity could be recovered. The explanation of this is obscure, since θ-toxin does not hydrolyse lecithin or cause turbidity in a saline extract of egg-yolk. The effect may have been due to 'non-specific' protein-lipid interaction involving electrostatic binding, dispersion binding or hydrophobic binding.

Antisera to other oxygen-labile haemolysins all neutralised the haemolytic activity of θ-toxin, although the avidity of antiserum for θ-toxin was lower in the heterologous system (Todd, 1941).

e. Cholesterol Inhibition:

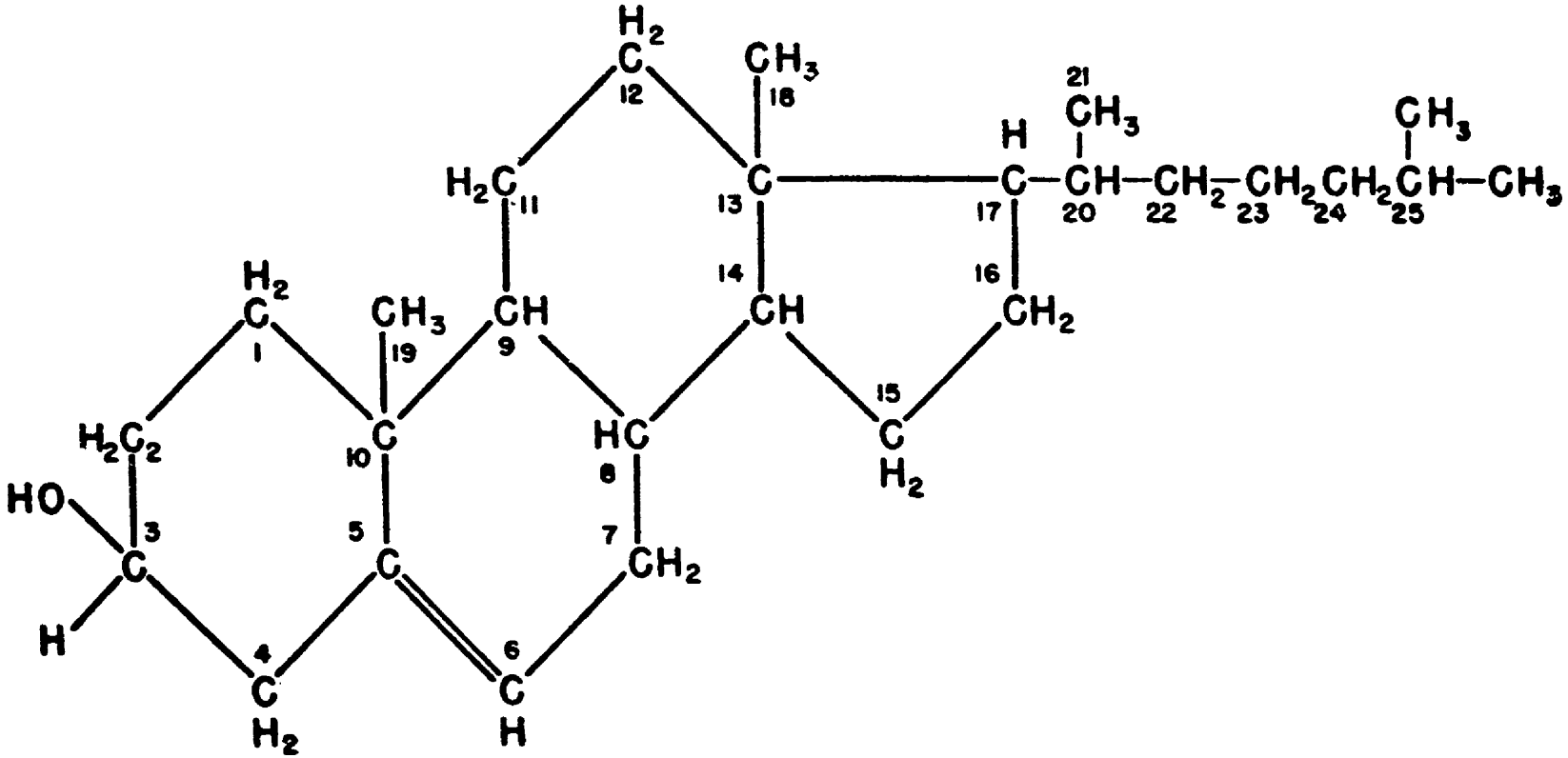
Hewitt and Todd (1939) demonstrated that SLO was inhibited by cholesterol, and the work of Cohen and Shwachman (1937) established this for pneumolysin. This inhibition was demonstrated for θ-toxin by Howard et al., (1953). From the work of Cohen et al., (1937) and Howard et al., it appears that all sterols tested possessing a β-hydroxyl group at position 3 of the sterol nucleus and a hydrophobic group attached to position 17 exert an inhibition effect on oxygen-labile haemolysins (see Figure 3). Despite this the nature of the cholesterol/θ-toxin interaction is not understood. High resolution proton magnetic resonance may help to resolve this problem.

Mason (1947) found that mouse erythrocytes were resistant to θ-toxin, a finding which Howard and Wallace (1953) confirmed for SLO. They could not explain the observed resistance of these erythrocytes in terms of a significant difference in cholesterol content.

Figure 3 : Inhibition of Cl. perfringens θ-toxin

Structure of Cholesterol

Inhibition of θ-toxin is associated with the β-hydroxyl group on carbon 3 and the hydrophobic side chain attached to carbon 17.



Cholesterol

Inhibition of θ-toxin by normal sera is due to the serum cholesterol content, delipidated serum being non-inhibitory. Turner et al., (1942) showed that if type A strains were passaged through cooked-brain medium the organisms lost their capacity to produce θ-toxin. This was due to the adsorption of the toxin onto the meat particles. It seems highly probable that the cholesterol in the meat particles was responsible for this.

f. Cytotoxicity of θ-Toxin:

There is a dearth of knowledge concerning any effects θ-toxin may have on cells and membrane-bound organelles. By comparison, the cytotoxicity of SLO for a wide range of cell types in vitro has been extensively studied. There appears to be a correlation between the sensitivity of cells to SLO and the presence of cholesterol in their cellular membranes. By analogy, it would be expected that other oxygen-labile haemolysins might have similar effects. It is conceivable, however, that although these toxins display great similarities, summarised in Table 15 , there may be differences in their affinity for some membrane-bound structures.

Bernheimer and Schwartz (1965) concluded that θ-toxin contaminating a preparation of ε-protoxin had been responsible for the cytolytic activity of the preparation towards rabbit blood platelets. θ-toxin also destroyed mast cells of the rat mesentery (Habermann, 1960). What contributions this toxin may have played in the observed effects of crude α-toxin preparations on other cells and organelles in vitro cannot be answered definitively.

Table 15 : Properties of Bacterial Oxygen-Labile Haemolysins

1.	Reversibly activated by sulphydryl reducing agents.
2.	Irreversibly inactivated by cholesterol.
3.	Lethal and cardiotoxic.
4.	Similar pH and temperature optima.
5.	Mice injected with sublethal doses of O-labile haemolysins rapidly develop a temporary resistance specifically to lethal doses of these toxins.
6.	Mouse erythrocytes are insensitive to these haemolysins.
7.	Cross neutralised by hyperimmune horse antiserum to each.
8.	Inactivated by erythrocyte stroma.

Todd (1941) referred briefly to some studies on the effect of SLO and θ-toxin on leucocytes. He stated that both of these oxygen labile haemolysins 'appear to be definite leucocidins when tested on leucocytes free from red cells'. Regrettably these studies were not subsequently published in detail. These observations may be important in considering the work of Robb-Smith (1945) and Sytnik (1960) on deoxyribonuclease and leucotoxin respectively (see pages 97 and 98).

The only investigation to date on the effect of θ-toxin on isolated membranes has been that of Habermann and Pohlmann (1959). Highly purified θ-toxin (Habermann, 1959 - see Table 13) produced morphological changes in human erythrocyte ghosts, which were dependent on the concentration of toxin employed, and the ionic environment of the treated ghost. The toxin was not however free of α-toxin. Indeed addition of Ca^{2+} enhanced the effect produced.

The participation of cholesterol in the formation of the ring structures produced on treatment of membranes with crude α-toxin, together with the heat lability of the factor responsible suggests the possible involvement of θ-toxin in these observations. Moreover, Dourmashkin and Rosse (1966) reported the formation of identical structures after treatment of erythrocyte ghosts with SLO. In addition, not all preparations of α-toxin caused formation of these rings, which could have been due to oxidation of the θ-toxin. They thus concluded that "whether or not these defects might be due to a contaminating enzyme is not clear".

In view of these observations, the interaction of highly purified θ-toxin with natural membranes and cholesterol was determined.

C. COLLAGENASE (KAPPA TOXIN)

Bacterial collagenase is a specific proteinase acting only on native and denatured collagen. Until recently, Cl. perfringens and Cl. histolyticum were the only micro-organisms shown to produce true collagenases. However, its production has now been demonstrated in Streptomyces madurae (Rippon, 1968; Rippon and Lorincz, 1964), Pseudomonas aeruginosa (Schoellmann and Fisher, 1966) and Trichophyton schoenleinii (Rippon and Lorincz, 1964).

The first description of collagenase has been attributed to Maschmann (1937; 1938a; 1938b). Macfarlane and MacLennan (1945) attributed the disintegration of isolated rabbit muscles by culture filtrates of type A to collagenase. This also agreed with the description of Robb-Smith (1945) that ' ... there is partial to complete destruction of the reticulin fibrils and collagen fibres' of infected muscle. Yet, it was left to Oakley et al., (1946) to provide immunological evidence for the distinct nature of collagenase.

Most studies on the purification and mechanism of action of collagenase have been carried out with Cl. histolyticum collagenase, because fewer extracellular factors are elaborated by this organism. Also the β-toxin of Cl. histolyticum is the most powerful collagenase known to date.

1. Purification.

A few isolated attempts to purify the collagenase of Cl. perfringens have been made (see Table 16). Levdikova's preparation is probably the purest yet obtained. It was completely free from lecithinase, hyaluronidase

Table 16 Purification studies on Cl. perfringens Collagenase (Kappa Toxin).

Authors	Year	Strain	Purification Procedure	Purity†	Comments
Bidwell and van Heyningen	1948	S 107	1. 90% $(NH_4)_2SO_4$ saturation ppt. 2. Adsorption onto calcium phosphate gel in 3.75% $(NH_4)_2SO_4$. 3. Elution from gel with 10% $(NH_4)_2SO_4$. 4. Absorption with 1% (w/v) Charcoal. 5. Repeat stages (2) and (3). 6. 35% $(NH_4)_2SO_4$ saturation ppt. 7. Acid dialysis against borate-sodium carbonate buffer pH=10.2.	° -	Purification - 200 fold (culture filtra Overall Yield - 13-18% Purified collagenase contained detectable α-toxin, but no detectable θ-toxin.
Boyer et al	1958	-	Type A toxin from Wellcome Research Laboratories as freeze dried culture filtrate. Purified by continuous flow paper electrophoresis	N.T.	Purified collagenase contained hyaluronidase. Overall Yield - no data available.
Habermann	1959	EP6K	1. 35% methanol ppt; pH=4.8. 2. Zone electrophoresis in starch columns; pH=9.0. 3. Hydroxylapitite chromatography with gradient elution at pH=6.8.	1	Purification - 60 fold (Stage 3) Overall Yield = 50% Homogeneous by membrane electrophoresis.
Levdikova	1966	EP6K	1. 65% $(NH_4)_2SO_4$ saturation ppt. 2. 25% $(NH_4)_2SO_4$ saturation ppt. 3. Fractional ppt. 20-28% $(NH_4)_2SO_4$ saturation. 4. Absorption with DEAE-cellulose, pH=5.6 5. Fractional elution of DEAE-cellulose with buffer pH=5.6 (0.4M acetate elution). 6. 40% $(NH_4)_2SO_4$ ppt. 7. Lyophilisation. 8. Fractional ppt. 29%-50% Acetone; -10°C; protein concentration =0.5%. 9. Lyophilisation. 10. Sephadex G-75 gel filtration. 11. Repeat Stage (10).	1	Purification 130 fold relative to Stage 1. Overall Yield - no data available. Purified material was free of detectable α-toxin and hyaluronidase.

†) °) Symbols as in Table 5

N.T. - not tested.

and θ-toxin, and appeared to be homogeneous in the ultracentrifuge. The preparation of Bidwell and van Heyningen was found to be heterogeneous when examined by Tiselius free zone electrophoresis (Charlwood, 1948), confirming their finding of small amounts of α- and θ-toxins in their preparations.

2. Physical and Chemical Properties.

Physical parameters for Cl. perfringens collagenase are available solely from the data of Levdikova (1966). These are summarised in Table 17.

Ca^{2+} have been shown to activate both clostridial collagenases, (Bidwell and van Heyningen, 1948; Mandl et al., 1958; Seifter et al., 1959; Levdikova, 1966), yet many authors have used either hide powder (azocoll) or tendon as substrates without exogenous addition of Ca^{2+}. It appeared that these two substrates contained sufficient calcium salts for activation of the enzyme (Gallop et al., 1957). The Ca^{2+} are believed to act in creating the necessary electrostatic conditions for adsorption of the enzyme to the substrate.

As with α-toxin the metal chelating agents OP and 8-hydroxyquinoline inactivate the enzyme irreversibly (Seifter et al., 1959; Levdikova, 1966). Addition of Ca^{2+} did not restore activity. Likewise Hg^{2+}, Ag^{2+} and Cu^{2+} were irreversibly inhibitory (Seifter et al., 1959; Levdikova, 1966). Cysteine and EDTA inhibition could be reversed by the addition of Ca^{2+}. It appears from current work on Cl. histolyticum collagenase that it is a zinc metallo-enzyme (see page 44). Work with Cl. perfringens collagenase has not yet progressed to the stage where this has been determined.

Table 17 : Physical and Chemical Characteristics of Cl. perfringens Type A Collagenase

Molecular Parameter †	Value
Molecular Weight	113,000
Sedimentation Coefficient (S_{20})	5.3
Isoelectric point	circa pH 5.0
Diffusion Coefficient	4.23
Electrophoretic Mobility	5.0

† Unitage as in Table 6 From Levdikova (1966)

Table 18 : Properties of purified collagenase

Amino acid analysis	see Levdikova (1966)
pH optimum	6.5 - 7.5
Heat sensitivity	Inactivated by heating at 60°C/10 min.
Inactivated by:	O-phenanthroline; 8-hydroxyquinoline.
Inhibited by:	Cysteine; EDTA.
Effect of Divalent Cations on activity:	
Activation:	Ca^{2+}
Inhibition:	Hg^{2+}, Cu^{2+}, Ag^{2+}.
No observed increase or decrease in activity	Zn^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}.

Collagenase was sensitive to heat (see Table 18) and to extremes of pH, although it was more stable to acid pH than to alkaline pH. Lyophilised preparations lost activity on storage (Levdikova, 1966).

3. Substrate Specificity.

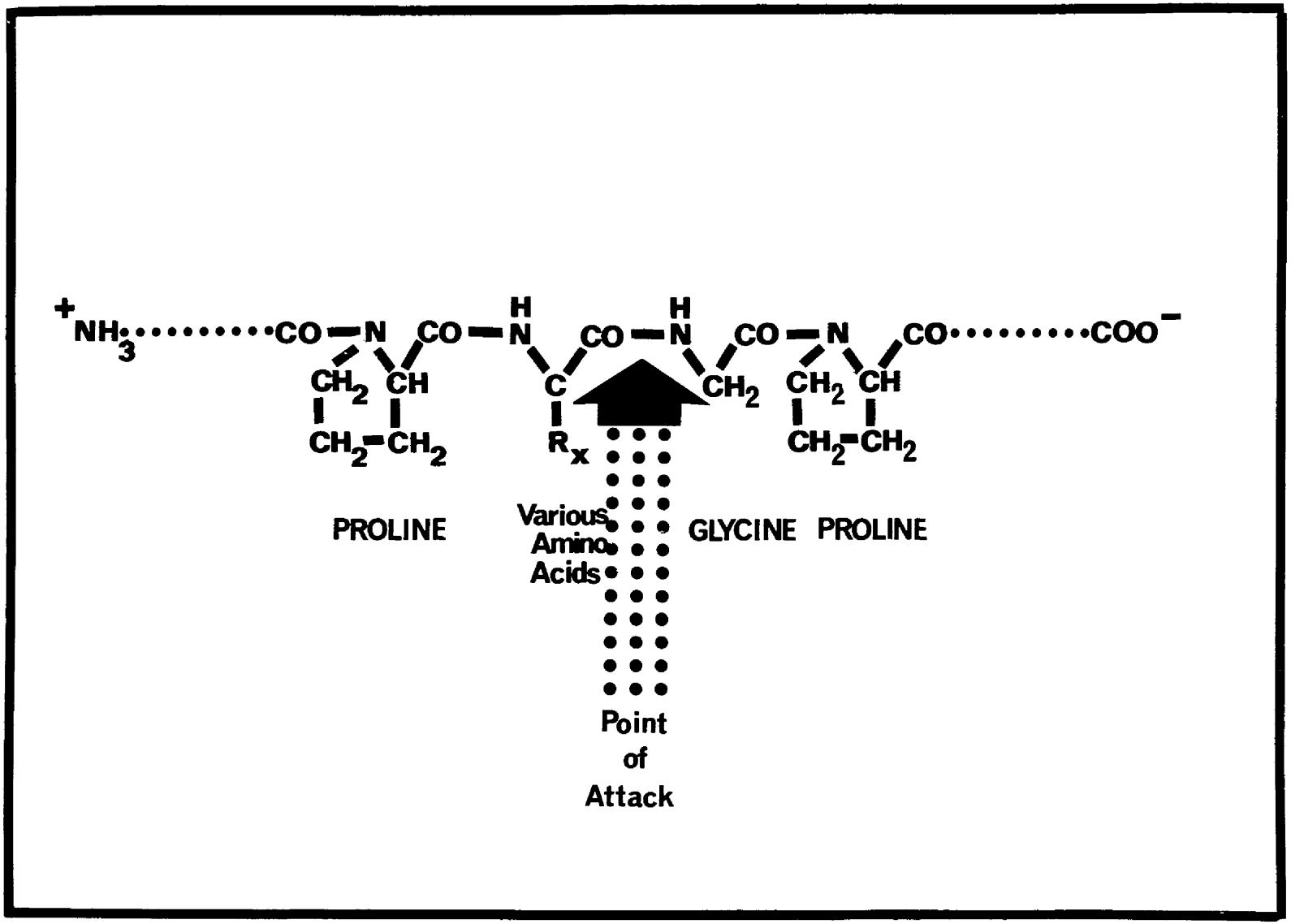
Bidwell and van Heyningen (1948) showed that Cl. perfringens collagenase had no proteolytic action on casein, urea-denatured haemoglobin, human serum, urea denatured human serum, horse serum or clupein sulphate (a basic protein from herring sperm), either in the presence or absence of cysteine. Levdikova (1966) confirmed this finding with casein and haemoglobin. Further, homogeneous preparations of collagenase contained no peptidase activity on the synthetic peptides leu-gly-gly, gly-pro-ala or leu-gly. Thus the enzyme appears to be specific for native and denatured collagens from a variety of sources.

4. Mechanism of Action of Collagenase.

Initial studies on the mode of action of collagenase were carried out using purified preparations from Cl. histolyticum and the results obtained in such studies have been applied to Cl. perfringens collagenase by direct comparison. Collagen is unique amongst proteins found in animal tissues in that it contains a high percentage of proline and hydroxyproline (14-15% of each) and hydroxylysine (1-2%) (Fruton and Simmonds, 1958). The studies of Michaels et al., (1958), Seifter et al., (1959) and Mandl (1961) showed that Cl. histolyticum collagenase was specific for peptide units in the collagen molecule having the sequence shown in Figure 4.

Levdikova (1966) compared in detail the mechanism of attack of Cl. histolyticum collagenase and Cl. perfringens collagenase on native

Figure 4 : Structure of peptide bond susceptible to hydrolysis by clostridial collagenase



collagen and synthetic hexapeptides. This revealed that although the point of attack of both enzymes was identical, the composition of residue R_x (see Figure 4) influenced the rate of hydrolysis by Cl. perfringens collagenase. Confirmation of this has come from further studies with synthetic hexapeptides (Orekhovich et al., 1970).

D. OTHER EXTRACELLULAR FACTORS

1. Hyaluronidase (μ-Antigen).

Hyaluronidase hydrolyses the intercellular cementing substance, hyaluronate. The differences between bacterial and mammalian hyaluronidases have been reviewed by Walker (1961).

The production of a spreading factor by Cl. perfringens was established by Duran-Reynals (1933) and McClean (1936), and the identification of this factor with hyaluronidase was shown by Chain and Duthie (1940) and Meyer et al., (1940b). Its serological identity was established by Oakley and Warrack (1951).

The purification studies reported in the literature are summarised in Table 19, but physical characterisation of this protein has not yet been undertaken. Current information on this enzyme is shown in Table 20, summarised from the work of Robertson et al., (1940), Rogers (1948), McClean (1943), Hale (1944) and Webster et al., (1954). A rich supply of nitrogenous nutrients was required for good yields. The enzyme appeared to be stable to storage (Byers et al., 1949).

Studies have indicated that the enzyme is inducible. The presence of potassium hyaluronate in the culture medium enhanced

Table 19 Purification Studies on *Clostridium perfringens* type A Hyaluronidase

Authors	Year	Strain	Method	Purity†	Comments
Robertson *et al.*	1940	Laboratory isolate	1. Acetone precipitation 50% v/v; -10°C. 2. Calcium phosphate adsorption 3. Dissolution of adsorbent with 3N acetic acid 4. Dialysis against cold distilled water and clarification by centrifugation.	?	Purification = 900 fold Recovery - not stated Substrate used in assay was horse synovial fluid mucin.
Rogers	1946	S 107	1. 70% $(NH_4)_2SO_4$ saturation of culture filtrate at pH=4.6; precipitate harvested. 2. Chromatography on Al_2O_3 column eluted with 0.1M phosphate buffer pH = 7.0.	?	Purification = 16 fold Recovery = 50% with respect to culture filtrate. Method difficult to reproduce.
Baker *et al.*	1956	2278	1. Culture filtrates adjusted to pH = 9.0; 30% $(NH_4)_2SO_4$ saturation; 24 hours at 4°C; precipitate discarded. 2. $(NH_4)_2SO_4$ saturation increased to 70%; 24 hours at 4°C; precipitate harvested and dissolved in 0.02M veronal buffer pH = 9.0 containing 0.45% NaCl. 3. 0.1M - 0.2M fractional ethanol precipitate pH = 9.0. 4. 0.05M - 0.1M fractional ethanol precipitate at pH = 6.8; protein concentration 1 mg/ml. 5. 0.05M - 0.1M fractional ethanol precipitate at pH = 7.5; protein concentration 1 mg/ml.	?	Purification = 1200 fold Overall Recovery = 45% with respect to culture filtrate. Specific Activity = 33,900 IU/mg protein.
Habermann	1959	BP6K	1. 35% Methanol precipitate at pH = 4.65. 2. Electrophoresis in starch column at pH = 9.0; anodic fraction harvested. 3. Dialysis against 0.1M phosphate buffer pH = 6.8. 4. Hydroxylapatite chromatography; gradient elution at pH = 6.8.	1	Purification = 35 fold Overall Recovery = 82% Specific Activity = 6560 IU/mg protein. Not separated from neuraminidase.

†Purity = No. of precipitin lines observed by immunodiffusion or immunoelectrophoresis against standard antiserum.

Table 20 . Properties of Hyaluronidase of Cl. perfringens type A.

Physical parameters	No data available from literature.
pH optimum	6.0
pH range of activity	3.9 - 8.5
Stability:	
(a) to Heat	Inactivated rapidly above 50°C.
(b) to Oxygen	Not inactivated by oxygen.
Activators	Phosphate, citrate, sulphate, chloride.
Inhibitors	Irreversibly inhibited by cyanide. Inhibited by chloroform, heparin, bile salts, cortisone, detergents and haemin.
Effect of ions on Activity	Inactive in the absence of electrolytes. Cu^{2+} ions do not inhibit.
Substrate specificity	Enzyme hydrolyses mucopolysaccharides present in horse synovial fluid, vitreous humour, umbilical cord, Achilles tendon connective tissue and gastric mucosa. Does not hydrolyse glandular mucin. Hydrolyses chondroitin.

production (Rogers, 1946; McClean and Hale, 1941; Hale, 1944), there being a direct relationship between hyaluronidase production and hyaluronate concentrations up to 0.5%.

Lack of work on this enzyme probably stems from the findings of Evans (1943b;1945a) that antihyaluronidase did not increase the protective power of α-antitoxin and that strains which did not produce hyaluronidase were equally virulent for guinea pigs as those that did produce this enzyme.

2. Deoxyribonuclease (ν-Antigen).

Deoxyribonuclease (DNase) remains uncharacterised physically and chemically. Its description and distribution have been dealt with elsewhere in this thesis (see page 12). It was first assayed by the use of the leucocyte nuclei disintegration test in fixed films of rabbit leucocytes (Macfarlane, cited by Warrack et al., 1951). Here the observations of Sytnik (1960) are relevant. He described a leucotoxin in culture filtrates which appeared to be distinct from α-toxin. However this author did not describe morphological damage caused by the leucotoxin and did not test for the presence of DNase in the culture filtrates. Thus the relationship of leucotoxin to DNase is uncertain (see page 87).

Whether the DNase of Cl. perfringens can be regarded as a leucocidin is debatable. Leucocidin produced by Staphylococcus aureus exhibited a high degree of specificity for polymorphonuclear leucocytes only (Gladstone, 1966). By contrast, damage to the nuclei of renal tubule epithelial cells, liver cells, connective tissue cells and muscle cells has been attributed to DNase (Weinberg and Combiesco, 1930; Robb-Smith, 1945)

in addition to its effects on leucocyte nuclei (Butler, 1942; 1943; Robb-Smith, 1945; Warrack et al., 1951). Whether DNase requires the synergistic action of α- or θ-toxin to penetrate susceptible cells or whether it penetrates by diffusion or pinocytosis remains to be elucidated. The possibility that nuclear karyolysis could be caused through lysosomal damage by α- or θ-toxin, particularly in liver cells and leucocytes, requires investigation. Indeed Oakley and Warrack (1951) showed that DNase was non-haemolytic and possessed no necrotising activity. Thus, in the meantime, DNase should be regarded as potentially leucotoxic rather than leucocidal.

Oakley and Warrack (1951) devised the so-called A.C.R.A. (acid-congo red-alcohol) test for the quantitation of this enzyme. No authors have reported its assay by methods involving hyperchromicity at 260 nm or the determination of nucleotides released by DNase as deoxyribose (Burton, 1956).

3. Bursting Factor.

A factor exerting an 'aggressin-like or infection promoting activity' on Cl. perfringens was described by Fredette et al., (1946; 1962). Most of their studies were carried out with strain SMG 121. This factor was neither lethal nor haemolytic and did not produce any of the effects associated with the intramuscular injection of collagenase. Cultures in which none of the known toxic or enzymic activities could be detected either in vivo or in vitro, but producing so-called 'bursting' factor, were virulent. The factor was stated to be immunogenic in guinea pigs and pigeons, but not rabbits. It was heat labile and non-filtrable, and was not neutralised by standard antitoxin.

Fredette and her coworkers maintained that this factor acted specifically on muscle tissue producing the characteristic lesion of gas gangrene, although they present no evidence to substantiate this claim. They said of their factor: 'Filtration through Berkefeld candles, however, removes this important pathogenetic agent, although allowing so-called toxin to flow through. Thus it would appear that fundamental research concerning the mechanism of Cl. perfringens gas gangrene has been sidetracked since this era (World War II) ....'. Their work may serve to account for some of the discrepancies of Evan's work (1945a) on virulence.

4. η-Toxin.

This toxin is one of the minor antigens produced by some Cl. perfringens type A strains and was first described by Ipsen and Davoli (1939) as being produced by the Lechien strain. It was reported to be lethal, but very little is known about this agent. Indeed, but for mentions in research on toxinological typing, the term would probably have fallen out of use. Its presence has so far been detected only by the use of immunological neutralisation tests on culture filtrates. No extensive study has been undertaken to characterise η-toxin, but because of its reported lethal action this factor warrants further investigation.

5. Neuraminidase.

McCrea (1947) demonstrated that culture filtrates of Cl. perfringens contained an enzyme that destroyed erythrocyte receptor sites rendering them inagglutinable by myxoviruses, similar to the effect

that had been observed by Stone (1947) with cultures of Vibrio cholerae. Evidence has accumulated from several workers that 'receptor destroying enzyme' is a hydrolase cleaving glycosidically bound sialic acid, such enzymes being called sialidases or neuraminidases when the sialic acid released is neuraminic acid (Heimer and Meyer, 1956; Popenoe and Drew, 1957; Cassidy et al., 1965; Collee, 1965b). That this enzyme was distinct from the haemagglutinin and the blood group A destroying enzyme was clearly demonstrated by Collee (1965a; 1965b).

The non-identity of this enzyme with α-toxin was evidenced by the lack of requirement for divalent cations, its different optimum pH and the fact that the peaks of neuraminidase activity and lecithinase activity did not co-incide on gel filtration (Collee, 1965b; Satterlee and Walker, 1969).

Although this enzyme was extensively purified by Cassidy et al., (1965), no physical characteristics were reported. Gel filtration studies suggested that it had a higher molecular weight than α-toxin (Satterlee and Walker, 1969). Known properties are summarised in Table 21.

Collee (1965b) found that the Hobbs food-poisoning strains he examined did not produce neuraminidase. In a more comprehensive study of its distribution among type A strains, Moss et al., (1967) were unable to detect neuraminidase production in all of the Hobbs serotyped strains tested (types 1-3 and 5-13), but found no such consistent correlation between neuraminidase production and food-poisoning strains of American origin. Of the American strains serologically related to Hobbs types, 45% produced neuraminidase, whereas 73% of the non-typable strains produced this enzyme. Moreover, these authors showed that 85% of classical type A strains produced the enzyme.

Table 21 Properties of Cl. perfringens type A neuraminidase.†

Parameter	Authors		
	Popenoe & Drew (1957)	Burton (1963)	Cassidy et al (1965)
pH Optimum	5.0 - 5.5 (a)	5.0 - 5.1 (p);(a)	5.2 - 5.5 (p);(c) 4.4 - 4.6 (a)
Effect of divalent cations on activity:	Ca^{2+}, Mn^{2+}, Mg^{2+} - no effect Fe^{2+}, inhibitory	-	All ions tested had no effect on activity
Effect of EDTA on activity:	None	-	None
Stability:			
(a) to Heat	-	Inactivated by heating at 50°C/1 min at pH 5-9	Rapidly inactivated
(b) to Storage	-	-	Activated
(c) to Dilution	-	-	Rapidly inactivated
K_m	-	0.4×10^{-3} M	2.4×10^{-3} M
Substrate Specificity:	Orosomucoid	NANA-lactose; -ganglioside	Bovine sialyl lactose; Human sialyl lactose.
Substrates not readily attacked	-	Klenk's fast ganglioside	Colominic acid

† Strain ATCC 10543 was used to prepare this enzyme by each group of authors

(a): in acetate buffer.
(p): in phosphate buffer.
(c): in citrate-phosphate buffer.

Neuraminidase was reported to be inducible in Vibrio cholerae (French and Ada, 1959) and in type II pneumococci (Kelly et al., 1966). However, attempts to induce neuraminidase production in Hobbs type strains and classical type A strains with N-acetylneuramin-lactose, N-acetylneuraminic acid and N-acetylmannosamine proved negative (Moss et al., 1967).

With the exception of Stephen (1961) no author has commented on the presence or absence of neuraminidase in purified preparations of α-toxin. As glycolipids and glycoproteins with terminal sialic acid residues are components of body fluids and tissues, it seems necessary to test for the presence of neuraminidase in preparations of α-toxin used in biological studies. Neuraminidase containing culture filtrates have been shown to cause profound changes in the surface of human erythrocytes. The non-group specific T antigen, which is thought to be situated near the surface of human erythrocytes, was exposed. Erythrocytes so treated were rendered agglutinable by sera containing T-antiserum. This phenomenon is termed panagglutination. However, the relationship of this phenomenon to neuraminidase activity has not been definitely established. The release of neuraminic acid residues will alter the surface charge on cells which may or may not assist in the approach of α-toxin, quite apart from eliminating possible steric hindrance and increasing substrate accessibility. Clearly studies on the possible interaction of purified α-toxin and neuraminidase require to be carried out.

6. Non Alpha, Theta Delta Haemolysins.

These were described by Brooks et al., (1957) as haemolysins which were non-neutralisable by antisera to the α-, θ- and δ-haemolysins of

Cl. perfringens. They were only produced by a very few strains of Cl. perfringens type A (see "Toxinological typing", pages 13 and 16), and were detected on horse and ox blood agar plates by means of neutralisation techniques. Strains of type A producing these factors produced zones of haemolysis around their colonies in the presence of antitoxic sera. Their presence in culture supernatants and culture filtrates has never been demonstrated. Whether or not they are produced in vivo and whether they play any significant role in pathogenesis is an open question.

It is worth recording here, however, that van Heyningen (1941b) predicted the existence of two further haemolysins, X and Y, to explain some observations on the additivity of haemolytic activities in culture filtrates. These factors were detected in one batch of fresh culture filtrate from strain S 107 as being unabsorbed by sheep erythrocytes. The activity of factor X was unstable, could not be reactivated by reducing agents, and did not require Ca^{2+}. Factor Y was more stable than X and seemed to require Ca^{2+}. The haemolytic activities of α, X and Y were additive unlike those of α and θ.

These haemolysins were not further reported on by van Heyningen and their nature remains unresolved. Whether these findings can be accounted for in terms of multiple forms of toxins or in terms of mutant forms of the α- and θ-haemolysins or to one contaminated batch of culture is difficult to assess.

7. Fibrinolysin.

In 1941 Reed et al., reported that strains of Cl. perfringens of undefined type, but probably type A, produced a factor that broke down

coagulated plasma. Coagulated plasma from different species varied in sensitivity to liquefaction (Table 22). Tillett (1937) had observed that streptococci could cause similar dissolution of plasma clots. He proposed that acid production or the production of a fatty acid caused this. Reed et al., argued that this was not the case in clostridia as both saccharolytic and proteolytic species exhibited clot lysis. Reed and Orr (1942) and Reed et al., (1942) were unable to raise antibodies to this factor in rabbits. Neither was the fibrinolytic activity neutralisable by commercial antitoxin. The fibrinolytic factor was heat stable, 50% of the original activity remaining after boiling for 80 minutes. They thus proposed that this factor was not protein in nature.

Although collagenase has a strict substrate specificity, no one yet has tested it out in this system. The possible relationship between collagenase or the alkaline proteinase of Shemanova et al., (1970) and fibrinolytic activity is not clear. No attempt has been made to characterise the factor responsible for fibrinolysis.

8. Haemagglutinins.

The production of haemagglutinins has been reported in a number of bacteria and viruses. All types of Cl. perfringens produce haemagglutinin. That this was intimately associated with the bacterial body was shown by Gürtler (1952) and Dafalla and Soltys (1953), who found that haemagglutinating activity could not be Seitz filtered. Wickham (1956) on the other hand, found that at least part of the haemagglutinin could be filtered. Production of this factor was associated with ageing or sporulating cultures (Collee, 1961). Wickham (1956) proposed that

Table 22 . Fibrinolytic Activity of Cl. perfringens type A.

No. of strains tested	Plasma	% of cultures producing solution of fibrin clot in	
		7 hours or less	24 hours or less
26	Human	17	83
33	Rabbit	62	67
5	Guinea pig	80	100
5	Sheep	0	0

Adapted from Reed, Orr and Smith (1941).

haemagglutination and the destruction of blood Group A substance and virus receptor substance of red cells were manifestations of the same enzyme (see pages 100 and below). Most of our information on the haemagglutinin of Cl. perfringens comes from the work of Collee (1961; 1965a; 1965b). It had a broad temperature optimum, and a pH optimum of 6.8. All ABO groups of human red cells were agglutinated. Mouse and horse red cells were relatively insensitive; guinea pig leucocytes were also agglutinated. Haemagglutinin was relatively thermostable and was insensitive to oxygenation and reduction. Treatment with lipase and lysozyme had no effect, but pancreatin caused partial inactivation. Charcoal, calcium phosphate gel, kieselguhr and Seitz asbestos adsorbed activity. It was not inhibited by a wide selection of carbohydrates distinguishing it from fimbrial agglutination (Duguid, 1957; 1959).

Because, characteristically, haemagglutinin was not produced by freshly isolated strains, Collee (1961) proposed that the haemagglutinin was a degraded form of one or more enzymes active on cell-surface polysaccharides. It would not appear to play a significant role, if any at all, in gas gangrene as it is not detected in fresh isolates.

9. Enzymes Destroying Blood Group Substances.

Two such enzymes have been reported in the literature but have not been well characterised or purified. Schiff (1939) described an exoenzyme produced by Cl. perfringens type A which destroyed blood group A substance as judged by the inhibition of the iso-agglutination reaction. The enzyme was heat labile and neutralisable by standard antitoxin. It was distinct from the α- and θ-toxins, neither of which affect blood group A substance. Moreover, selective neutralisation of the α- and θ-toxins in

culture filtrates failed to neutralise this activity. There are no reports in the literature of authors testing hyaluronidase or collagenase on this substrate. Collee (1965b)showed that this enzyme was separable from the haemagglutinin and neuraminidase of type A strains.

'H' enzyme was described in culture filtrates as destroying the H-substance of human blood group O erythrocytes (Morgan, 1946). It was reported to be thermostable and non-neutralisable by standard antitoxin. It therefore appears to be distinct from other toxins and enzymes in type A filtrates.

10. Histidine Decarboxylase and Histamine Production.

Gale (1941) in a study of amino acid decarboxylases produced by bacteria described the production of histidine decarboxylase by Cl. perfringens type A. This enzyme converts histidine to histamine. The enzyme was only produced, however, when the pH of the medium dropped below pH 5.0 and the enzyme had an observed pH optimum of between pH 2.5 - 3.0. The production of histamine had been described by Kendall and Gebauer (1930) and Eggerth (1939). The pharmacological action of histamine was elucidated by Dale and Hartley (1916). In an appendix to Gale's paper Brown et al., (1941) sought to determine whether the production of histamine could play some part in the pathogenesis of Cl. perfringens gas gangrene. Their data showed that the pH of infected tissue could be lowered sufficiently both for the production of enzyme and its activity. However, although they demonstrated a significant rise in the histamine level of infected muscle in experimental animals, the histamine level of the blood seldom reached twice the normal level. Thus these authors asserted that the absorption of histamine into the blood from infected

tissues was insufficient to produce any profound systemic effects although the local action of histamine could not be excluded.

11. Lipase.

Matsumoto (1961) observed that diglyceride released from egg yolk PTE by α-toxin underwent further degradation with the liberation of free fatty acids. He attributed this to the action of a lipase in his α-toxin preparation (an ammonium sulphate precipitate of culture filtrates of strain BP6K). He further showed that his toxin preparation hydrolysed Tween 80 (sorbitan mono-oleate polyoxyethylene) at the ester linkage. To my knowledge this report represents the only demonstration of extracellular lipase from Cl. perfringens, although Fleming and Neil (1927) demonstrated a tributyrinase in cellular extracts of this organism and Gonzalez and Sierra (1961) showed that type A strains produced lipolysis after prolonged growth on nutrient agar containing Tween 40 and Tween 60, but not on Tween 80. Lipolysis could therefore be important in membrane damage as a secondary event to the release of diglyceride by α-toxin. Indeed, Aikat and Dible (1956) reported the appearance of fatty acid in histological preparations of adipose tissue that had been treated with culture filtrates of Cl. perfringens.

12. Circulation Factor.

Ganley et al., (1955) observed a factor in crude culture filtrates that was antigenically distinct from α-toxin, θ-toxin, collagenase and hyaluronidase but which was inhibited by standard type A antitoxin. It was reported to inhibit phagocytosis and to cause an increase in the sensitivity of capillaries of the rat to adrenalin.

However, MacLennan (1962) considered that the existence of this agent had not been established. No further studies on this factor have been reported and its nature and existence remain unconfirmed.

MATERIALS AND METHODS

SECTION I : PREPARATION AND ASSAY OF TOXINS AND ENZYMES

A. TOXIN PRODUCTION

1. Strains of Organism.

Only two strains of Cl. perfringens type A were used during this study for the production of toxin. Strain S107 was obtained from the National Collection of Type Cultures, Colindale, London (NCTC 8237) which, according to Keppie and Robertson (1944), was isolated from the intestine of a sheep. However, NCTC states that their strain originated from the Lister Institute in 1941 and was originally designated (NCTC 6125). Moreover, the American Type Culture Collection Catalogue (1968) listed strain NCTC 8237 as Wellcome Laboratory Strain CN 1491.

Strain BP6K which has most commonly been used by American, Japanese and Russian workers was obtained from the National Collection of Industrial Bacteria, Aberdeen (NCIB 8875). This culture originated from the American Type Culture Collection (ATCC 10543). It was donated to ATCC by M.J. Boyd. This strain required L-histidine and a temperature of 45°C for growth in the defined medium of Boyd et al., (1948).

2. Media.

a. Medium of Adams et al., (1947) Modified by Bangham and Dawson (1962):

Details of the preparation of this medium are given in Appendix I. The medium which I used differed in certain respects from that of the above authors :-

(a) DL-tryptophan and L-cystine hydrochloride were used in the basal salts solution. Neither of the above specified the D or L stereoisomer or racemic mixture of these amino acids.

(b) Bangham and Dawson substituted "Tryptone" (Oxo Ltd., London) for the enzymic digests of casein and gelatin and extracts of pancreas and gastric mucosa added by Adams et al. In this study Tryptone (Oxoid Limited, London) was substituted.

(c) The thioglycollic acid was adjusted to neutrality prior to filtration and subsequent addition to the medium, a step specified by neither authors.

(d) The pH of the medium was adjusted to pH 7.5 prior to autoclaving.

Hereinafter this medium is referred to as M-I.

b. Medium of Murata et al., (1956) Modified by Murata et al., (1965):

Details of the preparation of this medium are given in Appendix I. The following differences in preparation were followed:

(a) Fructose, the carbohydrate source, was 'Millipore' filtered and added to the autoclaved medium.

(b) The basal medium was sterilised by autoclaving at 15 lb/in^2.

(c) The thioglycollic acid was prepared as for M-I and added to the autoclaved basal medium.

Hereinafter this medium is referred to as M-II.

c. Addition of Antifoam to Media:

Growth of strains S107 and BP6K in both M-I and M-II resulted in vigorous foaming of these media due to prolific gas production. The addition of an antifoam (Silcolapse 5000, I.C.I. Ltd.,) to either medium

prevented this, enabling easier sampling of culture bottles without affecting toxin yields. Some points of caution require to be given with regard to the use of antifoam in these media for toxin production by either strain of Cl. perfringens type A :

i) Caps of culture bottles must not be screwed down tightly during growth as bottles can explode in the incubator.

ii) Unscrewing tight caps causes a sudden release of gas pressure resulting in a violent aerosol of bacteria and an immediate excessive foaming of the culture medium out of the neck of the bottle.

iii) Care must be taken when observing aseptic technique, as flaming the neck of the culture bottle can often ignite the hydrogen in the bottle with a violent explosion which can crack the bottle or frighten the experimenter into dropping it. Sampling at frequent intervals helps to prevent large pockets of hydrogen accumulating.

3. Harvesting of Toxin.

For the production of a batch of toxin a freeze-dried ampoule of S107 or BP6K was opened and subcultured twice through Robertson's meat medium with added dextrin or fructose for M-I and M-II respectively (Appendix I) for 16-24 hours at 37°C. A starter culture of M-I or M-II was inoculated for each 500 ml of complete medium. These were incubated for 6-8 hours together with culture bottles of complete medium at 37°C. The starter cultures were sedimented in an MSE "Super Minor" centrifuge with swing out head at 2750g for 10 min, the supernatant fluid discarded, the bacterial pellets resuspended in 5 ml volumes of fresh

medium and added to the culture bottles. Incubation was carried out for 4-5 hours at 37°C. The cultures were then cooled in an ice-bath for 10 min and centrifuged at 13,000g for 15 min in an MSE 25 refrigerated centrifuge. Supernatant fluids were pooled to 4°C.

4. Measurement of Bacterial Growth.

Bacterial growth was monitored as the E_{650} of samples of the culture medium withdrawn at ½ hour or one hour intervals. Where the E_{650} exceeded 0.400 the culture was diluted with normal saline until a reading within the range 0.000 - 0.400 was obtained. Measurements were made in an SP600 spectrophotometer (Pye Unicam, Cambridge, England) in cells of 1 cm light path. Uninoculated medium or an appropriate dilution thereof served as a blank. Growth was recorded as the E_{650} x the dilution.

5. Concentration of Toxin Preparations.

Several methods were used to obtain concentrated toxin preparations.

a. Acetone Precipitation after van Heyningen (1941b):

Culture supernatant fluid	1000 ml
0.2M Sodium phosphate buffer, pH 6.5	75 ml
Acetone (Analar)	650 ml
1M Calcium acetate	15 ml

The culture supernatant was cooled in a refrigerated water-bath at -5°C and the phosphate buffer added. When the temperature fell to just below 0°C, the acetone which had been cooled to -20°C in a deep freeze was added slowly with constant stirring, such that the temperature of the mixture remained about -5°C. The calcium acetate was then added

with stirring of the mixture. The resulting calcium phosphate precipitate to which the toxins adsorb was harvested by centrifugation in an MSE 18 centrifuge at 23,000 g for 15 min after the mixture had stood at -5°C for 30 min. The precipitate was eluted twice with successive 50 ml volumes of ice-cold 20% saturated ammonium sulphate. After dialysis overnight at 4°C in 5/8" wide Visking tubing against distilled water, the above procedure was repeated.

b. Ammonium sulphate precipitation at 75% Saturation (after Bangham and Dawson, 1962):

Precipitation was carried out at 4°C. To culture supernatant fluids 470g of ammonium sulphate was added gradually per litre. The mixture was agitated by means of a magnetic stirrer. When the ammonium sulphate had dissolved, the mixture was allowed to stand at 4°C overnight prior to harvesting the precipitate by centrifugation at 23,000 g in an MSE 18 refrigerated centrifuge for 20 min. This precipitate was dissolved in ice-cold distilled water and dialysed overnight in 5/8" wide Visking tubing against several changes of stirred dialysate. The material was then clarified if necessary by centrifugation at 23,000 g for 15 min.

c. Methanol Precipitation (after Roth and Pillemer, 1953):

Culture supernatant was cooled to 0°C and the pH adjusted to 4.6 with 1N acetic acid. Methanol was added slowly to a final concentration of 35%(v/v). During this period the mixture was further cooled to -5°C in a refrigerated water bath. The resulting precipitate was harvested after 24 hours by centrifugation at 23,000 g for 20 min. Precipitates were dissolved in ice-cold distilled water, dialysed overnight against several changes of distilled water and clarified by centrifugation if required.

d. Sodium Chloride Saturation (Shemanova et al., 1965):

Culture supernatant fluid was cooled to 0°C and saturated with sodium chloride (357g/litre). The temperature was lowered to -5°C and the pH of the mixture adjusted to 4.3 - 4.4. The precipitate was harvested after 24 hours by centrifugation at 23,000 *g* for 15 min, dissolved in distilled water by addition of 0.1N NaOH and dialysed against distilled water overnight at 4°C.

e. Fractional Ammonium Sulphate Precipitation:

Culture supernatant fluids were cooled to 4°C and stirred by means of a magnetic stirrer. Solid ammonium sulphate was added to give an initial 20% saturation. No precipitate usually formed. Saturation was increased by 10% saturation steps to 70% saturation (Dawson et al., 1969). The resulting fractional precipitates were harvested by centrifugation in an MSE 25 refrigerated centrifuge at 38,000 *g* for 20 min. At each stage the mixture was stirred at 4°C for 2 hours prior to harvesting of the precipitate. Precipitates were dissolved in ice-cold distilled water and dialysed overnight against several changes of distilled water, the dialysate being stirred by a magnetic stirrer. After dialysis solutions were centrifuged to clarify them, if necessary.

f. Cl. perfringens Type A Toxin (Wellcome):

Laboratory standard type A test toxin, batch No. AGX 1846, was kindly supplied by I. Batty of Wellcome Research Laboratories, Beckenham, Kent. The toxin was freeze-dried in sealed ampoules each containing 10 mg. This material when reconstituted in 10 ml was stated to contain 4 units of α-toxin per 0.23 ml (mouse test). One LV unit was contained in 0.52 ml

(1 LV unit is the amount of toxin that releases 100 μg of acid-soluble phosphorus from a lecithin emulsion in 15 min at 37°C (Macfarlane and Knight, 1941)). By the phospholipase assay used in this thesis 5 LV units were approximately equal to 1 PCU of α-toxin (see page 123).

B. ASSAYS

1. α-Toxin.

a. Egg Yolk Turbidity Method:

i. Preparation of egg yolk extract: (a) The method of Kushner (1957) - The yolk of an egg was separated from the white, washed with isotonic saline to remove as much of the white as possible and then beaten with 500 ml 0.85% (w/v) sodium chloride (NaCl). Kaolin (20g) was added and stirred thoroughly into the mixture. After 10 min the solution was clarified by centrifugation at 18,000 *g* for 30 min. The pH of the saline extract was adjusted to pH 7.0 and the preparation Seitz filtered. (b) Method II - It was found that a more convenient substitute could be employed in the preparation of saline egg yolk extract. Egg yolk emulsion (Oxoid Limited, London - Code No. SR 47) was diluted 1:10 with 0.85% NaCl and stirred vigorously. The pH was adjusted to neutrality and the solution clarified by Seitz filtration. Both of these preparations were made on the day of use and were stored at 4°C until required.

ii. Spot tests: The following incubation mixture was used in all cases -

0.1 ml Test fraction

0.9 ml DICaB (see Appendix II)

1.0 ml Egg yolk extract.

This was incubated at 37°C for up to an hour and the presence or absence of opacity recorded. Only those fractions showing distinct turbidity or separation of fat globules were selected for titration.

iii. Doubling dilution titrations: Serial doubling dilutions of α-toxin were made in 1.0 ml volumes of DICaB in 4" x ½" test tubes. Pipettes were changed every third tube. Tubes without α-toxin acted as negative control tubes. To each tube 1.0 ml of egg yolk extract was added. Incubation was carried out in a water-bath at 37°C for 30 min. The highest dilution causing distinct turbidity was taken as the end point of the titration and the inverse of this dilution recorded as the titre for the purposes of gradient dilution titrations.

iv. Gradient dilution titrations: On the basis of the results obtained by doubling dilution titrations, test samples were suitably diluted in DICaB. The gradient dilution was set up as shown in Table 23.

Tubes 1-11 were pre-incubated at 37°C for 2 min before addition of 1 ml of egg yolk extract to each. Each series of tubes was incubated at 37°C for 30 min, after which time the tubes were immediately placed in an ice-bath. The E_{520} of each dilution was read in 0.5 cm glass cells against the control tube (tube 11) in an SP 600 Series 2 spectrophotometer and these results plotted against the volume of diluted toxin in each tube. One Unit of Egg Yolk Turbidity Activity (ETU) was defined as that dilution of α-toxin which produced an optical density of 0.500 at 520 nm in a light path of 0.5 cm, 30 min after mixing 1 ml of diluted toxin with 1 ml of egg yolk extract at 37°C.

b. Hot-Cold Haemolytic Activity:

i. Preparation of standardised sheep erythrocyte suspension (SSES): Sheep erythrocytes were obtained from Oxoid Limited, London, in Alsever's

Table 23 : Gradient Dilution Titration of α- and θ-Toxins

Tube No.	Volume of Diluent (ml)	Volume of Diluted Toxin (ml)
1	0.0	1.0
2	0.1	0.9
3	0.2	0.8
4	0.3	0.7
5	0.4	0.6
6	0.5	0.5
7	0.6	0.4
8	0.7	0.3
9	0.8	0.2
10	0.9	0.1
11*	1.0	0.0

* Control tube

Solution (Code No. SR 53) and stored refrigerated at 4°C until used. Blood more than three weeks old (according to the batch preparation date on the label) or blood showing considerable haemolysis on the first sedimentation by centrifuging was discarded.

Erythrocytes were washed three times with 0.85% NaCl and the packed erythrocytes resuspended as a 1% suspension (v/v) in 0.85% NaCl. The concentration of erythrocytes was then adjusted as follows. To 3 ml of DICaB, 1 ml of 1% sheep erythrocyte suspension was added and the resulting suspension haemolysed by the addition of saponin powder (BDH Chemicals Ltd.). After centrifugation at 2100 g in the swing out head of an MSE "Super Minor" centrifuge the E_{550} of the supernatant fluid was read in an SP 600 spectrophotometer in glass cells of 0.5 cm light path. This was equivalent to the E_{550} upon 50% lysis in titrations. The erythrocyte suspension was then adjusted with 0.85% NaCl such that the E_{550} = 50% Haemolysis = 0.250.

ii. Doubling dilution titrations: Serial doubling dilutions of α-toxin were made as previously described for the egg yolk turbidity assay. To each tube 1 ml of SSES was added. Tubes without α-toxin served as controls. Sets of tubes were first incubated at 37°C for 30 min, then at 0°C in an ice-bath for a further 30 min. Settled erythrocytes were resuspended every 10 min during both phases of the titration. The titre was read visually by comparison of tubes showing haemolysis with the control tube (0% haemolysis). The dilution causing a visual 50% haemolysis after these incubation periods was designated to contain one hot-cold haemolytic unit of α-toxin.

Alternatively the 50% end point was determined by centrifuging

test tubes at 2100 g for 5 min and comparing the haemoglobin released into the supernatant with 40%, 50% and 60% haemolysis standards prepared by dilution of a 100% haemolysed suspension.

iii. Gradient dilution titrations: These were carried out in the manner of Table 23 , except that 1.0 ml volumes of SSES were added to each tube. Each set of tubes was incubated as described for doubling dilution titrations. The tubes were then spun at 2100 g and the haemoglobin released measured as the E_{550} of supernatant fluids in an SP 600 in 0.5 cm cells. E_{550} was plotted against the volume of diluted toxin in each tube and the dilution causing 50% haemolysis determined by interpolation of the graph. One Haemolytic Unit (HU) of α-toxin was defined as the dilution of α-toxin causing haemolysis of 50% of the erythrocytes in 1.0 ml of SSES after incubation at 37°C for 30 min, followed by 30 min at 0°C.

c. Lethality Titration in Mice:

Fractions of α-toxin were diluted to contain 1 ETU per ml in DICaB. This was found by preliminary experiment to provide a suitable starting dilution for such titrations. Each test was performed with ten male mice. Pairs of mice (Tuck, Rayleigh, Essex, weighing 20-22 g) were injected intravenously via the caudal veins at the base of the tail with 0.2, 0.25, 0.3, 0.4 and 0.5 ml volumes of diluted toxin. Injections were made using a 23 gauge 1.1/4" needle from a 2.5 ml sterile polypropylene syringe (Becton and Dickinson, Drogheda, Ireland). Dilutions of toxin preparations were made just prior to injection. Diluted toxin was maintained in an ice-bath during the period of injection.

i. Control fluids: In all cases, pairs of mice were given 0.5 ml of control

fluid. In the electrofocusing experiments the controls were as follows:-

For the titrations in Figure 15 -

a. 60% sucrose containing 1% ampholine pH 5-8 in distilled water.

b. As in (a) diluted 1:4 with DICaB.

For the titrations in Figure 16 -

a. 60% sucrose containing 1% ampholine pH 5-8, 6M with respect to urea in distilled water.

b. As in (a) diluted 1:4 with DICaB.

On other occasions the control fluid for injections was DICaB.

The volume of injected preparation killing both or one of the mice in 24 hours was taken to contain one Lethal Unit (LU) of α-toxin.

ii. Comments on method: It is normal practice when testing the lethal action of toxin preparations to use groups of 4 - 10 mice per dose administered to minimise the effect of differences in individual resistance for the determination of the Minimal Lethal Dose (MLD) or LD_{50}, the dose killing 50% of a group of test animals. The above method of titration was devised because the animal house facilities did not permit the breeding of large numbers of mice. The resultant expense of buying in mice necessitated economy in the number of mice used in the titration of a single fraction. Even so approximately 200 mice were required to screen each electrofocusing column (see pages 181 and 186).

d. Release of Water-Soluble Phosphate from a Phospholipid Emulsion:

i. Preparation of egg yolk phospholipid: Egg yolk phospholipid was prepared after the method of Hanahan et al., (1951). Yolks from fresh eggs were separated and mixed in a Waring blender with two volumes of

acetone. After standing for 3 hours at room temperature, the mixture was centrifuged at 23,000 g in an MSE 18 refrigerated centrifuge and the supernatant fluid retained. The residue was similarly extracted with 2 volumes of 95% ethyl alcohol and then 1 volume of 95% ethyl alcohol, and the residue discarded.

Extracts were combined and concentrated to a small volume at 45°C under reduced pressure in a nitrogen atmosphere in a thermostatically controlled vaccuum chromatography oven. The concentrate was extracted with 2 volumes of petroleum ether (b.p. 30-60°C) and the extract retained. The aqueous layer was re-extracted twice with petroleum ether. Petroleum ether extracts were combined, concentrated to a small volume under reduced pressure under nitrogen gas and 4 volumes of acetone added. The precipitate was removed by centrifugation at 23,000 g in an MSE 18 centrifuge and the supernatant fluid discarded. The precipitate was suspended in acetone and centrifuged. Again the supernatant fluid was discarded. This acetone washing procedure was repeated 6 times. The precipitate was then dissolved in petroleum ether and any insoluble material removed by centrifugation. Four volumes of acetone were added to the petroleum ether soluble fraction and the above washing procedure repeated. After the final wash the phospholipid was stored as a precipitate under nitrogen in a -70°C deep-freeze. All solvents used were 'Analar' grade.

ii. Analysis of phospholipid substrate: Thin layer chromatography was carried out as described on page 158 . After development of the spots (see also page 159) this substrate was shown to contain in addition to lecithin, phosphatidyl ethanolamine, sphingomyelin and lysolecithin.

iii. Preparation of the phospholipid emulsion substrate: 120 mg of egg yolk phospholipid prepared as above were emulsified in 20 ml of 0.02M

Dimethylglutaric acid - NaOH buffer pH 7.2 which was 0.0025M with respect to calcium acetate and contained 1 mg/ml bovine serum albumin fraction V (BSA) (Armour Pharmaceuticals, Eastbourne, England), by sonication in a 50 ml round-bottomed quickfit flask using an ultrasonic cleaner (Allen and Hanbury - Surgical Instruments, London, Model No. 150).

iv. Assay procedure: All assays were carried out in triplicate and the results obtained averaged. Into chrome-cleaned 4 x ½" test tubes, 0.5 ml volumes of the phospholipid emulsion were pipetted and the tubes incubated at 37°C for 5 min, 0.1 ml volumes of test fractions were added at ½ min intervals. The assays were incubated at 37°C for 30 min without shaking. Control tubes contained 0.1 ml volumes of buffer or suspending fluid. The tubes were then removed at ½ min intervals; 0.2 ml 5% BSA in distilled water and 1 ml of 10% trichloroacetic acid were then added. The tubes were placed in an ice-bath until centrifuged at 4°C in a swing-out head of an MSE 'Super Minor' centrifuge at 2,100 g for 5 min to sediment the precipitated protein. This precipitation/centrifugation procedure greatly aided removal of the diglyceride and unhydrolysed phospholipid emulsion. Supernatant fluids were decanted into labelled micro-Kjeldahl digestion flasks (30 ml) and the precipitates washed by resuspension in 1.0 ml volumes of ice-cold 10% TCA. After centrifugation, the washes were combined with the parent supernatant fluids. The necks of the flasks were washed down with distilled water, three glass beads added to each flask and phosphate analyses carried out by a modified ½ Allen procedure (see below). One Phospholipase C Unit (PCU) was defined as that volume or dilution of α-toxin that released 1 μmole of phosphate (30.2 μg) from a phospholipid emulsion per minute. This was in accordance with the unitage supplied with all commercial preparations of phospholipase C.

v. Determination of phosphate by modified Allen method (1940):

Principle: The determination depends on the conversion of all the phosphorus into inorganic orthophosphate by digestion with sulphuric acid and hydrogen peroxide. In this case the phosphocholine released from the phospholipid emulsion by α-toxin is converted to inorganic phosphate. The orthophosphate forms a phosphomolybdate complex with ammonium molybdate which is reduced to a blue chromogen with the reducing agent amidol (2:4 diaminophenol hydrochloride). The intensity of the blue colour is measured spectrophotometrically as the optical density at 640 nm. The ½ Allen procedure allows determination of quantities of phosphate between 0-150 µg.

Reagents:

Standard Phosphorus Solution

- 2.193 g of KH_2PO_4 (Anhydrous) dissolved in 500 ml distilled water (1 mg/ml).

10N H_2SO_4 - 280 ml concentrated H_2SO_4 (MAR Grade) to 720 ml distilled water.

1% Amidol in 20% sodium metabisulphite

- 1g of amidol dissolved in 100 ml of 20% (w/v) sodium metabisulphite and filtered; store in dark bottle; prepare daily as required.

8.3% (w/v) Ammonium molybdate.

H_2O_2 - 10 vol MAR Grade.

Procedure: To each flask 1 ml 10N H_2SO_4 was added and the flasks heated gently on a digestion rack until the glass beads started vibrating, at which point vigorous heating could be applied. Heating was continued until all the water had evaporated and the sulphuric acid started to fume. When an appreciable quantity of organic matter was present, the contents

of the flask blackened. After cooling, a few drops of H_2O_2 were added. The flasks were reheated until bubbling stopped and sulphuric acid started to fume again. If the contents of a flask were still discoloured the procedure was repeated until the contents were clear.

To each flask the following were added and mixed thoroughly:

10.8 ml	Distilled water
1.0 ml	Amidol reagent
0.5 ml	Ammonium molybdate.

The tubes were allowed to stand for 10 min, but not more than 30 min before the optical densities at 640 nm were read against distilled water in glass cells of 1 cm light path. After correction for the control blank value, the µg phosphate in each tube was obtained from a standard curve prepared with standard inorganic phosphate solution.

2. θ-Toxin.

A haemolytic assay based on the method of Roth and Pillemer (1955) was used throughout this thesis. A standardised sheep erythrocyte suspension was prepared as described previously for α-toxin (see page 116).

a. Activation of θ-toxin:

Fractions or preparations assayed for θ-toxin were first reactivated as follows:

0.1 ml	Test material
0.9 ml	θ-diluent (see Appendix II)
0.25ml	0.1M Cysteine hydrochloride (neutralised) (see Appendix II)

This mixture was heated in a water-bath at 37°C for 15 min and then diluted with diluent to a final volume of 2 ml.

b. Doubling Dilution Titrations:

Serial doubling dilutions of activated toxin (starting at a dilution of 1:20) were set up in 1.0 ml volumes of diluent in a manner similar to that previously described for α-toxin (see page 119). To each tube 1 ml of SSES was added and the tubes incubated at 37°C for 30 min. The highest dilution causing 50% haemolysis visually was recorded as the titre. Two controls were included with each set of titrations :-

(a) contained diluent and erythrocytes

(b) contained diluent treated as for toxin activation and erythrocytes.

c. Gradient Dilution Titrations:

Activated toxin was diluted appropriately in θ-diluent. Gradient dilutions were set up as described for α-toxin (see page 118) using θ-diluent instead of DICaB. Tubes were incubated at 37°C for 30 min, cooled in an ice-bath to stop the reaction and the erythrocytes sedimented at 2100 g for 5 min in an MSE 'Super Minor' centrifuge with a swing out head. The E_{550} of each supernatant fluid was measured in 0.5 cm glass cells in an SP 600 spectrophotometer. These values were plotted against the volume of diluted toxin in each assay. The volume of diluted toxin causing 50% haemolysis was then determined by interpolation of the graph. One Haemolytic Unit (HU) of θ-toxin was defined as that dilution of activated toxin causing haemolysis of 50% of the erythrocytes in 1 ml of SSES.

3. Hyaluronidase.

Hyaluronidase was assayed by the method of Dorfmann (1955) as described by Servac Laboratories in their pamphlet 'Enzymes and Related Biochemicals' modified in terms of the final volumes in the assay. The bacterial enzyme was compared against an International Standard preparation of testicular hyaluronidase standardised by the method of Humphrey (1957).

a. Basis of the Assay:

Hyaluronic acid consists of N-acetyl-glucosamine and glucuronic acid disaccharide units linked β, 1 - 4, polymerised through β, 1 - 3 bonds (see Figure 5). Hydrolysis of this mucopolysaccharide occurs at the β, 1 - 4 linkages, resulting in the formation of disaccharide products in the case of bacterial hyaluronidases.

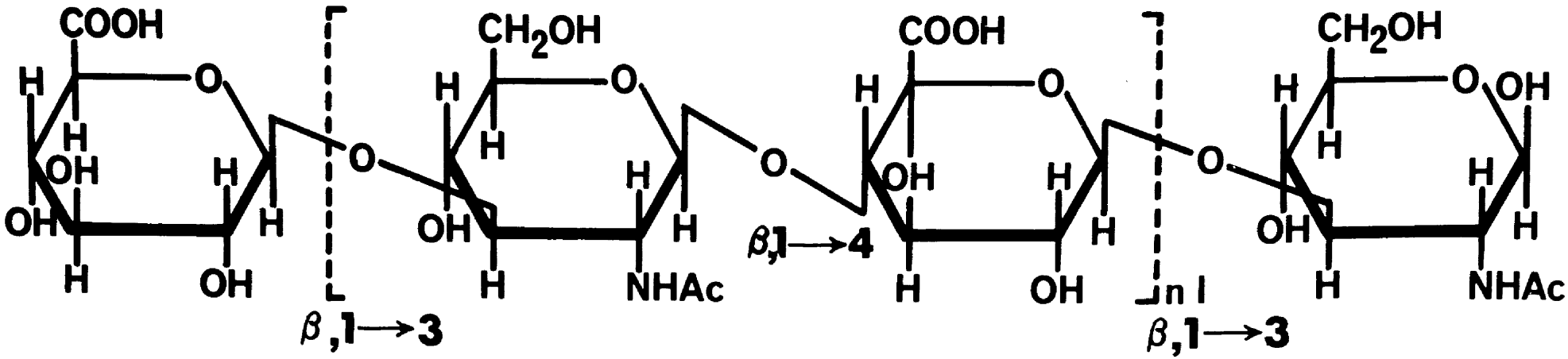
The hyaluronic acid molecule is negatively charged. This negative charge results in the mutual precipitation of cationic proteins such as albumin at acid pH. Under these conditions the solution of hyaluronic acid becomes turbid. This turbidity fails to develop if the hyaluronic acid is digested by hyaluronidase.

b. Substrate Preparation:

Highly polymerised hyaluronic acid (Batch No. 47) was obtained from Miles-Seravac, Maidenhead, England. It was prepared from human umbilical cord and was supplied as a potassium salt. It contained less than 2% protein and less than 3% chondroitin sulphate.

Hyaluronic acid was dissolved at a concentration of 4 mg/ml in 0.30 M phosphate buffer pH 5.30. A 1:10 dilution was made in

Figure 5 : Structure of Hyaluronic Acid



Hyaluronate η [3 (β-D-gluco-4, 5-en-urono)-2-acetamido-2-deoxy-D-glucose]

hyaluronidase diluent (see Appendix II); to 0.5 ml of this was added 0.5 ml of diluent and this mixture incubated at 37°C for 5 min. After incubation, 5 ml of acid albumin solution (see Appendix II) were added and the turbidity which developed after exactly 5 min further incubation was measured as E_{600} in an SP 600 spectrophotometer in a glass cell of 1 cm light path. The parent solution of hyaluronic acid was then diluted further such that a standardised solution gave an E_{600} = 0.250 when 0.5 ml of this solution was treated as described above.

c. Procedure:

Suitable dilutions of test material were made in hyaluronidase diluent. To 0.5 ml volumes of standardised hyaluronic acid solution dispensed in 6" x 5/8" test-tubes, 0.5 ml volumes of test solutions were added at $\frac{1}{2}$ min intervals. Tubes were incubated at 37°C for 45 min, at the end of which period 5 ml of acid albumin solution were added to each tube at $\frac{1}{2}$ min intervals. The E_{600} of each solution was read in an SP 600 spectrophotometer against an appropriate diluent/acid albumin blank. A substrate control was included with each set of titrations.

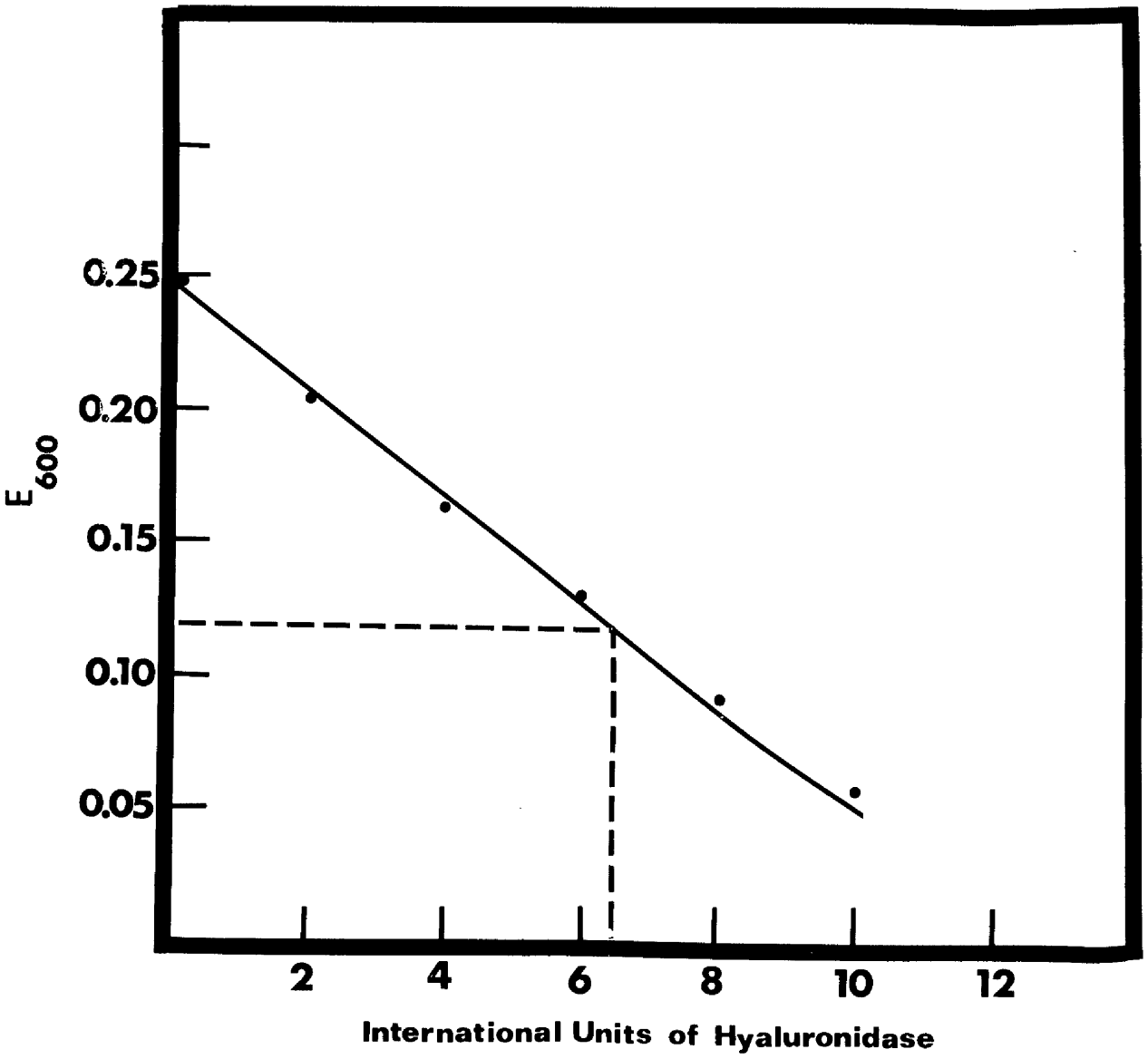
A standard curve was obtained by plotting optical density against enzyme concentration, using an International Standard preparation of ovine hyaluronidase (see Appendix III) and unknown samples read against this curve (Figure 6). Test samples giving an $E_{600} > 0.200$ were repeated using higher concentrations of test material. Similarly those giving an $E_{600} < 0.050$ were repeated at a lower concentration. Results were expressed in International Units (IU).

Figure 6 : Assay for Hyaluronidase Activity by the Turbidity Reduction Method of Dorfmann

Determination of Hyaluronidase Activity with reference to Standard Hyaluronidase Preparation

The resultant E_{600} of a standardised hyaluronic acid solution after digestion with increasing amounts of ovine testicular hyaluronidase are shown in Figure opposi Note the decrease in E_{600} with increasing hyaluronidase activity.

A 1:50 dilution of a toxin preparation of unknown hyaluronidase activity assayed as described on page 129 reduced the turbidity of the hyaluronic acid to an $E_{600} = 0.12$. As shown in the figure, this corresponds to the activity of 6.5 IU of hyaluronidase. The preparation thus contains 6.5 x 50 x 2 IU/ml = 650 IU/ml.


0.25
0.20
0.15
0.10
0.05
E 600
2
4
6
8
10
12
International Units of Hyaluronidase

4. Collagenase.

a. Azocoll Digestion Method:

i. Basis of method: Collagenase was assayed quantitatively using azocoll powder, and the specificity of this reaction was checked by testing fractions qualitatively on collagen paper discs. Azocoll is a hide collagen powder conjugated with a red azo-dye, prepared from tetra-azotised benzidine and "R salt", sodium 2-naphthol-3 ; 6 disulphonic acid. When mixed with proteins diazo compounds react with the phenolic ring of tyrosine, with the imidazole ring of histidine and the -NH group of proline and hydroxyproline. Azocoll powder is insoluble and reddish-purple in colour. When incubated with collagenase the red dye is released into solution and this can be measured spectrophotometrically.

ii. Procedure: Ten milligram samples of azocoll powder (Wellcome Reagents, Beckenham, England) were weighed out into 4" x $\frac{1}{2}$" test-tubes and 1.9 ml of collagenase diluent A (see Appendix II) added. These tubes were incubated at 37°C for 5 min in a water-bath and 0.1 ml samples of test solutions of collagenase added. Assays were carried out in duplicate. Incubation was continued for 1 hour at 37°C. The tubes were agitated every 10 min to resuspend the settled azocoll powder. At the end of the incubation period the tubes were centrifuged in an MSE "Super Minor" centrifuge at 2100 g for 2 min to sediment the azocoll powder. The E_{510} values of supernatant fluids were measured in 0.5 cm glass cells in an SP 600 spectrophotometer against a water blank. The average E_{510} of control tubes comprising 10 mg azocoll powder + 2 ml of diluent A, incubated under the above conditions, was subtracted from the values obtained with test solutions of enzyme. The relationship between dye release (E_{510}) and collagenase concentration, using a standard

preparation of Cl. histolyticum collagenase, was linear for E_{510} values between 0.000 and 0.250. Test samples giving readings higher than an OD = 0.250 were further diluted and repeated. One Azocoll Digesting Unit (AU) was defined as that amount of enzyme which caused an increase in the E_{510} of supernatant fluids of 0.010 per hour under the above conditions.

iii. Substrate specificity: Although providing a quick and convenient substrate for assay, azocoll is not a specific substrate for collagenase. Sreebny et al., (1955) reported that it was readily attacked by trypsin, chymotrypsin, papain and pepsin. Table 24 demonstrates the relative susceptibility to attack of azocoll by a number of proteolytic enzymes titrated as above from stock solutions of 1 mg/ml, confirming the findings of Sreebny et al. (see Appendix III).

b. Collagen Disc Method:

To confirm the identity of the enzyme which had been purified by isoelectric focusing and characterised as digesting azocoll powder, as a true collagenase, acid soluble collagen was prepared from rat tail tendons.

i. Preparation of acid soluble collagen and collagen paper discs (After I. Batty, personal communication. cf. Delaunay et al., 1949):

Twenty-eight rat tails (kindly supplied by W. Melvin of the Biochemistry Department, University of Glasgow) of varying sizes and from animals of different ages were skinned and the four tendons in each dissected out. This was most easily accomplished by one person stretching the tails with pinch-grip forceps whilst another dissected. Muscle and cartilage were removed as far as possible from the stripped

Table 24 : Susceptibility of Azocoll Powder to Attack by Proteolytic Enzymes°

Enzyme *	AU/mg Protein
Protease	17,760
Trypsin	15,300
Proteinase	14,460
Pronase	3,420
Papain	1,690
Ficin	1,350
α-Chymotrypsin	770
Collagenase	210

° See Appendix III.

* All assays were carried out on stock solutions containing 1 mg/ml of each enzyme in collagenase diluent A.

tendons which were then immersed in 2 litres of ice-cold 1:10,000 (v/v) acetic acid (1.75mM) and allowed to swell at 4°C for 48 hours with occasional stirring. The tendons became gelatinous, whereas any muscle or cartilage tissue remained insoluble.

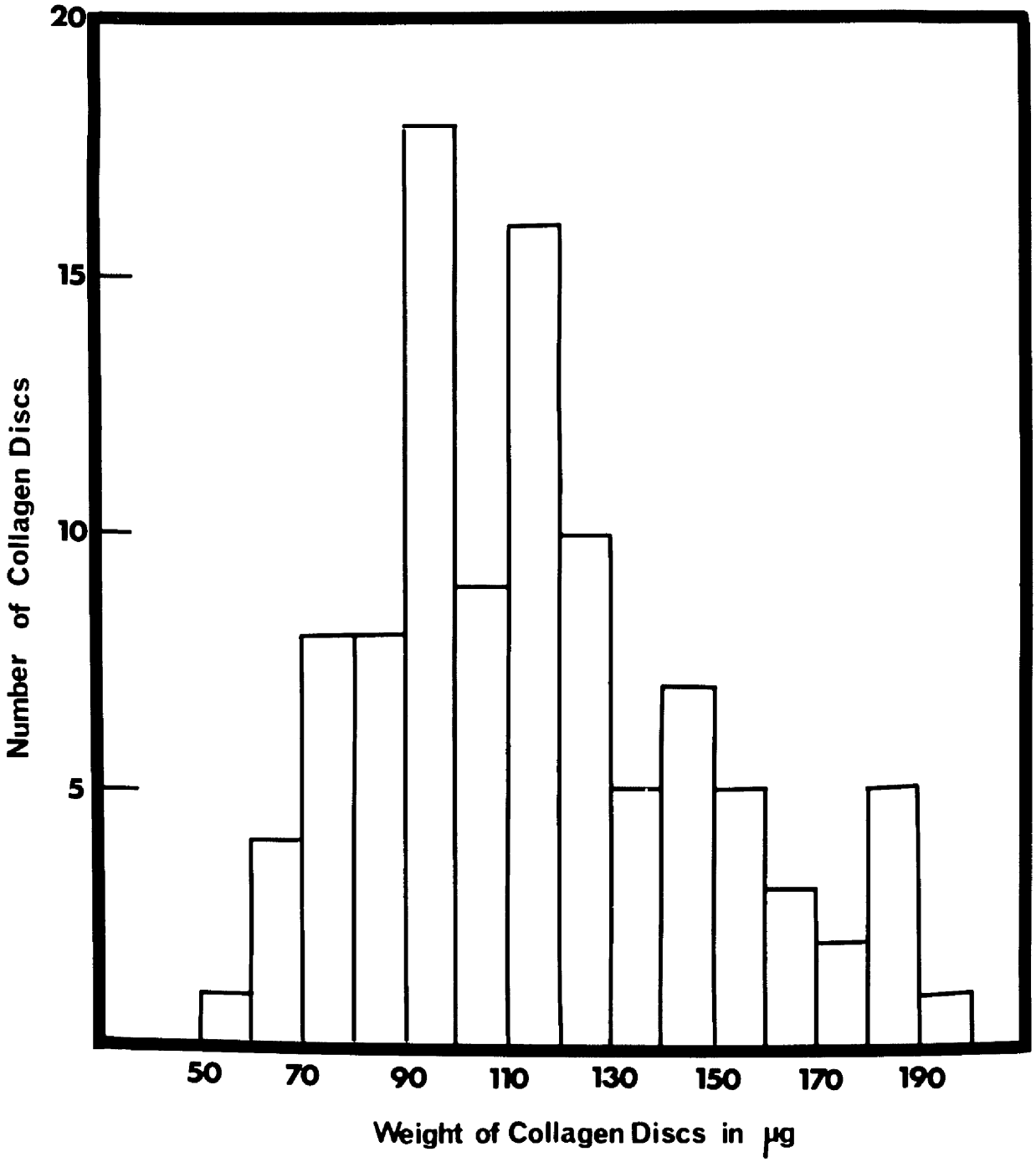
The viscous solution was then passed through several layers of butter muslin and stored overnight at 4°C. 200 ml of this filtrate were mixed with 60 ml of 10% (w/v) NaCl. A white floccular precipitate appeared. This solution was poured into a 13 cm Buchner funnel fitted with a 14.2 cm cellulose acetate membrane cut to size (Millipore (UK), London, Pore Size 1.2μ). Suction was applied at 10 lb/sq in and the filtrate discarded. A gelatinous layer of collagen formed on the membrane as it dried. The collagen paper could be stripped off the membrane when completely dry. It was stored between the protective sheets supplied with the Millipore filter membranes. Discs of collagen paper were cut using a No. 5 cork borer (Fisons, Loughborough, England - 8 mm diam.) against a glass surface.

A distribution analysis by weight of 100 such discs gave the pattern shown in Figure 7 . As a result of this finding only discs within the weight range 90-140 μg were used and within any group of tests all the discs used fell within a 10 μg range.

ii. Procedure: Spot tests were carried out in WHO complement titration trays. Each well contained 0.1 ml of test preparation and 0.9 ml of collagenase diluent B (see Appendix II). The sodium chloride in this buffer prevented spontaneous dissolution of the collagen. A collagen disc was added to each well and the wells sealed with sellotape. Incubation was carried out in an incubator at 37°C and the plate examined every hour for dissolution of the discs.

Figure 7 : Distribution Analysis of Collagen Discs by Weight

Discs were prepared by the method described on page 132 . Each disc was weighed on a five place single pan balance (Sartorius). Discs were grouped according to 10 µg ranges from 50-59 µg, 60-69 µg, 70-79 µg inclusive and so on. The number of discs in each weight grouping is shown for a weight analysis of 100 collagen discs picked at random. The average weight of discs was 117 ± 33 µg.


20
15
10
5
Number of Collagen Discs
50
70
90
110
130
150
170
190
Weight of Collagen Discs in µg

iii. Substrate specificity: Collagenase ex Cl. histolyticum, papain, trypsin, chymotrypsin, ficin, pronase, proteinase and subtilisin (see Appendix III) were each dissolved in collagenase diluent B at a concentration of 1 mg/ml. Volumes of 1 ml were pipetted into the wells of a WHO complement fixation tray and collagen discs (100-110 µg) added to each. Incubation was carried out as described above. A control disc was suspended in buffer alone. The opacity of discs was compared with that of the control after 1 - 48 hours incubation. The discs were readily attacked by pronase and collagenase, and more slowly by ficin within the first 8 hours. After 24 hours papain was also active. After prolonged incubation (48 - 60 hours) other proteolytic enzymes appeared effective, but since control discs incubated with buffer became gelatinous and dissolved, the significance of these findings was doubtful. In view of this, all tests were recorded as positive or negative only up to 8 hours of incubation at 37°C.

5. Neuraminidase.

a. Basis of Method:

Neuraminidase cleaves terminal neuraminic acid residues from glycoproteins, glycopeptides and glycolipids. The surface charge of these substances is associated with the carboxyl group of neuraminic acid. Orosomucoid, a glycoprotein occurring in normal human plasma has a molecular weight of about 44,100 (Smith et al., 1950) and contains some 11 - 12% sialic acid (Popenoe and Drew, 1957). This represents some 15 or 16 residues per molecule which account for its acid pI 2.7 (Popenoe and Drew, 1957). Neuraminic acid has been shown to be the terminal component in the glycopeptide of human

Figure 8 : Action of Cl. perfringens neuraminidase

Structure of Human Erythrocyte Glycopeptide

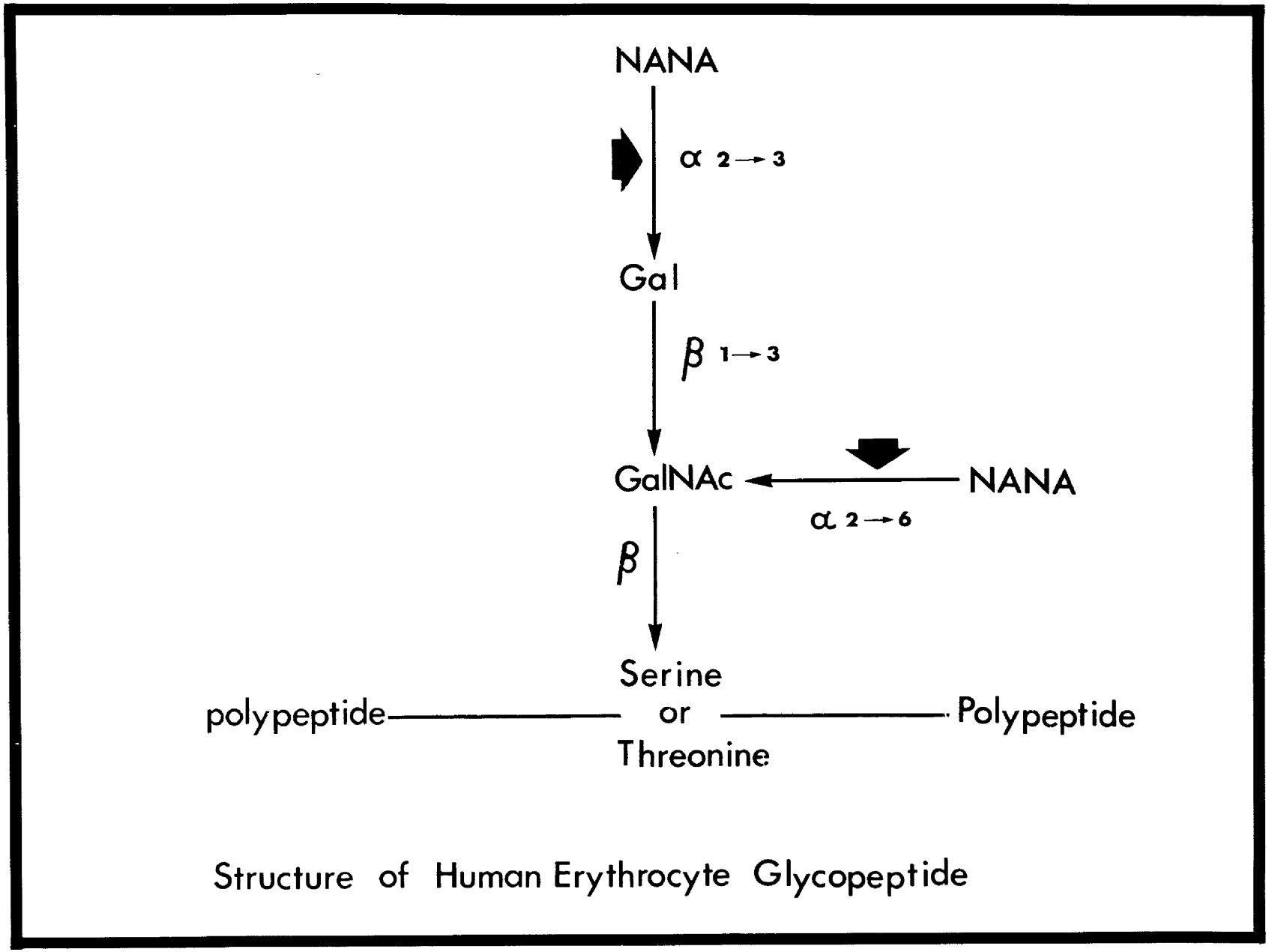
(Winzler, 1969).

NANA = N-acetyl-Neuraminic acid

Gal = Galactose

GalNAc = N-acetyl-galactosamine

Glycopeptide is associated with the carbohydrate rich amino terminus of stromal glycoprotein contributing M, N or MN specificity to the blood group of a donor. Hydrolysis of NANA by neuraminidase at the points shown by closed arrow heads is associated with a reduction in negative charge and loss of M, N or MN specificity and renders erythrocytes non-agglutinable by myxoviruses.



Structure of Human Erythrocyte Glycopeptide

erythrocytes possessing M, N or MN blood group specificity. Cleavage of this sugar from the glycoprotein by neuraminidase destroys the MN group specificity. The specificity limits of the glycosidic linkages are at present unknown (see Figure 8).

b. Method of Collee (1965b):

i. Substrate preparation: The whites of fresh hens' eggs were separated from the yolks. To 100 ml of separated egg white 400 ml of 0.85% NaCl buffered at pH 7.2 by 0.005M phosphate buffer were added. The mixture was homogenised in a Waring blender and centrifuged in an MSE 18 refrigerated centrifuge at 18,000 g for 15 min to remove particulate debris. The clear solution was stored in bijoux bottles at -70°C.

ii. Assay procedure: Each reaction mixture contained the following:

0.1 ml Enzyme preparation

0.4 ml Neuraminidase diluent A or B (see Appendix II)

1.0 ml Substrate

Tubes were incubated at 37°C for 30 min. A substrate control without enzyme and a water blank were included in each series of tests. The reaction was stopped by the addition of 0.2 ml Warren's Reagent A (vide infra). Assays were carried out in duplicate.

iii. Determination of neuraminic acid (Warren, 1959):

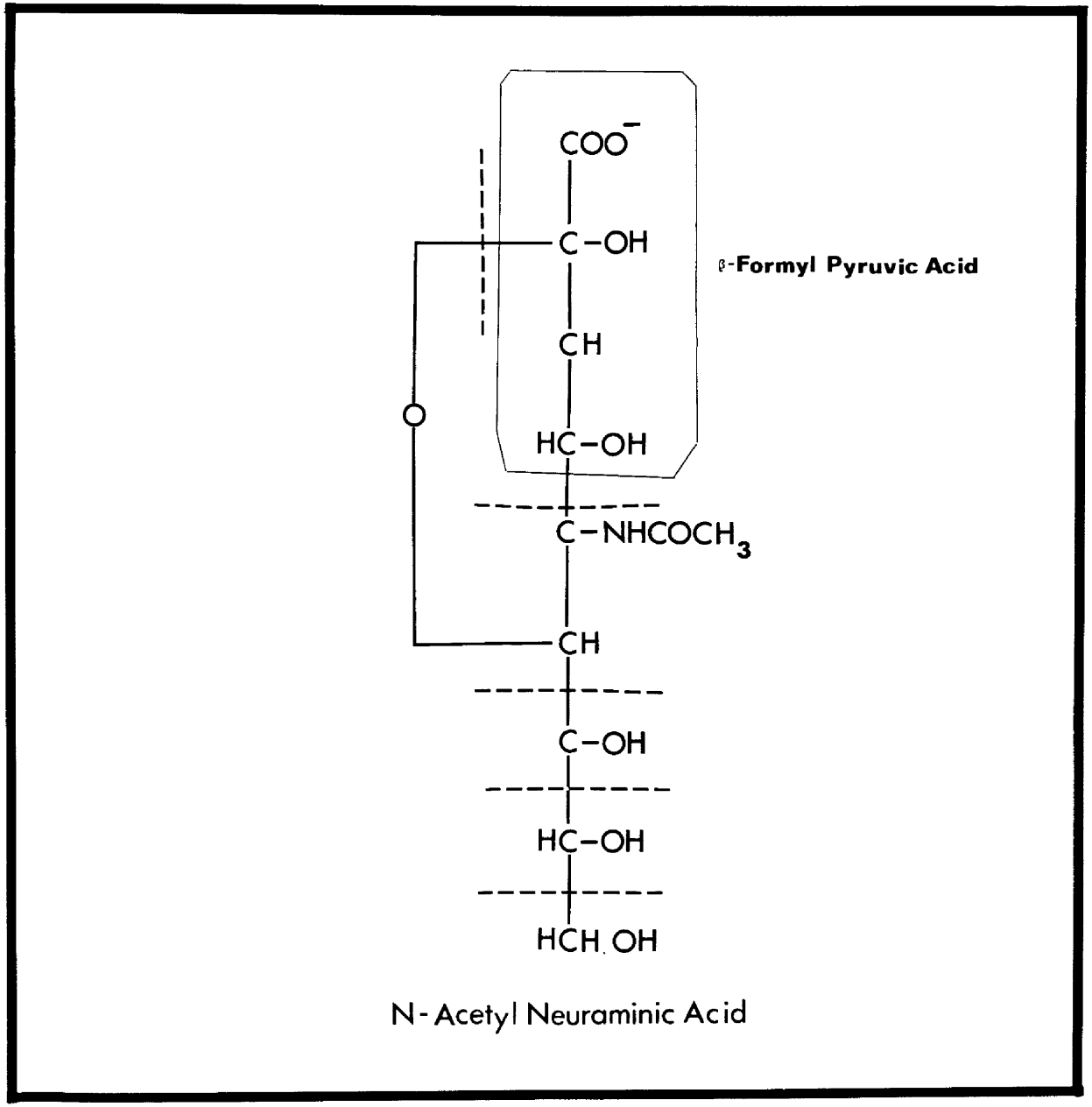
Principle: Periodate oxidation of N-acetyl neuraminic acid results in the formation of β-formyl pyruvic acid. Oxidation by periodate splits C - C bonds having adjacent OH groups or an NH_2 group adjacent to an OH group. β-Formyl pyruvic acid is then coupled to 2-thiobarbituric acid to form a pink chromogen with a maximum absorption at 549 nm (see Figure 9).

Figure 9 : Assay for Neuraminidase Activity

Structure of N-acetyl-Neuraminic Acid (NANA)

- - - - - - - Points of periodate cleavage

Note the carboxyl group on carbon 1 responsible for the negative charge of glycoproteins containing NANA and the formation of β-formyl pyruvic acid from Carbons 1, 2 and 3 of the hemiacetal ring.



N-Acetyl Neuraminic Acid

Reagents:

A. Sodium metaperiodate (0.2M) in 9M phosphoric acid (53% w/v).

B. Sodium arsenite (10% w/v) in 0.5M sodium sulphate solution.

C. 2-Thiobarbituric acid (0.6% w/v) in 0.5M sodium sulphate solution.

D. "Analar" Cyclohexanone.

All solutions are stable at room temperature for several months.

Procedure: The tubes were allowed to stand for 20 min at room temperature after addition of the periodate. 1.0 ml of reagent B was then added to each assay and the contents of each tube mixed thoroughly. After 2 min the yellow-brown colour was usually completely discharged. To each tube 3 ml of reagent C were then added, mixed by inversion and the tubes placed in a boiling water-bath for 15 min. After cooling to room temperature, 4 ml of reagent D were added to each tube and the contents mixed by agitation with a "Whirlimix" (Gallenkamp Limited, Widnes, England). The tubes were then centrifuged at 3000 rpm for 5 min to separate the phases. The E_{549} of the cyclohexanone extracts was measured in 1 cm cuvettes against the water blank extracted as above in an SP600 spectrophotometer. Spectrophotometer cells were rinsed with absolute alcohol between readings. A standard curve for N-acetylneuraminic acid (NANA) - (L. Light, Colnbrook, England) was prepared by this procedure (0-100 µg) and the NANA in test samples obtained by reference to this, after subtraction of substrate control values. One Unit of Neuraminidase was defined as the amount of enzyme releasing 1 µmole (309 µg) of NANA per 30 min from the egg white substrate (Collee, 1965b). Activity was expressed in milliunits of enzyme.

b. Method of Holding and Collee (1971):

i. Substrate: Human serum glycoprotein, pasteurised to destroy any Australia antigen present, acted as substrate. The preparation designated HGP_{11} was kindly supplied by Dr. J.G. Collee, Department of Bacteriology, University Medical School, University of Edinburgh.

ii. Assay: Each assay mixture contained the following:

0.1 ml Enzyme preparation

0.15ml Neuraminidase diluent B (see Appendix II)

0.25ml Substrate.

All assays included three controls - a substrate control with no added enzyme, an enzyme control with no substrate and a buffer control. The assays were incubated at 37°C for 30 min and the reactions stopped by the addition of Aminoff's Reagent A (vide infra). Assays were carried out in duplicate.

iii. Determination of NANA by modified Aminoff's method (Holding and Collee, 1971): (see also Cassidy et al., 1965, and Aminoff, 1961):

Reagents:

A. 0.025M Periodic acid in 0.125N H_2SO_4.

B. 2% (w/v) Sodium arsenite in 0.5N HCl.

C. 0.1M 2-thiobarbituric acid adjusted to pH 9.0 with 1N NaOH.

D. n-Butanol containing 5% (v/v) concentrated HCl.

Procedure: After addition of reagent A tubes left to stand at room temperature for 30 min; 0.2 ml of reagent B was then added to each tube. If the yellow-brown colour was not discharged in 2 min, a further 0.2 ml of reagent B was added. To each tube 2.0 ml of reagent C were then added and the tubes placed in a boiling water-bath for 7½ min. After cooling the pink chromogen was extracted into 4 ml of reagent D by

vigorous mixing. The phases were separated by centrifugation at 2100 g in an MSE "Super Minor" centrifuge in a swing out head. The E_{549} of supernatant phases were read in 1 cm cuvettes. A standard curve was prepared using the above procedure with solutions containing 0-30 µg NANA. This method for the determination of NANA was far more sensitive than that of Warren. One Unit of Neuraminidase activity was defined as under method 1.

SECTION II : PURIFICATION AND ASSESSMENT OF PURITY

A. ISOELECTRIC FOCUSING

Although the theory and technique of isoelectric focusing have been the subject of three recent reviews (Peeters, 1970; Haglund, 1970; Vesterberg, 1971), the essential features of this method will be summarised.

The method of electrofocusing in its present form owes much to the theoretical treatment of Svensson (1961, 1962a, 1962b), although the basic principles of the technique have been traced back to 1912 to two Japanese chemists, Ikeda and Suzuki, by Vesterberg (1968b). The successes and failures of the technique up to 1948 have been reviewed by Svensson (1948).

1. Principle of Electrofocusing.

Amino acids are examples of ampholytes, compounds which behave as acids and bases, since they each contain at least one carboxyl group and one amino group. These become negatively and positively charged respectively on dissociation. Proteins and polypeptides are thus also ampholytes. The net charge on an ampholyte is dependent on the number of free carboxyl and amino groups, the dissociation constants of these groupings and the pH of the suspending liquid. The pH at which the number of positive charges is equal to the number of negative charges is called the isoelectric point (pI) i.e. the pH at which the molecule is electrically neutral.

LKB Produkter, Bromma 1, Sweden have synthesised a special class of ampholytes having good conductance at the pIs. These are called 'carrier ampholytes'. These carrier ampholytes are also characterised by possessing buffering capacity at their isoelectric points. If they are electrolysed between an acid anode and alkaline cathode they migrate to their respective pIs, where they determine the pH by their buffering capacity and a pH gradient is thereby set up extending from anode to cathode. Separation of these ampholytes is maintained if mixing is prevented on termination of electrolysis. This can be achieved by the use of a stabilising density gradient of a highly water-soluble nonelectrolyte such as sucrose. A pH gradient set up in such a convection free environment is termed a 'natural pH gradient'. If proteins are also present in such a system they concentrate or focus at their respective pIs in the pH gradient set up by the carrier ampholytes. Hence the name given to this technique - isoelectric focusing.

The method differs from conventional zone electrophoresis in two respects in that electrophoresis takes place (a) in a buffer with varying pH, this increasing from anode to cathode, in contrast to electrophoresis at constant pH (b) in the absence of salts which could otherwise affect the ionisation of the protein one wishes to separate.

2. Carrier Ampholytes.

LKB carrier ampholytes are marketed under the trade name of 'Ampholine'. Ampholines are available for the formation of pH gradients

between pH 3-10 and fractions thereof. These carrier ampholytes are suitable for electrofocusing because they are efficient buffers at their pIs, have good conductance at their pIs and form a smooth pH gradient. Furthermore, they have a low E_{280}, essential when monitoring protein elution.

Ampholines consist of a mixture of synthetic aliphatic polyaminopolycarboxylic acids obtained by coupling acrylic acid to a mixture of polyalkalene-polyamines under conditions in which no amide bonds (including peptide bonds) are formed. The synthetic reaction leads to the formation of a series of at least 300 homologues and isomers of ampholytes with differing pIs in the pH range 3-10. These are separated into narrow pH ranges using an electrolysis apparatus. The practice of adding glutamic and aspartic acids and lysine and arginine to extend the pH ranges covering pH3 and pH 10 respectively has now been discontinued.

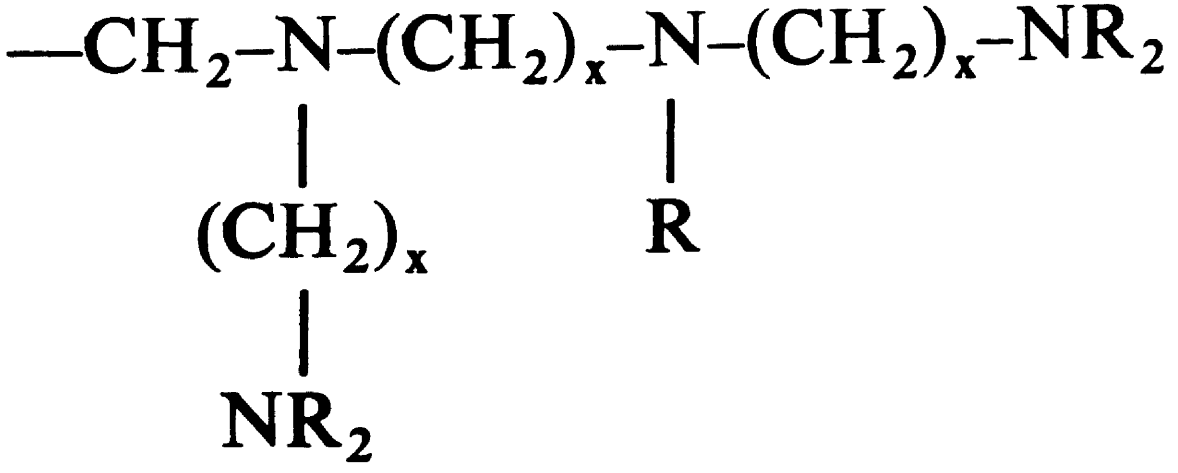
The generalised formula of carrier ampholytes is given in Figure 10 . The reader is referred to Davies (1970) for discussion of the physical and chemical properties of Ampholine chemicals.

3. Apparatus Used.

The specially designed columns supplied by LKB-Produkter AB were used throughout the electrofocusing studies in this thesis. Both the 110 ml (LKB 8101) and 440 ml (LKB 8102) were used. Figure 11 is a schematic diagram of the apparatus. Compartment (17) which is annular is cross section, is the electrofocusing compartment. Shaded areas represent the cooling water compartments. It should be noted

Figure 10 : Generalised Formula for the Structure of Carrier Ampholytes: Aliphatic Polyamino-Polycarboxylic Acids

(Davies, 1970)

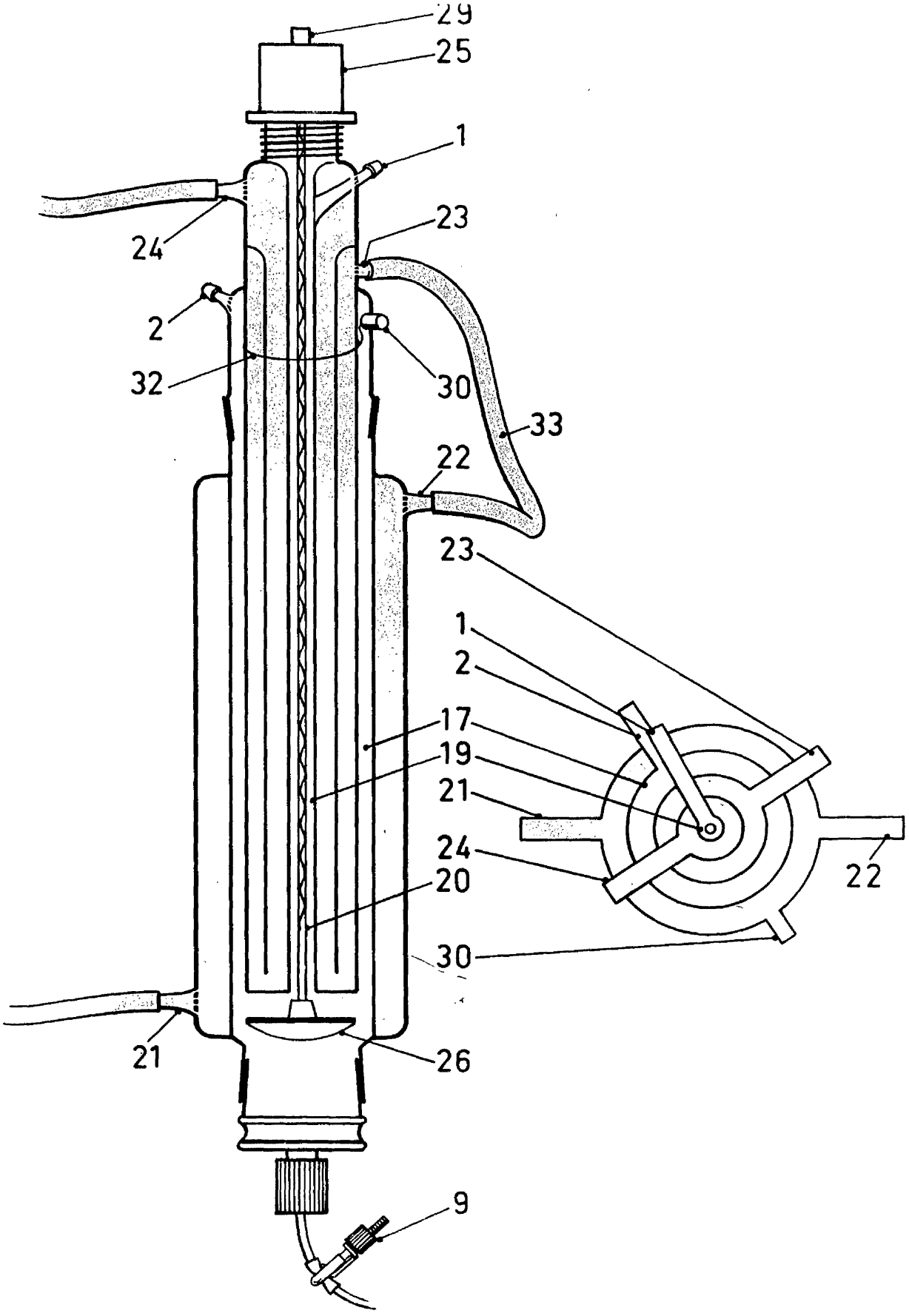


$x = 2$ or 3

$R = H$ or $-CH_2-CH_2-COOH$

Figure 11 : A Sketch of the LKB Electrofocusing Columns

Note the annular cross-section of the electrofocusing compartment thermostatted from both sides by cooling water compartments (shaded areas).


29
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9

that the separation compartment is thus cooled on both sides. Power was supplied by means of an LKB 3371D power pack which gives the voltage range 0 - 1200V, variable in two ranges 0 - 600V and 0 - 1200V DC. A thermostatically controlled waterbath (Grant Instruments, Cambridge, England, or Techne, Duxford, England) having a circulating pump was cooled by means of a cooling coil (Grant Instruments or Townson and Mercer, Croydon, England), and the thermostat of the waterbath adjusted such that water was maintained at 4°C with minimal accumulation of ice on the cooling coil. Water was pumped from this reservoir around the column by means of a water pump (Techne). Water circulated through the column by entering the column at nipple (21) of the outer cooling jacket leaving via nipple (23) and entering the inner cooling jacket via nipple (23) returning to the reservoir via nipple (24) - see Figure 11.

All pH measurements were made using Vibret Model 46A expanded scale pH meter (E.I.L., Richmond, Surrey) fitted with a combination glass micro-electrode (Activion Glass Limited, Kinglassie, Scotland - Type No. M.27DP) and a pH temperature compensator immersed in the waterbath at 4°C.

Although density gradients for the columns were made up manually during preliminary studies as described in the 'LKB 8100 Ampholine Electrofocusing Equipment Instruction Manual', the rest of the column gradients described in this thesis were made using the LKB 8121 (110 ml) and LKB 8122 (440 ml) gradient mixers with columns of the appropriate capacity. The mixers were driven by a stirrer motor (LKB 8121). These mixers were specially designed to give reproducible continuously linear gradients.

The 110 ml column was loaded and drained by the use of an LKB 10200 perspex pump fitted with a 9:100 ratio gear box (LKB 10233) giving a flow rate of 80 ml/hour. With the 440 ml column an LKB 4912A peristaltic pump, adjusted to give a flow rate of 180 ml/hour, was used for loading and draining. All fractions were collected by the use of an LKB 7000 UltroRac fraction collector.

4. Preparation of Material for Electrofocusing.

Only low concentrations of salt are permissible in the protein solutions applied to electrofocusing columns. If the salt is not removed the pH gradient is disturbed. In the case of the 110 ml column the maximum permissible salt concentration in the sample loaded is 0.5mM, and for the 440 ml column, 1.5mM. All toxin fractions were dialysed against a large excess of distilled water or a 1% (w/v) solution of glycine (BDH 'Analar') - employing several changes of dialysate which was stirred. This was carried out overnight at 4°C prior to loading of the material onto the column.

5. Preparation of Density Gradients.

The dense and light solutions used to make up the sucrose density gradient were prepared after the method of Bernheimer et al., (1968a) as the volumes used by these authors ensured loading of the total sample. Some material was always left in the mixer using the volumes outlined by LKB-Produkter AB in their electrofocusing manual. Preparation of the sucrose gradient solutions is outlined in Appendix IV. Electrofocusing experiments in urea were only carried out in the 110 ml column. Both the dense and the light solutions were made 6M with respect to urea. Preparation of these solutions is dealt with in Appendix IV.

Solutions of sample were added to the light and/or dense solutions. Initially the toxin preparations were applied in the light solutions as recommended in the LKB electrofocusing manual. However, a large amount of the applied material in the crude preparations focused at the anode and precipitated out of solution. Therefore in all subsequent experiments toxin was applied in the dense solution with the anode placed at the bottom of the column. These insoluble precipitates did not adhere to the glass or trail on draining of the columns. In preparative electrofocusing columns, use was made of both the dense and the light solutions to maximise application of toxin to any one column.

Ampholine carrier ampholytes obtained as 40% (w/v) solutions were used to prepare pH gradients between pH 3 - 10(LKB 8141), pH 4 - 6(LKB 8152) and pH 5 - 8 (LKB 8143). Concentrated ampholine was diluted 1:4 with distilled water to give a 10% (w/v) ampholine solution for addition to the dense and light solutions (see Appendix IV). The final concentration of the ampholines in the dense solution was 1.5% and in the light solution 0.5%. This initial distribution of the carrier ampholytes aids conductance in the dense sucrose and thus rapid establishment of the pH gradient.

6. Preparation of Electrode Solutions.

Electrode solutions for the 110 ml and 440 ml columns were prepared according to the instructions in the LKB electrofocusing manual, when the anode was placed at the bottom of the column. The anode solution contained sulphuric acid at a final concentration of 1% (v/v);

the cathode solution contained sodium hydroxide at a final concentration of 1% (w/v). For details of their composition see Appendix IV. For 'Urea' columns both the anode and the cathode solutions were made 6M with respect to Urea (see Appendix IV). In the 110 ml columns approximately 16 ml of anode solution was placed at the base of the column and 65 ml, in the case of the 440 ml column.

7. Loading of Columns.

Columns were supported vertically by means of three or four pinch clamps to lab-block scaffolding (Gallenkamp Ltd.). The columns were loaded as described in the LKB electrofocusing manual with valve (26) in the open position as in Figure 11. The anode solution was loaded through nipple (1), the gradient and cathode solutions, through nipple (2).

8. Placement of Electrodes.

In all electrofocusing experiments the anode was placed at the bottom of the column by attachment of the appropriate lead to the plug (29) giving the central electrode wound round the teflon rod (20) a positive charge. The upper electrode (32) consists of a loop of platinum wire located at the top of the electrofocusing compartment (17). The cathode lead was attached to plug (30).

9. Electrofocusing Run Conditions.

Electrofocusing was obtained using a final potential of 800V in all experiments. In the case of broad pH gradient runs only 150-200V could be applied initially. With narrow pH gradients in both the 110 ml

and 440 ml columns, initial potentials of the order of 300-400V could be applied. A maximum power of 0.5 - 1.0W for the LKB 8101 column and 2 - 4W for the LKB 8102 column was applied at the start of any run. As has been stated above, focusing was carried out at 4°C. Only slight, if any, temperature fluctations were noted during runs.

10. Draining of Columns.

Initially columns were drained by gravity by opening clamp (9) after switching off the power supply, disconnecting electrodes from the supply, closing valve (26) and draining the central electrode compartment tube (19) by pumping out through nipple (1). However, this resulted in a variable flow rate during draining, the rate increasing with the decreasing sucrose density. Attempts at draining the column by forcing out the contents by pumping water into the top of the column through nipple (2), proved inconvenient and did not result in an even flow rate (see Figure 11).

In most experiments, therefore, columns were drained by introducing the appropriate peristaltic pump in the outlet line. Fractions of 2 ml from column 8101 and of 4 ml from column 8102 were collected in 3" x $\frac{1}{2}$" plastic test tubes or 2" x $\frac{1}{2}$" glass test tubes. Fractions of these volumes gave convenient volumes of material to work with, whilst maintaining the separation.

11. Cleaning of Columns.

The electrofocusing columns were cleaned by soaking overnight in a bath of pyroneg (Diversey Ltd., Barnet, Herts.), or Decon 90 (Decon

Laboratories Ltd., Brighton) followed by thorough rinsing in tap water and distilled water. Columns were not siliconised as has been recommended by some authors to prevent distortion of banded proteins (Flatmark and Vesterberg, 1966; Ui, 1971a).

12. Measurement of pH.

Immediately after draining of columns fractions were placed in the water-bath containing the column coolant and allowed to equilibrate for 10 min. The pH meter was calibrated against 2 ml volumes of pH 7.0 Standard Buffer prepared from buffer sachets (Electronic Instruments Ltd.,) on the day of use and cooled to 4°C with the fractions under test. The calibration of the pH meter was checked against the standard buffer after every four pH determinations. Measurements of pH were made by immersion of the electrode in the fractions. Approximately $\frac{1}{2}$ min was allowed for equilibration before the pH was read off on the appropriate expanded range scale. Between readings the electrode was thoroughly rinsed with distilled water and wiped dry with absorbent cotton wool or tissue.

a. The Effect of Urea on pH and the pH of Carrier Ampholytes:

Urea is known to raise the pH of solutions (Burk and Greenberg, 1930; Levy, 1958; Bull et al., 1964), but its effect on the pH of carrier ampholytes used to form the pH gradient for isoelectric focusing has only recently been considered in the author's experience. Tests in this laboratory showed that the pH of the original unfocused solution containing the complete mixture of carrier ampholytes giving the pH range 5 - 8 was raised by 0.34 of a pH unit by the addition of 6M urea whether the pH was determined at 4°C or 25°C (see Table 25).

Table 25 : Effect of 6M Urea on the pH of Carrier Ampholytes

Mixture	pH** measured at + 4°C	pH** measured at + 25°C
AS [oo]	6.85	6.48
AS + 6M Urea	7.19	6.82

** pH meter calibrated against pH 7.0 standard Buffer prepared from E.I.L. sachet using the manufacturer's temperature correction.

[oo] AS = Ampholines pH range 5 - 8 (1% w/v) + 50% (w/v) Sucrose.

More recently the effect of urea on the pI of carrier ampholytes throughout the entire pH gradient has been investigated by Ui (1971a, 1971b). He observed an increase of 0.42 pH units in the pI of carrier ampholytes independent of the pH range used in columns containing 6M urea. Josephson et al., (1971) have reported a larger increase of 0.9 pH units in the pI of carrier ampholytes throughout the pH range 3 - 6 in 7M urea. Thus although differing by degree both groups have confirmed that urea causes an upward shift in the pH of the ampholyte gradient, and that correction must be made for the observed pIs of proteins when electrofocusing is carried out in urea.

At present the absolute value of the correction factor to be applied to the observed pIs is far from clear and this subject obviously merits more intensive investigation in a number of laboratories. In light of the above findings I feel it is more appropriate at present to correct for the effect of urea by its inclusion in the standard buffer used to calibrate the pH meter.

For this reason the standard buffer was made 6M with respect to urea in the case of columns containing 6M urea, and the pH meter calibrated according to the pH value given by the manufacturer for the buffer in the absence of urea at 4°C.

13. Measurement of UV Absorbance of Fractions.

The E_{280} of all fractions was measured using an SP 500 UV spectrophotometer (Pye Unicam, Cambridge, England), using quartz microcells of 1.0 cm light path. The lamp housing was always adjusted

such that the light passed through the 12 x 12 mm slit and the lens before entering the cell. In all cases distilled water was used as the blank solution.

B. IMMUNOLOGICAL METHODS

1. Immunoelectrophoresis.

Immunoelectrophoresis was carried out by the method of Stewart-Tull (personal communication). Into a rectangular tray was poured 500 ml of 5% ionagar No. 2 (Oxoid) containing 5% (w/v) phenol as a preservative and this allowed to set on a Shandon levelling platform to form a horizontal thick-bed surface. Siliconised glass sheets 20 x 10 cm were placed on this surface and 1% electrophoresis agar (see Appendix V) poured over the plate to form an agar layer 1.0 - 1.5 mm thick. This was allowed to set and the plates then refrigerated overnight. Troughs and wells were cut out of the agar using a No. 1 cork borer, a scalpel and a ruler. The wells were 0.4 cm in diameter, the troughs 0.4 cm wide by 7 cm long and the distance between the wells and the troughs was 0.4 cm.

Electrophoresis was carried out in a Shandon electrophoresis tank. Lint wicks were applied to the edges of the gel and immersed in 0.05 M barbitone buffer (see Appendix V). A drop of bromothymol blue was added to one well to act as an electrophoretic marker. A voltage of 100-150V was applied to obtain electrophoretic separation. When the dye marker had migrated to the ends of the troughs, electrophoresis was stopped, the agar removed from the troughs and the troughs filled with antiserum. Diffusion of antiserum and antigens took place

over the central compartment of the electrophoretic tank or in a plastic food container lined with wet blotting paper at room temperature.

2. Ouchterlony Double Diffusion Tests.

Immunodiffusion tests were carried out on agar plates containing 1% Ionagar (Oxoid Ltd.,) in 0.03M borate/0.14M sodium chloride buffer pH 8.3. The agar layer was approximately 3-4 mm thick. Porcelain cups of 0.04 ml volume were filled with antiserum or test fractions and placed 7 mm apart on the agar surface. The plates were incubated overnight at 37°C and then at room temperature in a plastic food box lined with wet blotting paper.

3. Standard Antiserum.

Clostridium perfringens Type A diagnostic serum was obtained from Wellcome Laboratories, Beckenham. The batch no. used throughout this thesis was K7739. The serum contained 0.35% cresol as a preservative. It was stored at 4°C.

C. DISC-GEL ELECTROPHORESIS

The procedure used for electrophoresis was a modification of the method of Davis (1964) as used by McNiven et al., (1972). Disc-gel tubes were soaked in photoflow (Kodak Ltd., London) and dried. Separating gels contained 11.7% (w/v) acrylamide (B.D.H., Poole) and 0.153% (w/v) NN'-methylenebisacrylamide (B.D.H., Poole). Stacking gels were prepared by a modification of the method of De Vito and Santome (1965) omitting EDTA and contained 4% acrylamide and 1% NN'-methylenebisacrylamide. Both the gels and the buffer were made

0.1% (w/v) and the toxin samples 0.2% (w/v) with respect to sodium dodecyl sulphate (SDS). Details of tank buffer, gel buffers and catalysts are given in Appendix VI. Electrophoresis was performed at 1 mamp per gel until the tracking dye added to the cathode compartment had migrated to within 1 cm of the bottom of the gel at which time the current was boosted to 5 mamp per gel. Gels were extruded using a syringe, and were fixed and stained by immersion overnight in a solution containing amido black dissolved in acetic acid/methanol. They were rehydrated in 7% acetic acid prior to electrophoretic destaining in 7% acetic acid [redacted] at 6 mamp per column. R_f values were obtained by comparison with the track dye front.

Molecular weight determinations were carried by the method of Shapiro et al., (1967) from a standard curve of the logarithm of molecular weights of standard proteins against R_f values determined under identical conditions (see Appendix VI). Shandon disc electrophoresis tanks were used throughout.

D. THIN LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS

Thin layer chromatography plates were prepared using a Shandon semi-automatic Unoplan Leveller. Glass plates 20 x 20 cm were cleaned by soaking in Decon 90 followed by rinsing in tap and distilled water and dried in a hot air oven. The plates were aligned in the leveller and the spreader blade adjusted to give a layer thickness of 0.25 mm. The slurry was evenly mixed by shaking 36 g Silica Gel G (Merck), 48 ml distilled water and 24 ml 'Analar' methanol

in a stoppered bottle for 1 min. The slurry was poured into the spreader and the layers spread in a smooth sweep of the spreader. After drying *in situ* the plates were placed in a storage rack in a desiccator cabinet until required. Silica gel G plates were activated by heating in a drying oven at 110°C for 20-30 min.

Samples were applied 2 cm from the bottom edge of the plate. Plates were developed in chromatography tanks (Shandon, London) with chloroform:methanol:water in the ratio 130:70:10. Development was terminated 14 cm from the origin at a rule across the plate. After drying, plates were sprayed with 50% (v/v) sulphuric acid and heated at 110°C for 10 min. Phospholipids were detected as charred spots.

E. PROTEIN ESTIMATIONS

Protein estimations were carried out by the method of Lowry *et al.*, (1951) using lysozyme or bovine serum albumin as a standard (see page 8A). The protein content of fractions was monitored by ultraviolet extinction measurements at 280 nm using an SP 500 spectrophotometer with quartz cells of 10 mm light path.

SECTION III : CELLS AND MEMBRANES

A. PROPAGATION AND TREATMENT OF KREBS 2 ASCITES TUMOUR CELLS

1. Source of Cells.

Mice containing Krebs 2 ascites tumour cells were the gift of Drs. Janet Smillie and R.H. Burdon of the Biochemistry Department, University of Glasgow, to Drs. Dorothy A. Symington and J.P. Arbuthnott of this Department (Symington, 1969). This cell line arose spontaneously in the inguinal region of a hybrid male mouse from a solid carcinoma and the cells were converted to the ascitic form by Klein and Klein (1951).

As a cell model system they have the distinct advantage over normal tissue culture techniques that they can be easily propagated by intraperitoneal injection in mice. They grow in the peritoneal fluid in the form of a homogeneous suspension of cells. Although neoplastic, these cells possess the electron transport system of normal mammalian cells (Symington, 1969).

2. Propagation.

Porton white mice weighing 25-40 g were injected intraperitoneally with 0.3 ml of ascites fluid. Cells were harvested from the mice 7 to 10 days later by opening up the peritoneal cavity and withdrawing the fluid with a sterile plastic syringe. This was found to be easier than needle aspiration of the peritoneum. Cells which were contaminated with erythrocytes through haemorrhaging were discarded.

3. Washing of Cells.

Cells were washed by a modification of the method of

Symington (1969). To freshly harvested cells an equal volume of 7% (w/v) polyvinyl pyrrolidone (PVP) buffered with 0.02M Tris/HCl pH 7.3 was added, and the cells sedimented by centrifugation in an MSE "Super Minor" centrifuge with a swing out head at 2100 g for 5 min. The cells were then resuspended in PVP-Tris buffer and sedimented. On the second wash the cells required to be centrifuged for 10 min. The wash in Ringer's solution used by Symington (1969) was omitted because of the presence of Ca^{2+} in this solution. Cells were resuspended to their original volume in buffered PVP. PVP had several advantages as a suspending fluid :-

(a) because of its high molecular weight the suspending buffer had a high viscosity which prevented the cells from clumping.

(b) cells did not readily settle out in this suspending medium.

(c) the structural integrity of the cells was maintained.

(d) it was also found that cells were maintained in a metabolically active state at $4^{\circ}C$ for up to 48 hours in this suspending fluid, with little effect on the respiration rate of the cells using succinate as substrate.

Normally, cells for manometric studies and respiration studies with the oxygen electrode were used within 2 hours of harvesting.

4. Measurement of Respiration of Ascites Cells.

Respiration of Krebs ascites cells was followed by two methods.

a. The Warburg Manometric Technique:

i. Principle: The Warburg apparatus used was manufactured by Townson and Mercer, Croydon, England. It consists of two parts, a flask and a manometer. The former is immersed in a water bath at constant

temperature and the latter contains Krebs manometer fluid (Umbreit et al., 1964). The flasks were calibrated with mercury, flask constants being calculated by the method of Umbreit et al., (1964). Oxygen uptake was monitored by observing changes in the level of the manometer fluid in the closed manometer-flask system and was recorded as a pressure change with reference to a set point on the manometer scale to which the level of the manometer fluid was adjusted before readings. As pressure varies with temperature all readings in mm were corrected with reference to a thermobarometer control. The amount of oxygen consumed per time interval was then calculated by multiplication of this value by the appropriate flask constant.

This technique was used in the early stages of the biological studies undertaken in this thesis. Its main disadvantages were the time taken to set up the experiment, the limited number of experiments that could be performed on any one batch of ascites cells and that one was unable to perform a repeat confirmatory experiment immediately, on the same batch of ascites cells.

ii. Standard Warburg assay: Each assay consisted of seven flasks - duplicate flasks for respiration after toxin treatment, control respiration and endogenous respiration. The seventh flask was a thermobarometer control for changes in atmospheric pressure. Table 26 gives the set up of experiments in which Krebs 2 ascites cells were pretreated with AS/G100 α-toxin in the presence of Ca^{2+} (see page 228). After pretreatment the substrate was tipped from the side arm into the main vessel. Table 27 gives the set up of experiments in which Krebs 2 ascites cells were pretreated with AS/G100 α-toxin in the absence of Ca^{2+}. Substrate was tipped into the main vessel after

Table 26 : Experimental protocol for the Effect of AS/G100 α-Toxin on Succinate Respiration of Krebs 2 Ascites Cells in the Presence of Ca^{2+} as measured in the Warburg Apparatus.

Flask No.	1	2	3	4	5	6	TB
Main Vessel: *							
PVP/Tris Buffer	1.4	1.4	1.4	1.4	1.4	1.4	2.2
0.025M Calcium Acetate	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Toxin	0.5	0.5	-	-	-	-	-
Control Fluid	-	-	0.5	0.5	0.5	0.5	0.5
Ascites Cells	0.5	0.5	0.5	0.5	0.5	0.5	-
Side Arm: *							
Succinate (0.2M) in PVP/Tris Buffer	0.3	0.3	0.3	0.3	-	-	-

* All volumes in millilitres

Table 27 : Experimental protocol for the Effect of AS/G100 α-Toxin on Succinate Respiration by Krebs 2 Ascites Cells in the Absence of Ca^{2+} as measured in the Warburg Apparatus.

Flask No:	1	2	3	4	5	6	TB
Main Vessel: *							
PVP/Tris Buffer	1.7	1.7	1.7	1.7	1.7	1.7	2.5
Ascites cells	0.5	0.5	0.5	0.5	0.5	0.5	-
Toxin	0.5	0.5	-	-	-	-	-
Control Fluid	-	-	0.5	0.5	0.5	0.5	0.5
Side Arm: *							
Succinate (0.2M) in PVP/Tris Buffer	0.3	0.3	0.3	0.3	-	-	-

* All volumes in millilitres

pretreatment of the cells for 30 min.

b. Polarography:

This technique has the decided advantage over Warburg manometry that it allows the measurement of reactions occurring immediately after addition of substrate to cells. Such measurements are impossible with the Warburg technique because of the lack of sensitivity of the system.

The sensor, the polarographic oxygen electrode, is a membrane electrode comprising a 0.025" diameter platinum cathode and a U-shaped silver anode encased in an epoxy block except at the end of the probe, over which a thin teflon membrane is stretched. The electrode is filled with KCl and the membrane is oxygen permeable.

When two distinct liquid systems are separated by a membrane that is permeable to oxygen, in this case the KCl of the electrode and the test buffer solution in which the sensor is immersed, and only one of these is exposed to oxygen - the test solution - this gas will diffuse across the membrane until the oxygen content of each of the liquids is in equilibrium. The driving force for the transfer of oxygen across the membrane is the difference between the partial pressures of oxygen in the gas phase which would be in equilibrium with the oxygen concentrations in each of the liquid phases. This difference is termed oxygen tension.

The oxygen electrode is a device which measures the rate of oxygen transfer across the membrane. Application of a potential between two electrodes submerged in a liquid results in a flow of current.

At characteristic potentials this current increases markedly due to reduction reactions at the cathode. At a constant potential of 0.8V in the case of the oxygen electrode the current is a measure of the number of molecules of oxygen being reduced at the cathode per unit time i.e. of the rate of oxygen transfer to the cathode, resulting from the diffusion of oxygen across the membrane. Since the rate of diffusion is dependent on the oxygen tension across the membrane, the method acts as a measure of oxygen uptake by cells because the oxygen tension falls with its utilisation in respiration. Because the oxygen electrode is consuming oxygen in solution it must be continually exposed to new solution. This is achieved by stirring of the sample with a magnetic stirring bar.

The oxygen electrode requires to be calibrated with a solution of known oxygen content at a known barometric pressure. It was shown by calibration of the apparatus that the buffered suspending solution used, PVP-Tris/HCl, had the same oxygen content when saturated as Ringer's solution at 37°C. Ringer's solution at 37°C contains 5 µl of oxygen/ml at 100% saturation at ambient barometric pressure. A change of 50% in saturation in a 3 ml sample therefore means that $\frac{3 \times 5 \times 50}{100} = 7.5$ µl of oxygen was consumed in the time interval studied.

The biological oxygen monitor used was manufactured by Yellow Springs Instrument Co. Inc., and comprised an oxygen electrode and an electronic signal amplifier. The constant temperature water bath/ circulating pump assembly was manufactured by MGW LAUDA, West Germany. The rate of change of oxygen saturation was recorded by means of a constant speed chart recorder (Metrohm, Switzerland).

B. PREPARATION OF ERYTHROCYTE GHOSTS

The erythrocytes were sedimented by centrifugation at 3000 g for 10 min in an MSE "Super Minor" bench centrifuge in swing out buckets. The serum was removed with a Pasteur pipette and the buffy coat removed gently with a spatula. The erythrocytes were then washed three times with 0.85% NaCl.

Packed erythrocytes were lysed in 200 volumes of 0.01M Sørensen's phosphate buffer pH 7.4 (Documenta Geigy, 1962). The cells were lysed by dropwise addition to stirred buffer at room temperature. The ghosts were harvested by centrifugation at 48,000 g for 15 min in an MSE 25 refrigerated centrifuge. The packed ghosts were washed twice with 0.01M Tris-HCl buffer pH 7.4 (Documenta Geigy, 1962) and harvested by centrifugation at 48,000 g for 15 min. This procedure yielded ghosts that were free of haemoglobin. The final ghost pellet was maintained for up to 2 hr at $4^{\circ}C$ until required.

RESULTS

SECTION I : TOXIN PRODUCTION

A. TOXINOGENESIS DURING THE GROWTH CYCLE

Experiments were carried out to study the relationship between α-toxin, θ-toxin, collagenase and hyaluronidase production and the growth phase of the culture. A typical experiment is summarised in Figure 12. Growth, toxin production and enzyme production were monitored by methods described earlier (see pages 113 and 116). Samples of the culture medium (M-II) were taken at intervals over a period of 5 hours.

It can be seen from Figure 12 that :-

i) exponential growth of the culture commenced immediately upon inoculation

ii) θ-toxin and hyaluronidase production commenced shortly after initiation of logarithmic growth

iii) there was a significant lag before α-toxin and collagenase were detected in the culture medium supernatant fluids

iv) production of all four extracellular products ceased or tailed off as the culture entered the stationary phase of growth. This sequence of toxin-enzyme production was observed in the case of both the S107 and BP6K strains.

Continued incubation for up to ten hours resulted in little further growth. The titre of α-toxin declined, but not that of θ-toxin. The effect of prolonged incubation on hyaluronidase and collagenase was

Figure 12 : Toxinogenesis of Cl. perfringens Strain BP6K

Symbols:

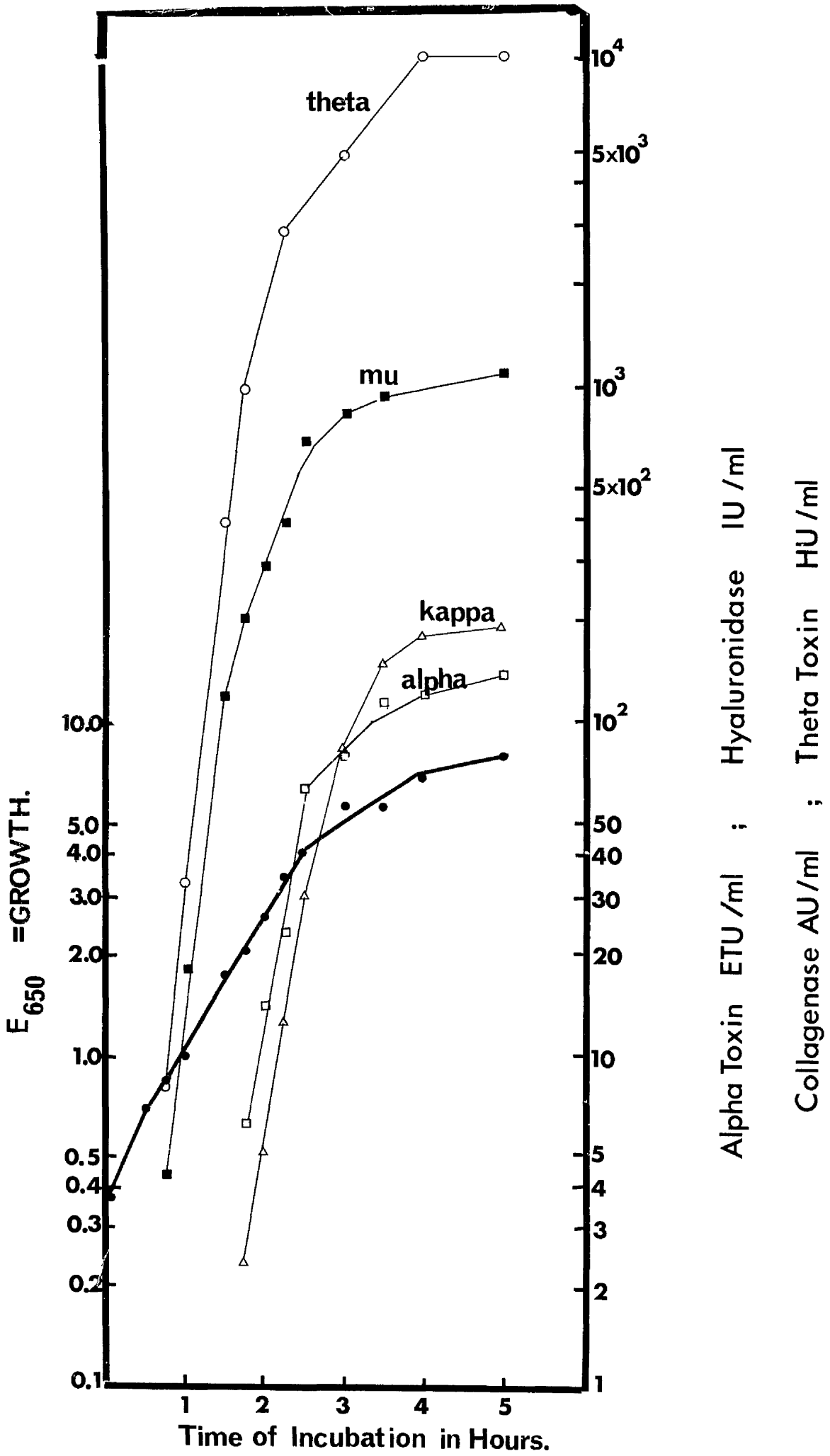
(●) Growth as E_{650}

(○) θ-Toxin (HU)

(□) α-Toxin (ETU)

(■) Hyaluronidase Activity (IU)

(△) Collagenase Activity (AU)


theta
mu
kappa
alpha
10^4
5×10^3
10^3
5×10^2
10^2
50
40
30
20
10
5
4
3
2
1
10.0
5.0
4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
E_{650} =GROWTH.
1
2
3
4
5
Time of Incubation in Hours.
Alpha Toxin ETU/ml ; Hyaluronidase IU/ml
Collagenase AU/ml ; Theta Toxin HU/ml

not determined. Assays for the determination of deoxyribonuclease or neuraminidase were not available at the time these experiments were carried out and thus their distribution with time was not determined.

In culture medium M-II the E_{650} was found to increase to 14.0 - 15.0 in 4 - 5 hours in a number of experiments. This increased growth did not always result in a similar increase in toxin production. Generally α-toxin yields in culture medium M-II were higher by a factor of 2 or 3 than those observed in M-I.

B. CONCENTRATION OF α-TOXIN

Methods used by previous authors for concentrating α-toxin from culture supernatant fluids or filtrates were compared to determine which would prove the most convenient for preparing toxin for the initial electrofocusing experiments. A summary of one such study on culture supernatant fluid from strain BP6K is shown in Table 28.

From these results it is clear that methods c and d gave poor yields, whereas methods a and b provided yields of approximately 50%. In handling small volumes of culture supernatant up to 1 litre, method a had several advantages over method b :-

(a) for a marginally smaller yield in activity, an 6 fold increase in purity was obtained

(b) the ammonium sulphate precipitate from method b was difficult to dissolve in a minimal volume of distilled water; moreover, it stuck readily to glass vessels and plastic centrifuge bottles and tubes making it difficult to harvest

Table 28 : Partial Purification Studies on BP6K α-Toxin from M-II Culture Supernatant Fluids[oo]

Fraction	Total Initial Activity (PCU)	Total Activity Recovered (PCU)	Recovery (%)	Total Protein* Recovered (mg)	Specific Activity (PCU/mg)	Purification
M-II Culture Supernatant Fluid	262	-	-	2610	0.1	-
2nd Acetone Precipitate - Method a	-	141	54	43	3.3	33 X
75% Ammonium Sulphate Precipitate - Method b	-	146	56	302	0.5	5 X
36% Methanol Precipitate - Method c	-	35	14	33	1.1	11 X
NaCl Saturation Precipitate - Method d	-	37	14	79	0.5	5 X

[oo] 100 ml volumes were harvested by the procedures outlined on page 113 of 'Materials and Methods'; all precipitates were dissolved in distilled water and dialysed overnight against distilled water prior to assay.

* Protein was determined by the method of Lowry.

(c) toxin was easily eluted from the calcium phosphate precipitate which had been used to adsorb toxin in the presence of acetone.

Both methods a and b were, however, used to harvest toxin for electrofocusing as a means of checking for alterations that might have occurred in the proteins harvested by one or other of these techniques. In handling large volumes of culture supernatant, both of these methods had drawbacks. On the one hand, method a involved the use of large volumes of acetone and incurred considerable cooling problems when handling large volumes of culture supernatant. On the other hand, method b gave material of low specific activity. Ideally for electrofocusing of crude toxin, the amount of contaminating material should be minimal in order to maximise the amount of toxic protein processed at any one time.

Culture supernatant fluids were precipitated by method e (see page 115). Each of the fractional precipitates was assayed for α-toxin, θ-toxin, collagenase and hyaluronidase. The total activity detected in each fraction and the corresponding % recoveries are given in Table 29 . Whereas most of the α-toxin and collagenase were precipitated between 40-50% saturation, the major portionsof θ-toxin activity and hyaluronidase were not precipitated until a saturation value above 50%.

Further investigations were carried out to determine the % saturation between 30-50% giving the best combination of recovery and purification of α-toxin. Culture supernatant was saturated to 30% by addition of solid ammonium sulphate, the precipitate harvested and

Table 29 : Fractional Ammonium Sulphate Precipitation of M-II Culture Supernatant Fluids of Strain BP6K

Toxin	Saturation with $(NH_4)_2SO_4$						
	0%	0-30%	30-40%	40-50%	50-60%	60-70%	Total Recovery of Activity
Total α-Toxin Recovered[oo]	47,500	790	4,660	21,100	340	0	26,890
(ETU)	(100)	(1.7)	(9.7)	(44.5)	(0.7)	-	(56.6)
Total θ-Toxin Recovered[oo]	1.28	0.02	0.18	0.23	0.65	0.14	1.22
(HU x 10^6)	(100)	(1.9)	(14.4)	(18.3)	(50.4)	(11)	(circa 95)
Total Collagenase Recovered[oo]	120,000	1330	6050	22,700	10,100	3620	43,800
(AU)	(100)	(1.1)	(5.0)	(18.9)	(8.3)	(3.0)	(36.5)
Total Hyaluronidase Activity[oo]	630,000	10,400	7300	30,700	425,900	111,900	586,200
Recovered (IU)	(100)	(1.8)	(1.3)	(4.5)	(67.7)	(17.7)	(93.0)
Total Protein Recovered (mg)[oo]	33,600	500	550	760	1,320	1,760	4,890
	(100)	(1.5)	(1.6)	(2.3)	(3.9)	(4.6)	(14.5)

[oo]Percentage recoveries are given in brackets below each total recovery
* Protein was determined by the method of Lowry

discarded. The supernatant fluid was divided into three 500 ml volumes. Fractional precipitates between 30-45%, 35-50% and 30-50% saturation were harvested, dialysed and assayed by egg yolk turbidity activity. The results are shown in Table 30 . It was decided to sacrifice the marginal gain in recovery observed in the 30-50% saturation precipitate for the slightly higher purification obtained by using 35-50% saturation. In all preparative scale electrofocusing experiments this fractional ammonium sulphate precipitate of culture supernatant fluids was used.

Table 30 : Fractional Precipitation of α-Toxin from M-II Culture Supernatant Fluids of Strain BP6K with Ammonium Sulphate

$(NH_4)_2SO_4$ Saturation (%)	Volume of Sample (ml)	Protein (mg/ml)	ETU/ml	Total Recovery (ETU)	Total Protein (mg)	Specific Activity (ETU/mg)	Purification	Recove (%)
0	500	30	50	25,000	15,100	1.6	-	100
30-45	51	8.1	137	7,000	410	17	10 X	28
30-50	46	10	301	14,300	490	29	18 X	57
35-50	48	9.5	282	13,500	450	30	19 X	54

SECTION II : ELECTROFOCUSING STUDIES

A. α-TOXIN

1. Initial Observations.

Initial electrofocusing studies centred on whether this technique would separate the α- and θ-toxins of Cl. perfringens type A. Figure 13 shows a typical electrofocusing experiment in a broad pH gradient using crude culture supernatant concentrate from strain S107.

It can be seen from this figure that the α- and θ-toxins were separated by electrofocusing in such gradients, although some overlap of their activities was evident. Of the total θ-toxin recovered, 10% was found in fractions containing α-toxin. The pIs of the α- and θ-toxins were 5.15 and 6.38 respectively in this experiment. In considering this technique as a practical method of purification, the principal problem encountered in these early studies was the low recovery of α-toxin. For instance, only 13.4% of the original α-toxin activity was recovered in the experiment shown in Figure 13 , but, generally, yields were even lower than this. Indeed on some occasions no α-toxin activity was recovered. Some initial data are shown in Table 31.

Despite low yields, attempts were also made at this stage in the electrofocusing studies to determine whether heterogeneity could be found in α-toxin preparations using the higher resolution of narrow pH gradients. With three different preparations of α-toxin in experiments in which yields were of the order of 10% of input activity, several

Figure 13 : Separation of the α- and θ-toxins of Cl.perfringens type A by Electrofocusing in Broad pH gradients.

Electrofocusing of crude ammonium sulphate precipitated toxin prepared from strain S 107 culture supernatant fluid. 20.2 mg of a 70% $(NH_4)_2SO_4$ saturation precipitate were applied to the LKB 8101 column. The pH gradient was from pH 3-10. Electrofocusing was carried out for 56 hours at 4°C with a final potential of 800 V. Fractions of 2 ml were collected.

(•••) pH gradient

(●) E_{280}

(□) α-Toxin (PCU)

(○) θ-Toxin (HU)

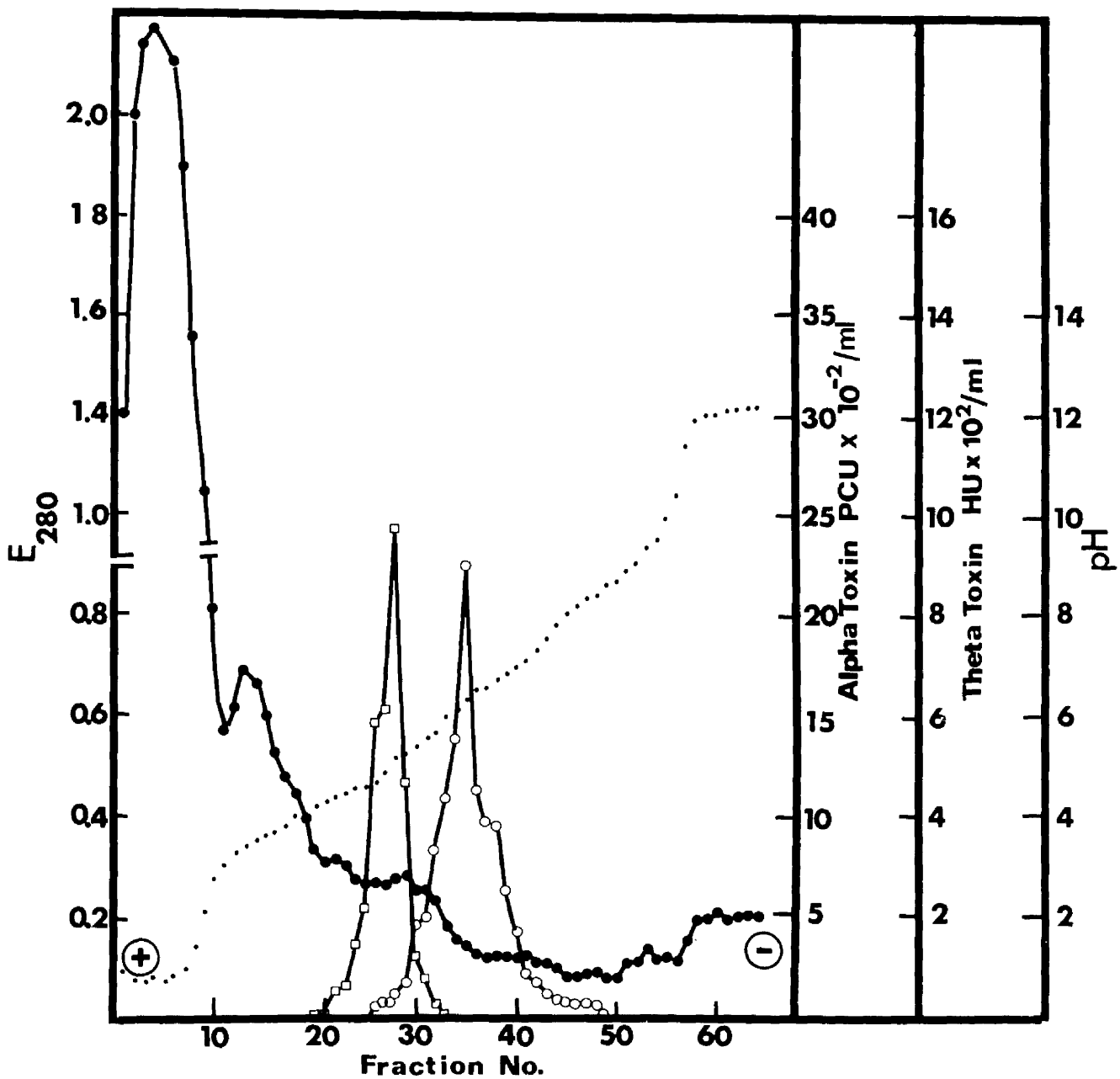

E_{280}
2.0
1.8
1.6
1.4
1.0
0.8
0.6
0.4
0.2
40
35
30
25
20
15
10
5
Alpha Toxin PCU x 10^{-2}/ml
16
14
12
10
8
6
4
2
Theta Toxin HU x 10^{2}/ml
14
12
10
8
6
4
2
pH
+
−
10
20
30
40
50
60
Fraction No.

Table 31 : Recoveries of α-Toxin in Initial Electrofocusing Experiments

Strain	pH Gradient	Activity Applied	Activity Recovered	% Recovery
S107°	5-8	8.3 PCU	0.9 PCU	11
BP6K*	3-10	305 ETU	22 ETU	7
BP6K*	3-10	764 ETU	—	0
S107°	3-10	12.8 PCU	1.7 PCU	13
S107°	3-10	27.8 PCU	—	0
Koch-Light Phospholipase C	3-10	15 PCU	—	0

° Sample prepared by Method b page 114 of 'Materials & Methods'

* Sample prepared by Method a page 113 of 'Materials & Methods'

— No activity detected.

peaks of activity were observed (see Table 32). It was clear that the major peak of activity occurred at a pH between 5.40 and 5.60 in these preparations. The occurrence of other peaks of activity varied from one preparation to another. However, these results did suggest the occurrence of three forms of α-toxin with pIs between 5.00-5.10, 5.20-5.30 and 5.40-5.60.

2. Electrofocusing Studies with Wellcome Toxin (AGX 1846).

During the above preliminary studies, a short note by Bernheimer et al., (1968a) came to my attention. These workers reported the recovery of 64% of α-toxin activity applied to an electrofocusing column. This contrasted with the low yields which I had found. In their study Bernheimer and his coworkers used a lyophilised culture filtrate supplied by R.O. Thomson of Wellcome Research Laboratories. This material gave two peaks of α-toxin activity having pIs of 5.2 and 5.5. Both components possessed egg-yolk turbidity activity and hot-cold haemolytic activity.

A similar sample of freeze-dried culture filtrate, preparation AGX 1846, was supplied to me by Wellcome Research Laboratories, Beckenham, Kent. Because of the limited amount of material obtained, only a small number of experiments could be carried out. Apart from one refocusing experiment described below, all findings were verified by duplicate experiments.

This toxin preparation was focused under conditions similar to those described by Bernheimer et al., (1968a) except that (a) the toxin was applied to the column in the heavy solution, (b) focusing was

Table 32 : Detection of Heterogeneity in α-Toxin Preparations

Experiment	Strain	Nature of Material Electrofocused	Weight of Material Applied (mg)	pH Gradient	pIs of Peaks of Activity	Assay Used
A	S107	70% $(NH_4)_2SO_4$ saturation precipitate of M-I culture supernatant fluid	12.1	5-8	5.30 5.42 5.52	Phospholipase C Activity
B	S107	As above further purified by (a) Chromatography on Sephadex G-100 (b) 70% Methanol saturation precipitation	ND	5-8	5.06 5.29 5.42 5.60	Phospholipase C Activity
C	BP6K	Calcium phosphate adsorption in the presence of acetone from M-II culture supernatant fluid. Material further purified by Sephadex G-100 chromatography	212	5-8	5.00 5.28 5.42	Hot-cold Haemolysis Phospholipase C Activity

ND = Not determined

carried out for 46 hours, (c) the final potential was 800V, and (d) fractions of 2 ml rather than 4 ml were collected on draining the electrofocusing column. The elution profile is shown in Figure 14. Two peaks of α-toxin activity were detected having pIs of 5.26 and 5.55. The recovery of activity, however, was only 31% in contrast to the reported finding of Bernheimer et al.

A further experiment was carried out to determine whether these two forms of α-toxin were reproducible and whether they possessed the three activities accorded to α-toxin by van Heyningen (see 'Unitarian Hypothesis', page 72). Focusing was carried out under the same conditions described above except for the amount of material applied to the column and the duration of the experiment. Fractions were assayed for both lecithinase and hot-cold haemolytic activity. Only those fractions containing these activities were tested for lethal activity in mice for reasons outlined on page 121 . The results of these tests are shown in Figure 15 . For clarity, only those fractions in which α-toxin activity was detected are shown. The relationship between the titres of the three activities is shown in Table 33 and the total activity recovered as detected by each assay in Table 34.

These findings demonstrate the following points :-

(a) that two peaks of α-toxin activity were found on electrofocusing of Wellcome toxin AGX 1846 by the use of each of the three assays employed

(b) that the peaks of activity by each assay were co-incident - fractions 13 and 17 of Figure 15 with pIs of 5.20 and 5.59 respectively

Figure 14 : Identification of Two Forms of α-Toxin in Wellcome type A Toxin AGX 1846 by Electrofocusing in Narrow pH Gradients

15 mg of lyophilised toxin was dissolved in 1% glycine and dialysed overnight against 1% glycine. The pH gradient was from pH 5-8. Electrofocusing was carried out for 46 hours at 4°C with a final potential of 800 V. 2 ml fractions were collected.

(•••) pH gradient

(⊙) E_{280}

(□) α-Toxin (ETU)

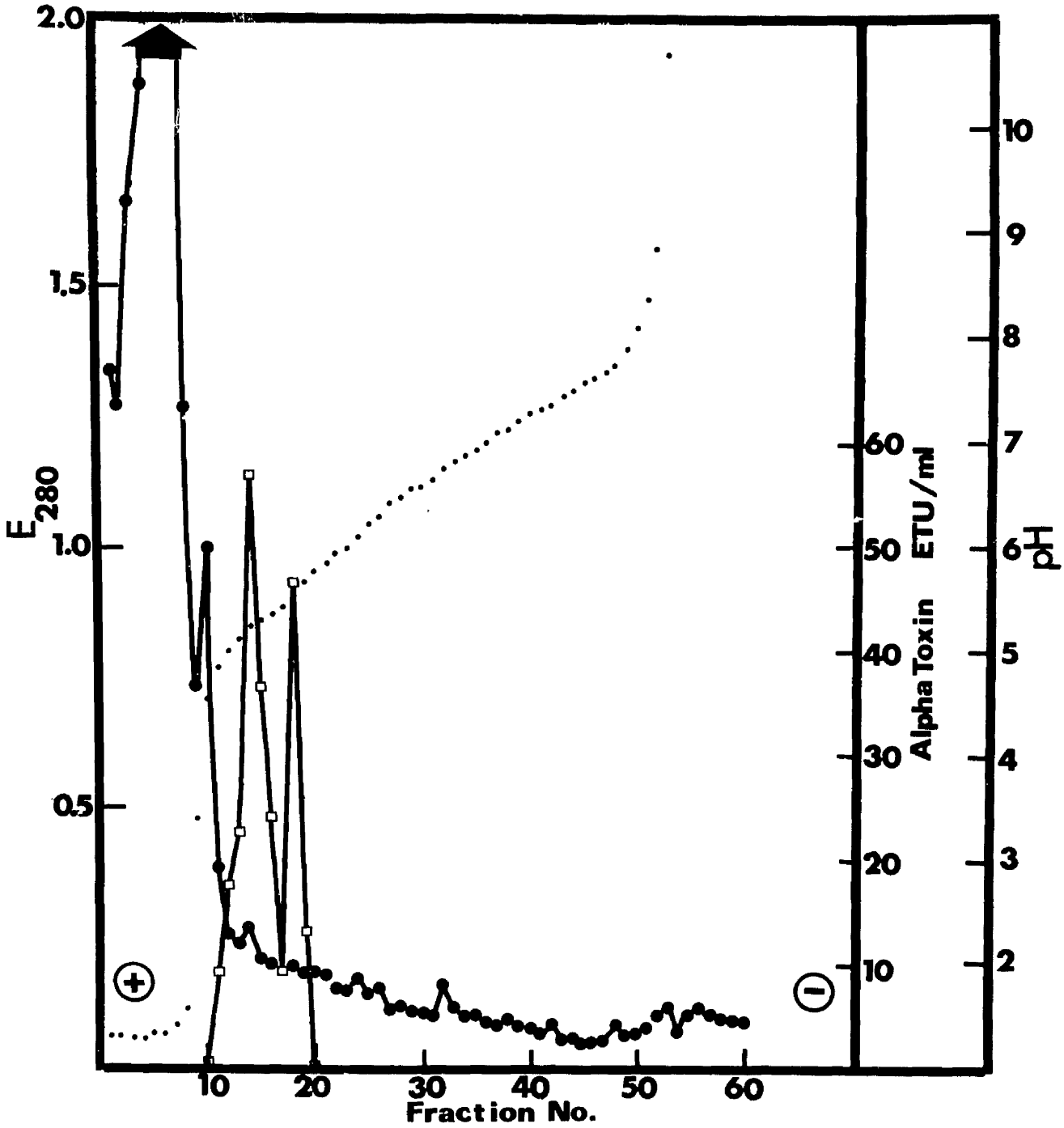

2.0
1.5
1.0
0.5
E_{280}
10
20
30
40
50
60
Fraction No.
Alpha Toxin ETU/ml
pH
+
−

Figure 15 : Elution Profile of the Egg-Yolk Turbidity, Hot-Cold Haemolytic and Lethal Activities of α-Toxin on Electrofocusing of Wellcome type A Toxin AGX 1846 in Narrow pH Gradients.

10 mg of AGX 1846 were dissolved in 1% glycine and dialysed against 1% glycine for 24 hours. The resulting 5 ml of dialysed toxin were applied in the dense solution to the LKB 8101 column. Electrofocusing was carried out for 40 hours at 4°C with a final potential of 800 V. Fractions of 2 ml volume were collected.

(•••) pH gradient

(□) α-Toxin Activity (ETU; HU; LU)

(●) E_{280}

The three parts of this figure represent different measures of α-toxin activity from one experiment viz. from top to bottom: egg-yolk turbidity activity, hot-cold haemolytic activity and lethal activity.

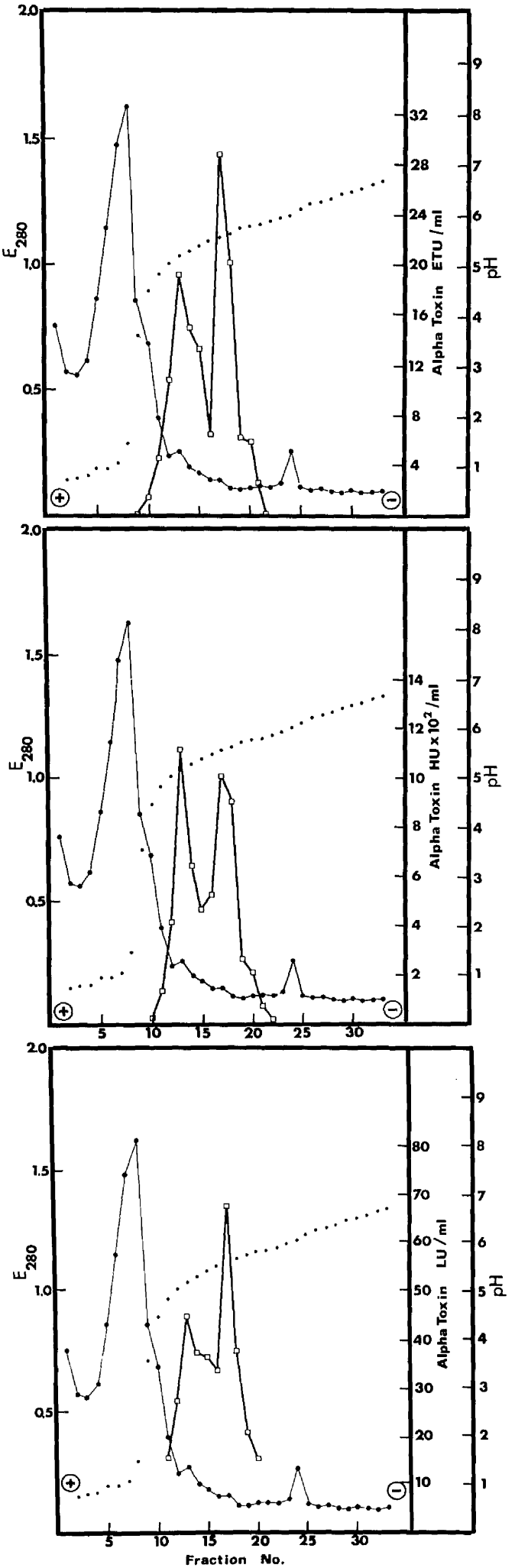

E280
Alpha Toxin ETU/ml
pH
Alpha Toxin HU x 10² /ml
Alpha Toxin LU/ml
Fraction No.

Table 33 : Isoelectric Focusing of AGX 1846 Cl. perfringens type A Toxin

Relationship between Titres of Various Activities of α-Toxin

Fraction No.	ETU/ml	HU/ml	LU/ml	Ratio of ETU/HU/LU
10	1.3	21	ND	1 / 12 / -
11	4.5	132	15.0	1 / 29 / 3.3
12	10.9	412	27.2	1 / 38 / 2.5
13**	19.2	1115	44.5	1 / 58 / 2.3
14	14.9	668	37.2	1 / 45 / 2.5
15	14.6	472	36.4	1 / 33 / 2.5
16	6.5	526	33.3	1 / 81 / 5.1
17**	28.8	1004	67.3	1 / 35 / 2.3
18	20.2	900	37.5	1 / 45 / 1.9
19	6.2	264	20.6	1 / 43 / 3.3
20	6.0	207	15.0	1 / 35 / 2.5
21	2.5	68	ND	1 / 27 / -

** Peaks of activity

ND - Not determined

Isoelectric Focusing of AGX 1846 Cl. perfringens type A Toxin

Table 34 : Recovery of α-Toxin from Experiment shown in Figure 15

	ETUs	HUs	LUs	Ratio of ETU/HU/LU
Total Recovered Activity [o]	270	11,600	670	1 / 43 / 2.5
Total Activity Applied	1230	37,200	2420	1 / 40 / 2.0
Recovery of Activity %	22	31	28	

Table 35 : Recovery of α-Toxin from Experiment shown in Figure 16

Urea Column

	ETUs	HUs	LUs	Ratio of ETU/HU/LU
Total Recovered Activity [o]	560	67,530	2050	1 / 120 / 3.7
Total Activity Applied	1570	125,300	3150	1 / 80 / 2.0
Recovery of Activity %	36	54	65	

[o] Fractions of 2 ml volume

(c) that recoveries of the three activities were of the same order (Table 34)

(d) that the ratios of the three activities in each fraction were of the same order with the possible exception of fraction 16

(e) and that these ratios compared well with those found for the three activities in the material applied to the column indicating simultaneous purification of each activity.

These observations support the unitarian view that lecithinase activity, hot-cold haemolytic activity and lethal activity are manifestations of the action of α-toxin. The average pIs of the above two forms of α-toxin were found to be 5.57 ± 0.04 and 5.23 ± 0.09. For convenience these forms shall be designated α_A and α_B respectively.

The presence of these two forms of α-toxin raised the question of the relationship between α_A and α_B. It seemed possible that they could be related as conformers (molecules with the same amino acid sequence, but different three dimensional structures and/or charge) or as polymers (molecules or aggregates formed from a different number of sub-units). Urea is known to cause disaggregation of proteins to sub-units and unfolding of the three dimensional structure of proteins by rupture of hydrogen bonding. It is also electrophoretically neutral which allows its incorporation into systems undergoing electrophoresis. Furthermore, Ispolatovskaya and Klimacheva (1966) have shown that α-toxin activity is not inactivated by urea.

AGX 1346 toxin was thus focused in the same pH gradient used to detect α_A and α_B, but in the presence of 6M urea to determine whether

any change occurred in the isoelectric point of one or both of these forms of α-toxin. Again, for clarity, only those fractions in the region of the pH gradient in which α-toxin activity was detected are shown in Figure 16. In contrast to the picture obtained in the absence of urea (Figure 15), only one peak of α-toxin activity was detected by assays for the egg yolk turbidity, hot-cold haemolytic and lethal activities of this toxin. Table 36 presents the titres of each activity per fraction and the ratio of these activities to egg yolk turbidity activity.

It can be seen that the peak of activity for each assay occurred in fraction 17 which had a pI of 5.56. Furthermore with the exception of fractions 14 and 19, comparable ratios were obtained for each of these activities in each fraction. However, Table 35 shows that in contrast to the experiments carried out in the absence of urea (see Table 34), the % recovery of egg yolk turbidity was considerably lower than the recoveries of the hot-cold haemolytic and lethal activities of α-toxin.

The main conclusion of these experiments was that this form of α-toxin, α_{Urea}, possessed all three activities associated with α-toxin and corresponded to the form α_A found by focusing experiments in the absence of urea.

It was decided to test whether the α_B component reappeared on removal of the urea. Fractions 13-18 inclusive from the experiment shown in Figure 17A were pooled, dialysed exhaustively against several changes of 1% glycine for 48 hours at 4°C to remove the urea, and

Figure 16 : Elution Profile of the Egg-Yolk Turbidity, Hot-Cold Haemolytic and Lethal Activities of α-Toxin on Electrofocusing of Wellcome type A Toxin AGX 1846 in Narrow pH Gradients in the presence of 6M Urea.

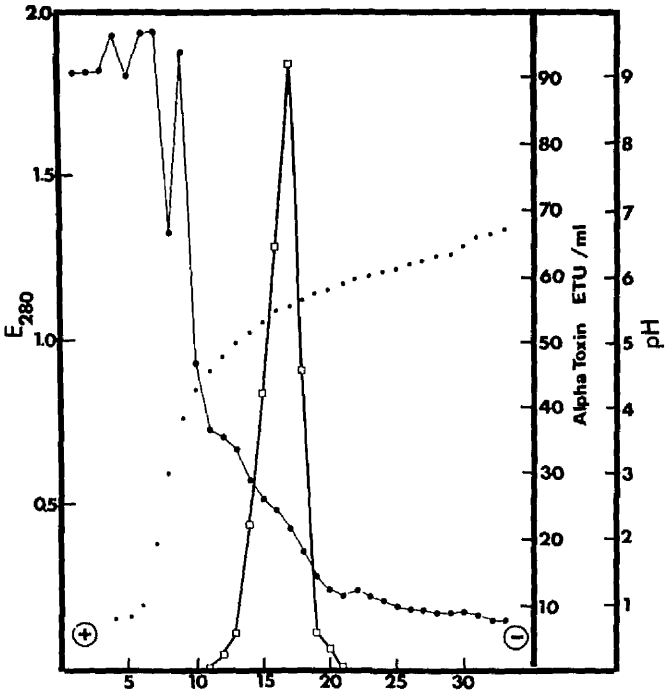
20 mg of AGX 1846 were dissolved in 6M urea and dialysed against 1% glycine containing 6M urea for 24 hours. The resulting 5 ml of toxin were applied to the column as 2 ml in the heavy solution and 3 ml in the light solution. Electrofocusing was carried out for 46 hours at 4°C with a final potential of 800 V. 2 ml Fractions were collected.

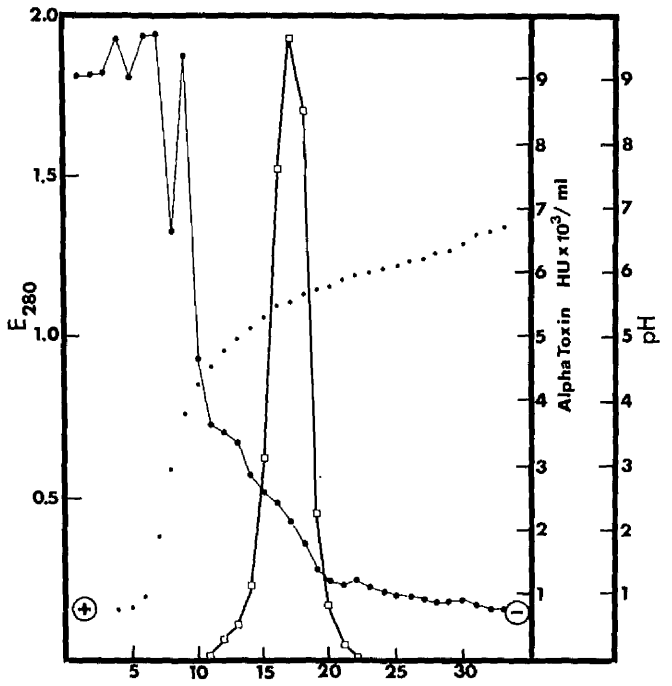
(•••) pH gradient

(□) α-Toxin Activity (ETU; HU; LU)

(●) E_{280}

The three parts of this figure represent different measures of α-toxin activity from one experiment viz. from top to bottom: egg-yolk turbidity activity, hot-cold haemolytic activity and lethal activity.


2.0
1.5
1.0
0.5
E_{280}
90
80
70
60
50
40
30
20
10
Alpha Toxin ETU /ml
9
8
7
6
5
4
3
2
1
pH
⊕
⊖
5
10
15
20
25
30


2.0
1.5
1.0
0.5
E_{280}
9
8
7
6
5
4
3
2
1
Alpha Toxin HU x 10^3/ ml
pH
⊕
⊖
5
10
15
20
25
30

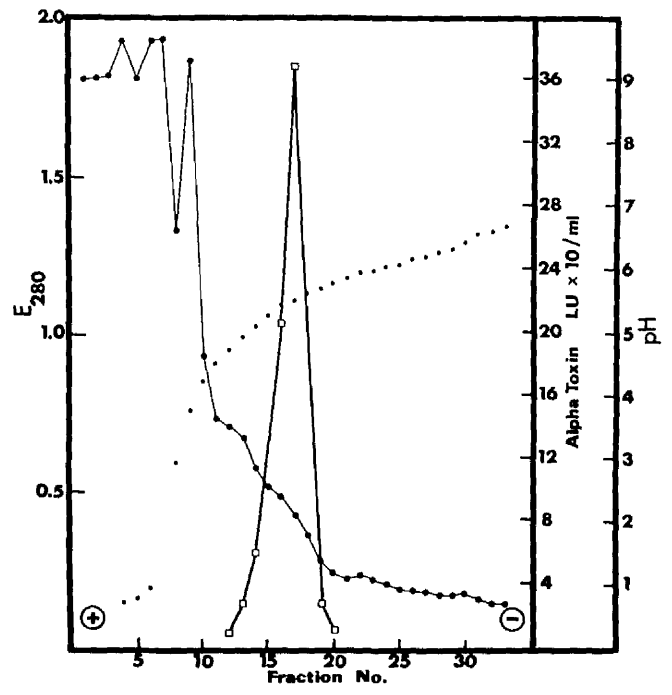

2.0
1.5
1.0
0.5
E_{280}
36
32
28
24
20
16
12
8
4
Alpha Toxin LU x 10/ ml
9
8
7
6
5
4
3
2
1
pH
⊕
⊖
5
10
15
20
25
30
Fraction No.

Table 36 : Isoelectric Focusing of AGX 1846 Cl. perfringens type A Toxin in Urea

Relationship Between Titres of Various Activities of α-Toxin

Fraction No.	ETU/ml	HU/ml[oo]	LU/ml	Ratio of ETU/HU/LU
12	ND	160	ND	
13	5.3	610	27	1 / 115 / 5.1
14	23.0	1120	59	1 / 49 / 2.6
15	42.1	3110	105	1 / 74 / 2.5
16	64.5	7620	215	1 / 118 / 3.3
17**	91.2	9600	368	1 / 105 / 4.0
18	45.5	8480	223	1 / 186 / 4.9
19	5.5	2260	27	1 / 411 / 4.9
20	3.0	810	ND	1 / 269 / -

** Peak of Activity

ND Not determined

oo Hot-Cold Haemolytic Titrations carried out with a different batch of sheep erythrocytes from that used in the experiment described in Table 33 and Figure 15.

refocused in the same narrow pH gradient. Figure 17B illustrates the elution profile of α-toxin as assayed by hot-cold haemolysis. A major peak corresponding to α_A was detected having a pI of 5.48. A minor peak with a pI 5.23 appeared corresponding to that observed for α_B. Thus the component α_B undergoes a reversible change in the presence of urea.

Unfortunately, further studies on this interesting observation with the α_B form of α-toxin derived from AGX 1846 toxin were not possible as I was unable to obtain more of the AGX 1846 preparation from the Wellcome Research Laboratories.

3. Preparative Scale Electrofocusing Experiments.

The results obtained with AGX 1846 toxin were more encouraging in terms of the α-toxin activity recovered than those mentioned earlier. It was decided to reinvestigate the possibility of α-toxin purification on a preparative scale.

In an initial preparative experiment toxin was electrofocused in the 110 ml column using a pH 4 - 6 gradient, as experiments in broad pH gradients had indicated that such a gradient would provide maximum separation of α-toxin both from precipitates forming at the anode and other extracellular factors (see 'Purity of electrofocused α-toxin', page 194 , for fuller explanation of this point). After assay of fractions for hot-cold haemolysis, the elution profile shown in Figure 18 was obtained. Most of the recovered activity resided in a peak having a pI 5.50 corresponding to the pI of the α_A form determined in AGX 1846 toxin. A pronounced shoulder on the acid side

Figure 17 : Refocusing of α_{urea} in the Absence of Urea

A. Electrofocusing of AGX 1846 in 6M Urea:

20 mg of AGX 1846 dissolved in 6M Urea and dialysed overnight against 1% glycine in 6M Urea. Of the resulting 8 ml, 5 ml were applied in the dense solution and 3 ml in the light. Focusing was carried out for 46 hours at 4°C with a final potential of 800 V. Fractions of 2 ml volume were collected.

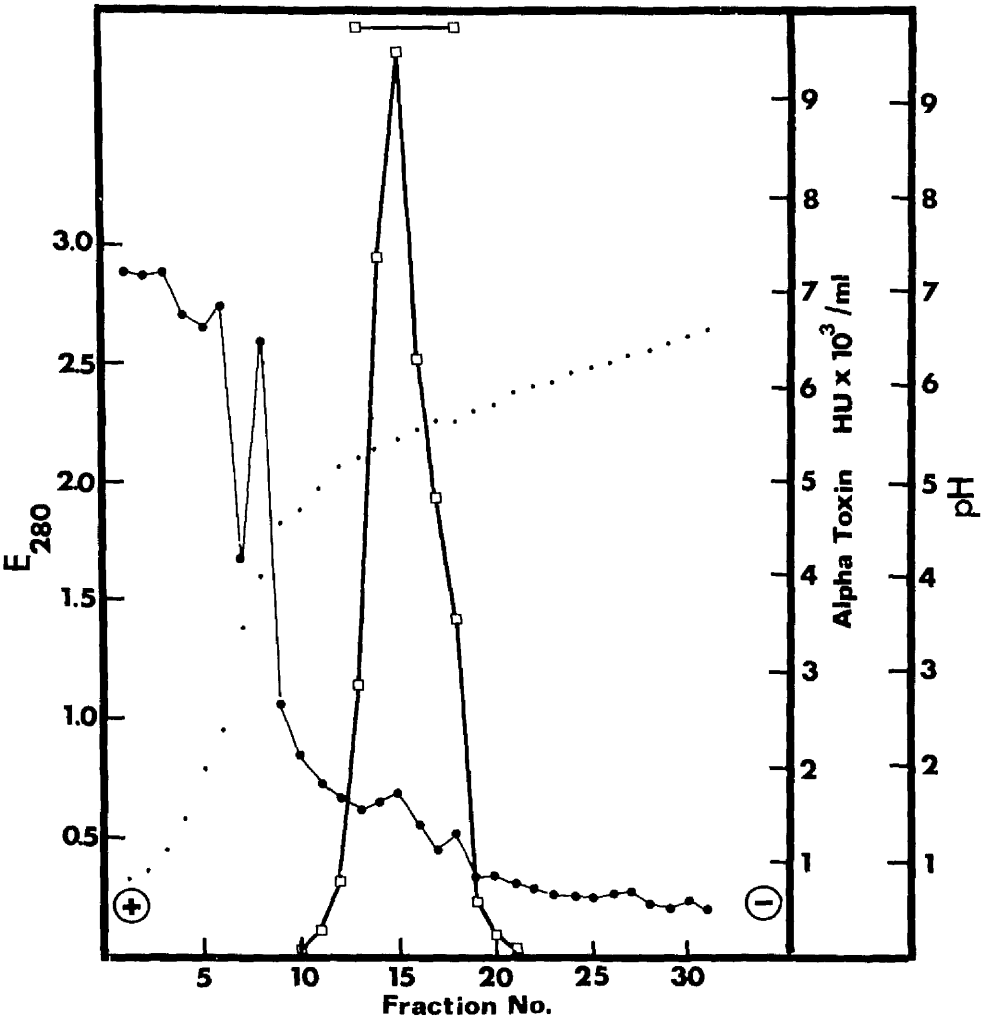
B. Refocusing of peak fractions of α-toxin activity in the absence of urea:

Fractions 13-18 inclusive from the experiment above (□ ----- □) were pooled and dialysed free of urea. Of the resulting 15 ml of toxin, 10 ml were applied in the dense solution and 5 ml in the light solution to the LKB 8101 column. Focusing was carried out for 49 hours at 4°C with a final potential of 800 V. Fractions of 2 ml were collected.

(•••) pH Gradient

(□) α-Toxin Activity (HU)

(⊙) E_{280}


E_{280}
3.0
2.5
2.0
1.5
1.0
0.5
Alpha Toxin HU x 10^3/ml
pH
9
8
7
6
5
4
3
2
1
⊕
⊖
5
10
15
20
25
30
Fraction No.

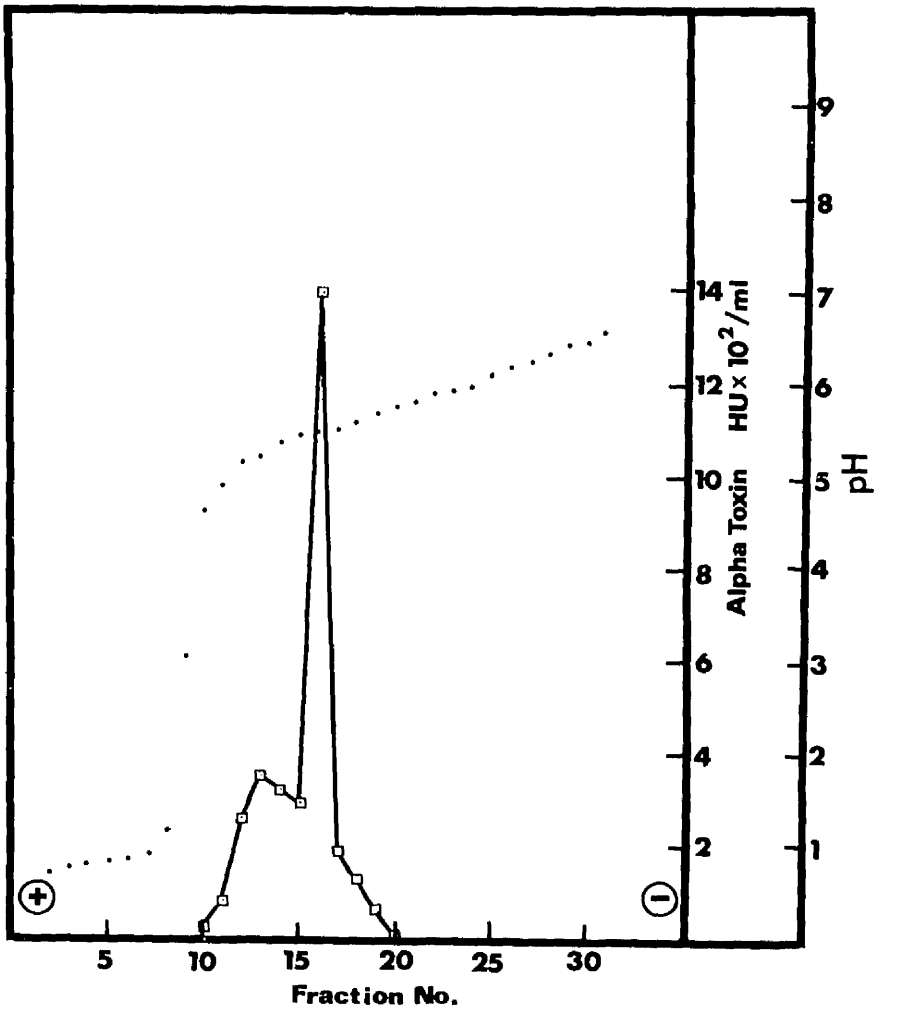

Alpha Toxin HU x 10^2/ml
pH
14
12
10
8
6
4
2
9
8
7
6
5
4
3
2
1
⊕
⊖
5
10
15
20
25
30
Fraction No.

Figure 18 : Electrofocusing of α-Toxin from Strain BP6K in Narrow pH Gradients

A 35 - 50% fractional $(NH_4)_2SO_4$ saturation precipitate was prepared from culture supernatant fluids of strain BP6K grown in M II medium.
652 mg of crude toxin were applied to the LKB 8101 column as glycine dialysed material in ampholines giving a narrow pH gradient from 4 - 6. Electrofocusing was carried out at 4°C for 70 hours with a final potential of 800 V. Fractions of 2 ml were collected.

Figure 19 : Refocusing of the α-Toxin of Strain BP6K

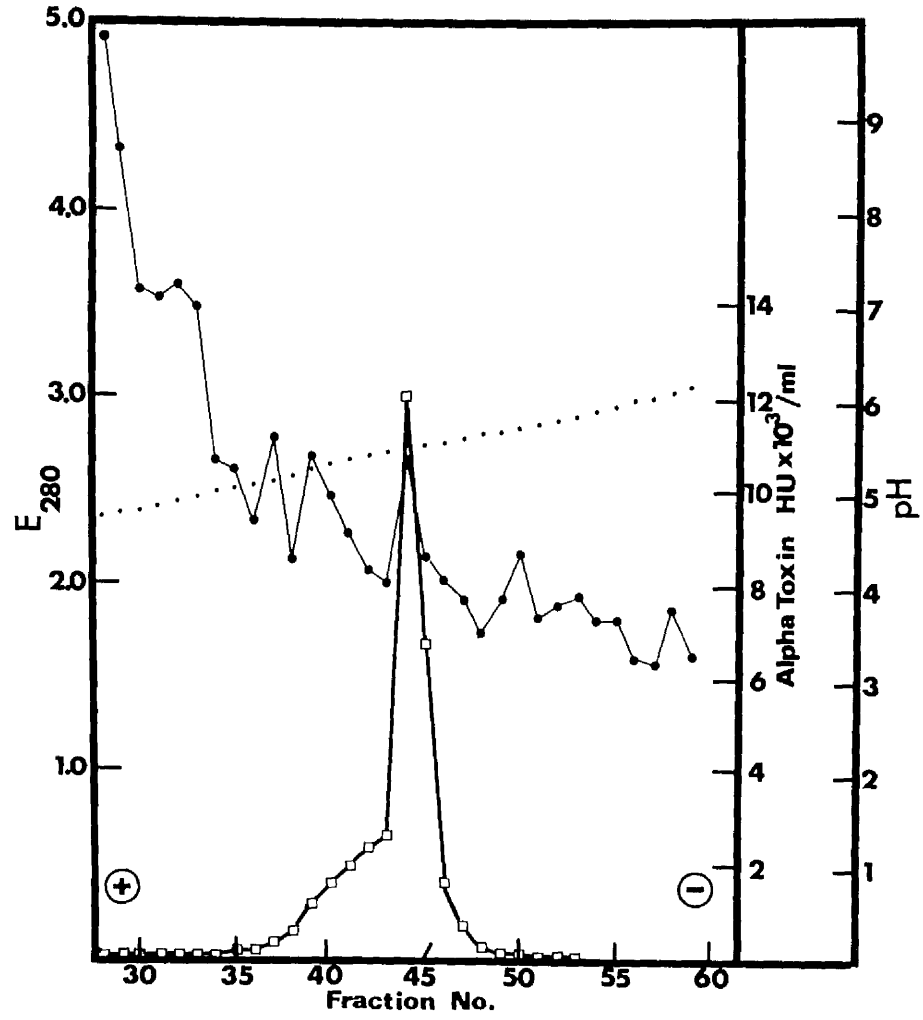
Fractions 38 - 47 inclusive from Figure 18 were pooled, concentrated and dialysed against 1% glycine. 15 ml of the glycine dialysed material were applied, 10 ml in heavy solution, 5 ml in the light. The pH gradient was from 5 - 8. Electrofocusing was carried out for 49 hours at 4°C with a final potential of 800 V.
2 ml fractions were collected.

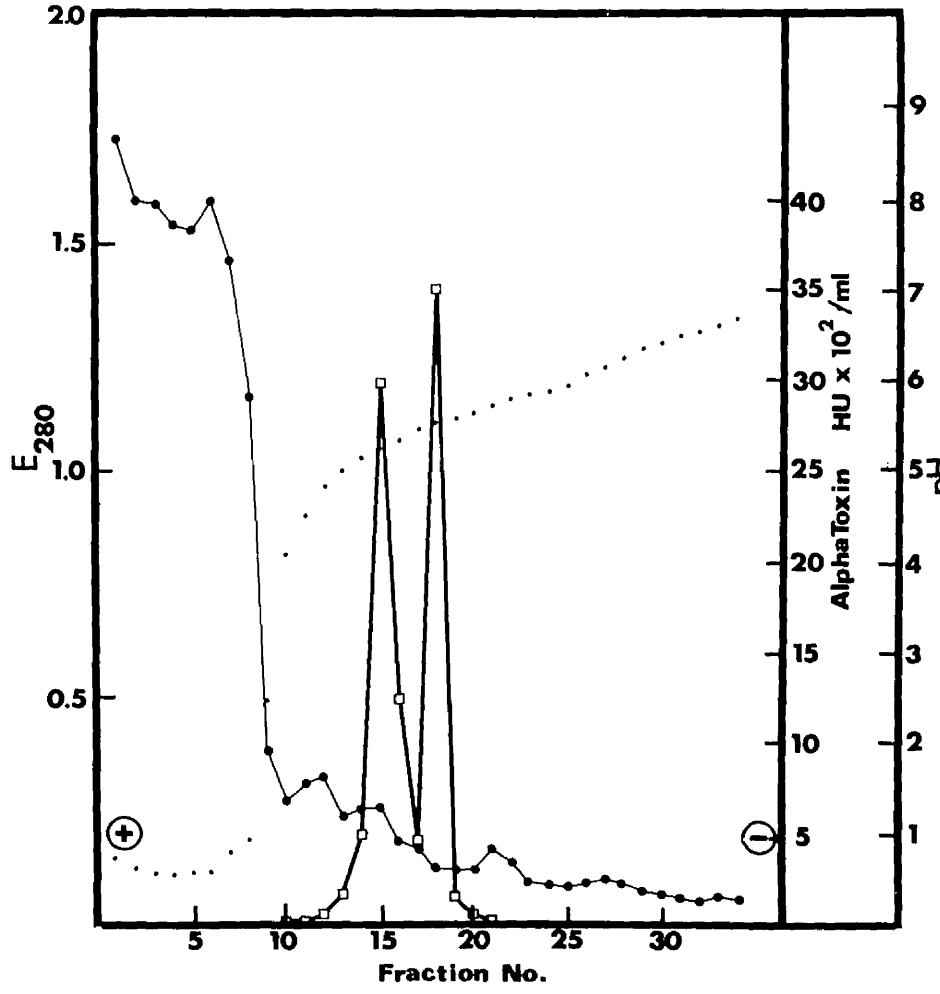
Symbols for both figures:

(•••) pH gradient

(□) α-Toxin Activity (HU)

(⊙) E_{280}


5.0
4.0
3.0
2.0
1.0
E_{280}
14
12
10
8
6
4
2
Alpha Toxin HU x 10^3/ml
9
8
7
6
5
4
3
2
1
pH
⊕
⊖
30
35
40
45
50
55
60
Fraction No.


2.0
1.5
1.0
0.5
E_{280}
40
35
30
25
20
15
10
5
AlphaToxin HU x 10^2/ml
9
8
7
6
5
4
3
2
1
pH
⊕
⊖
5
10
15
20
25
30
Fraction No.

of this peak from pH 5.11 to 5.42 should be noted. Fractions 38 to 47 from Figure 18 were pooled, concentrated by precipitation with 70% $(NH_4)_2SO_4$ saturation, dialysed against 1% glycine and refocused in a pH 5 - 8 gradient. The elution profile of α-toxin in this experiment is shown in Figure 19 . Again two peaks of hot-cold haemolytic activity were found with pIs of 5.25 and 5.49 in fractions 15 and 18 respectively. These correspond with the pIs found for α_B and α_A in AGX 1846. In contrast to previous experience, however, yields of α-toxin from both of these columns were high, 80% and 84% respectively.

Toxin concentrates prepared by fractional ammonium sulphate precipitation (see pages 115 and 175) have since been focused on a preparative scale in the 440 ml column. Up to 3.87 g of such preparations have been applied with a recovery of 60% of activity to one column utilising both the heavy and light solutions for the application of a large volume of sample - 312 ml. During this type of electrofocusing experiment, a large amount of precipitation occurred at the anode. Nevertheless, such precipitates neither interfered with drainage of the columns nor caused trailing of this acidic material. When such precipitates were harvested from the anodic region of electrofocusing columns and analysed for protein, DNA and RNA, only protein was detected in such material. The nature of these acidic proteins has not been investigated.

Thus these electrofocusing experiments have revealed heterogeneity in the α-toxin of Cl. perfringens type A. The existence of a major electrophoretic component α_A having a pI 5.49 $\pm$ 0.06, the average of

10 determinations in narrow pH gradients, together with a component α_B with a pI 5.25 $\pm$ 0.03, the average of eight determinations, has been established.

4. Purity of Electrofocused α-Toxin.

a. Separation of α-Toxin from Other Toxins and Enzymes Produced by Clostridium perfringens type A by Electrofocusing:

In an assessment of the purity of α-toxin prepared by isoelectric focusing it was decided to investigate the distribution of other extracellular products in this area of the pH gradient. Such investigations, in addition to providing information about the purity of α-toxin allowed the determination of the isoelectric points of these factors, and also an assessment of the usefulness of this technique in the preparation of each.

i. θ-Toxin, hyaluronidase and collagenase: The distribution of α-toxin, θ-toxin, hyaluronidase and collagenase in a broad pH gradient is illustrated by the experiment shown in Figure 20. It should be noted that

(a) all four major components of the toxin of Cl. perfringens type A are acidic proteins focusing in a narrow region of the pH gradient from pH 4.5 to 6.5

(b) that separation of these toxins is incomplete by focusing in broad pH gradients and

(c) that most of the protein in such crude preparations of toxin focuses at the anode.

The pI and recovery of each component is summarised in Table 37. On the basis of such observations with toxin from strains BP6K and S107

Figure 20 : Separation of α-Toxin, θ-Toxin, Collagenase and Hyaluronidase on Electrofocusing in Broad pH Gradients

Isoelectric focusing of toxin prepared from culture supernatant fluids of strain BP6K grown in M II medium by method a (see page 113).
The pH gradient was from 3 - 10. 47 mg of toxin were applied in the less dense solution. Focusing was carried out for 67 hours at 4°C in the LKB 8101 column, with a final potential of 800 V. Fractions of 2 ml volume were collected.

(•••) pH gradient
(●) E_{280}
(□) α-Toxin Activity (ETU)
(△) Collagenase Activity (AU)
(▣) Hyaluronidase Activity (IU)
(○) θ-Toxin Activity (HU)

The two parts of this figure illustrate the elution profiles of these factors from one experiment viz. top part: collagenase and α-toxin; bottom part: hyaluronidase and θ-toxin.

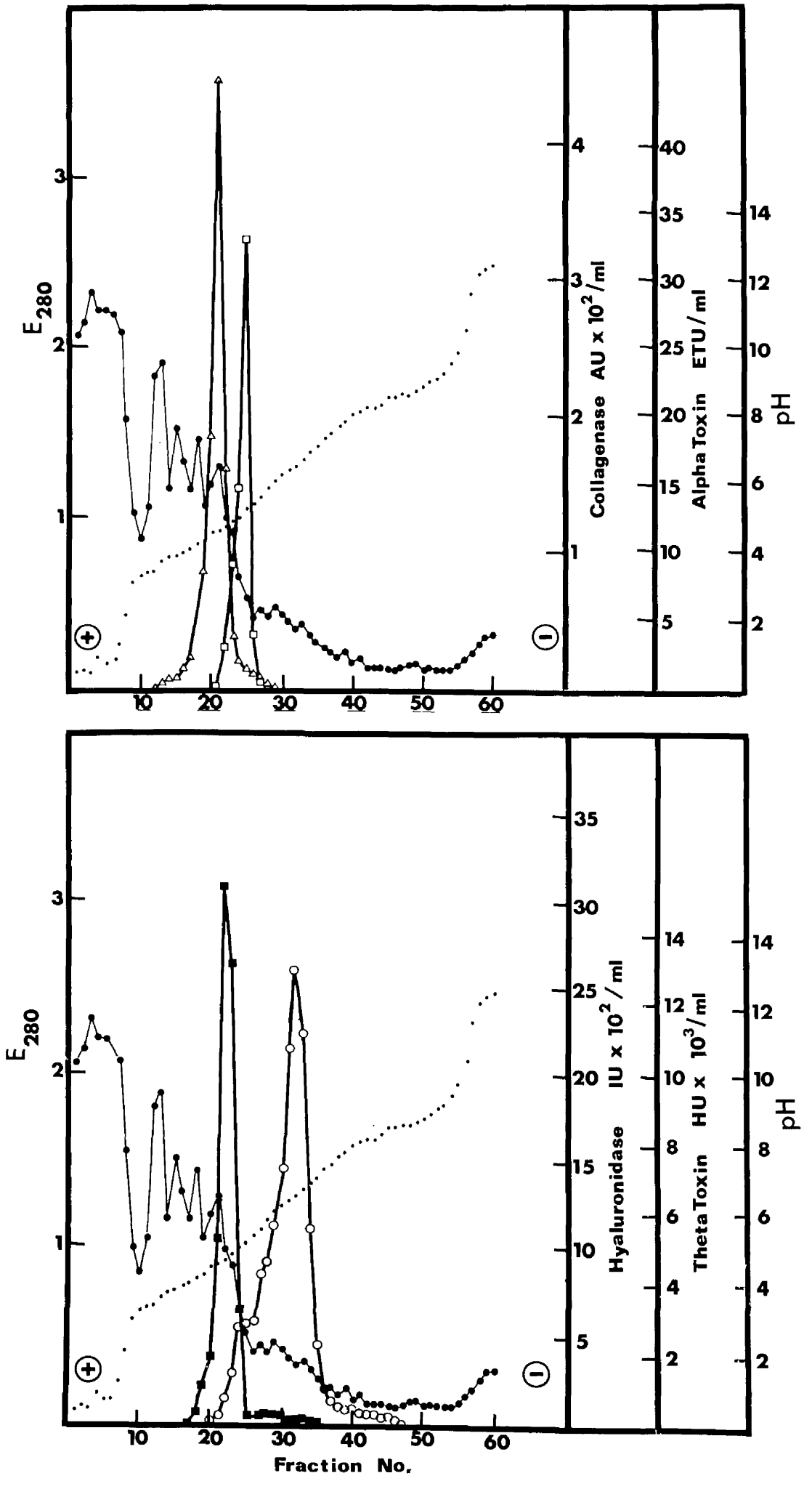

E280
Collagenase AU x 10²/ml
Alpha Toxin ETU/ml
pH
Hyaluronidase IU x 10²/ml
Theta Toxin HU x 10³/ml
Fraction No.

Table 37 : Electrofocusing of Cl. perfringens BP6K Toxin in Broad pH Gradient of 3-10. - pI and Recovery of Toxins.

Component	pI	Activity Applied	Activity Recovered	Recovery %
α-Toxin	5.20	380 ETU	120 ETU	32
θ-Toxin	6.54	220, 200 HU	146, 500 HU	66
Collagenase	4.61	5400 AU	2200 AU	40
Hyaluronidase	4.72	22300 IU	16200 IU	73

and with Wellcome toxin AGX 1846 a pH gradient of 4 - 6 was chosen as that likely to achieve optimal separation of these components on a preparative scale.

The suitability of such a gradient has been justified by preparative scale electrofocusing experiments. Figure 21 demonstrates the elution profile of these components in such an experiment using toxin prepared from strain BP6K. The high degree of resolution of each component should be noted. More detailed studies of hyaluronidase, collagenase and θ-toxin are dealt with under individual sections (vide infra).

ii. Deoxyribonuclease: Attempts at assaying culture supernatants or partially purified toxin for deoxyribonuclease activity by the methods of Kunitz (1950), Alexander et al., (1961) and Wadstrom (1967) proved unsuccessful. The ACRA method of assay or assay by rabbit leucocyte nuclei disintegration were not attempted. Thus the distribution of this enzyme in electrofocusing experiments was not determined, although both strains BP6K and S107 are known to produce this factor (Masui et al., 1956).

iii. Neuraminidase: Unlike the other factors mentioned above, neuraminidase focused in the α-toxin region of the pH gradient and for this reason a summary of the contamination of α-toxin with neuraminidase must be considered in this section.

In view of the possible effect of neuraminidase on the erythrocyte surface (see 'Neuraminidase', page 102 and 'Neuraminidase Assay', page 136) it was considered necessary to determine the location of this enzyme in pH gradients on electrofocusing. Culture supernatants

Figure 21 : Separation of α-Toxin, θ-Toxin, Collagenase and Hyaluronidase on Electrofocusing in Narrow pH Gradients

Isoelectric focusing of 35-50% fractional $(NH_4)_2SO_4$ saturation precipitate prepared from culture supernatant fluids of strain BP6K grown in M II medium.

650 mg of crude toxin were applied to the LKB 8101 column as 54 ml of glycine dialysed material. The less dense solution contained 26 ml and the heavy solution, 28 ml. Ampholines giving a narrow pH gradient from 4 - 6 were used. Electrofocusing was carried out for 70 hours at 4°C with a final potential of 800 V. Fractions of 2 ml volume were collected.

(•••) pH gradient

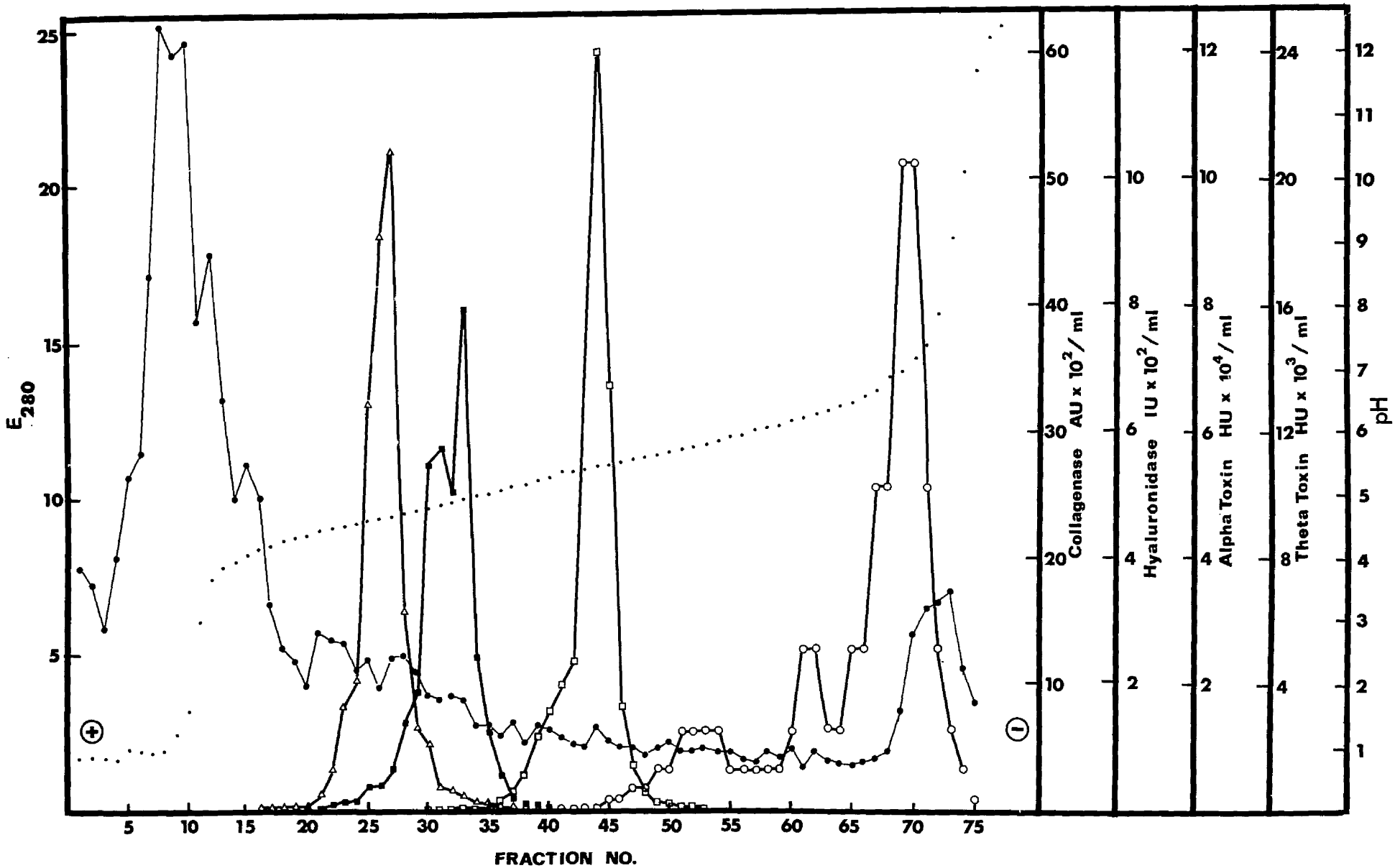
(⊙) E_{280}

(□) α-Toxin Activity (HU)

(△) Collagenase Activity (AU)

(▣) Hyaluronidase Activity (IU)

(○) θ-Toxin Activity (HU)


E280
25
20
15
10
5
⊕
⊖
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
FRACTION NO.
60 50 40 30 20 10
Collagenase AU x 10² / ml
10 8 6 4 2
Hyaluronidase IU x 10² / ml
12 10 8 6 4 2
Alpha Toxin HU x 10⁴ / ml
24 20 16 12 8 4
Theta Toxin HU x 10³ / ml
12 11 10 9 8 7 6 5 4 3 2 1
pH

of both strains S107 and BP6K contained this enzyme as did concentrates obtained by ammonium sulphate precipitation. This is shown in Table 38 ; although the amount of neuraminidase applied in the starting material to a focusing column may not be very large, it must be borne in mind that the very nature of the isoelectric focusing technique will concentrate this activity into a narrow zone of the pH gradient.

Initial attempts at assaying fractions from preparative focusing columns for neuraminidase proved fruitless. Instead of obtaining the characteristic N-acetyl-neuraminic acid chromogen with Warren's assay procedure, bright orange chromogens were produced which sometimes precipitated out of solution. Absorption spectra of such chromogens revealed absorbance peaks at 450 nm and 524 nm masking the production of the NANA chromogen at 549 nm (see Figure 22). It was subsequently shown that these chromogens were caused by periodate cleavage of the sucrose forming the density gradient in columns and the subsequent reaction of these products with thiobarbituric acid. Thus all fractions from electrofocusing columns had to be dialysed free of sucrose prior to assay. Personal communication with Dr. J.G. Collee, Bacteriology Department, University Medical School, University of Edinburgh, led to the use of a more sensitive assay system that he and his colleagues have developed. Again, however, sucrose interfered with the chemical determination of NANA by Aminoff's method. Indeed, concentrations of sucrose as low as 0.05% caused appreciable chromogenicity at 450 nm swamping the reaction of thiobarbituric acid with NANA. Thus although this assay for neuraminidase was more sensitive in detecting NANA it did not obviate the necessity to dialyse all fractions free of sucrose.

Table 38 : Detection by the Method of Collee (1965) of Neuraminidase Activity produced by Strains BP6K and S 107

Strain	Material Assayed	Milliunits of Neuraminidase/ml
BP6K	MII Culture Supernatant Fluid	35
BP6K	35-50% Saturation Fractional $(NH_4)_2SO_4$ Precipitate	113
S107	M-I Culture Supernatant Fluid	40
S107	70% Saturation $(NH_4)_2SO_4$ Precipitate	56

Table 39 : Assay of Fractions for Neuraminidase Activity

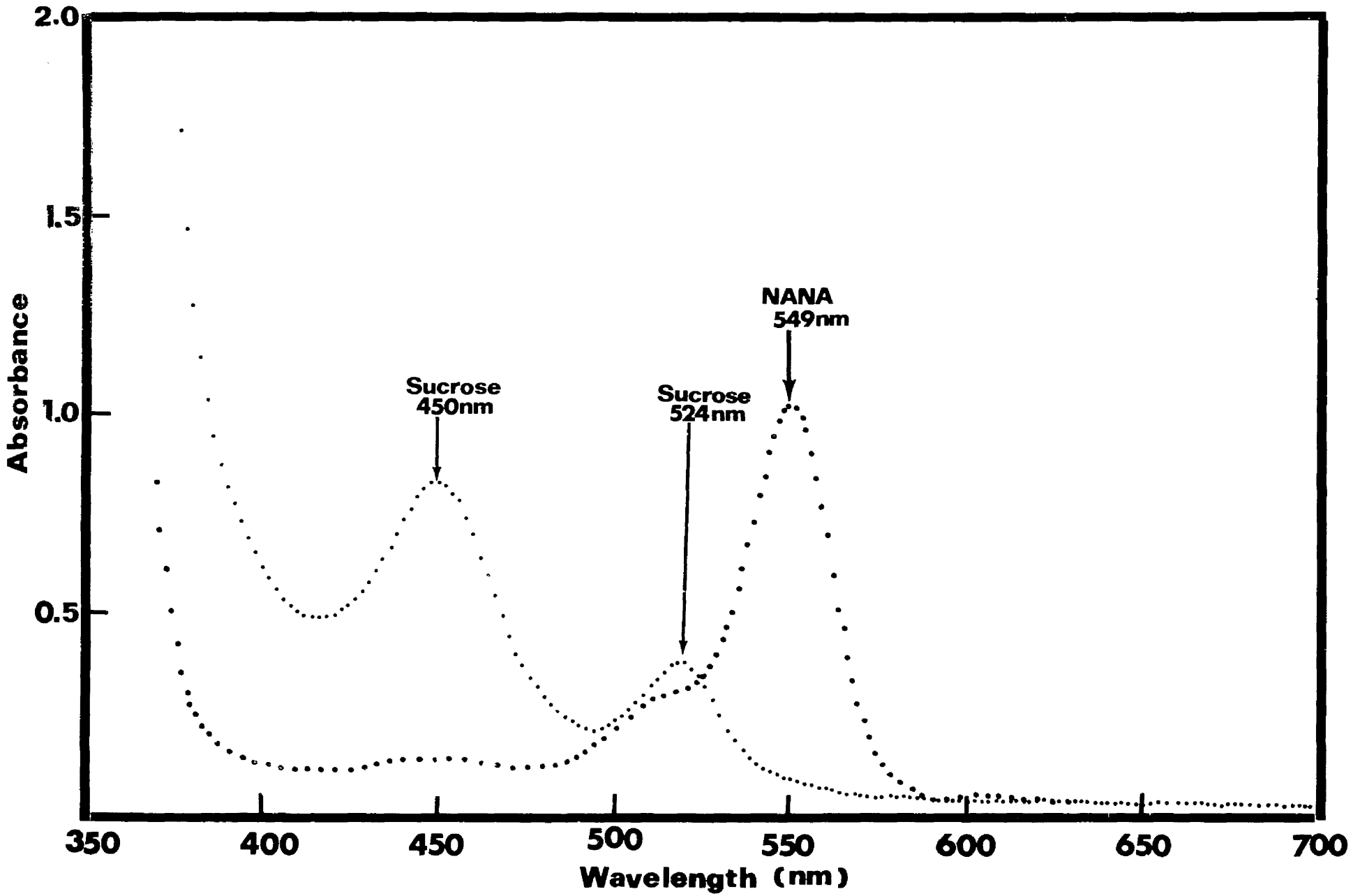
Fraction No.	Material Assayed	Milliunits of Neuraminidase/ml
83	α_B	104
88	Intermediate hot-cold haemolysis peak	498
93	α_A	<35

Figure 22 : Assay for Neuraminidase Activity –

Effect of Sucrose on Chromogen Development in NANA Assay

●●●●●● Normal Absorption Spectrum of NANA as assayed by Warren's Procedure showing absorption maximum at 549 nm

..... Absorption Spectrum of Sucrose after assay by Warren's procedure for NANA showing absorption peaks at 450 nm and 524 nm


2.0
1.5
1.0
0.5
Absorbance
Sucrose
450nm
Sucrose
524nm
NANA
549nm
350
400
450
500
550
600
650
700
Wavelength (nm)

Assay of dialysed fractions demonstrated that neuraminidase focused in the same area of the pH gradient as α-toxin. At the same time preparative electrofocusing experiments in pH 4 - 6 gradients revealed the appearance of an intermediate peak of α-toxin activity (between α_A and α_B) as detected by hot-cold haemolysis. The experiment shown in Figure 23 epitomises such observations. Only that area of the pH gradient in which α-toxin was detected is shown and the α_B and intermediate peaks of activity are emphasised by plotting these titres on an expanded scale. Fractions 83, 88 and 93, the three peak activity fractions, were concentrated by dialysis against 70% saturated ammonium sulphate at 4°C. The harvested precipitates were dissolved in 1 ml volumes of distilled water, dialysed overnight against several changes of distilled water and assayed for neuraminidase by the method of Holding and Collee (1971). The results of these assays are shown in Table 39 . Thus it appeared that α_A contained a negligible amount of neuraminidase, whereas α_B contained an appreciable quantity. Significantly, however, the intermediate form of α-toxin is associated with 4 times the quantity of neuraminidase found in fraction 83. Thus the pI of neuraminidase lay between pH 5.20 to 5.50. Unfortunately I was unable to determine the pI more accurately because of the lack of substrate.

This initial observation has since been confirmed during a collaborative study on Cl. perfringens neuraminidase in conjunction with Dr. Collee and Dr. Fraser of Edinburgh University and Dr. Arbuthnott of this department. Culture filtrate of strain L2A supplied by the Edinburgh group was dialysed, freeze-dried and electrofocused in a broad

Figure 23 : Observation of a Peak of Hot-Cold Haemolytic Activity between α_A and α_B during Preparative Scale Electrofocusing Experiments

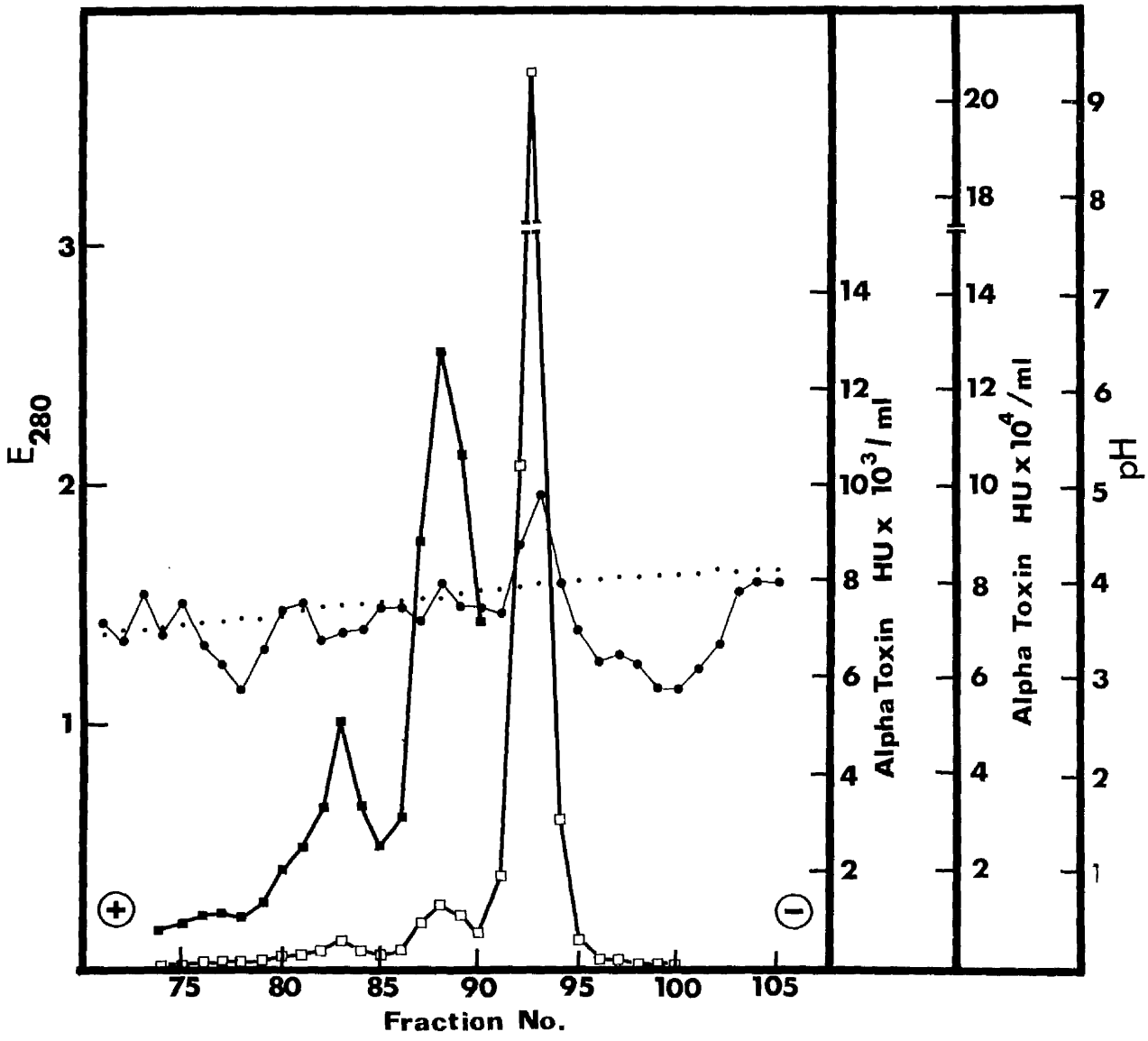
3.87 g of a 35-50% fractional $(NH_4)_2SO_4$ saturation precipitate of culture supernatant fluids of strain BP6K were applied in 170 ml of dialysed toxin to the LKB 8102 column. 100 ml of this were placed in the dense solution. Focusing was carried out for 67 hours at 4°C with a final potential of 800 V. Fractions of 4 ml volume were collected.

(•••) pH gradient

(●) E_{280}

(■) α-Toxin Activity (HU x 10^3)

(□) α-Toxin Activity (HU x 10^4)


E_{280}
3
2
1
14
12
10
8
6
4
2
Alpha Toxin HU x 10^3/ml
20
18
14
12
10
8
6
4
2
Alpha Toxin HU x 10^4/ml
9
8
7
6
5
4
3
2
1
pH
⊕
⊖
75
80
85
90
95
100
105
Fraction No.

pH gradient 3 - 10, the material being applied to the 110 ml column in the heavy solution. The density gradient was of glycerol instead of sucrose, from 65% (v/v) to 0%. Fractions again had to be dialysed free of glycerol, for although glycerol does not produce the intense orange chromogens associated with sucrose, it reacts readily with periodate preventing the cleavage of NANA (see page 138). Neuraminidase from strain L2A had a pI of 5.27.

In summary this method of purification of α-toxin enables its quantitative separation from θ-toxin, hyaluronidase, collagenase and at least in the case of α_A, the major electrophoretic component of culture concentrates tested in this thesis, from neuraminidase.

b. Immunological Homogeneity: Immunoelectrophoresis of α_A and α_B preparations from preparative electrofocusing experiments with toxin prepared from strain BP6K has revealed that such preparations are homogeneous. Plate 2 shows immunoelectrophoresis of fraction 83 (α_B) and fraction 93 (α_A). Only one precipitin arc was observed anodic to the origin (Plate 2). Antistreptolysin O did not give rise to a precipitin arc in either of these systems indicating the absence of θ-toxin.

In immunodiffusion studies in Ouchterlony double diffusion tests α_A and α_B showed a reaction of identity with only one line of precipitation.

By contrast immunoelectrophoresis of fraction 88, the intermediate peak of α-toxin activity detected during preparative electrofocusing experiment as shown in Figure 23, revealed the

Plate 2 : Immunoelectrophoresis of Electrofocused α- and θ-Toxins of Cl. perfringens Type A

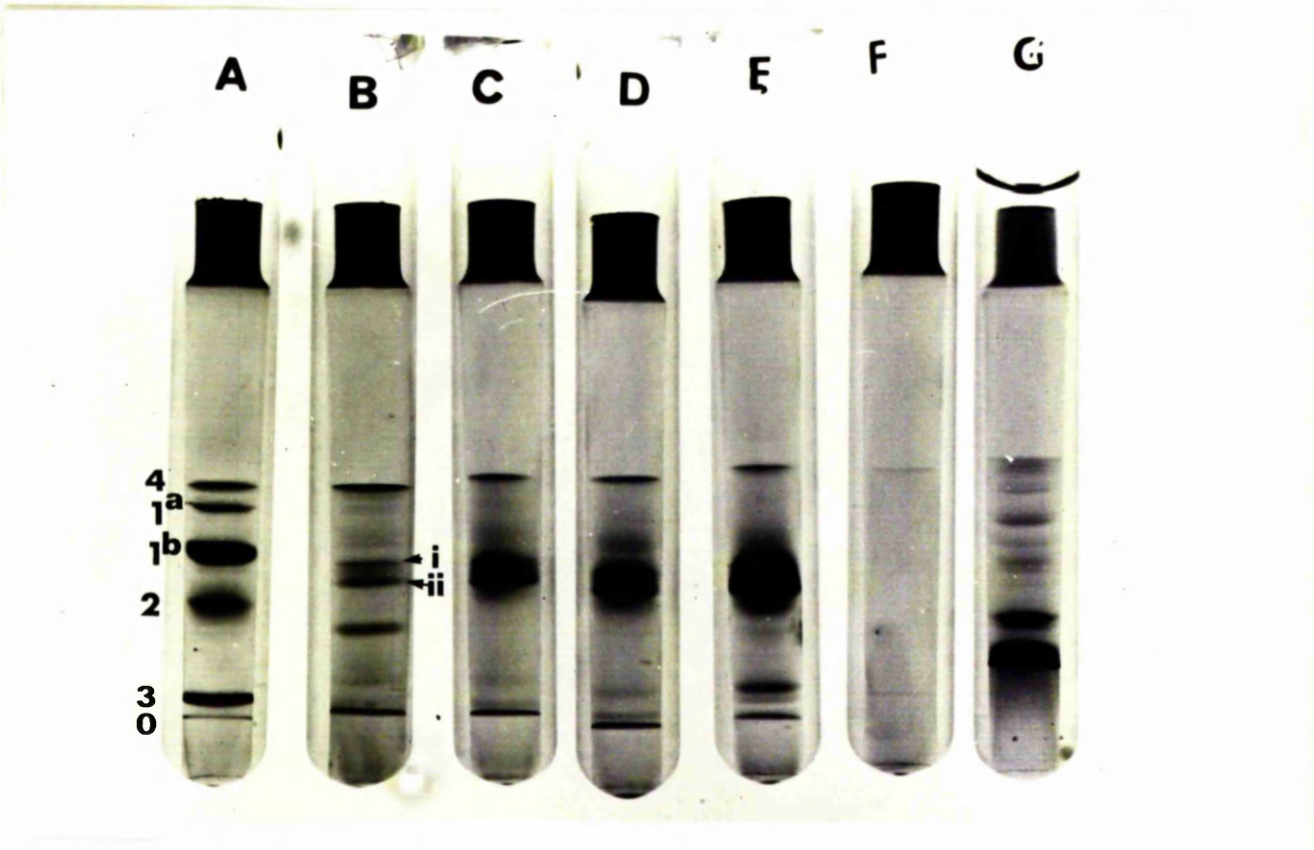
Well contents
1. α_B - Fraction 83 of Figure 23
2. Intermediate α-toxin peak detected by hot-cold haemolysis - Fraction 88 of Figure 23
3. α_A - Fraction 93 of Figure 23
4. θ-toxin

The precipitin arcs were developed with Cl. perfringens type A antiserum K7739 in troughs (A).
Anode to the left of photograph.

Plate 3 : SDS-Polyacrylamide Electrophoresis of Cl. perfringens α-Toxin Fractions purified by Electrofocusing

Gels:
A. Standard reference protein mixture - (o) track dye front (1^a) BSA dimer (1^b) BSA (2) ovalbumin (3) Chymotrypsinogen (4) Artefact band
B. α_B - Fraction 83 of Figure 23 (40 µg)
C & D. Intermediate α-toxin peak detected by hot-cold haemolysis - Fractions 88 and 89 respectively of Figure 23 (72 and 84 µg respectively).
E. α_A - Fraction 93 of Figure 23 (96 µg)
F. Blank control gel with artefact band (4)
G. Crude BP6K α-toxin (103 µg) - 35-40% $(NH_4)_2SO_4$ saturation precipitate.


A
1
A
2
A
3
A
4
A


A
B
C
D
E
F
G
4
1a
1b
2
3
0
i
ii

presence of two precipitin arcs one of which corresponded to that observed with α_A and α_B preparations (Plate 2).

c. Polyacrylamide Gel Electrophoresis in the Presence of SDS.

Disc gel electrophoresis of α_A in 11% polyacrylamide containing SDS showed one major protein band with two minor bands (Plate 3). Sometimes with more protein on a gel other minor bands were detected. The molecular weight of α_A was estimated by reference to three standard proteins-BSA, ovalbumin and chymotrypsinogen. Its molecular weight was determined as $53,800 \pm 1,400$.

When α_B fractions and fractions from the intermediate peak of α-toxin activity were electrophoresed similarly, two major bands (i and ii) were observed (see Plate 2 fractions 83, 88 and 89 from Figure 23). These bands lay close together, the lower of the two bands corresponding to that seen in α_A gels. The second band had a slightly higher molecular weight determined as $60,400 \pm 1,000$. A gel showing electrophoresis of crude toxin is given for comparison. A total of 12 components can be seen although many more minor bands were visible in the original gel.

5. Substrate Specificity of the α_A and α_B Forms of α-Toxin

The ability of α_A and α_B to hydrolyse lecithin and sphingomyelin was tested. L-α-Lecithin (General Biochemicals, Lot No. 84781) and sphingomyelin (L. Light & Co. Ltd., Colnbrook) were emulsified by sonication in an identical manner to that used in the preparation of the phospholipid emulsion for the assay of α-toxin. TLC of these substrates as outlined on page 158 revealed a single charred spot in

the case of the L-α-Lecithin, but two additional spots at the solvent front were present in the sphingomyelin. The nature of these spots was not resolved, but they were not due to contaminating amounts of PTC, PTE, PTI, PTS or lysolecithin. Assays contained 1 mg of lecithin (42.6 µg phosphorus) or 1.32 mg of sphingomyelin (51.0 µg phosphorus) (Tattrie, 1959; Dawson et al., 1969) in a final volume of 1.0 ml. The following reaction mixture was used :-

0.5 ml Phospholipid emulsion

0.4 ml Emulsion buffer (Ca^{2+} = 2.5 mM)

0.1 ml α-Toxin fraction.

Tests were set up in duplicate. Controls contained substrate + distilled water instead of α-toxin. Tubes were incubated at 37°C/30 min. One tube of each pair was assayed for phospholipid breakdown by determination of water-soluble phosphorus by Allen procedure. To the duplicate tube 10 ml of chloroform (Analar)were added, the contents thoroughly mixed, and the phases allowed to separate. The chloroform extracts were evaporated to dryness on a rotary evaporator under nitrogen and redissolved in 0.2 ml chloroform. TLC of 50 µl volumes of each of these extracts was carried out.

The results obtained for phosphate release by two preparations of α_A and α_B obtained by electrofocusing of AGX 1846 toxin are shown in Table 40. It can be seen that both forms of α-toxin hydrolysed lecithin and sphingomyelin as did the starting material, AGX 1846. Moreover these results could be correlated with the picture obtained by TLC of chloroform extracts of their duplicate assays. This is demonstrated in Plate 4.

Table 40 : Release of Water-Soluble Phosphorus from Lecithin and Sphingomyelin Emulsions by α_A and α_B

α-Toxin*	µg Phosphorus released from	
	Lecithin	Sphingomyelin
α_{B_1}	4	14
α_{A_1} *	13	23
α_{B_2}	6	19
α_{A_2} *	12	21
AGX 1846 (1 mg/ml)	34	49
Total Phosphorus in Control Emulsion	43	51

* Subscript numbering refers to preparations of α-Toxin from different electrofocusing experiments

Plate 4 : Thin Layer Chromatography of Chloroform Extracts of Lecithin and Sphingomyelin Emulsions treated with α_A and α_B Preparations of Cl. perfringens α-Toxin in the presence of Ca^{2+}

Plates spread with silica gel G.

50 µl samples applied at the origin

Chromatogram developed with Chloroform:Methanol:H_2O = 65:35:5

Phospholipid spots developed by charring with concentrated H_2SO_4.

A = Control sphingomyelin

B = Control lecithin

C = Sphingomyelin treated with AGX 1846 (1mg/ml)

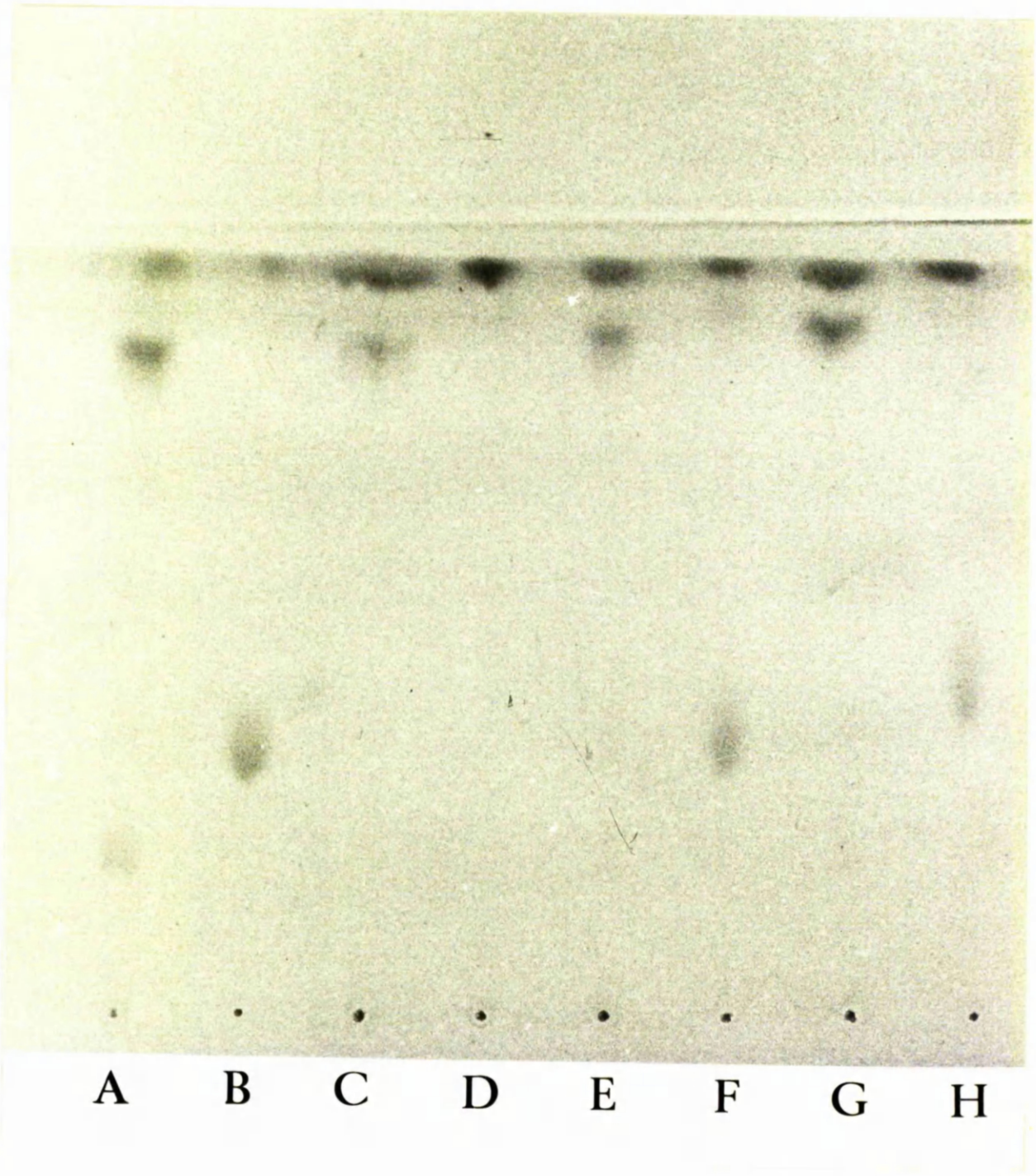
D = Lecithin treated with AGX 1846 (1mg/ml)

E = Sphingomyelin treated with α_{B_1}

F = Lecithin treated with α_{B_1}

G = Sphingomyelin treated with α_{A_2}

H = Lecithin treated with α_{A_2}


A
B
C
D
E
F
G
H

6. Zinc Content of α-Toxin Preparations.

Specimens for Zn analysis were concentrated by dialysis against 70% saturated ammonium sulphate at 4°C; the precipitates were harvested by centrifugation at 25,000 g for 15 min at 4°C and redissolved in distilled water. Samples were then dialysed exhaustively against deionised water for 48 hours with several changes of the stirred dialysate. All glassware used had been cleaned with Decon 90 (Decon Laboratories Ltd., Brighton, England) and rinsed thoroughly with glass distilled water and then deionised water (conductivity less than 0.1 micromhos).

Zinc was determined by atomic emission spectroscopy by Mr. D. Miller of the Geology Department, University of Glasgow, using a Hilger Large Quartz/Glass E742 Spectrograph. Electrodes were made of graphite. The dialysed samples were loaded into wells drilled in the ends of these electrodes (Volume 0.1 ml) and dried into the wells using a chromatography sample drier. Protein applied to each electrode was estimated by the Lowry method. Specimens were excited in the carbon arc at 5 amp DC with an electrode gap of 4 mm. The UV spectra were recorded between 370 - 240 nm using quartz optics.

Table 41 lists the samples analysed. In none of these samples were the characteristic emission lines of zinc detected.

Table 41 : Samples of α-Toxin Analysed for Zinc Content by Atomic Emission Spectroscopy

Sample	Preparation or Strain	Fraction No(s)	Figure in this thesis	Weight of sample analysed (μg)	Amount of Zn detected † (μg)
α_A	AGX 1846	17 + 18	15	182	< 0.2
α_B	AGX 1846	12 + 13 + 14	15	146	< 0.2
α_A	AGX 1846	15 + 16 + 17	16	136	< 0.2
$\alpha_A + \alpha_B$	BP6K*	35-43 inclusive	-	3,500	< 0.2
α	S 107°	24 + 25 + 26 + 27 + 28 + 29	13	845	< 0.2

* Preparative column experiment (4 ml fractions) pH gradient 4 - 6

o Pooled fractions from broad pH gradient 3 - 10

† Sensitivity limit of analysis = 0.2 μg Zn

B. θ-TOXIN

Results under 'Initial Studies' (see page 176) have already indicated that the technique of electrofocusing can separate the two cytolytic agents produced by Cl. perfringens viz. the α- and θ-toxins. The yield of θ-toxin from strain S107 in that experiment was 67.5% of that applied (see Figure 13). Similar yields have been obtained with the θ-toxin of strain BP6K illustrated in Figure 20 and Table 37. Electrofocusing studies with θ-toxin in broad pH gradients 3 - 10 are summarised in Table 42 . These studies have revealed that θ-toxin has a pI of 6.56 ± 0.13, the average of 8 determinations comprising experiments with preparations from strains BP6K and S107 and Wellcome preparation AGX 1846. The pI of θ-toxin could not be determined accurately in pH 4 - 6 gradients because of the loss of linearity in such pH gradients in the region of the pI of θ-toxin (see Figure 21).

Furthermore, it has been shown that θ-toxin can be refocused in broad pH gradients of 3 - 10 with good recovery of activity. Figures 24 and 25 show the elution profiles of θ-toxin on primary electrofocusing and on refocusing of the peak fractions respectively. The recoveries are given in Table 42 (Experiment No. 2 and No. 3 respectively).

It should be noted that the elution profiles of θ-toxin in Figures 13 and 20 exhibit "shoulders" on the acid side of their elution profiles. The pH at which these "shoulders" occurred are summarised in Table 43 and are designated I and II.

Attempts to resolve θ-toxin by refocusing on a narrow pH

Table 42 : Summary of Electrofocusing Studies on Cl. perfringens θ -Toxin*

Experiment No.	Strain or Preparation	Culture Medium Supernatant	Preparation of Sample[o]	Protein Applied to Column (mg)	pI	Activity Applied (HU)	Activity Recovered (HU)	Recovery (%)
1	BP6K	M-II	a	25	6.79	60,500	37,900	58
2	BP6K	M-II	a	ND	6.42	69,800	62,200	89
3	BP6K	M-II	x	ND	6.38	20,600	12,500	61
4	S 107	M-I	c	20	6.38	14,400	9,700	68
5	S 107	M-I	c	40	6.52	23,600	20,200	91
6	BP6K	M-II	a	135	6.61	629,000	449,000	72
7	BP6K	M-II	a	47	6.54	220,200	145,500	66
8	AGX 1846	?	f	20	6.56†	ND	ND	ND

[o] See pages 113 - 115 of 'Materials and Methods'
x Refocus of Peak Fractions 29-36 inclusive from experiment 2
* All experiments were carried out in pH gradients from 3-10 in the LKB 8101 (110 ml) column
ND Not determined
† Determined by doubling dilution titrations only

Figure 24 : Isoelectric Focusing of θ-toxin in Broad pH Gradients

The pH gradient was from 3 - 10. 12.7 mg of θ-toxin prepared from culture supernatant fluid of strain BP6K grown in M II medium by the acetone/calcium phosphate procedure of van Heyningen were applied in the dense solution to the LKB 8101 column. Focusing was carried out for 46 hours at 4°C with a final potential of 800 V. Fractions of 2 ml volume were collected.

Figure 25 : Refocusing of θ-Toxin in Broad pH Gradients

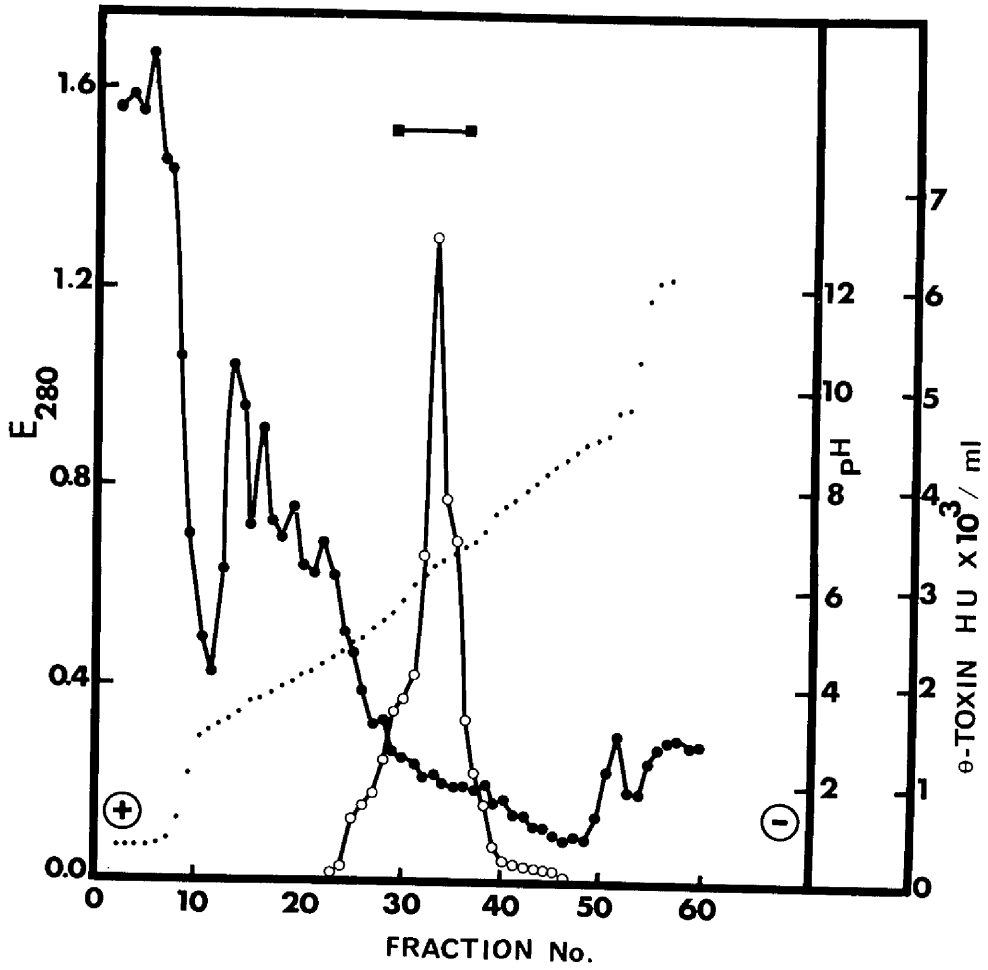
Fractions 29 - 36 inclusive from Figure 24 (■——■) were pooled, dialysed overnight against 1% glycine and applied to column LKB 8101 in the light solution (13 ml). Focusing was carried out for 37 hours at 4°C with a final potential of 800 V. Fractions of 2 ml were collected.

Symbols for Both Figures:

(•••) pH gradient

(O) θ-Toxin Activity (HU)

(●) E_{280}


1.6
1.2
0.8
0.4
0.0
E_{280}
0
10
20
30
40
50
60
FRACTION No.
12
10
8
6
4
2
pH
7
6
5
4
3
2
1
0
θ-TOXIN HU x 10^3/ ml

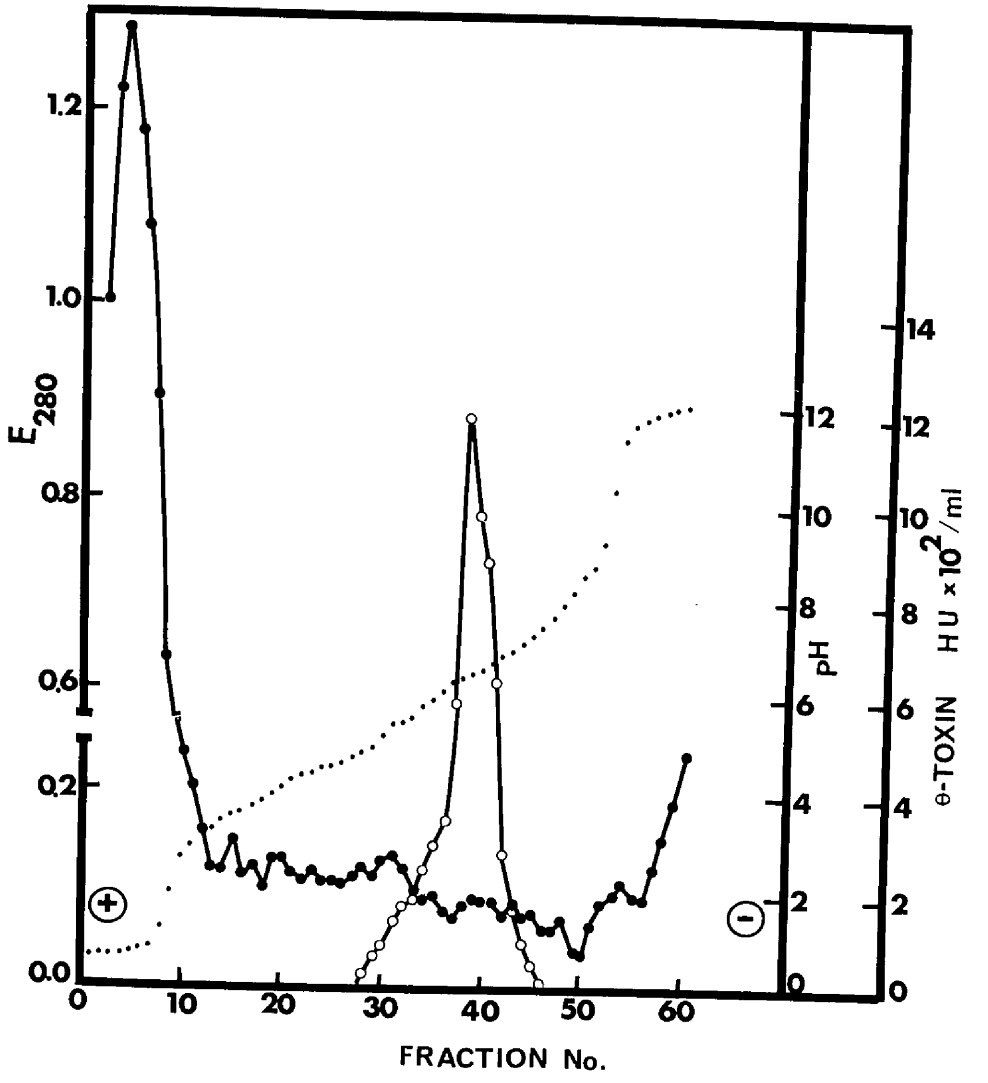

1.2
1.0
0.8
0.6
0.2
0.0
E_{280}
0
10
20
30
40
50
60
FRACTION No.
12
10
8
6
4
2
pH
14
12
10
8
6
4
2
0
θ-TOXIN HU x 10^2/ml

Table 43 : Electrofocusing Studies on Cl. perfringens θ-Toxin

Observation of "Shoulders" in Broad pH Gradients of 3-10

Experiment No.†	Strain	pH Range of "Shoulders" I	II
4	S107	-	5.40-5.60
5	S107	4.95-5.05	5.50-5.70
7	BP6K	5.00-5.30	5.60-5.90
6	BP6K	5.25-5.30	5.50-5.90

†See Table 42.

gradient of pH 5 - 8, all fractions from a broad gradient of pH 3 - 10 containing detectable θ-toxin, proved unsuccessful. The haemolytic activity was distributed throughout the pH range 5.9 - 7.0 with no clear peak or peaks of activity.

1. Purity of Electrofocused θ-Toxin.

Insufficient material was recovered in the peak fractions of θ-toxin from analytical column experiments in pH 3 - 10 gradients for the purity of such preparations to be studied. However, θ-toxin from peak fractions in preparative column experiments in pH 4 - 6 gradients has provided evidence that this toxin is obtained in a highly purified state by electrofocusing.

a. Polyacrylamide Gel Electrophoresis in the Presence of SDS:

The θ-toxin peak fractions from Figure 21 (fractions 69 and 70) were pooled and the toxin harvested by the method described on page 202 for α-toxin. Electrophoresis in 11% polyacrylamide disc gels in the presence of SDS revealed two bands of protein - a major component with a molecular weight of 61,500 and a minor band with a molecular weight of 36,300 (see Plate 5).

b. Immunoelectrophoresis:

Immunoelectrophoresis of θ-toxin from pH 4 - 6 gradients has revealed that such preparations were homogeneous. For example, immunoelectrophoresis of fractions 69 and 70 from Figure 21 gave only one precipitin arc against standard Cl. perfringens type A antiserum (see Plate 2). It can be seen that under the electrophoretic conditions used θ-toxin hardly migrated from the origin.

Plate 5 : SDS-Polyacrylamide Electrophoresis of Cl. perfringens Toxins and Enzymes obtained by Electrofocusing

Gels:
- U. α_A (30 µg)
- W. θ-toxin (55 µg)
- X. Collagenase peak Fraction I (82 µg)
- Y. Collagenase peak Fraction II (111 µg)
- Z. Standard Protein Mixture
- B. Blank gel
- BP6K. 35-50% $(NH_4)_2SO_4$ saturation precipitated toxin (90 µg)

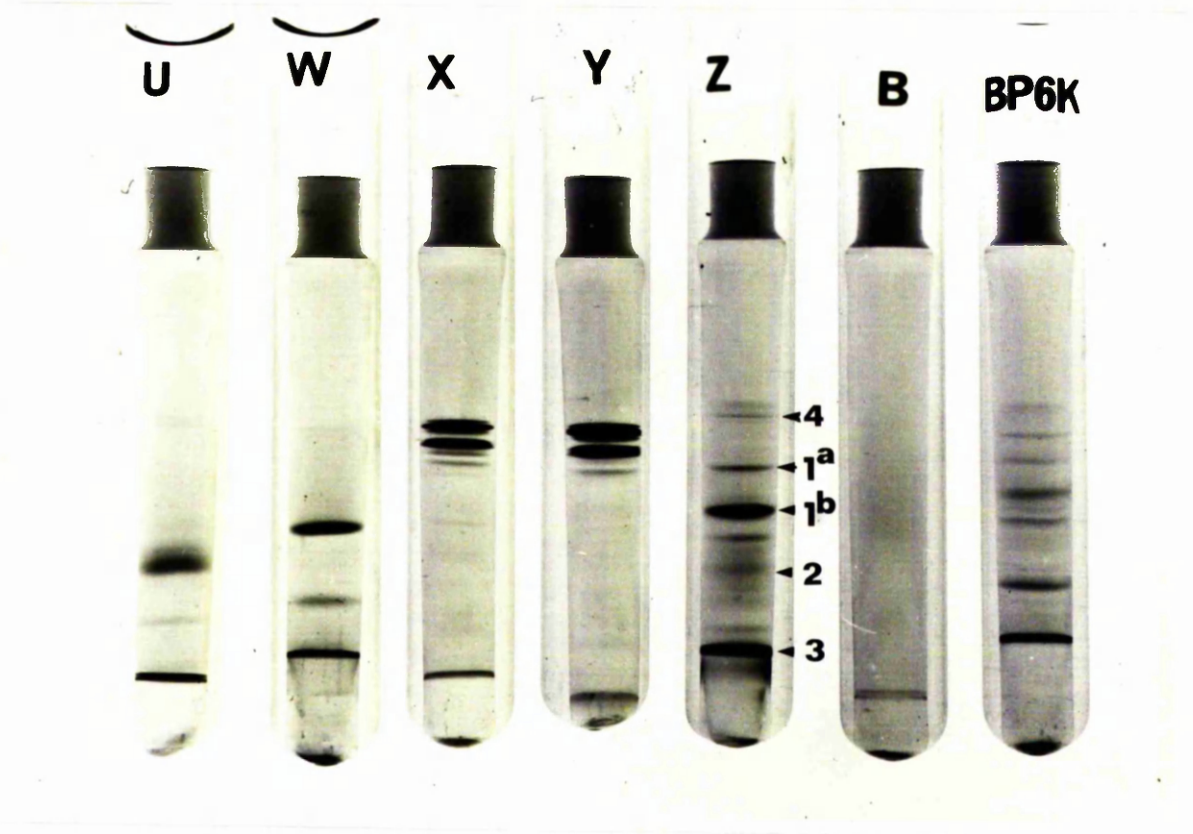
Standard Proteins: 1b = BSA 1a = BSA dimer 2 = Ovalbumin 3 = Chymotrypsinogen 4 = Artefact band

Plate 6 : Immunoelectrophoresis of Electrofocused Cl. perfringens Collagenase

Wells X and Y contain collagenase fractions I and II respectively as above.

A - Standard diagnostic Cl. perfringens type A antiserum K7739.

Anode to left of photograph.


U
W
X
Y
Z
B
BP6K
4
1a
1b
2
3


X
A
Y
A

C. COLLAGENASE

It has already been shown in Figures 20 and 21 that an enzyme capable of digesting azocoll powder was found in toxin obtained from culture supernatants of strain BP6K and focused at pIs of 4.61 and 4.65 respectively. That this enzyme was indeed a true collagenase, namely the kappa toxin of Cl. perfringens, was shown by its ability to digest native collagen by use of the collagen disc technique (see page 132).

Tests carried out on fractions 13 to 26 of the electrofocusing experiment shown in Figure 20 are shown in Plate 7 . After two hours' incubation at 37°C fractions 20, 21 and 22 had caused dissolution of the collagen discs. These three fractions contained maximum azocoll digesting activity with the peak in fraction 21. By 4 hours the disc treated with fraction 21 was completely degraded and discs in wells with material from fractions 18, 19 and 23 were becoming digested. After 8 hours discs of collagen tested with fractions 18 - 23 inclusive had been completely or almost completely digested. Those incubated with fractions 24 - 26 had also become thinner. Thus all fractions containing 40 AU of collagenase per ml or above digested 110 - 120 µg of collagen in 8 hours. Similar studies with fractions from other electrofocusing experiments confirmed in all cases that only those fractions containing azocoll digesting activity caused dissolution of collagen discs. Moreover, fractions with peak azocoll digesting activity corresponded with the fractions in which dissolution of the collagen discs first became evident. Thus the pI of the azocoll digesting enzyme was also that for the collagenase.

Plate 7 : Assay of Fractions of Focused BP6K Toxin for Collagenase by the Collagen Disc Method

Fractions 13-26 inclusive from the experiment shown in Figure 20 in which azocoll-digesting activity was determined. WHO plate incubated at 37°C.

C = control collagen disc suspended in buffer alone.

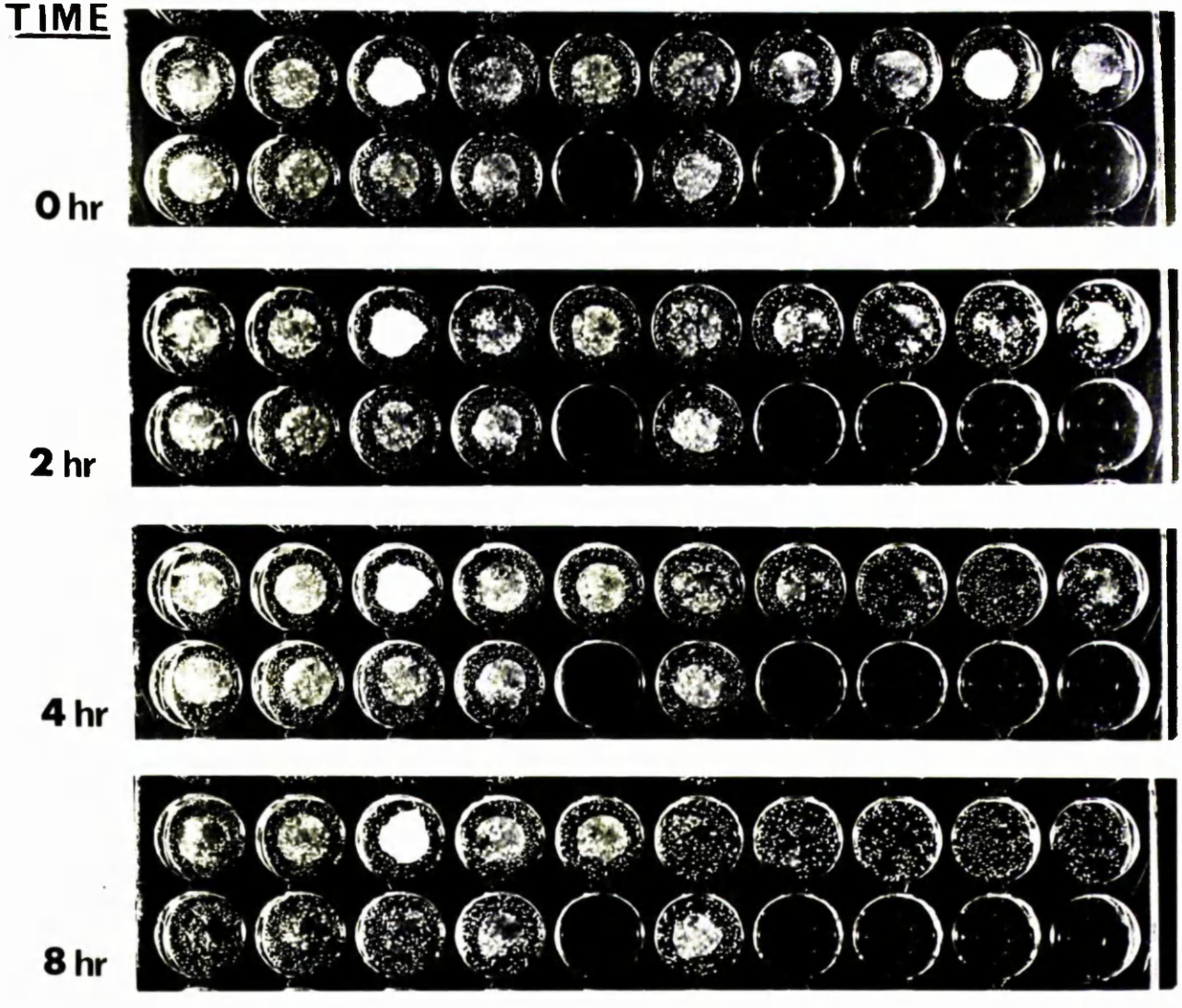

TIME
0 hr
2 hr
4 hr
8 hr
Fraction 13 14 15 16 17 18 19 20 21 22
Number 23 24 25 26 C

Table 44 summarises those electrofocusing experiments in which the pI and recovery of collagenase were determined. The average pI from 7 determinations was 4.54 $\pm$ 0.14 with a recovery of 57%.

In none of the electrofocusing experiments carried out in either broad range or narrow range pH gradients were multiple peaks of activity observed. In most experiments the collagenase peak was associated with a definite protein peak as monitored by E_{280} (see Figures 20 and 21).

In view of the findings of Levdikova (1966) that the collagenases of Cl. perfringens and Cl. histolyticum had almost identical molecular weights as determined by sedimentation velocity on ultracentrifugation, but different amino acid compositions and isoelectric points as determined by electrophoresis with a free boundary apparatus (Entveiler); the isoelectric point of Cl. histolyticum collagenase was investigated by isoelectric focusing.

Figures 26 and 27 show experiments in which Cl. histolyticum collagenase (see Appendix III) was first electrofocused in a pH 3 - 10 gradient and then those fractions containing detectable activity refocused in a pH 5 - 8 gradient. The pIs determined were respectively 5.55 and 5.58, with recoveries of the order of 80% of the applied activity in both cases. Again peak azocoll digesting activity coincided with the peak of collagen dissolution activity.

1. Purity of Electrofocused Collagenase.

Peak fractions of collagenase activity from preparative electrofocusing experiments were examined by disc electrophoresis and immunoelectrophoresis.

Table 44 : Summary of the Electrofocusing Studies on Cl. perfringens Collagenase

Experiment No.	pH Gradient	Strain or Preparation	Culture Medium Supernatant	Preparation of sample[o]	Column Used	Protein Applied to Column (mg)	pI	Activity Applied to Column (AU)	Activity Recovered from Column (AU)	Recovery (%)
I	3-10	BP6K	M-II	a	LKB 8101	135	4.34	15,500	8,100	52
II	3-10	BP6K	M-II	a	LKB 8101	47	4.61	5,400	2,200	40
III	3-10	AGX 1846	?	f	LKB 8101	20	4.32	15,200	9,500	62
IV	3-6	BP6K	M-II	a	LKB 8101	54	4.48	6,200	3,700	58
V	4-6	S 107	M-I	a	LKB 8101	20	4.59	1,300	370	29
VI	4-6	BP6K	M-II	e	LKB 8102	652	4.65	45,900	37,600	82
VII	4-6	BP6K	M-II	e	LKB 8102	2900	4.68	168,800	132,100	78

[o] See pages 113 - 115 of 'Materials and Methods'

Figure 26 : Isoelectric Focusing of Cl.histolyticum collagenase

12 mg of freeze-dried collagenase (Appendix III) applied in the heavy solution. The pH gradient was from pH 3 - 10. Focusing was carried out at 4°C for 48 hours, with a final potential of 800 V. 2 ml fractions were collected.

(•••) pH Gradient

(⊙) E_{280}

(▲) Collagenase-azocoll digesting activity

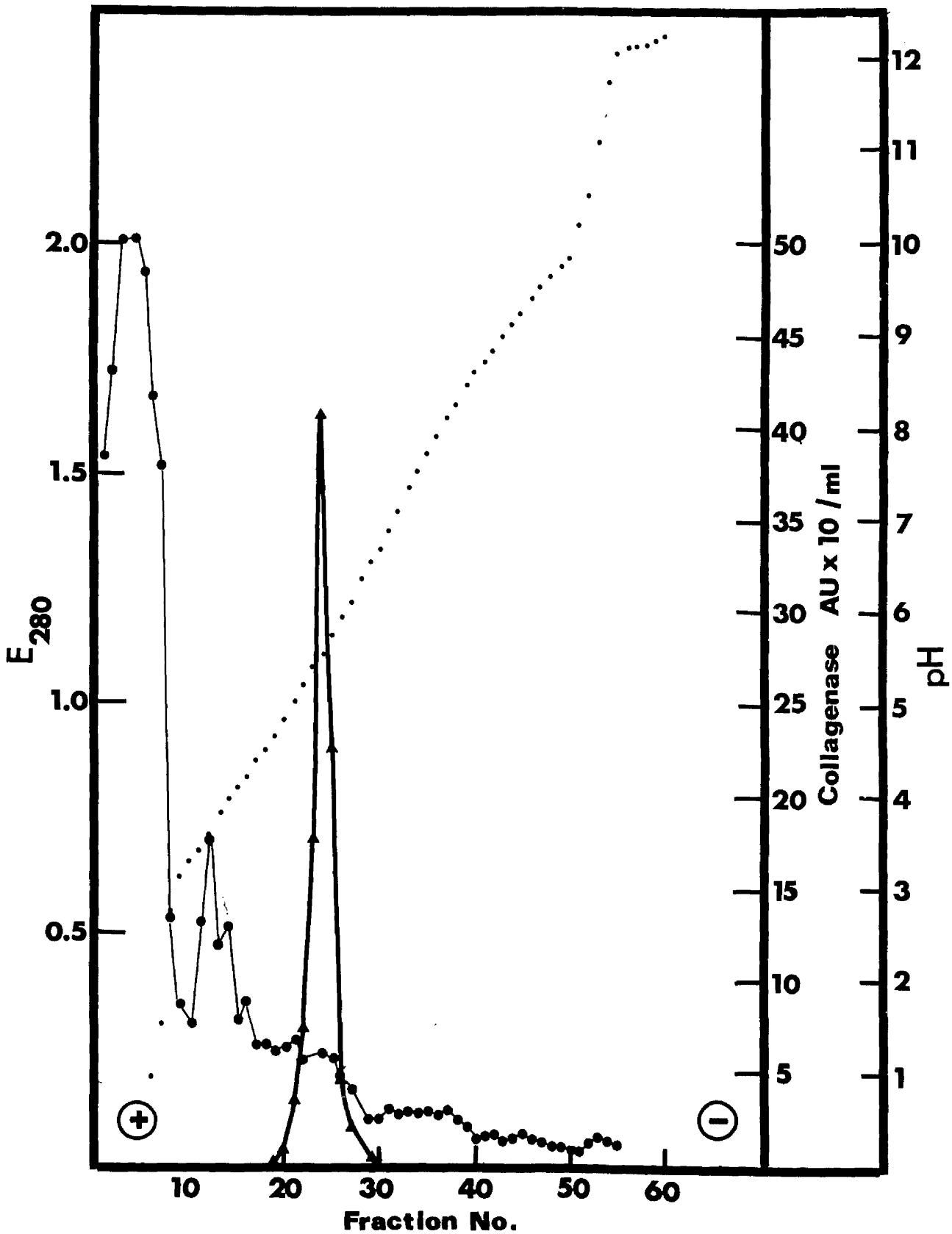

2.0
1.5
1.0
0.5
E_{280}
10
20
30
40
50
60
Fraction No.
⊕
⊖
50
45
40
35
30
25
20
15
10
5
Collagenase AU x 10 / ml
12
11
10
9
8
7
6
5
4
3
2
1
pH

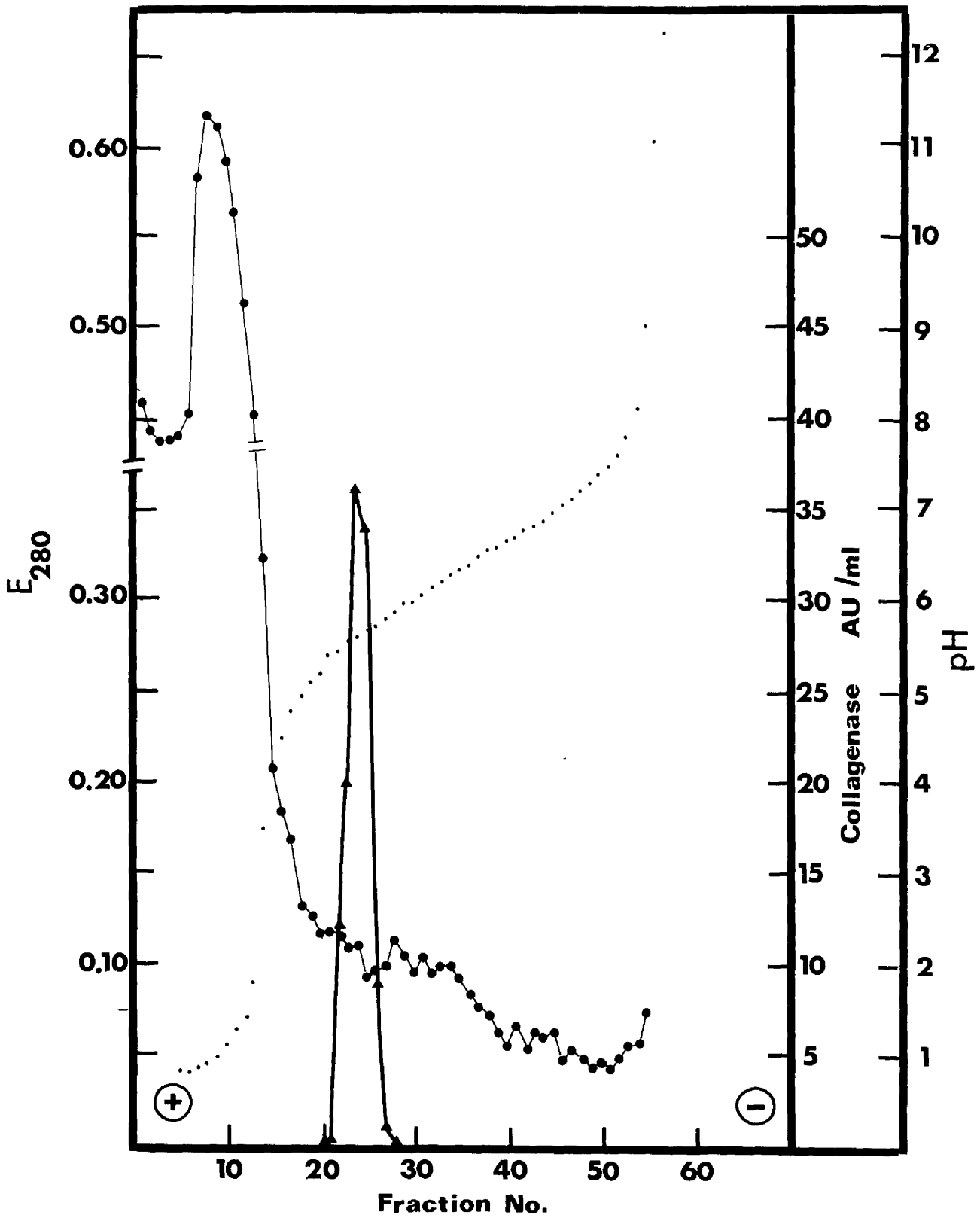
Figure 27: Refocusing of Cl. histolyticum collagenase

Fractions 20-28 inclusive from the experiment shown in Figure 26 were refocused in a narrow pH gradient, pH 5 - 8. Electrofocusing was carried out at 4°C for 38 hours with a final potential of 1200 V. 2 ml fractions were collected.

(•••) pH Gradient

(●) E_{280}

(▲) Collagenase-azocoll digesting activity


0.60
0.50
0.30
0.20
0.10
E_{280}
50
45
40
35
30
25
20
15
10
5
Collagenase AU /ml
12
11
10
9
8
7
6
5
4
3
2
1
pH
+
−
10
20
30
40
50
60
Fraction No.

a. Polyacrylamide Gel Electrophoresis in the Presence of SDS:

Disc electrophoresis in the presence of SDS of two preparations of collagenase from different electrofocusing experiments together with a gel containing the BP6K toxin applied to these columns is shown in Plate 5 . It can be seen that both preparations contained 6 components. These had molecular weights ranging from 107,000 - 144,000 as indicated on Plate 5 . The identity of one or more of these bands with collagenase has not yet been established.

b. Immunoelectrophoresis:

Immunoelectrophoresis of collagenase fractions has revealed a single precipitin line in both cases as is shown in Plate 6 . Of the purified toxins examined by immunoelectrophoresis it was the most anodic of the proteins. The observation of one precipitin line warns against using this criterion alone as a gauge of homogeneity of preparations.

D. HYALURONIDASE

Electrofocusing of hyaluronidase has been referred to previously (Figures 20 and 21). Those experiments in which the pI of hyaluronidase was determined by electrofocusing on an analytical scale are summarised in Table 45 . The average pI from 7 determinations was 4.73 ± 0.05 with an average recovery of 66%.

Hyaluronidase thus appeared to be homogeneous, but later experiments on a preparative scale in narrow pH gradients with toxin prepared from strain BP6K have led to the observation of multiple forms. For example in the experiment shown in Figure 21 two peaks of activity

Table 45 : Summary of Electrofocusing Studies on Cl. perfringens Hyaluronidase*

Experiment No.	pH Gradient	Strain or Preparation	Culture Medium Supernatant	Preparation of sample[o]	Protein Applied to Column (mg)	pI	Activity Applied to Column (IU)	Activity Recovered from Column (IU)	Recovery (%)
A	3-10	S 107	M-I	c	43	4.78	4,100	1,600	38
B	3-10	BP6K	M-II	f	420	4.74	3,200	2,000	63
C	3-10	S 107	M-II	c	101	4.75	22,800	13,500	59
D	3-10	BP6K	M-II	a	135	4.63	63,800	54,600	86
E	3-10	BP6K	M-II	a	47	4.72	22,300	16,200	73
F	3-6	BP6K	M-II	a	54	4.76	30,400	17,300	57
G	4-6	S 107	M-I	c	20	4.78	9,300	6,700	71
H	3-10	AGX 1846	?	f	20	4.69	720	610	85

* All experiments were carried out in the LKB 8101 (110 ml) column

[o] See pages 113 - 115 of 'Materials and Methods'

were detected having pIs of 4.88 and 4.98. Moreover, in another experiment in a pH 4 - 6 gradient, 3 peaks of activity were observed (see Figure 28). The peak fractions 63, 68 and 75 had pIs of 4.83, 4.94 and 5.07 respectively.

1. Purity of Electrofocused Hyaluronidase.

Attempts to assess the purity of hyaluronidase by immunoelectrophoresis and SDS gel electrophoresis proved unsuccessful. No precipitin lines were obtained when concentrated hyaluronidase fractions were immunoelectrophoresed against standard type A antiserum. Nor were distinct protein staining bands observed on gel electrophoresis in the presence of SDS.

Figure 28: Observation of Multiple Forms of Hyaluronidase in Preparative Scale Electrofocusing Experiments.

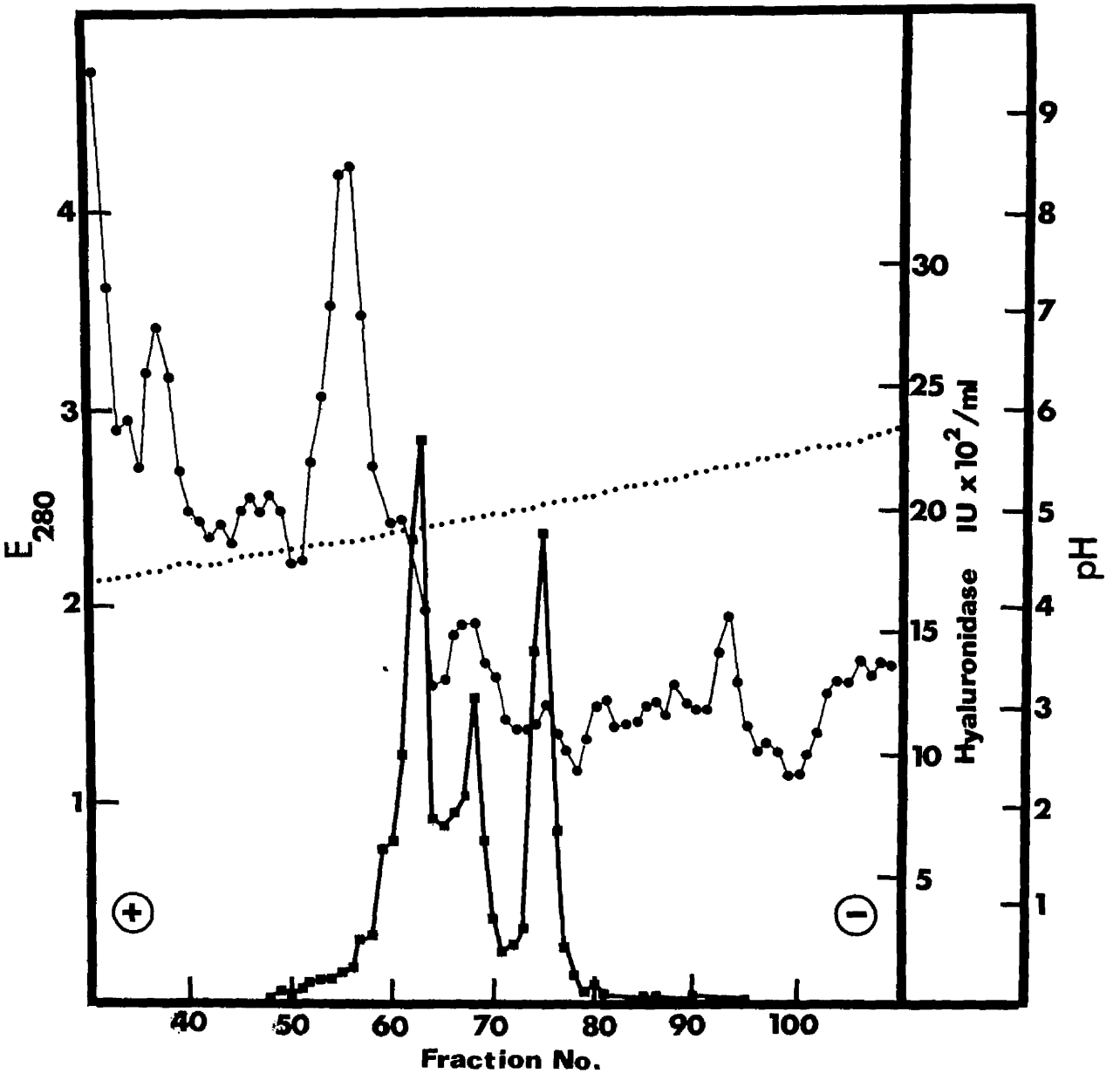
Isoelectric focusing of 35-50% fractional $(NH_4)_2SO_4$ saturation precipitate prepared from culture supernatant fluids of strain BP6K grown in M II medium.

The pH gradient was from 4 - 6. 2.95 g of toxin were applied to the LKB 8102 preparative electrofocusing column in 312 ml of dialysed material, 152 ml of which were applied in the dense solution. Focusing was carried out for 80 hours at 4°C with a final potential of 800 V. Fractions of 4 ml were collected.

(•••) pH gradient

(o) E_{280}

(▪) Hyaluronidase Activity


E_{280}
Hyaluronidase IU x 10^2/ml
pH
Fraction No.
40
50
60
70
80
90
100
1
2
3
4
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
+
−

SECTION III : EFFECTS OF PURIFIED TOXINS ON MAMMALIAN CELLS AND CELL MEMBRANES

A. THE EFFECT OF α-TOXIN ON THE OXIDATION OF SUCCINATE BY KREBS 2 ASCITES TUMOUR CELLS

1. Warburg Experiments.

α-Toxin purified by method a (see page 113) and Sephadex G-100 gel filtration chromatography (hereinafter referred to as AS/G100 α-toxin) had two distinct effects on the oxidation of succinate by Krebs 2 ascites tumour cells, according to whether these experiments were carried out in the presence or the absence of Ca^{2+}.

a. Experiments in the Presence of Ca^{2+}:

Ascites cells were pre-incubated with purified α-toxin or control fluid for 30 min at 37°C in the Warburg flasks. In each of four experiments (Table 46) succinate oxidation by toxin treated cells was abolished, respiration falling to the level of a cell suspension respiring endogenously. This is demonstrated in Figure 29A.

b. Experiments in the Absence of Ca^{2+}:

Krebs 2 ascites tumour cells were pretreated with α-toxin in the same way as above. However, addition of exogenous substrate to these cells caused a marked increase in the rate of oxygen uptake compared with ascites cells treated with control fluid. This effect is shown in Figure 29B and a summary of these experiments is presented in Table 46.

Thus this preparation of α-toxin caused inhibition of succinate

Table 46 : Effect of AS/G-100 α-Toxin on the Respiration of Krebs 2 Ascites cells (Warburg Experiments)

Experiment No.	$*Ca^{2+}$	µlitres O_2 uptake/hour			Stimulation	Inhibition
		Toxin treated	Control Respiration	Endogenous Respiration	%	%
1	+	16	102	20	-	100
2	+	45	138	31	-	87
3	+	5	81	20	-	100
4	+	16	62	43	-	100
5	-°	282	189	114	124	-
6	-°	222	113	22	120	-
7	-°	252	128	25	120	-
8	-°	269	141	46	136	-

* + = present
  - = absent
  Concentration of Ca^{2+} = 2.8 mM

o = Incubation mixture contained 283 HU of θ-toxin/ml cf. Tables47, 26 and 27

Figure 29 : Effect of AS/G100 α-Toxin on Succinate Respiration of Krebs 2 Ascites Cells

A. Ascites cells preincubated with 35 µg (3.3 PCU) of α-toxin for 30 min at 37°C with Ca^{2+} = 2.8 mM before adding 60 µmoles of substrate.

B. Ascites cells preincubated with 35 µg (3.3 PCU) of α-toxin for 30 min at 37°C in the absence of Ca^{2+} before adding 60 µmoles substrate.

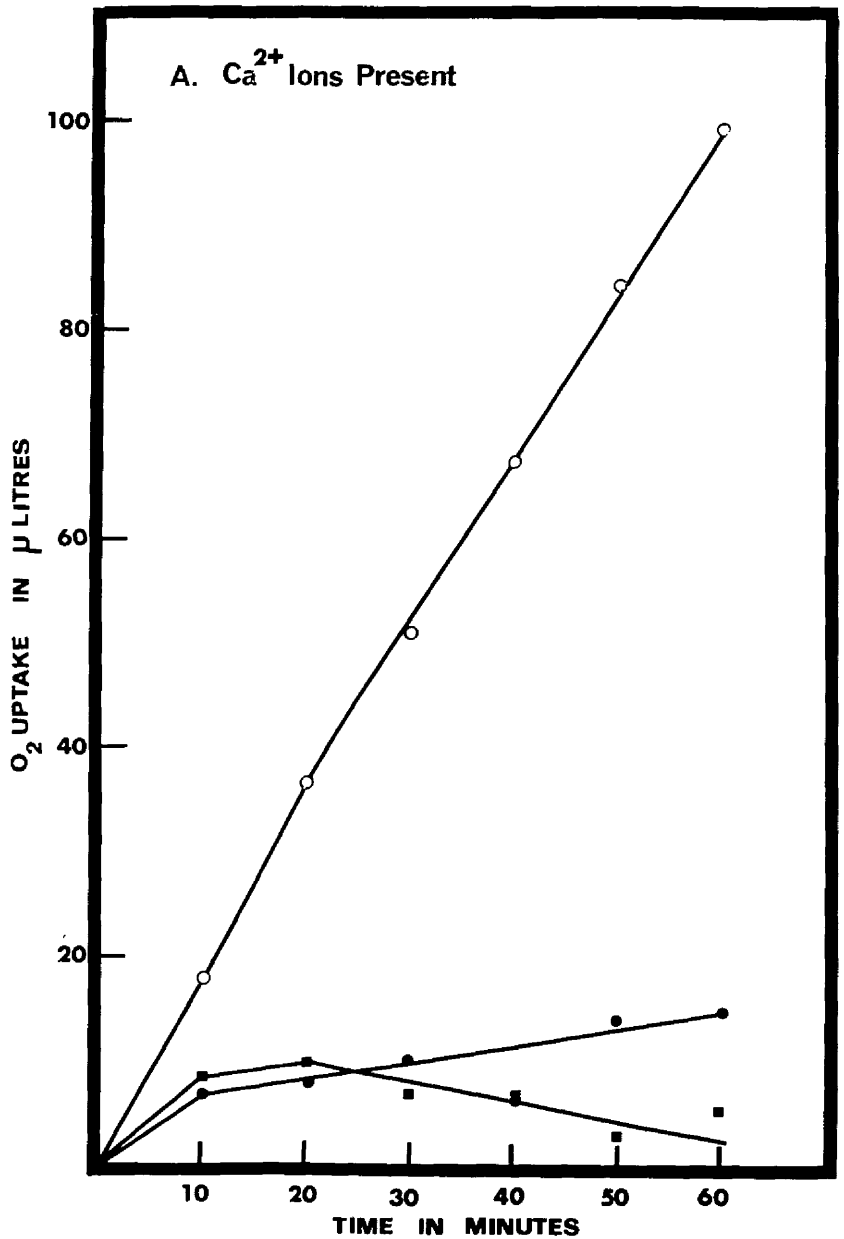
Symbols for both A and B:

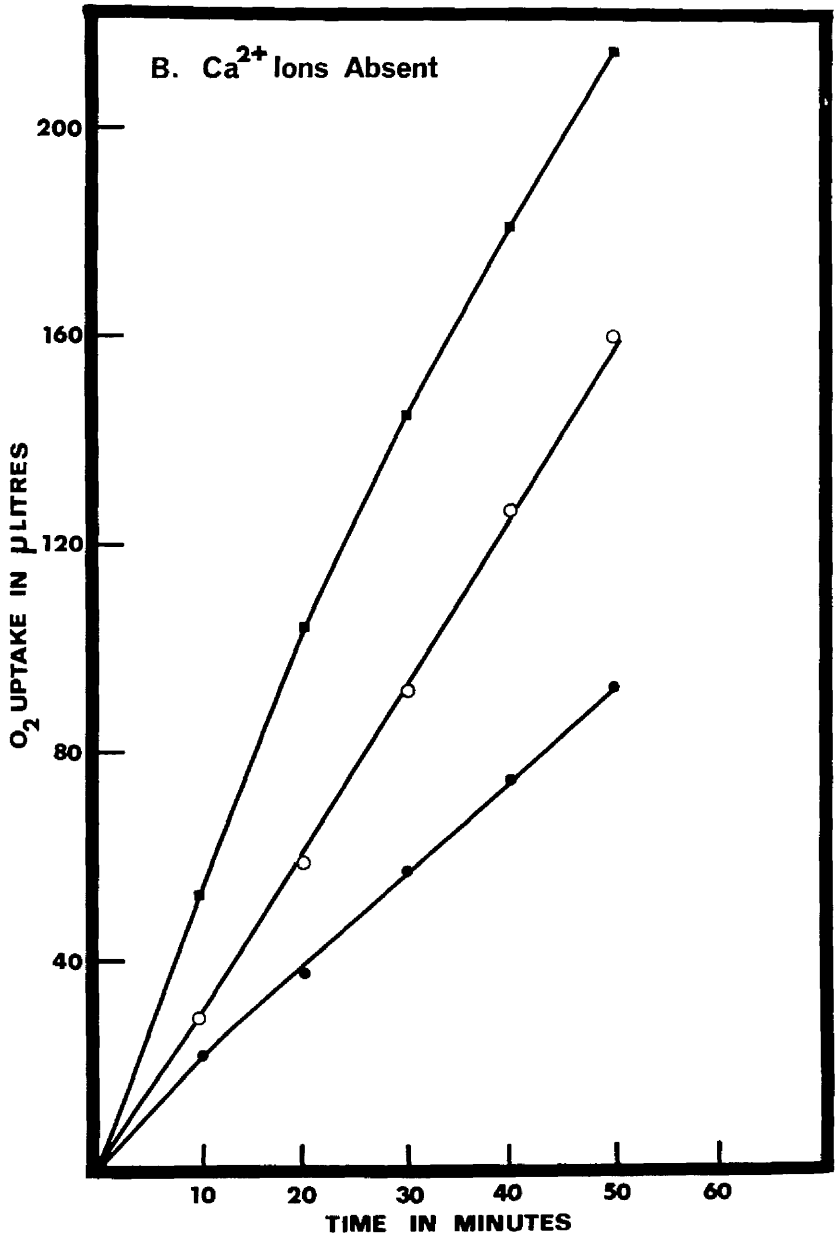
(O) Respiration of Control cells

(▣) Respiration of toxin treated cells

(⊘) Endogenous respiration of ascites cells

N.B. Experiments A and B were performed on different batches of ascites cells.


A. Ca^{2+} Ions Present
100
80
60
40
20
O_2 UPTAKE IN μ LITRES
10
20
30
40
50
60
TIME IN MINUTES


B. Ca^{2+} Ions Absent
200
160
120
80
40
O_2 UPTAKE IN μ LITRES
10
20
30
40
50
60
TIME IN MINUTES

respiration in the presence of Ca^{2+}, but appeared to stimulate succinate respiration in their absence.

The AS/G100 preparation used in these experiments was titrated for the presence of θ-toxin, hyaluronidase and collagenase. The titres of each product are shown in Table 47 . From this table it is evident that the observed effects on the respiration of Krebs 2 ascites cells could have been due to α-toxin or θ-toxin or the synergistic action of both cytolytic agents. The possible participation of hyaluronidase or collagenase seemed unlikely because of the low titres.

2. Oxygen Electrode Experiments.

The oxygen electrode was used to monitor the respiration of ascites cells treated with toxin preparations obtained by preparative electrofocusing. In all experiments the final volume was 3.0 ml. As in Warburg experiments the amounts of ascites cells and succinate were adjusted depending on the required composition of the reaction mixture. The cells were always pre-incubated at 37°C for 30 min with the test toxin before addition of substrate. The control cells were similarly pre-incubated.

Figure 30 illustrates the results obtained when Krebs 2 ascites cells were treated with α- and θ-toxin. The presence or absence of Ca^{2+} neither affected the rate of succinate respiration nor the rate of oxygen uptake due to endogenous metabolism. It can be seen that whereas α_A caused complete inhibition of succinate respiration, θ-toxin, by contrast, stimulated the respiration rate.

Table 47 : Titres of Toxins and Enzymes in AS/G100

α-Toxin Preparations

Toxin or Enzyme	Titre per ml
α-Toxin	6.6 PCU
θ-Toxin	1710 HU
Collagenase	9.5 AU
Hyaluronidase	4.0 IU

Figure 30 : Effect of Electrofocused α-Toxin and θ-Toxin on Succinate Respiration of Krebs 2 Ascites Tumour Cells

Oxygen uptake measured in the oxygen electrode.

(1) Endogenous respiration of ascites cells.

(2) α-Toxin treated ascites cells - pretreatment with 20 μg of α_A for 30 min at 37°C before addition of substrate. Ca^{2+} = 2.8 mM.

(3) Control respiration of ascites cells on succinate.

(4) θ-toxin treated ascites cells - pretreatment with 242 HU/ml for 30 min at 37°C before addition of substrate.

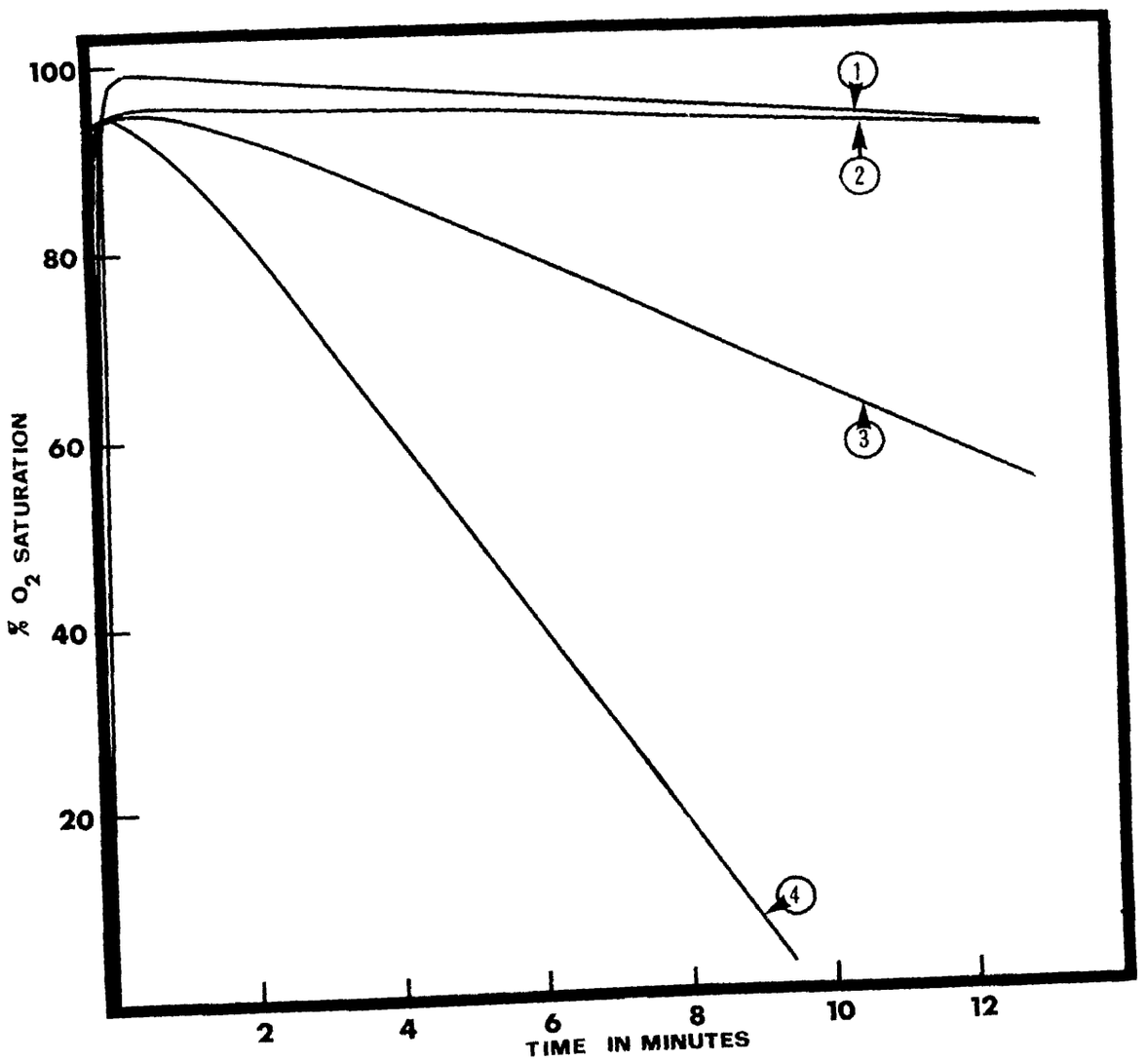

100
80
60
40
20
% O_2 SATURATION
1
2
3
4
2
4
6
8
10
12
TIME IN MINUTES

Table 48 (A) shows that α_A inhibited respiration only in the presence of Ca^{2+}. Furthermore, α-toxin failed to stimulate in the absence of Ca^{2+}, a finding which contrasted with earlier experiments in which AS/G100 preparations were employed. Also, the amounts of α_A used in these experiments contained no detectable collagenase, hyaluronidase or θ-toxin.

In the earlier experiments, 0.5 ml of the AS/G100 preparation used contained 855 HU of θ-toxin. In order to investigate the possible role of θ-toxin in these experiments, electrofocused θ-toxin was activated, titrated and diluted such that 700-800 HUs of θ-toxin were added in 0.1 ml of buffer. It is obvious from Table 48 (B) that exposure of Krebs 2 ascites cells to such quantities of toxin prior to the addition of substrate resulted in a marked increase in the respiration of these cells on succinate.

A disparity between the actual percentage stimulation observed in individual experiments will be noted.

The possibility that this effect was caused by a variation in cell numbers between experiments was tested. Symington (1969) showed that the number of cells in preparations of ascites cells obtained by the method described on page 160 was of the order of 1-2 x 10^8/ml. No significant change in the degree of stimulation of succinate respiration was observed over a 10 fold increase in cell numbers with a fixed concentration of θ-toxin/experiment (see Table 49). Moreover, following pre-treatment of ascites cells with equal amounts of θ-toxin for varying periods between 1-30 min, the % stimulation of O_2 uptake increased with time to reach a plateau in 15 min.

Table 48 : Effect of Electrofocused α- and θ-Toxins on the Respiration of Krebs 2 Ascites Cells

(Oxygen Electrode Experiments)

Toxin	Experiment No.	*Ca^{2+}	μ litres O_2 uptake/hour			Stimulation	Inhibition
			Toxin Treated	Control Respiration	Endogenous Respiration	%	%
(A) α_A	1 F	+	4	31	6	-	100
α_A	2 F	+	4	56	3	-	99
α_A	3 F	-	55	56	3	-	0
α_A	4 F	-	116	119	10	-	2
(B)	5 F°	-	96	33	5	227	-
	6 F°	-	109	65	26	112	-
	7 F°	-	142	85	16	84	-
	8 F°	-	219	106	11	119	-

* + = present, - = absent } Concentration of Ca^{2+} = 2.8 mM

o = Incubation mixture contained 242 HU of θ-toxin/ml cf. Table 46

Table 49 : Effect of Cell Numbers on the Stimulation of Succinate Respiration of Krebs 2 Ascites Cells by Cl. perfringens Type A θ-Toxin

No. of Ascites Cells/Experiment x 10^7	μ litres of O_2 uptake/hour†			% Stimulation of Succinate Respiration
	Toxin § Treated	Control	Endogenous	
1.3	15.4	5.1	0.7	234
2.0	32.8	11.4	1.5	216
5.6	100.1	33.5	3.1	219
12.8	183.8	60.6	5.7	234

†Average of duplicate determinations

§θ-Toxin concentration = 520 HU/Experiment - final volume 3 ml. Cells treated with θ-toxin for 30 min at 37°C prior to the addition of succinate

The effect of collagenase on succinate respiration was also tested. A preparation containing 8000 AU of collagenase/mg was employed. In experiments the protocol was devised such that 4800 AU of collagenase were added in 0.1 ml. Calcium acetate was added to a final concentration of 1 mM. The results obtained are shown in Table 50. Clearly collagenase caused no significant change in the rate of succinate respiration of the ascites cells, the average respective rates of respiration of toxin treated and control cells being within one standard deviation of each other.

B. EFFECT OF ELECTROFOCUSED α- AND θ-TOXINS ON ERYTHROCYTES AND ERYTHROCYTE GHOSTS

1. Interaction of α-Toxin with Mammalian Erythrocytes.

a. Haemolytic Spectrum:

α-Toxin purified by isoelectric focusing was tested for its ability to haemolyse some species of mammalian erythrocytes. Doubling dilution titrations were carried out as described on page 117. Erythrocyte suspensions of each species were prepared as for the SSES used for hot-cold haemolytic titrations (see page 117).

i. α-Toxin purified by electrofocusing in 6M urea: The peak fraction of α-toxin activity from the experiment shown in Figure 16 was dialysed overnight against several changes of saline to remove the urea. This preparation was referred to as α_{Urea}. It was diluted 1:20 with DICaB to provide the starting dilution for each titration. Control titrations were set up using α_{Urea} similarly diluted in α-toxin diluent lacking Ca^{2+}. Titrations were carried out in 0.5 ml volumes. An equal volume of each of the

Table 50 : The Effect of Cl. perfringens Collagenase on the Respiration of Krebs 2 Ascites Cells

Respiration	Consumption of O_2 µlites/hour	Mean Rate of Respiration (± 1SD)
Endogenous	24	24
	24	
Control cells	93	
	103	
	98	97 ± 4
	98	
	92	
Treated cells*	100	
	93	103 ± 7
	108	
	112	

* 4.26×10^6 cells/ml treated with 1600 AU collagenase/ml

SD = Standard deviation

erythrocyte suspensions was added to each set of tubes. Horse blood was defibrinated and contained no preservative (Oxoid Ltd.); the sheep blood was in Alsever's solution (Oxoid Ltd.); rabbit blood was obtained on the day of the experiment from the animal house; human blood was outdated blood group O obtained from the blood bank of the Western Infirmary, Glasgow, or blood group O freshly withdrawn from myself.

After hot-cold haemolysis the titres of α_{Urea} on each species of erythrocyte were read as visual 50% haemolysis end points. None of the control titrations showed haemolysis at the initial dilution of 1:20. All of the test titrations showed haemolysis in the first tubes of each titration after incubation at 37°C. The results are shown in Table 51.

ii. α_A from preparative electrofocusing experiments: The titrations were carried out as under (i). The starting dilution in this case was a 1:50 dilution of α_A. Bovine erythrocytes were used instead of human erythrocytes and were obtained from the Veterinary School, University of Glasgow. The titres were recorded after the "Hot-cold" phase of the titrations and are shown in Table 51.

From Table 51 it appears that bovine and human erythrocytes are equally susceptible to lysis by α-toxin preparations although from other experiments it has been shown that human erythrocytes lyse principally during the "hot" phase of incubation. Horse and rabbit erythrocytes were less susceptible to haemolysis, lysis occurring principally during the "hot" phase of the incubation period.

b. Kinetics of Hot Haemolysis by α-Toxin:

All the experiments in this section utilised the α_{Urea}

Table 51 : Haemolytic Spectrum of Electrofocused α-Toxin On Mammalian Erythrocyte Species

Erythrocyte Species	Hot-Cold Haemolytic Titre (HU/ml)	
	α_{Urea}	α_{A}
Bovine	ND	6400
Horse	80	400
Human	2560	ND
Rabbit	320	1600
Sheep	2560	6400

ND = Not determined

preparation from AGX 1846 Wellcome toxin. The experiments were performed in stoppered spectrophotometer cuvettes of 1 cm light path placed in the constant temperature housing (SP 874) of an SP 800 recording spectrophotometer (Pye Unicam) fitted with a scale expansion accessory (SP 850) and programme controller, series 2 (SP 825). Lysis of the erythrocyte suspension was followed as a decrease in E_{650} of the suspension, and the rate of change recorded on a potentiometric slave recorder (SP 22) using a scale expansion factor set at X 2. Water was circulated through the constant temperature housing from a thermostatically controlled water-bath at 37°C.

i. Observations on "hot" haemolysis: The following reaction mixture was used :

2.5 ml Buffer

0.2 ml of Standardised erythrocyte suspension

20 µl of α_{Urea} or Normal saline (Control fluid).

The results are shown in Figure 31. Only when sheep erythrocytes were treated in the presence of Ca^{2+} was hot haemolysis observed. Moreover, the rate of haemolysis was linear with time. In the absence of Ca^{2+}, in phosphate buffer or in borate buffer, no significant change in E_{650} was observed. Horse erythrocytes similarly treated in the presence of Ca^{2+} did not lyse at the concentration of α-toxin used. Moreover, α_{Urea} reactivated with 0.1 M cysteine-HCl did not cause lysis of sheep erythrocytes in the absence of Ca^{2+}. It can be concluded therefore, that hot lysis was due to α-toxin and not due to contaminating amounts of θ-toxin.

ii. Evidence for alterations in the sensitivity of sheep erythrocytes during the initial pre-lytic phase of hot lysis:

Observations on hot lysis of sheep erythrocytes by Cl. perfringens

Figure 31: Hot Haemolysis of Sheep Erythrocytes by Electrofocused α-Toxin

Toxin: α_{urea} from AGX 1846

Volume added: 20 µl = 51 HU

Buffer used:

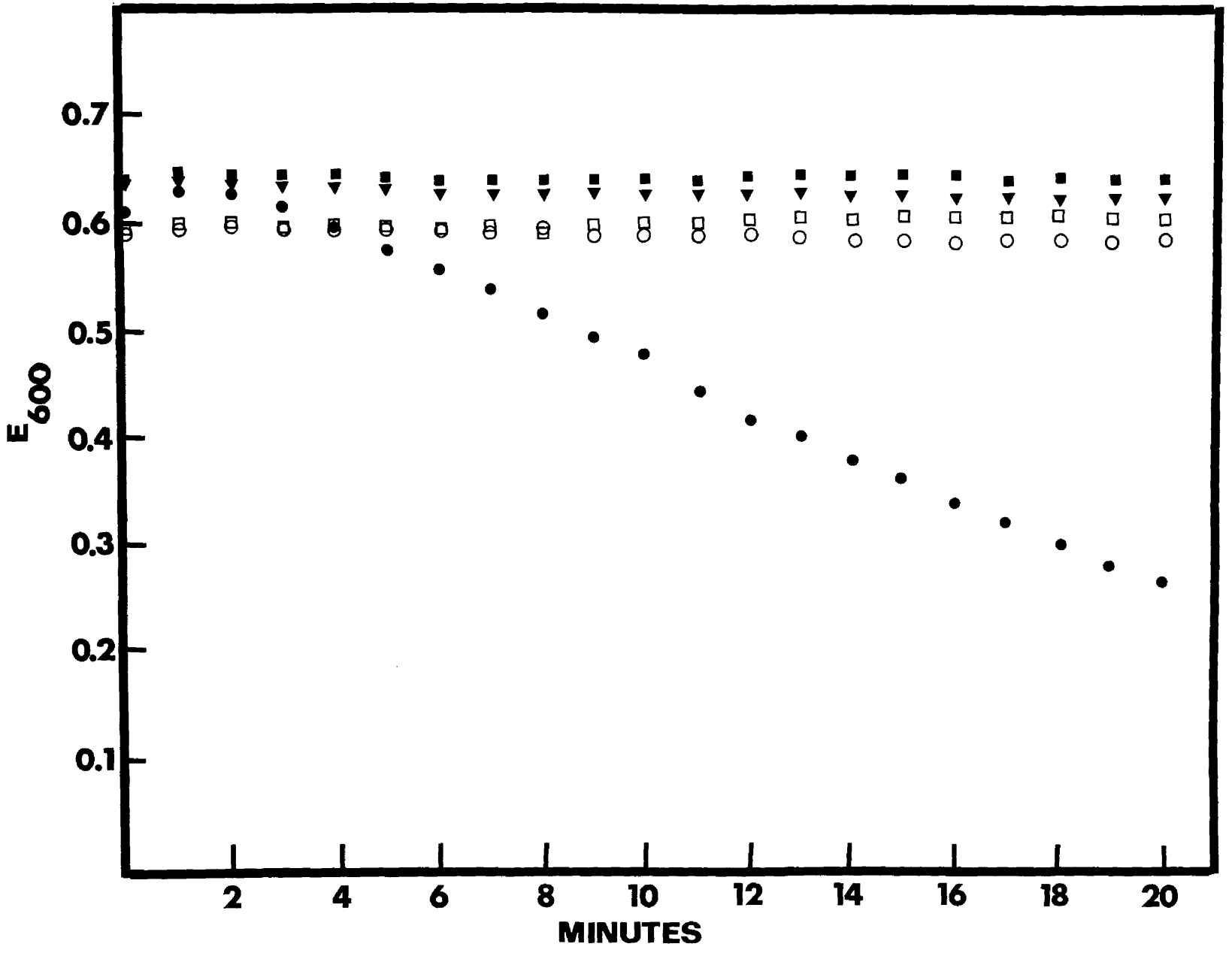
(●) DICaB

(■) Phosphate Buffer (θ-toxin diluent) - No added Ca^{2+}.

(▼) Phosphate Buffer - No added Ca^{2+}. α-Toxin reactivated with cysteine hydrochloride.

(□) Borate Buffer - No added Ca^{2+}.

(○) Controls sheep cells in DICaB - no toxin added.


0.7
0.6
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0.4
0.3
0.2
0.1
E600
2
4
6
8
10
12
14
16
18
20
MINUTES

α-toxin showed a pre-lytic lag phase of the order of 3 - 4 min with the quantity of α-toxin used. It would be difficult to observe quantitative changes in the phospholipid composition occurring in the quantity of erythrocytes present in the reaction mixture, except perhaps by the use of radio-active tracer techniques. Therefore an indirect approach was used to investigate possible alterations occurring in sheep erythrocytes during the lag phase. These were monitored by adding a constant amount of crude staphylococcus α-toxin from strain Wood 46 (W46-toxin) at different times during the lag. Although this preparation of staphylococcus α-toxin probably contained δ-haemolysin it proved satisfactory under the conditions of the experiment. Staphylococcus α-toxin was selected because it seemed likely to react synergistically with Cl. perfringens α-toxin, thus increasing the sensitivity of the haemolytic assay.

The SSES used in these experiments was the same as that used in the experiments illustrated in Figure 31. All suspensions were equilibrated for 2 min in the cell housing prior to the addition of toxin and gave the results shown in Figure 32. Haemolytic curves were superimposed as traces from data obtained from the potentiometric chart recorder. The chart speed was 3.5 cm/min. Only the initial portion of the Cl. perfringens α-toxin haemolytic curve is shown and this followed the time course of that in Figure 31.

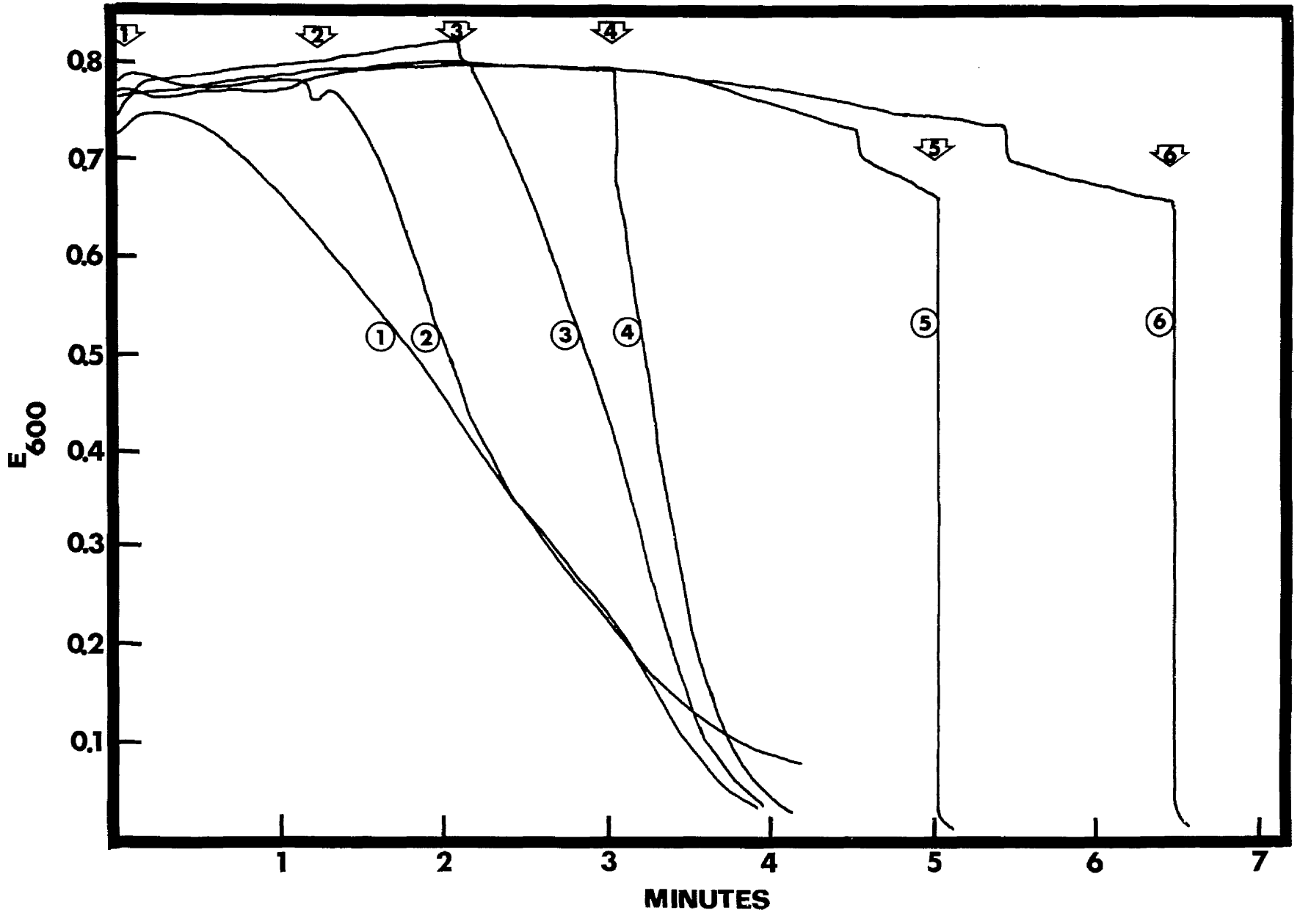
It is obvious that the α_{Urea} preparation caused profound changes in the susceptibility of the sheep erythrocytes during the first three minutes of incubation. The rate of haemolysis of the erythrocytes by W46-toxin increased as a function of the period of

Figure 32 : Alteration in the Sensitivity of Sheep Erythrocytes to W46 Toxin after pretreatment with Cl. perfringens α-toxin

1. Lysis of sheep erythrocytes by W46 toxin. No α-toxin treatment.

2-6. Sheep erythrocytes pretreated with α_{urea} (20 µl = 51 HU) for varying periods prior to the addition of W46 toxin.

Arrows mark points of addition of W46 toxin.


1
2
3
4
5
6
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
E600
1
2
3
4
5
6
7
MINUTES

pre-treatment of these cells with α_{Urea}, as indicated by a steepening of the W46 curves. Moreover once the erythrocytes began to lyse under the action of α_{Urea} itself (after 3.5 min in Figure 32) subsequent addition of W46-toxin caused flash lysis of the erythrocyte suspension.

c. Changes in the Phospholipid and Protein Composition of Erythrocyte Ghosts Treated with α-Toxin:

Rabbit erythrocyte ghosts were prepared from blood freshly drawn by ear puncture. The α-toxin used was an α_A peak fraction. The experimental protocol is shown in Table 52A. The test-tubes were incubated in a water-bath at 37°C for 45 min. At the end of the incubation period 5 or 8 ml of 0.01M Tris-HCl buffer, pH 7.4were added to each tube, the contents centrifuged at 48,000 g for 15 min and the supernatant fluids removed and frozen until assayed. The ghost pellets were resuspended in 3 or 5 ml of buffer and frozen. Protein was determined by the method of Lowry et al., (1951) and phosphate by the method of Allen (1940).

From Table 52B it can be seen that such treatment of rabbit erythrocyte ghosts released some 70-80% of the total membrane bound phosphorus into the supernatant fluid indicating an equivalent hydrolysis of phospholipid. By comparison, no change occurred in the protein content of the ghosts as a result of treatment with α-toxin.

d. Morphological Studies of α-Toxin Treated Mammalian Erythrocyte Ghosts:

Ghosts were prepared from rabbit, human, sheep and horse erythrocytes (sources as given on page 239). All ghost pellets were white. SDS-polyacrylamide electrophoresis of 100 µg of solubilised

Table 52 : Protein and Phosphorus Analysis of α_{Urea} Treated Rabbit Erythrocyte Ghosts and Ghost Supernatant Fluids

A. Experimental Protocol

Component	1 Ghost Control	2 Toxin Treated	3 Toxin Control
0.01M Tris-HCl Buffer, pH 7.4	0.1	0.1	0.2
0.05M Calcium Acetate	0.02	0.02	0.02
Distilled Water	0.1	-	-
Rabbit Erythrocyte Ghosts	0.2	0.2	-
α_{Urea}	-	0.1	0.1

B. Results

	Experimental Sample	Protein µg	Phosphate µg
1	Supernatant fluid	0	6.7
	Ghost Pellet	480	12.6
2	Supernatant Fluid	146	17.2
	Ghost Pellet	450	4.8
3	Supernatant Fluid	166	0
	Ghost Pellet	0	0

ghost protein revealed the presence of only a very faint haemoglobin band near the track dye front during electrophoresis (Arbuthnott, unpublished results). They were treated with 20 μg of α-toxin (α_A) in the manner of the protocol of Table 52A , and incubated at 37°C for 45 min. Ghost preparations for electron microscopy were mixed with negative stain and dried on to the support specimen grids.

All the electron micrographs shown were kindly supplied by Dr. J.H. Freer of this department. Control ghosts possessed common morphological features. They all had a fine granular appearance although the sheep ghosts were more complex than the other erythrocyte ghosts examined (compare Plates 8 and 10). In addition, the sheep erythrocyte ghosts appeared to have fragmented more than the other erythrocyte ghosts using the method of preparation employed. The human and horse erythrocyte ghosts contained many electron transparent rings (30-47 nm in diameter) into which the negative stain had penetrated (see Plate 8). These features are similar to those described by Harris and Agutter (1970) in human erythrocyte ghosts.

Exposure of these ghost preparations to α-toxin caused certain distinctive effects to all four species of erythrocyte ghosts tested :-

(1) The ghosts appeared less granular than controls.

(2) Electron transparent globules were formed which either remained associated with the residual ghost (Plates 9 and 11) or were released from the ghost (Plate 12).

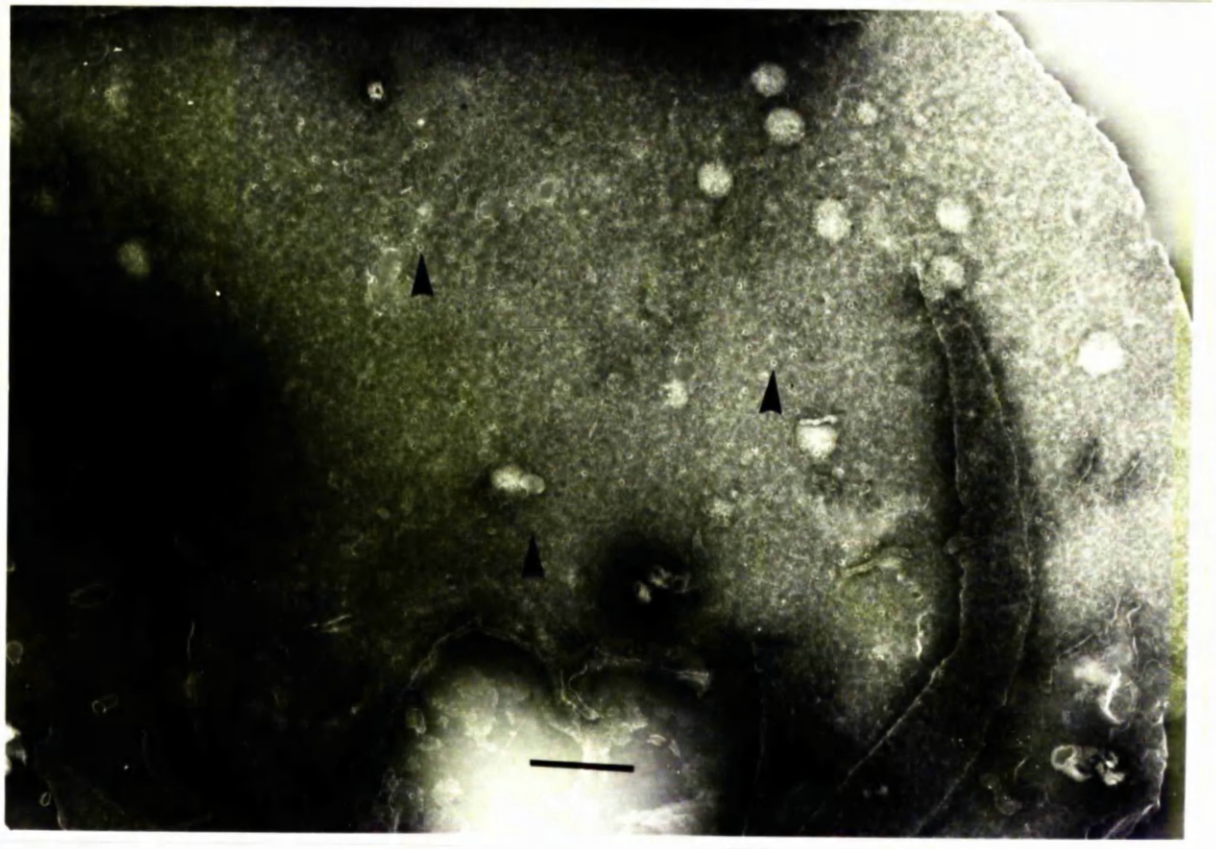
(3) No further fragmentation of the residual erythrocyte ghosts occurred.

(4) None of the ghost preparations examined showed any arcs or ring-like structures similar to those described on page 67.

Plates 8 - 15 : All preparations were negatively stained with 2% ammonium molybdate, pH 6.8.

Plate 8 : Electron micrograph of the surface structure of a control human erythrocyte ghost. The arrows indicate electron transparent rings or pitlike infoldings into which the negative stain has penetrated. These measure 31-46 nm in outer diameter and 13-24 nm in internal diameter. X 27,720. Dimension marker = 500nm

Plate 9 : Electron micrograph of a human erythrocyte ghost treated with α-toxin (20 μg of α_A to 0.2 ml of ghosts). Incubated at 37°C for 45 min. Ca^{2+} = 2.5 mM. Arrows indicate rings described in control ghosts (C). Electron transparent globules or vesicles are seen (G) - 200-300 nm in diameter. X 34,500. Dimension marker = 500nm.



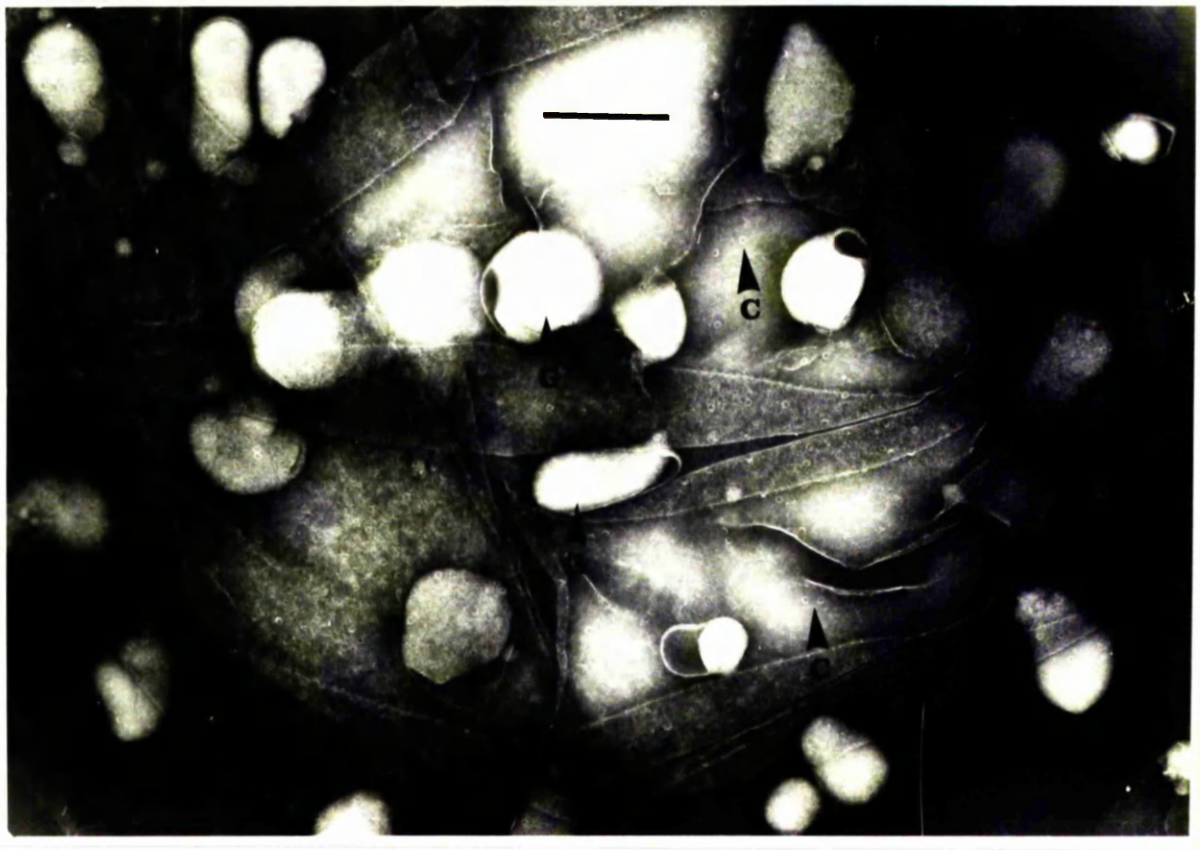
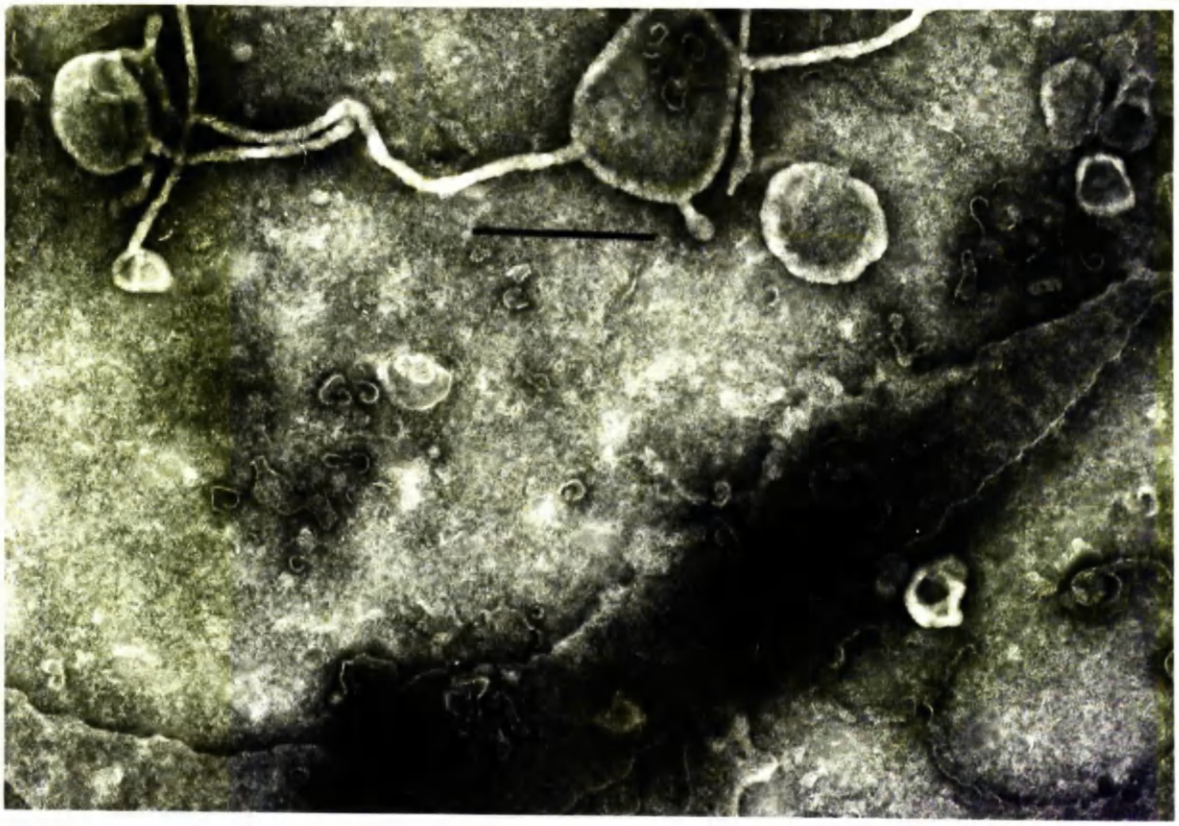

C

Plate 10 : Electron micrograph of the surface structure of a control sheep erythrocyte ghost. Note the complexity of this surface structure in comparison with that of the human erythrocyte ghost (see Plate 8). X 47,700.

Dimension marker = 500nm

Plate 11 : Electron micrograph of a sheep erythrocyte ghost treated with α-toxin (20 μg of α_A to 0.2 ml ghosts). Incubation at 37°C for 45 min. Ca^{2+} = 2.5 mM. Note electron transparent globules or vesicles in association with membrane or freed from ghost surface (G). X 47,700.

Dimension marker = 500nm



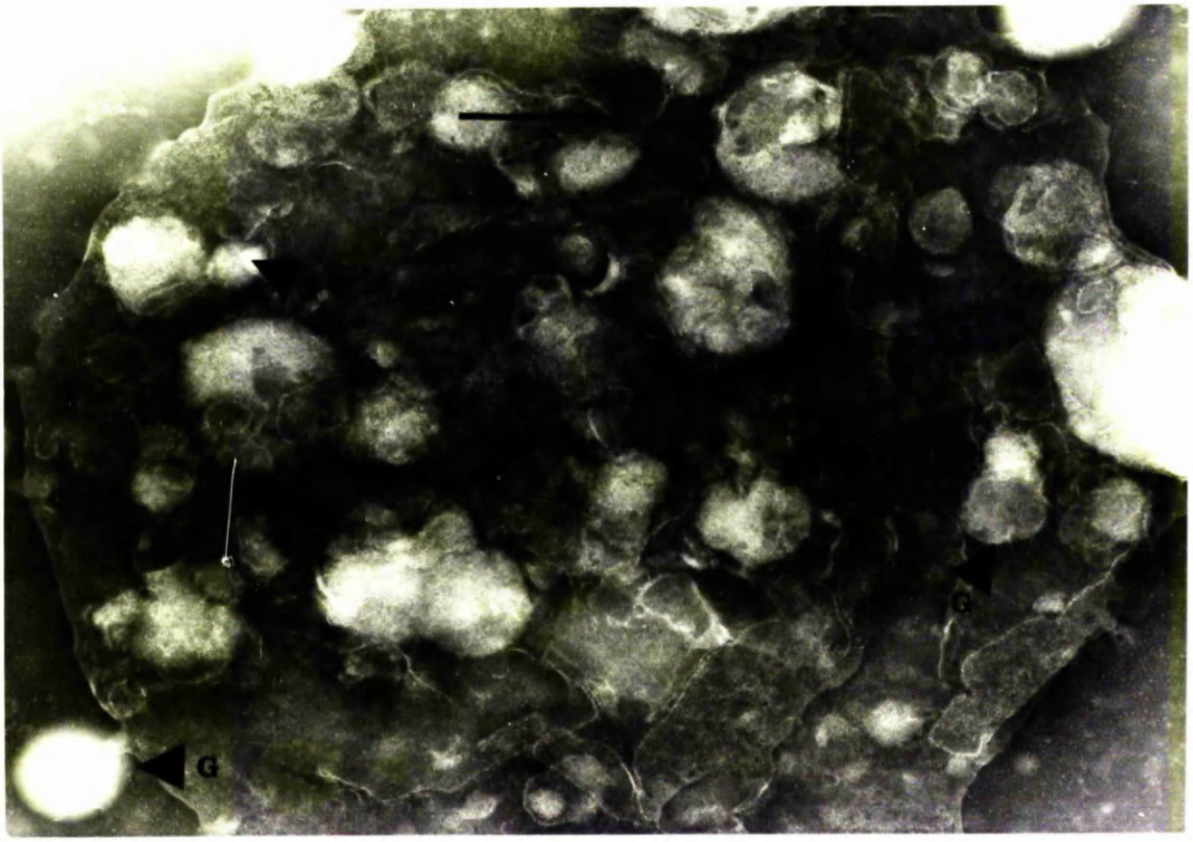
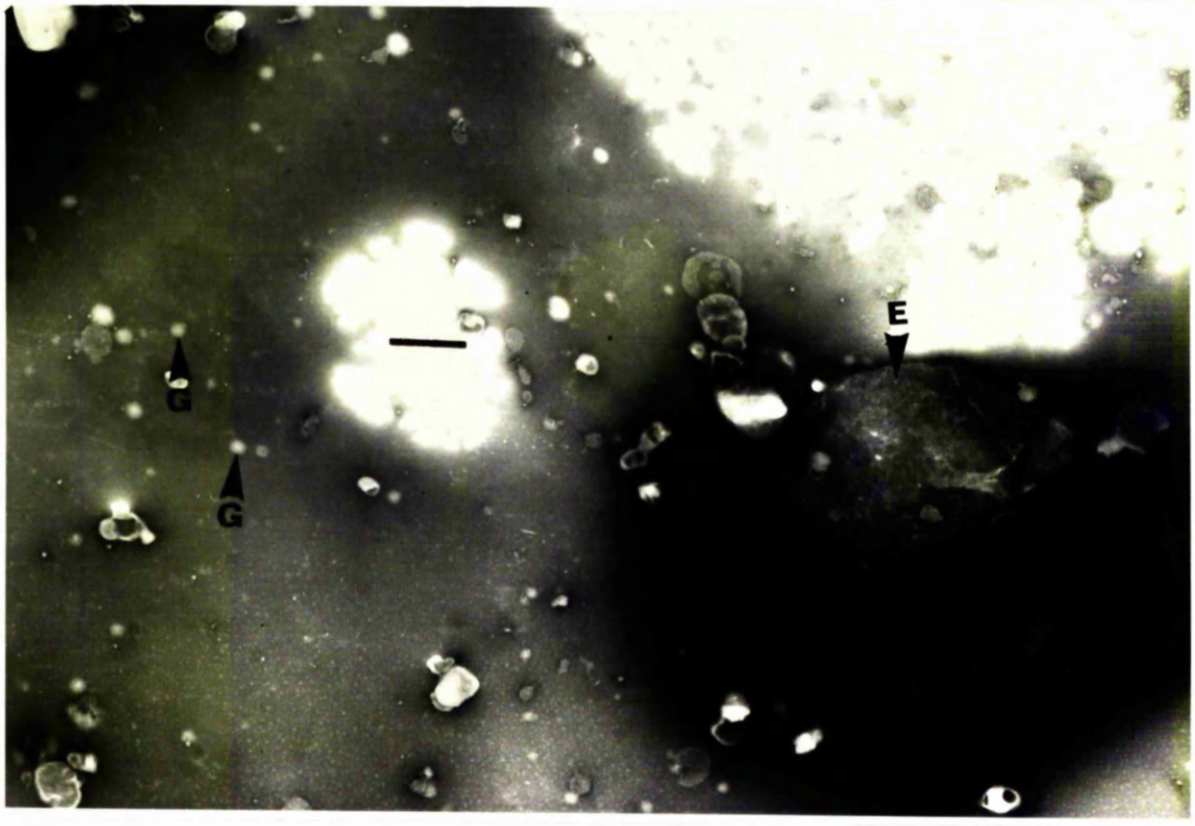

G
G

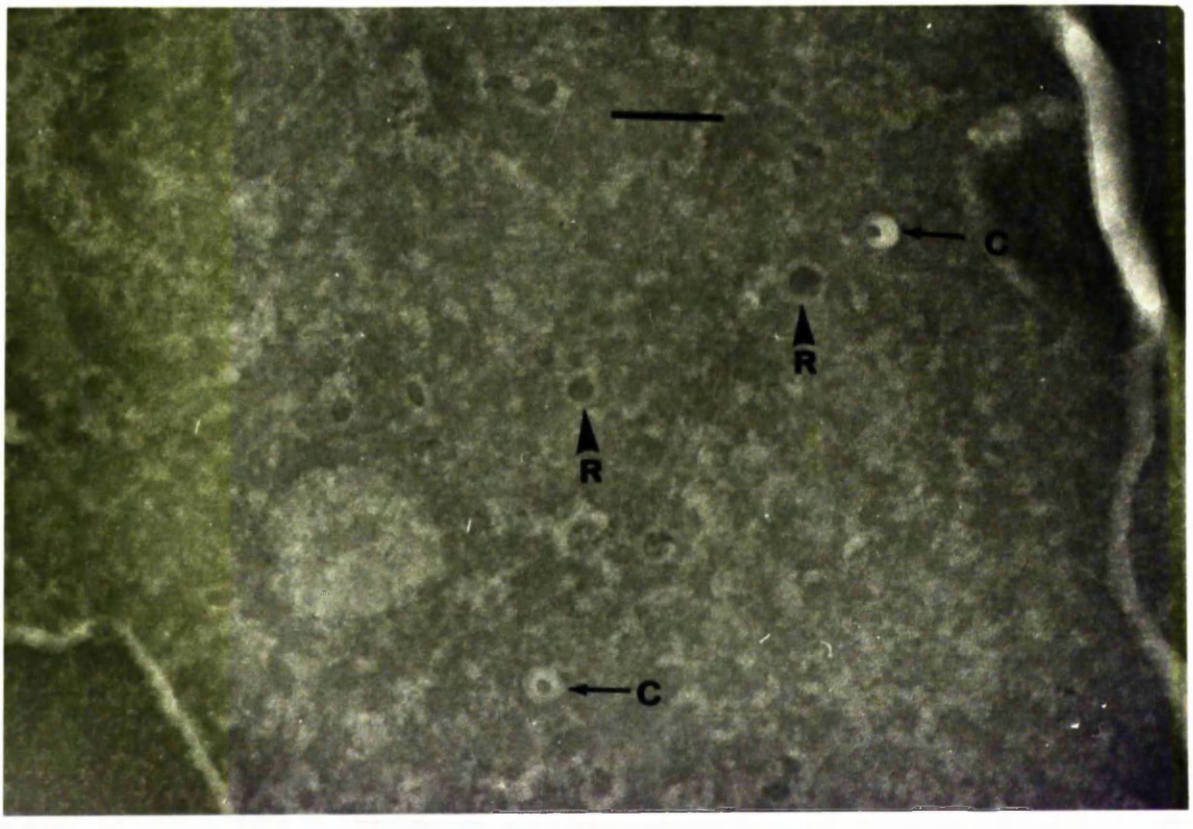
Plate 12 : Electron micrograph of horse erythrocyte ghost treated with α-toxin (20 µg α_A + 0.2 ml ghosts). Incubation at 37°C for 45 min. Ca^{2+} = 2.5 mM. Note the release of electron transparent globules (G) from the erythrocyte ghosts (E). X 18,870.

Dimension marker = 500nm

Plate 13 : Electron micrograph of a human erythrocyte ghost treated with θ-toxin (520 HU to 0.2 ml ghosts.) Control ghosts as in Plate 8 . Electron transparent ring structures of diameter 50–70 nm with a border 3 – 5 nm wide were formed (R). These ring-like structures were not observed on control preparations. The rings or pitlike infoldings as found on control ghosts are arrowed. (C) X 153,900.

Dimension marker = 100nm


E
G
G


C
R
R
C

(5) The electron transparent rings that were identified on control human and horse ghosts were still readily visible after α-toxin treatment.

2. Interaction of Electrofocused θ-Toxin with Ghosts and Cholesterol.

Ghosts were prepared from human "O" erythrocytes as previously described. 0.2 ml of packed ghosts was incubated with 0.2 ml of θ-toxin (520 HU) which had been reactivated as described on page 125 and dialysed overnight against distilled water to remove the cysteine hydrochloride. Control ghosts were treated with 0.2 ml of dialysed reactivation mixture without toxin.

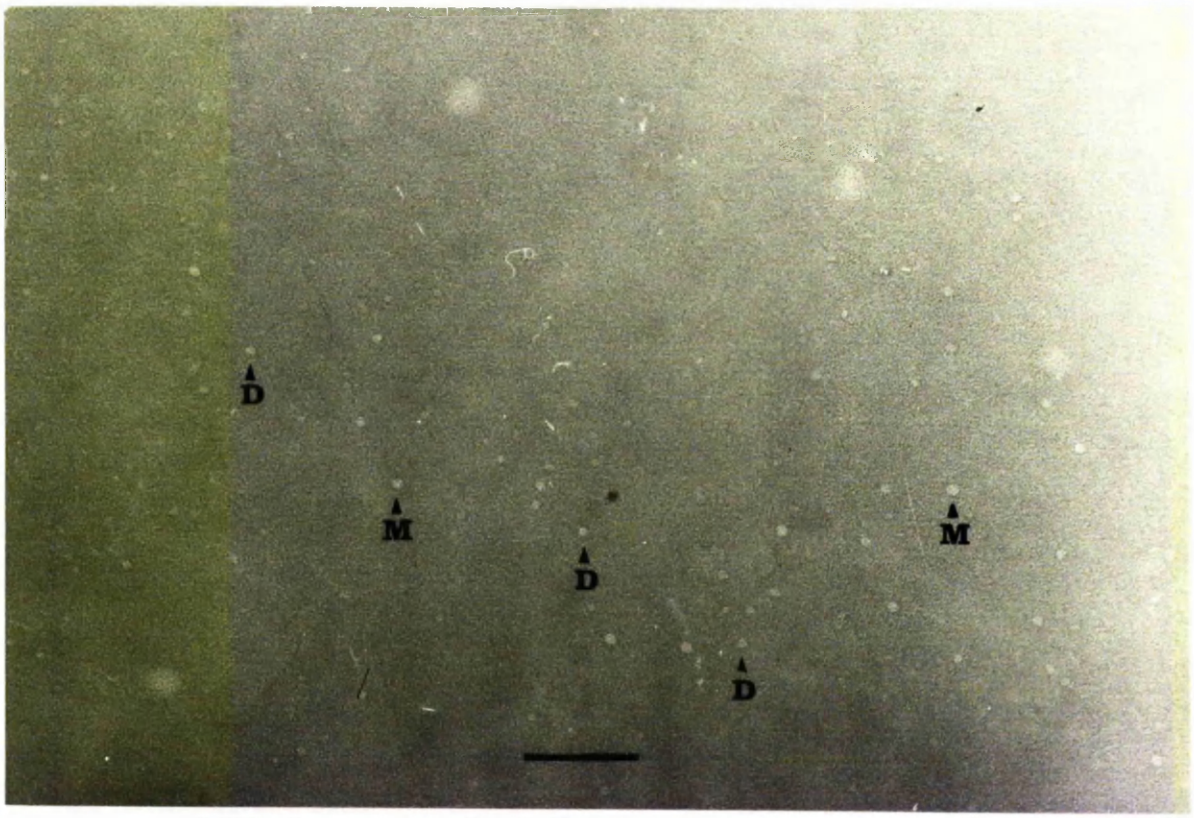
Examination of negatively stained preparations revealed the presence of ring-like structures of diameter 50-70 nm similar to those described by authors treating membranes with crude α-toxin (see page 250). This observation prompted an investigation of the interaction of θ-toxin with cholesterol. Cholesterol (0.5 g)-BDH reagent grade- was dissolved in 10 ml acetone with gentle heating and poured into 50 ml of boiling water. The resultant emulsion was filtered to remove non-emulsified cholesterol; 0.1 ml of this emulsion was treated with 0.1 ml of θ-toxin (260 HU) and the mixture incubated at 37°C for 10 min. Electron microscopy of this mixture revealed the presence of ribbons or groups of arc shaped structures possessing an apparent substructure. The arcs were 5.5-10 nm wide (Plate 14). The control cholesterol emulsion comprised droplets or micelles 5-13 nm in diameter similar to the substructural units (Plate 15).

Plate 14 : Electron micrograph of a cholesterol emulsion showing the formation of very small cholesterol micelles (M) some of which show an electron dense centre (D) Diameter of micelles = 5-13 nm. X 153,900.

Dimension marker = 100nm

Plate 15 : Electron micrograph of a cholesterol emulsion treated with θ-toxin (520 HU to 0.1 ml). Arc-like structures have formed which are 5.5-10 nm wide. Some possess a micellar substructure (M). X 153,900.

Dimension marker = 100nm


D
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D
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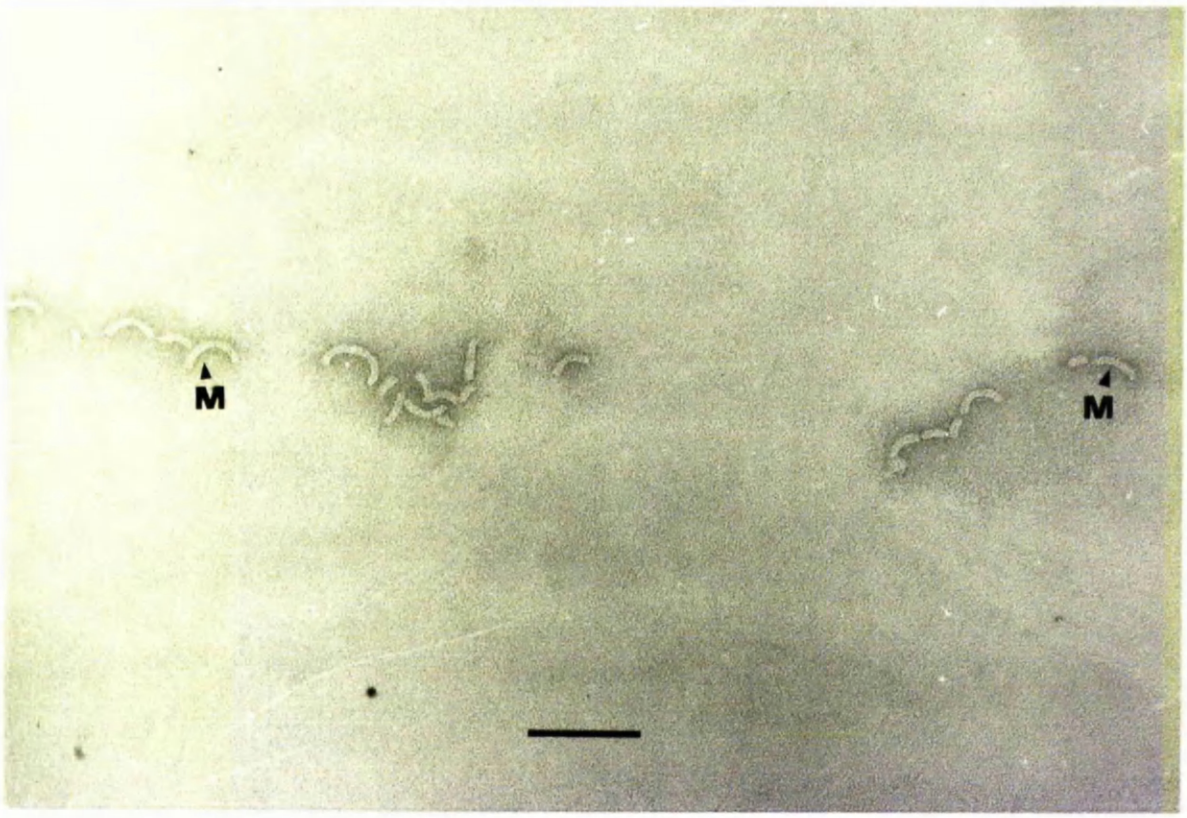

M
M

DISCUSSION

SECTION I : GROWTH AND TOXIN PRODUCTION

The growth conditions used in this investigation were developed to give maximal rate of growth in batch culture without impairing toxin yields. The maximum period of incubation was thus only 4 - 5 hours. The short incubation period had two other advantages :-

(a) A large number of samples could be taken over a short period of time to study toxinogenesis with growth, enabling one to set up several assays simultaneously for the same toxin.

(b) The harvesting of α-toxin from culture supernatant fluids by fractional ammonium sulphate precipitation could be executed easily within a prolonged working day and the resultant harvested material redissolved and dialysed overnight to enable electrofocusing of the toxin to begin the following morning.

The observation of a lag between the start of growth and α-toxin production in my system agrees with the findings of previous authors (see page 20). It is noteworthy that there has been but one previous report in which the production of more than one factor by type A cells was studied simultaneously during the growth cycle (Gale and van Heyningen, 1942), although Shemanova et al., (1970) studied the production of extracellular products individually in batch cultures of the same organism. Gale and van Heyningen grew strain S 107 in meat broth. No mention was made in this paper of the maintenance of this culture or the nature of the inoculum. They observed that θ-toxin appeared in the medium a short time before α-toxin and that both the

α- and the θ-toxin titres increased during growth until active cell division ceased. Thus although the cultural conditions are different my findings parallel these observations. However, by contrast with my findings, they observed an even longer lag in hyaluronidase production compared to α- and θ-toxin, although the lack of assays at regular time intervals as for α- and θ-toxin may obviate this conclusion. Collagenase had not been differentiated as a distinct component of culture filtrates of Cl. perfringens type A in 1942 (Oakley et al., 1946).

The discrepancy between my results and those of Gale and van Heyningen on hyaluronidase production may be a reflection of insufficient assays or differences in cultural conditions. It has already been noted that several workers have shown that hyaluronidase was inducible (see page 94). Thus it is possible that the presence of an effective inducer in my medium has influenced the time scale of production.

Ispolatovskaya (1971) stated that Mukhin reported in his dissertation thesis that deoxyribonuclease production paralleled that of lecithinase and that there was a correlation between the activities of both enzymes (see also Mukhin et al., 1966). Furthermore the work of Moss et al., (1967) showed that neuraminidase was produced during the logarithmic growth of type A cells. It would thus appear that more intensive investigation of toxinogenesis of this organism with time may reveal the significance of these factors in the physiology of Cl. perfringens.

SECTION II : ELECTROFOCUSING STUDIES

A. GENERAL CONCLUSIONS

Before dealing with aspects of electrofocusing as they apply to the results obtained with individual components of the toxic armoury of Cl. perfringens type A it is possible to draw some general conclusions about the suitability and applicability of this technique to the purification and preparation of individual components of the toxin complex.

In assessing the efficacy of a purification method several factors have to be borne in mind :-

(a) Capital costs

(b) Running costs

(c) Yields of individual components

(d) Purity of individual components

(e) Recoveries of individual components

(f) Ease of operation

(g) Speed of operation.

For a purification method to receive widespread usage and to be effective, factors (c) - (g) must in the end justify factors (a) and (b). In these respects this technique has clearly justified its application to the problem of toxin purification and has demonstrated the benefits to be gained from it in comparison with other techniques used by previous authors.

The isoelectric points and average recoveries of those components so far examined are summarised on Table 53 . It can be

Table 53 : Summary of Isoelectric Focusing Studies on the Toxins and Enzymes of Clostridium perfringens Type A

Toxin	Nature of Material		pI (± 1SD)	Average % Recovery of Activity
α-toxin *	AGX 1846	α_A	5.57 ± 0.04	27
		α_B	5.23 ± 0.09	
		α_{Urea}	5.52 ± 0.16	36 - 65
	S107/BP6K Toxin	α_A	5.49 ± 0.06	50 - 80
		α_B	5.25 ± 0.03	
θ-toxin	S107/BP6K Toxin		6.56 ± 0.13	72
Collagenase			4.54 ± 0.14	57
Hyaluronidase			4.73 ± 0.05	67
Neuraminidase	L2A Toxin		5.27	ND

ND = Not determined

* = See Table 32

SD = Standard deviation

readily seen that these principal extracellular products of Cl. perfringens type A are all acidic proteins with pIs between 4.50 - 6.50. This observation in no small way accounts for the difficulties experienced by previous authors in applying more conventional electrophoretic techniques to the purification of these products. The major problems have always been incomplete separation of components and the relatively small amounts of material that could be processed at any one time. On the latter score electrofocusing gains appreciably over other techniques. It not only allows the processing of gram quantities of material at a time, but also guarantees a 100% recovery of total material applied. Maximum separation of the components in Table 53 was obtained using a pH gradient from 4 - 6 giving optimum expansion of the pH scale range in which these interesting factors were found (see Figure 21).

It must be emphasised that although total recoveries of each toxin or enzyme studied were good, the material applied to electrofocusing columns had been processed to obtain maximum yields of α-toxin. As can be seen from Tables 29 and 30 the fractional ammonium sulphate precipitate chosen resulted in high yields of α-toxin from the culture medium supernatant fluids at the expense of recovery of other components. Thus the total overall recoveries of other factors obtained in this investigation are not necessarily reflections of the optimum yields that could have been achieved utilising suitably selected concentrates for other individual components. Under appropriate conditions milligram yields of purified material are possible by this one step process. Only 4 - 5 days were required to

prepare highly purified α-toxin from the time of inoculation of the culture medium.

The results have shown that the major α-toxin component was immunologically homogeneous. SDS-polyacrylamide disc gel electrophoresis also indicated a high degree of purity although when 100 μg amounts of purified toxin were applied minor contaminants were detected. The nature of the minor impurities observed has not been elucidated. With θ-toxin a higher degree of homogeneity was obtained. The observation of multiple bands in immunologically homogeneous collagenase is discussed later.

Not only were good recoveries of highly purified individual components obtained, but a number of these components could be processed to a similar degree of purity simultaneously. Only Habermann (1958; 1959) has previously attempted such simultaneous purification studies, which involved a number of steps specific for certain toxins or enzymes. By comparison, electrofocusing allows fractionation of several factors by a single process.

Apart from purification these studies have allowed the determination of an important physical characteristic of each of the products, viz. their pIs (see Table 53). The accuracy of the isoelectric points of θ-toxin in preparative runs was diminished by the fact that in pH 4 - 6 gradients it focused where the pH gradient was fairly steep. Isoelectric points should only be determined, where possible, from values obtained in the linear portion of the gradient. The accuracy of the result is increased on narrow pH range gradients.

One drawback of the technique is the difficulty of removing the carrier ampholytes after electrofocusing. Vesterberg (1969) claimed that less than 0.5% of the initial carrier ampholytes remain in a normal dialysis tube after 48 hours of stirred dialysis at 4°C. This has not been the experience in this laboratory. Although the concentration of ampholytes fell to 6 - 8% of the original concentration in the first 24 hours of dialysis, further removal of carrier ampholytes was slow. Prolonged dialysis is not conducive to the maintenance of biological activity but is necessary because the carrier ampholytes react readily with Lowry reagents in the determination of protein. It was found that dialysis against 80% saturated $(NH_4)_2SO_4$ did not precipitate the carrier ampholytes, but gave good recovery of toxin. Furthermore it was possible to wash the precipitate with 80% saturated ammonium sulphate. This has proved to be the most satisfactory way of separating the desired product from the ampholytes.

In summary therefore it can be said that electrofocusing allows the preparation of highly purified homogeneous toxic components of Cl. perfringens type A in high yield, which are suitable for detailed biological studies. In the author's view, this approach to the problem of the fractionating crude toxin preparations more than justifies the expense of the apparatus and ampholines.

B. MULTIPLE FORMS OF PROTEINS

Before dealing with the finding of multiple forms of Cl. perfringens α-toxin, I intend to discuss possible explanations or causes of molecular heterogeneity in proteins and the effect urea could

have on their observation.

Heterogeneity or the occurrence of multiple forms has been observed in enzymes and proteins with widely differing functions. By 1961 more than 30 enzymes had been shown to exist in multiple forms within individual organisms (Gregory, 1961). The existence of multiple forms of enzymes poses many questions, such as the possible advantage they may confer on an organism, and their role in the life of the cell producing them. This phenomenon could be due to

(a) proteins possessing identical substrate specificity and catalytic function, but differing in chemical composition presumably with separate genes determining the primary structure of each form

(b) identical molecular replicas of the same protein existing in different forms due to binding to other molecules, aggregation or conformational changes

(c) artefacts of preparation and/or purification, such as altered, degraded or denatured forms of the same protein, retaining certain properties of the parent or native form.

Some aspects of these possibilities are now dealt with in greater detail, with particular emphasis on points bearing on the observation of multiple forms by the use of isoelectric focusing. Although there have been three recent reviews on the theory and application of the technique of electrofocusing, no author has so far attempted to review the problems associated with its use. Most stress is therefore laid on the possible causes of artefactual multiple forms.

1. Conformational Changes and Polymer Formation.

Examination of the amino acid composition of bacterial exoenzymes and exotoxins has revealed characteristic features. These proteins have :-

(a) a high content of aspartic and glutamic acids (aspartic acid + asparagine; glutamic acid + glutamine) and

(b) an exceptionally low cyst(e)ine content, with the exception of diphtheria toxin and possibly tetanus toxin (Raynaud, 1968).

Moreover, cell bound proteins of organisms producing extracellular proteins possess a cyst(e)ine content of the order of 3 times greater than that found in exoenzymes or exotoxins (Pollock and Richmond, 1962). Pollock (1962) supposed that exoenzymes would have a low molecular weight (MW < 80,000) and that possessing little or no cysteine and few or no disulphide cross-linkages, would have poor rigidity. He also proposed that the flexible structure of these molecules allowed them to diffuse relatively easily through biological membranes.

This suggests that the native conformation of exoenzymes tends towards an open rather than a compact three dimensional structure. Since they lack disulphide bridges, the principal forces responsible for protein conformation will be hydrogen bonding, coulombic forces and hydrophobic interactions. These may possibly result in a number of preferred stable configurations for a particular protein. Such molecular species are termed conformers.

Susor et al., (1969) concluded that the molecular heterogeneity of crystalline preparations of glyceraldehyde-3-phosphate dehydrogenase,

rabbit muscle aldolase A and yeast aldolase revealed by isoelectric focusing was consistent with the random formation of tetrameric or dimeric molecules from two similar, but non-identical sub-units in each case. However, they were not able to relate heterogeneity to sub-unit composition in the case of all proteins examined. Their results further revealed that crystalline proteins designated as homogeneous by accepted criteria (SDS disc gel electrophoresis, ultracentrifugation, immunoelectrophoresis) could exhibit heterogeneity on isoelectric focusing. However, these authors were careful to point out that some of the heterogeneity detected could have been due to degradation or modification of the protein during isolation and purification (vide infra).

At this stage it is requisite to consider the effect of urea on protein structure in relation to these possible molecular interpretations of multiple forms of an enzyme. High concentrations of urea destroy the non-covalent linkages in proteins responsible for the maintenance of the three dimensional configuration of the molecule, viz. hydrogen bonding, coulombic forces and to some effect hydrophobic interactions. This results in unfolding of the molecule or disaggregation (Reithel, 1963).

Ui (1971a; 1971b) has pointed out that such changes in the three dimensional configuration of a native protein could alter its isoelectric point provided that the native configuration obscured some dissociable groups within the interior of the molecule which became exposed in the unfolded molecule. He studied native and urea "denatured"

proteins as a means of assessing the state of dissociable groups in native proteins. No changes were observed in the pI of human haemoglobin or bovine insulin on isoelectric focusing in the presence of 6M urea, whereas exposure of human serum albumin in a similar manner reduced the heterogeneity observed. He proposed that "if the degree of heterogeneity is almost the same whether 6M urea is present or absent, it is conceivable that heterogeneity is due to variation in chemical structure", whereas "if heterogeneity is reduced ..... the participation of conformational heterogeneity might have to be considered".

However, the possibility that urea might cause a reduction in heterogeneity by disaggregation of the multiple molecular forms of an enzyme to a smaller number of unit molecular species cannot be excluded. In this respect it is unfortunate that Susor et al., (1969) did not test their aggregate hypothesis to explain multiple forms by carrying out isoelectric focusing experiments in the presence of 6M urea.

Furthermore the possibility of activation of soluble inactive aggregates by urea must not be overlooked. Arbuthnott et al., (1967) have demonstrated this phenomenon with staphylococcus α-toxin. Soluble inactive aggregate (α_{12S}) could be disaggregated by urea to yield active α-toxin (α_{3S}).

2. Factors Responsible for the Formation of Artefactual Multiple Forms of Proteins.

Whereas conformational changes and aggregation may be responsible for the occurrence of 'natural' multiple forms of a protein,

certain treatments give rise to artefactual multiple forms. In essence they involve an alteration in the overall charge of the molecule.

a. Carbamylation of Proteins by Cyanate Formed in Urea Solutions:

The 'Analar' urea used in electrofocusing experiments was stated to contain not less than 99.5% urea. Nevertheless, cyanate is formed in concentrated urea solutions by the reaction A shown in Table 54. It can be removed slowly by the other reactions shown in the same table. There is a linear relationship between cyanate formation and urea concentration and the reaction is temperature dependent. Hence the need for care in the preparation of urea solutions for electrofocusing experiments (see Appendix IV).

Cyanate is known to react readily with free amino groups in proteins as is shown in Figure 33. Carbamylation causes a decreased positive charge on proteins, because the reaction product is essentially a substituted urea, the amino groups of which do not dissociate. Thus carbamylated proteins have a pI lower than that of the corresponding native proteins as the pH would have to be lowered to protonate free carboxyl groups to reduce the net negative charge on the protein to zero.

b. Deamidation:

The amino acid amides glutamine and asparagine are widely distributed in proteins. The amide amino groups of these molecules are more susceptible to hydrolysis than other amino groups. For example, as a result of this lability these amino acids cannot be determined directly in acid or alkali hydrolysates of proteins, but are recorded as total glutamic and aspartic acid respectively.

Table 54 : Formation and Hydrolysis of Cyanate in Urea Solutions

A. Formation

$(NH_2)_2\ CO \longrightarrow NH_4^+ + NCO^-$

urea — ammonium ion — Cyanate

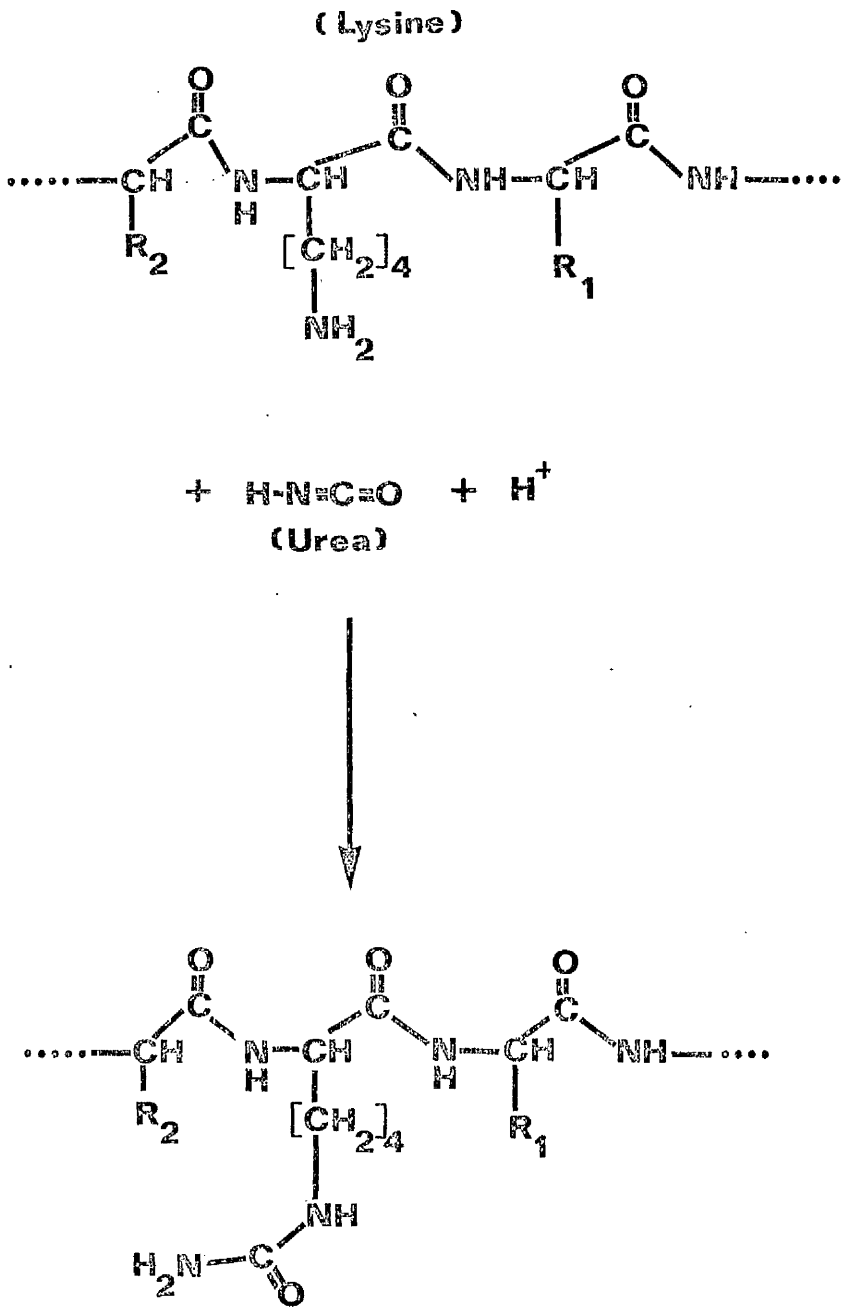
B. Hydrolysis

$HNCO + H_3^+O \longrightarrow NH_4^+ + CO_2$

$HNCO + H_2O \longrightarrow NH_3 + CO_2$

$NCO^- + 2H_2O \longrightarrow NH_3 + HCO_3^-$

Figure 33: CARBAMYLATION of AMINO GROUPS by UREA



Deamidation may arise through isolation and purification of proteins and even by storage in the frozen state, e.g. by storage of insulin at -15°C for 2 to 3 days (Berson and Yalow, 1966). It can also occur through lyophilisation (Lewin, 1969). Freezing and thawing of the main component of lactate dehydrogenase (LDH) gave rise to proteins having the same electrophoretic mobility as the isozymes of LDH (Chilson et al., 1965). Carlstrom (1966) and Carlstrom and Vesterberg (1967) proposed that the heterogeneity obtained with cow's milk lactoperoxidase (LPO) on isoelectric focusing could be explained by deamidation of glutamine and asparagine residues. They suggested that LPO 1 corresponded to the "fully amidated" molecule and that by the loss of one or both of two labile amide amino groups, LPO 2 or LPO 3 respectively were formed. LPO 4 and LPO 5 arose by subsequent hydrolysis of a third and fourth amide residue. The difference in pI between these forms of LPO was of the order of 0.1 -> 0.2 pH units and could in the opinion of Carlstrom and Vesterberg reflect differences of one unit of charge between each sub-fraction of LPO. Similar observations have been made with cytochromes (Flatmark and Vesterberg, 1966). Carlstrom considered that his multiple forms of LPO resulted from storage of milk in the udder above 35°C for many hours in the presence of hydrolytic enzymes, as opposed to transformations occurring during preparative manipulations at 4°C under mild conditions.

Thus deamidations can cause a decreased positive charge and a lowering of the isoelectric point of the protein. The change in the isoelectric point will depend on the positioning of the amide group within the protein molecule and its function. If it is buried within

the interior of the native protein, hydrolysis will most likely not cause a conformational change; but as amide groups can form strong bonds with other groups in a protein molecule (Tanford, 1962) hydrolysis of readily accessible amide groups could cause such changes.

c. Loss of a Carbohydrate Moiety:

Carlstrom (1969) has presented evidence which suggested that the conversion of the major subfractions of the LPO B-group** to the corresponding subfractions of the LPO A-group** of lactoperoxidase involved a change in the carbohydrate content of each. The LPO A-group contained less mannose, glucosamine and galactosamine. The author proposed that these sugars comprised a heterosaccharide residue which was bound to the protein moiety by a β-aspartyl-glycosylamine linkage involving the amide group of asparagine (Neuberger and Marshall, 1966). Hydrolysis of the amide group of the asparagine bound to the heterosaccharide would cause a conversion of the type LPO B ---->LPO A whilst hydrolysis of amide groups not linked to any carbohydrate would cause conversion within the LPO B-group (vide supra). This LPO B ---> LPO A conversion could be obtained by dialysis against cold distilled water for several days or incubation under alkaline conditions at room temperature. The LPO A components had lower isoelectric points than their corresponding LPO B-group subfractions.

** The heterogeneity of LPO originally described by Polis and Schmukler (1953) by moving boundary electrophoresis in acetate buffer pH 5.0, $\mu = 0.1$. LPO was separated into two main components LPO A and LPO B with mobilities equal to 3.94 and 2.85 x 10^{-5} cm $^{2}V^{-1}$. LPO B comprised LPO 1 - LPO 5.

d. Changes in the Charge of Metallic Ions Associated with Proteins:

Many proteins contain metal ions which are essential for the biological function of the macromolecules e.g. alkaline phosphatase, carboxypeptidase, insulin and myoglobin (Mb).

Vesterberg (1967) carried out electrofocusing experiments on both the ferri-Mb and ferro-Mb from horse skeletal muscle. The latter was less stable than the ferri form and susceptible to oxidation to the ferri form. The difference in pI between the ferric and ferrous forms was of the order of 0.5 pH units. The chemical agents employed to reduce ferri-Mb to ferro-Mb did not cause detectable changes in the protein moiety of the molecule, but only a shift in the valency of the iron from 3 to 2. Vesterberg concluded that the change in pI was reflective solely of the change in the charge on the metal ion of iron in myoglobin.

e. Lyophilisation:

For a summary of the possible consequences of freeze-drying the reader is referred to "Multiple forms of α-toxin", page 46 of this thesis.

f. Electrolysis:

French workers have recently made observations with the copper-containing protein ceruloplasmin that have led them to propose that the technique of electrofocusing per se can give rise to the formation of multiple forms (Pejaudier et al., 1971). This represents an isolated observation which may be extremely important but its general applicability cannot yet be assessed.

3. The Effect of Sucrose on Proteins and their Isoelectric Points.

It might be asserted that high concentrations of sucrose could cause conformational change in a protein by dehydration. There is no evidence in the literature for such an effect. Vesterberg (1967) electrofocused myoglobin to examine the possible dependence of observed pI values on sucrose concentration. No significant differences in the pI of myoglobin components were observed, whether they focused in areas of the pH gradient with high or low sucrose concentrations. However, although the influence of sucrose concentration can be neglected in the case of myoglobin, it may be dangerous to extrapolate these data to other proteins as has been done by most workers in the field. Vesterberg has proposed that all pIs should be checked by electrofocusing under conditions of reversed polarity, to avoid possible errors arising from the presence of sucrose.

C. MULTIPLE FORMS OF α-TOXIN

Results reported in this thesis indicate the presence of two forms of the α-toxin of Cl. perfringens type A which have been termed α_A and α_B. On isoelectric focusing these forms had pIs of 5.49 ± 0.06 and 5.25 ± 0.03 respectively. At no time was heterogeneity observed in broad pH gradients either with preparations from strains S 107 or BP6K (see Figures 13 and 20 and Table 37), or Wellcome toxin AGX 1846. In these broad gradients the α-toxin focused as a single peak of activity with a pI between 5.10 and 5.50. The two forms described would not be detected under the electrophoretic conditions of these broad gradients as the pH of the gradient rises by approximately 0.2 pH units/2 ml fraction collected. Even under optimal separation conditions this would

mean that α_A and α_B would appear in neighbouring fractions from such gradients. Similar results have been obtained with the staphylococcus enterotoxin by Chang and Dickie (1971). The two major components had isoelectric points of 8.55 and 8.25 on narrow pH gradients with the same pH difference as observed with the α_A and α_B forms of Cl. perfringens, but were not resolved on a broad pH gradient. In the AGX 1846 preparations of α-toxin obtained from Wellcome Laboratories these two forms were shown to be associated with egg-yolk turbidity, hot-cold haemolytic and lethal activities, thereby confirming and extending the observation of Bernheimer et al., (1968a) using a virtually identical preparation. Furthermore, these results provide strong evidence in favour of the unitarian hypothesis of van Heyningen (see page 72).

Moreover, the presence of both of these components has been demonstrated in freshly prepared toxin derived from strains S 107 and BP6K. That the form possessing the higher pI was the main component of α-toxin was evidenced by :-

(a) the observation that in freshly prepared toxin this form constituted some 80-90% of the total α-toxin activity recovered, with c.20-30% of this appearing in the peak fraction of α_A

(b) it was the only form observed in focusing experiments carried out in the presence of 6M urea.

For these reasons this form was given alphabetical precedence and designated α_A. This is also consistent with the nomenclature adopted by MacNiven et al., (1972) for multiple forms of staphylococcus α-toxin. These observations immediately pose the question of the nature of α_B and its relationship, if any, to α_A. Is α_B a mere artefact of

preparation? If so, how does it arise? Or is it a unique molecular species of α-toxin, an isozyme of phospholipase C?

The lower pI of α_B could be consistent with its formation from α_A by (i) deamidation (ii) loss of a carbohydrate moiety or (iii) reduction of a metal ion associated with the protein. Lyophilisation is known to cause deamidation as is purification or ageing. Indeed α_B has been identified in preparations that have undergone such treatments. It is also possible that α_B could arise in the culture medium by the action of hydrolytic enzymes acting on α_A by analogy with the effect of storage of LPO in the udder of the cow prior to harvesting. No metal ion was identified in α-toxin preparations examined by atomic emission spectroscopy, so that changes associated with reduction of metal ions are improbable on the basis of results in this investigation. Analysis for the presence of hexose sugar by the anthrone method proved negative. Nevertheless, much as these possibilities could account for experimental results, (i) and (ii) would result in a permanent change in the overall charge of the molecule.

In the presence of urea the α_B form disappeared. There are two possible explanations of this effect :-

(a) that α_B was more susceptible to the action of urea than α_A and was completely inactivated by such treatment, thereby leaving only detectable α_A activity

(b) that α_B was converted by urea into the α_A form.

Ispolatovskaya and Klimacheva (1966) showed that α-toxin was stable to the action of urea. Also, it is doubtful that the identical recoveries of egg-yolk activity of α-toxin in the presence and absence of urea were

produced by the urea stabilising the α_A form whilst inactivating the α_B form. The fact that dialysis of α_{Urea} to remove the urea resulted in the reappearance of α_B is *prima facie* evidence of the convertibility of α_A and α_B. It must be concluded therefore that α_B is related to α_A other than as an artefact since the convertibility of these forms cannot be reconciled with possibilities (i) and (ii) (*vide supra*).

Carbamylation in the presence of urea can be excluded on the grounds that it would have caused a decrease in the observed pI to a value less than that of α_B.

Thus in reconsidering the relationship between α_B and α_A two distinct possibilities remain :-

(i) α_B is an aggregate of α_A

or (ii) α_B is a conformer of α_A.

In the former it must be assumed that positive charges would be blocked or obscured in some way as a result of protein-protein association. Decreased positive charge would be accomplished in the latter case by shielding of dissociating groups as a result of folding of the molecule. The shielded groupings would then be re-exposed by disaggregation or unfolding respectively. Neither the immunological nor the molecular weight studies provide conclusive evidence to favour either hypothesis.

The total recovery of haemolytic and lethal activities was increased in experiments carried out in the presence of 6M urea; no comparable increase was noted for egg-yolk activity. Although causing no apparent change in overall charge, it is conceivable that the urea could still have unfolded the α_A form by disrupting hydrogen bonding.

In this condition the α_A molecule in urea may have increased membrane penetration properties which would only show up in the haemolytic and lethal assays. This hypothesis is consistent with the proposals of Pollock (1961) on exoenzyme structure and could explain the observed discrepancies in yield.

1. Observations made during Preliminary Studies.

Reference to Table 32 reveals that those preparations examined during preliminary studies which had undergone a fair degree of purification prior to electrofocusing, i.e. (B) and (C), exhibited an α-toxin peak at a pI between 5.00 - 5.10. It now seems highly probable that this arose as a result of deamidation of the α-toxin preparations either by prolonged exposure to 70% methanol or dilution on Sephadex G-100; moreover, in these purified states the protein may have been more susceptible to deamidation by dialysis. The absence of this form in experiment (A) of the same Table 32 with toxin from strain S 107 that had merely been precipitated with ammonium sulphate adds weight to this conclusion.

2. The Relationship of Current Observations on Multiple Forms to those Previously Described.

My findings concur with the observations of Russian and Polish workers reviewed on pages 46 - 50 in that :-

(a) freeze dried toxin contained two peaks of α-toxin activity (Ispolatovskaya and Levdikova, 1962)

(b) multiple step purification or storage led to the observation of multiple forms whereas fresh preparations were homogeneous (Ispolatovskaya, 1964; Uspenskaya and Meduski, 1957).

The observation of α_B in fresh preparations in this thesis can perhaps be explained by the large quantity of material examined in preparative scale work (see Figure 23) whereas Uspenskaya and Meduski and Ispolatovskaya and Larina (1959) could only examine milligram quantities of crude toxin by the techniques used. The complex heterogeneity observed by Ispolatovskaya (1964) and Uspenskaya and Meduski (1957) could in the light of my review on possible causes of multiple forms have been caused by deamidation due to lyophilisation, storage and complex purification procedures.

In summary, my results have shown that α-toxin can exist in two forms, but only further work on preparative quantities of these individual forms will resolve the question of their molecular relationship. Optical rotary dispersion and electrometric hydrogen ion titration curve analysis may reveal data relevant to conformation of these forms, whilst amino acid analysis, ultracentrifugation in the presence and absence of urea, and Sephadex gel filtration chromatography may confirm or disprove aggregate formation.

3. SDS-Polyacrylamide Disc Gel Electrophoresis of α-Toxin.

It was shown by Pitts-Rivers and Impiombato (1968) and Reynolds and Tanford (1970) that for those proteins so far studied in detail identical amounts of SDS were bound on a g/g basis with protein, under the conditions used for SDS-disc gel electrophoresis. It has been assumed that the SDS anions swamped all charge effects on proteins so that the proteins migrated according to the electrophoretic charge determined by the SDS bound to the protein. Under these conditions of

electrophoresis the unique structural configuration of proteins was lost as the SDS induced a conformational change in the protein which resulted in a uniformity of hydrodynamic shape. Mobility in the polyacrylamide gel was therefore determined by the molecular weight (MW). Preparations of α_B were shown to contain a component with an MW identical to that obtained with α_A preparations. However, that α_A and α_B may be related as conformers rather than by aggregation of α_A cannot be concluded from the use of this technique alone as SDS is also known to dissociate hydrophobic bonding between protein molecules. Thus the MW of a protein determined by SDS-gel electrophoresis may be the monomeric MW of sub-unit proteins or the unit protein MW of aggregated proteins. Therefore although the α_B component had a molecular weight identical to that of α_A the method does not clearly differentiate between a conformational or polymeric relationship.

The identical pI obtained for α_A on isoelectric focusing in the presence and absence of urea interpreted in terms of the proposals of Ui (1971a; 1971b) would indicate that this molecule had not undergone a change in conformation by exposure of masked dissociating groups within the molecule and that the molecule had a fairly open structure consistent with the view of Pollock (1961). The MW of α_A determined in this thesis (53,800 $\pm$ 1,300) is in good agreement with that determined by Shemanova et al., (1968) of 51,200 by ultracentrifugation. These MWs raise the possibility that α_B could be a dimer of α_A with an MW close to that determined by Meduski and Volkova (1958) with freeze-dried preparations (107,000). Yet, in their report, Bernheimer et al., (1968a) stated that their two forms of α-toxin had identical effluent volumes

on Sephadex G-75 gel filtration chromatography indicating that their MWs were similar; this lends support to the possibility that α_B is a conformer of α_A. Unfortunately no mention was made of the actual MW of their multiple forms which is important in view of the earlier finding of Bernheimer and Grushoff (1967) of a lower MW than that determined in this investigation. Sugahara and Ohsaka (1970) also failed to report on the MW of the two forms of α-toxin they observed during their electrofocusing study.

4. Serological Relationship of α_A to α_B.

Both α_A and α_B gave only one precipitin line on immuno-electrophoresis against standard antiserum. Furthermore, they produced a line of identity in Ouchterlony double diffusion gel tests. This indicates that these molecular species possessed common antigenic determinants, but does not exclude the possibility that the molecules were dissimilar in other respects. Further studies with monospecific antisera raised to homogeneous preparations of both of these forms of α-toxin could reveal differences in structural features which might act as antigenic determinants. These could be revealed by cross absorption neutralisation studies. Sugahara and Ohsaka (1970) proposed that failure of some antisera to neutralise α-toxin as effectively as others may be explained by the presence of these two forms of α-toxin.

5. Substrate Specificity of α_A and α_B.

Within the limits of the method used it does not appear that the α_A and α_B forms possessed differing substrate specificity or that either of them was a specific sphingomyelinase. Both forms hydrolysed

sphingomyelin and lecithin in the presence of Ca^{2+}. These findings agree with the recent electrofocusing studies of Sugahara and Ohsaka (1970) confirming the existence of two molecular forms of α-toxin. In their study both forms possessed the ability to hydrolyse these substrates and peaks of lecithin and sphingomyelin hydrolysing activity coincided.

It is difficult to reconcile my findings and those of Sugahara and Ohsaka with the observations of Macchia and Pastan (1967), Macchia et al., (1967) and Pastan et al., (1968). Substrate specificity tests were only carried out in my experiments on fractions with peak α-toxin activity. Only those fractions which possessed α-toxin activity were screened by Sugahara and Ohsaka (1970) for the ability to hydrolyse sphingomyelin and lecithin in the presence of Ca^{2+}. Thus both of these approaches have precluded the possibility that had every fraction from the electrofocusing column been screened for specific sphingomyelin hydrolysing activity in the presence and absence of Ca^{2+} such activity might have been detected elsewhere in the pH gradients.

If this possibility is not substantiated by experiment, several other possibilities remain. Macchia and Pastan, Sugahara and Ohsaka and myself have all been working with the same strain, BP6K, although it is possible that mutants may have been selected by sub-culturing. If one considers the possibility that phospholipase C possesses different attachment sites for lecithin and sphingomyelin, a mutation leading to the incorporation of a 'wrong' amino acid might cause a conformational change in the tertiary structure of the enzyme such that only the site of attachment of lecithin would be altered leading to inactivity on that substrate.

Both forms of α-toxin require to be tested for their ability to stimulate the oxidation of glucose by thyroid slices and the incorporation of inorganic phosphate into them. According to the experimental results of Macchia and Pastan (1967) neither α_A nor α_B should affect thyroid slices.

The studies of Zamecnik et al., (1947) showed that α-toxin did not hydrolyse glyceryl-phosphoryl choline, indicating that the non-polar substitutions on the α' and β carbons of the lecithin molecule (see Figure 1) played some role in determining substrate - enzyme interaction, although the ionic end group provided the major specificity of these molecules. Chain length and degree of saturation of the fatty acid substituents have been implicated by Graf and Stein (1966). They showed that α-toxin preferentially attacked lecithin molecules containing linoleic acid rather than arachidonic acid. Moreover it attacked β-oleoyl lecithin preferentially to β-linoleoyl (see also Coleman et al., 1970). Pastan et al., (1968) demonstrated that α-toxin hydrolysed egg-yolk lecithin more readily than dipalmitoyl lecithin. Furthermore the studies of van Golde et al., (1967) have shown that individual phospholipids within a single species can exhibit a high degree of heterogeneity. (See also Meduski et al., 1956; Gray and Macfarlane, 1959; de Haas and van Deenen, 1963; Glende and Cornatzer, 1966; Dyatlovitskaya et al., 1967b; Rosenberg and Condrea, 1968). Thus in assessing the substrate range of α-toxin I feel that a more guarded appraisal of substrate range is required.

Matsumoto (1961) noted that higher concentrations of substrate were required in the case of sphingomyelin and cephalin before hydrolysis

by α-toxin was marked. In addition Pastan et al., (1968) were considering the relative rates of hydrolysis of substrates under varying conditions, a more accurate means of assessing substrate specificity. However, even kinetic measurements of hydrolysis presented problems as sphingomyelin hydrolysis tailed off very quickly after an initial linear phase whereas lecithin hydrolysis remained linear over much longer periods (Matsumoto, 1961; Pastan et al., 1968).

A much more intensive investigation of the substrate-enzyme relationships of α_A and α_B is required, together with inhibitor studies, before it can be established conclusively that one or other of these forms is or is not related to the sphingomyelinase of Macchia and Pastan.

Finally it is not impossible that the sphingomyelinase of these authors is only produced under certain cultural conditions and that its identification in crude concentrates may therefore depend on the particular medium used. In this respect it must be noted that the medium of Macchia and Pastan (1967) differed from that used by Sugahara and Ohsaka (1970) and myself. Indeed Pastan et al., (1968) indicated that preliminary studies had demonstrated that growth of Cl. perfringens BP6K on a medium enriched with sphingomyelin increased the activity of sphingomyelinase. I have been unable to trace any subsequent report on this interesting footnote.

6. Neuraminidase in α-Toxin Preparations.

The detection of a second precipitin line on immunoelectrophoresis of fractions associated with neuraminidase activity occurring

between the peaks of α_A and α_B activity (see page 203) confirms that some major antigen of Cl. perfringens type A other than collagenase, hyaluronidase and θ-toxin focuses in this region of pH gradients. Thus far it has not been possible to conclude with certainty that this second precipitin line is associated with neuraminidase. Nor can it be asserted that the protein band with a molecular weight of 60,400 $\pm$ 1,100 observed during SDS-polyacrylamide disc gel electrophoresis of α_B and intermediate α-toxin peaks observed in preparative electrofocusing experiments is neuraminidase although the higher molecular weight than α-toxin is in agreement with other observations (Satterlee and Walker, 1969).

However it is hoped that collaborative studies at present under way with Drs. Collee and Fraser of Edinburgh University will eventually lead to an appropriate conclusion as to the nature of these observed components.

The importance of assessing the presence of neuraminidase in α-toxin preparations is emphasised by the observation of neuraminidase in association with a peak of α-toxin activity in preparative electrofocusing experiments (see Figure 23). This enzyme has only been previously tested for in α-toxin preparations by Stephen (1961), although Habermann (1958) reported its non-separability from hyaluronidase. The release of NANA from the surface of erythrocytes reduces the net negative charge on the cell surface. This could conceivably allow the α-toxin to approach its target areas more readily. Thus neuraminidase could accentuate the effect of phospholipase C on the erythrocyte membrane. Alternatively the neuraminidase could weaken the erythrocyte

membrane in such a way that lysis of the cell occurs when a smaller percentage of the total membrane phospholipid has been hydrolysed. This may provide a possible explanation for the occurrence of this prominent intermediate peak of α-toxin activity detected by hot-cold haemolysis. These findings justify the assertion that synergism may occur between neuraminidase and phospholipase C. Of note in this respect are the observations of Rodbell (1966) and Rodbell and Jones (1966) who observed that whereas neuraminidase had no effect per se on 'naked' isolated fat cells (see page 64), their α-toxin preparation stimulated glucose and amino acid metabolism. Their studies were not however extended to include the effect of both agents together on such cell preparations.

7. Zn^{2+} Content of α-Toxin.

In contrast to the observations of Ispolatovskaya and Klimacheva (1966) and Ispolatovskaya (1967), atomic emission spectroscopy of α-toxin prepared in this laboratory failed to reveal the presence of spectral lines characteristic of Zn^{2+} or the presence of any other metallic ion.

No obvious explanation of this discrepancy between my findings and those of the Russian workers can be offered at present except to say that my precautions against extraneous contamination of samples with metallic ions were more rigorous than the procedures used by Ispolatovskaya (1967). In view of the lack of evidence for the presence of Zn^{2+} in my studies it would be interesting to test the effect of OP and EDTA on the activity of α-toxin provided by isoelectric focusing.

Ispolatovskaya (1967) provided no quantitative data on the weight of each sample examined which led to the observation of spectral lines. In a study of B. cereus phospholipase C, Ottolenghi (1964) was able to detect as little as 0.6 μg of Zn^{2+} in 450 μg of protein (1 part in 760 i.e. 1.3 μg/mg protein). From his data and that of Johnson and Bonventre (1967) the molecular weight of B. cereus phospholipase C appears to lie between 40,000 - 70,000, approximately in the same MW range as Cl. perfringens α-toxin. On the basis of the molecular weight for α-toxin determined in this thesis (53,800) and assuming 100% purity and 1 Zn^{2+} per protein molecule, one would have expected to find 1.2 μg Zn^{2+}/mg protein. Thus although the quantities of protein examined in the case of AGX 1846 preparations would place the theoretical quantity of Zn^{2+} near the limit of sensitivity of the technique (0.1 - 0.2 μg) the BP6K and S 107 preparations examined should have contained quantities well above this limiting value i.e. approximately 4.2 μg and 1.0 μg respectively (see Table 41).

D. θ-TOXIN

The pI of θ-toxin determined here was 6.56 ± 0.13. This is close to the value of 6.50 obtained by Bernheimer et al., (1968a) for the pI of cereolysin, the oxygen labile haemolysin produced by B. cereus. The recovery of 70% of cereolysin activity by these authors is comparable with that found for θ-toxin. Moreover, cereolysin could be refocused with good recovery in broad pH gradients, but not in narrow pH gradients cf. θ-toxin page 212 . Somewhat more complex results were obtained with SLO (Bernheimer et al., 1968a). Again haemolytic activity was associated with a protein peak at pH 6.50, but the bulk

of the haemolytic activity migrated close to the anode in a pH 6 to 8 gradient. Of significance in this respect may be the observation of Halbert (1970) that SLO had a pI of 5.8 after dissolution in or dialysis against glycine. Unfortunately Bernheimer et al., did not extend their studies to include electrofocusing of SLO in broad pH gradients, or refocusing studies.

Nevertheless, there is sufficient evidence from my work and theirs to propose that oxygen labile haemolysins may have physical features in common in addition to the biological characteristics listed in Table 15. Their common pI may in some way explain some of these common features. An exception to this proposal has, however, already been demonstrated. Kreger and Bernheimer (1969) demonstrated that pneumolysin had a pI of 4.50 on electrofocusing in a broad pH gradient, but the pneumolysin used was obtained from whole bacteria by the lead acetate extraction technique of Cohen et al., (1942) as opposed to harvesting from culture supernatants as in the case of SLO, cereolysin and θ-toxin. This cell-associated form may differ from extracellular pneumolysin.

Bernheimer et al., and Kreger and Bernheimer did not report on the purity of their electrofocused oxygen-labile haemolysins. θ-Toxin obtained in the current study was serologically homogeneous although containing a minor additional protein band when examined by SDS polyacrylamide disc electrophoresis. The MW of 61,500 is similar to those estimated for other oxygen labile haemolysins (see Table 55). Thus in addition to having similar pIs these haemolysins appear to fall

Table 55 : Comparative Molecular Weights of Oxygen-Labile Haemolysins

Oxygen-labile	MW	Authors	Method
Streptolysin O	80,000	Alouf & Raynaud (1967)	gel filtration
	61,000	van Epps & Anderson (1968)	ultracentrifugation
	60,500	van Epps & Anderson (1968)	gel filtration
Cereolysin	54,000	Bernheimer & Grushoff (1967)	gel filtration
Tetanolysin	< 100,000	Hardegree(1965)	gel filtration
Listeriolysin	< 70,000	Jenkins & Watson (1971)	gel filtration
Thuringiolysin	c.50,000	Pendleton (personal communication)	gel filtration
Pneumolysin	63,000	Kreger & Bernheimer (1969)	gel filtration
θ-toxin	61,500	This thesis	SDS polyacrylamide electrophoresis

within a narrow molecular weight range. As a group they merit further comparative studies.

The "shoulders" on the acid side of the elution profile of θ-toxin invite comment. They could indicate heterogeneity but this aspect would form the subject of a separate study.

Finally, perhaps the one question that cannot be answered definitely from the electrofocusing studies on oxygen labile haemolysins so far is whether the pIs determined represent the pIs of the oxidised or reduced form of these haemolysins or whether both forms have the same pI. Nothing is yet known about possible changes that could occur in molecular structure as a result of activation with reducing agents. In my studies I was able to detect θ-toxin without prior activation especially in those fractions shown to be the peak fractions on activation. It would therefore appear that the reduced and oxidised forms of θ-toxin could have identical pIs. It could however be argued that the shoulders referred to above represent θ-toxin in stages of oxidation as they were detected by reactivation of the toxin. The observations of Bernheimer et al., (1968a) on SLO are relevant. The form of SLO having a pI of 6.5 may be the fully reduced form, whereas the activity that was more anodic may represent the fully oxidised form detected by subsequent reactivation. However, it must be remembered that SLO is found principally in the inactive or oxidised form in culture supernatant fluids, while the reverse is true in the case of θ-toxin and cereolysin. The incorporation of reducing agents such as dithioerythritol and mercaptoethanol into the electrode and gradient solutions should enable this problem to be investigated.

E. COLLAGENASE

No evidence was obtained in my electrofocusing studies to suggest the existence of heterogeneity in Cl. perfringens collagenase. In contrast such heterogeneity has been shown in other proteolytic enzymes of bacterial and fungal origin (Rebeyrotte and Labbe, 1969), although the contribution of lyophilisation to such observations cannot be excluded.

The collagen disc assay was introduced to confirm that the elution profile of azocoll digesting activity was associated with true collagenase activity. However this assay has since proved useful as a quick spot-test screening method. The azocoll method can then be used to quantitate collagenase activity. Purified preparations were serologically homogeneous but were notably heterogeneous when examined by SDS disc gel electrophoresis (see page 217). These results may be explained in two ways :-

(i) All the observed components in the peak fractions of collagenase activity were distinct high molecular weight proteins produced by the bacterium, possessing pIs so close as to render them inseparable by electrofocusing, but possessing different molecular weights, making them separable by SDS disc gel electrophoresis.

(ii) All 6 components were molecular species of collagenase with varying molecular weights which had arisen after electrofocusing by "auto-digestion" of a common parent molecule.

Possibility (i) seems improbable because of the high resolution of electrofocusing and the unlikelihood of one organism producing six extracellular proteins with identical pIs, all possessing high molecular weights.

On the other hand possibility (ii) is supported by certain data. Chymotrypsin is known to cleave chymotrypsinogen A and chymotrypsin to form a family of closely related active chymotrypsins (Reithel, 1963). Moreover the amino acid analysis data of Levdikova (1966) showed that Cl. perfringens collagenase contained some 7 moles % glycine and 2 moles % proline. It is therefore conceivable that the amino acid sequences susceptible to collagenase are present within the collagenase molecule itself (see Figure 4). Electrofocusing would favour autodigestion, if it does occur, since the technique concentrates proteins, thereby increasing the probability of protein-protein interaction. A study of activity decay on storage at $4^{o}C$ and refocusing of stored peak fractions requires to be done before these suggestions can be discarded or verified. The molecular weight range of these 6 components (107,000 - 132,000) is of the same order as the MW determined by Levdikova (113,000). However, Kameyama and Akama (1970) reported that their purified collagenase had an MW of 80,000 by Sephadex gel filtration. Clearly further studies are required to resolve this discrepancy.

The elution profile for both azocoll and collagen digesting activity coincided suggesting the absence of other proteolytic enzymes in the material examined. If present, such an enzyme must have a pI identical to that of collagenase. This possibility seems less likely in view of data available for Cl. histolyticum proteolytic enzymes; the collagenase had a pI of 5.57 whereas the γ-toxin (non-specific proteinase, cysteine-activated protease, clostripain, clostridiopeptidase B) was isoelectric at a pH between 4.80 - 4.90 (Mitchell, 1969).

Antisera raised against peak fractions of collagenase should facilitate the investigation of this problem.

Regarding the comparison of Cl. histolyticum and Cl. perfringens collagenase it must be noted that the difference in isoelectric points can be explained on the basis of the amino acid analyses of Levdikova (1966) on these two collagenases. Cl. histolyticum collagenase had a lower aspartic acid and higher lysine content which would make it the more cathodic.

F. HYALURONIDASE

In my experience it is often difficult to assess the purity of material prepared by electrofocusing unless it is produced on a large scale. This applies to hyaluronidase in particular since the kind of concentrate from culture supernatant fluids used for preparative scale experiments did not favour the harvesting of this enzyme (see Table 29). It can be seen that only about 6% of the total hyaluronidase activity in culture supernatant fluids was applied to preparative columns when 35-50% $(NH_4)_2SO_4$ saturation precipitates were employed. The small amounts of hyaluronidase obtained under these conditions were inadequate for studies on purity.

Heterogeneity of hyaluronidase was observed in preparative scale experiments. No satisfactory explanation can be offered for the difference between isoelectric points observed in broad and narrow pH gradients, but these findings require confirmation.

G. PROBLEMS OF TERMINOLOGY AND NOMENCLATURE IN ELECTROFOCUSING

Samuels (1961) proposed that the term "isoenzyme" or "isozyme" should only be applied to enzymes possessing identical substrate specificity, but different primary structures, whereas multiple forms arising through conformational or "non-nucleic acid mediated" changes which result in the formation of metastable states that can be changed back into the original form by varying conditions, should not be considered isozymes. Thus the principal difference between an isozyme and a conformer is that the former term is applied to molecular species possessing differing primary structures, with the latter being applied in the case of those with differing tertiary structures.

More recently the International Union of Biochemistry Commission on Biochemical Nomenclature has made recommendations on the nomenclature of multiple forms of enzymes (IUPAC - IUB Commission, 1971). Multiple forms of enzymes were grouped as shown in Table 56 . Genetically determined differences in primary structure are responsible for the multiplicity of groups 1 - 3, but not groups 4 - 7. They recommended that the term 'multiple forms of an enzyme' should be used as a broad term covering all proteins possessing the same enzymic activity and occurring naturally in a single species; the term "isoenzyme" or "isozyme" should apply only to those multiple forms arising from genetically determined differences in primary structure, and not to those derived by modification of the same primary sequence (i.e. Groups 1 - 3 of Table 56).

Table 56 : Multiple Forms of Enzymes

Group	Reason for multiplicity	Example
1	Genetically independent proteins	Malate dehydrogenase in mitochondria and cytosol
2	Heteropolymers (hybrids) of two or more polypeptide chains, noncovalently bound	Lactate dehydrogenase
3	Genetic variants (allelic)	Glucose-6-phosphate dehydrogenase in man
4	Proteins conjugated with other groups	Phosphorylase a and b
5	Proteins derived from one polypeptide chain	The family of chymotrypsins arising from chymotrypsinogen
6	Polymers of a single subunit	Glutamate dehydrogenase of MW 1,000,000 and 250,000
7	Conformationally different forms	All allosteric modifications of enzymes

IUPAC-IUB Commission on Biochemical Nomenclature (1971)

One of the criteria used to demonstrate whether multiple forms of an enzyme represent different molecules is non-convertibility into other forms on recycling through the same purification procedure. Results presented here indicate that the α_A and α_B forms of α-toxin cannot yet be regarded as true isozymes. However, experiments were not carried out to test the interconvertibility of α_A and α_B using single peak fractions of both forms, as has been done by MacNiven et al., (1972) with multiple forms of staphylococcus α-toxin, and with staphylococcus enterotoxin B (Chang and Dickie, 1971). Whereas the α_A and α_B forms of staphylococcus α-toxin and the pI 8.55 and pI 8.25 forms of enterotoxin B had identical molecular weights and were serologically identical in each case, these species of toxin differed in their convertibility. The α-toxin forms were interconvertible, whereas refocusing of the pI 8.55 form of enterotoxin B gave rise to the pI 8.25 form, but refocusing of the latter did not yield the former; in fact refocusing resulted in the formation of forms of lower pI between 7.80 and 8.20. Moreover, both the α_A and α_B forms of staphylococcus α-toxin were stable to refocusing in 6M urea. Stability of forms in 6M urea was not tested out in the case of enterotoxin B. Thus careful interconvertibility studies require to be undertaken to further our understanding of the relationship between the α_A and α_B forms of Cl. perfringens α-toxin.

Some differentiation of nomenclature has been used by authors to designate the particular heterogeneity observed. For example electrofocusing of staphylococcus ß-haemolysin (Wadström and Möllby, 1971) and δ-haemolysin (Kreger et al., 1971) revealed in both cases

an anionic and a cationic component with pIs of 3.0 and 9.4 and 5.0 and 9.5 respectively. However, by refocusing of β-haemolysin, the cationic species was further resolved by the use of narrow pH gradients into a number of multiple forms which were reproducible and had pIs of 8.8, 9.0, 9.4 and 9.8. This type of heterogeneity was termed "microheterogeneity" which accords with the usage of Carlström and Vesterberg (1967) and Carlström (1969). Care, however, must be taken in interpreting such results as natural or artefactual heterogeneity especially with prepurified or pre-electrofocused material. Nevertheless, the term "microheterogeneity" does serve a useful function in describing multiple forms that are only revealed on electrofocusing in narrow pH gradients and as such should be applied in the opinion of this author to the α_A and α_B forms described for Cl. perfringens α-toxin.

Finally the IUB (1964) proposed that isozymes be distinguished and numbered on the basis of electrophoretic mobility, with the number 1 being assigned as a subscript to that form having the highest mobility towards the anode. At present insufficient evidence is available to justify the adoption of this nomenclature in the case of α-toxin and this author feels that the system adopted assigns primary importance to the pI 5.49 form instead of the more anodic form.

SECTION III : STUDIES ON MAMMALIAN CELLS AND MEMBRANES

Before discussing the results presented as part of this thesis I feel it is necessary to express the immediate purpose of the investigations carried out. There were three distinct aims :

1. To clarify, confirm and extend certain observations regarding the interaction of α-toxin with membranes that have been reviewed earlier.
2. To initiate research into the interaction of θ-toxin with membranes, as the major portion of our current knowledge of the mechanism of action of oxygen labile haemolysins is restricted to studies carried out with streptolysin O.
3. To identify areas of research which might contribute useful information on the cytolytic action of these toxins.

Thus although my studies are incomplete, they do fulfil these criteria and do represent significant starting points for a number of investigations. Moreover the use of highly purified α- and θ-toxins obtained by isoelectric focusing has simplified the experimental approach to these problems.

A. ACTION OF α- AND θ-TOXINS ON CELLULAR METABOLISM

The object of these studies was to determine whether these toxins caused metabolic changes in whole cells. In the case of α-toxin it was pointed out in the introduction that studies on whole cells were restricted to morphological changes induced by its action, and that its effect on cellular metabolism had been solely considered with isolated mitochondria (see page 65). On the other hand such studies with

θ-toxin had never been carried out. Even investigations with SLO have been restricted largely to an examination of morphological changes resulting from its interaction with tissues, cells and mitochondria (Halbert, 1970).

1. Effect of α-Toxin on Succinate Oxidation by Krebs 2 Mouse Ascites Tumour Cells.

Experiments with highly purified α-toxin prepared by electrofocusing and free of detectable θ-toxin, collagenase, hyaluronidase and neuraminidase have demonstrated that α-toxin is cytotoxic and that cytotoxicity is associated with metabolic changes within the cell, in this case in succinate oxidation. Although the sensitivity of succinic oxidase activity of isolated mitochondria has been known for some years (Macfarlane and Datta, 1954) this is, to this author's knowledge, the first report of impairment of succinate oxidation after α-toxin treatment of whole cells.

Three possible mechanisms could account for the action of α-toxin on succinate oxidation (a) damage to the mitochondria of the ascites cells resulting from penetration of α-toxin to the interior of the cells (b) a loss of permeability of the cells to succinate as a result of the hydrolysis of phospholipid components of the cytoplasmic membrane (c) an indirect effect on mitochondria resulting from membrane damage. It would seem desirable that the observed metabolic changes of the ascites cells be correlated with possible morphological changes. Here the observations of Rodbell and Jones (1966) on the action of α-toxin with free fat cells are relevant (see page 64). High concentrations of α-toxin were shown to cause cell lysis whereas lower

concentrations caused increased cell permeability. In addition α-toxin treatment of muscle cells caused increased permeability of the membrane to potassium (Martonosi, 1968). On the other hand exposure of giant squid axons to high concentrations of toxin (20 mg/ml) did not impair normal permeability, but did render their membranes more susceptible to rupture (Rosenberg, 1970). Evidence was also presented by Rosenberg and Condrea (1968) and Rosenberg (1970) that α-toxin could penetrate the membrane of the Schwann cell and axolemma without causing membrane rupture or loss of permeability.

It seems likely that ascites cells would provide a useful model for studying the effect of α-toxin on cellular metabolism.

2. Effect of θ-Toxin on Succinate Oxidation by Krebs 2 Mouse Ascites Tumour Cells.

In early studies AS/G100 preparations of α-toxin were shown to cause a stimulation in the rate of O_2 uptake by ascites cells respiring on succinate in the absence of the exogenous addition of Ca^{2+}. This was later shown to be caused by contaminating amounts of θ-toxin, as θ-toxin purified by isoelectric focusing, in identical amounts to that found in AS/G100 α-toxin, caused stimulation. The involvement of θ-toxin was supported by the observations that toxin which had not been reactivated and dialysed or reactivated toxin which had been left exposed to the air for several days at $4^{o}C$ failed to cause stimulation of succinate oxidation.

The simplest hypothesis to explain this observation is that θ-toxin causes an alteration in the permeability of the cytoplasmic

membrane to succinate resulting in an influx of succinate to intact mitochondria. It could equally well be suggested that in addition to having an effect on the cytoplasmic membrane θ-toxin could also have a direct effect on the mitochondria assuming that it were able to penetrate to the interior of the cell.

In view of the similarities between Cl. perfringens θ-toxin and SLO, there follows a brief discussion of the action of SLO. All cells so far tested that possess cholesterol as a constituent of cellular membranes are sensitive to SLO (Halbert, 1970). By contrast, bacterial protoplasts and spheroplasts which lack cholesterol are insensitive. Together with the fact that SLO is inhibited by cholesterol, these findings suggest that the cytotoxicity of SLO depends on its capacity to interact with membrane cholesterol. That its cytotoxicity and haemolytic activity are properties of the same moiety was indicated by the finding that an extract of erythrocyte stroma neutralised both activities (Mastroeni et al., 1969). The available information on lipid analyses of mitochondria isolated from a limited number of tissues indicates that it forms by 1-5% of the total lipid (Rouser et al., 1968). By comparison cholesterol comprises some 25-30% of the total lipid of mammalian erythrocytes (Rouser et al., 1968). Keiser et al., (1964) reported that SLO caused swelling of rabbit liver mitochondria, but these authors did not go on to study metabolic changes in the isolated mitochondria associated with this morphological change. Alouf and Raynaud (1968c) also pointed out that microscopic examination of tissue culture cells treated with SLO revealed cytological changes in the mitochondria.

Also, Ginsburg and Grossowicz (1960) have shown that SLO caused Ehrlich ascites tumour cells to swell. These cells also produced "pseudopodium-like" structures from the cytoplasmic membranes. It is hoped to carry out experiments in the near future to investigate these aspects in relation to θ-toxin interaction with ascites cells.

It was noted that the percentage stimulation in succinate oxidation by ascites cells varied over a wide range. The reason(s) for this is/are not yet clear, although several inter-related possibilities exist; these observations could have been due to :-

1. Differences in the susceptibility of the SS^{35}S used to titrate the θ-toxin on separate days prior to dilution for addition to the ascites cells.
2. Differences in the number of ascites cells added to individual experiments.
3. Differences in the susceptibility of batches of ascites cells to θ-toxin.

Although comprehensive studies on intraspecies variation in the sensitivity of erythrocytes to θ-toxin have not yet been carried out, the data available for SLO indicate that such variation is minimal (Herbert, 1941; Alouf and Raynaud, 1968c). Indeed most species of mammalian erythrocyte appear to be equally susceptible (Bernheimer, 1970). Although the cholesterol content of an individual erythrocyte species can vary within wide limits ($\pm$ 30%) and has been shown to fluctuate even over as short a period as 8 - 12 hours, this would not appear from the above data to cause a significant change in its susceptibility to SLO (Cooper, 1969).

On the second point, it would appear from my studies that the stimulation in succinate respiration was not just a function of the number of cells, but due to an interplay between θ-toxin concentration, no. of cells and time of interaction.

On the third point it seems possible that similar fluctuations in the cholesterol content of ascites cell membranes to that observed in human erythrocyte membranes could occur with disturbances in the metabolism of the injected mouse caused by their intraperitoneal growth. Diet and meal times are known to affect serum cholesterol levels in man. It is therefore conceivable that the time of harvesting of ascites cells in relation to food consumption by the mouse could also affect the membrane cholesterol level. Symington and Arbuthnott (1969) observed a variation in the susceptibility of ascites cells to stimulation by streptolysin S which could not be accounted for simply in terms of minor differences in the ages of cells. However this does not exclude age as an important factor in the case of θ-toxin.

B. MAMMALIAN ERYTHROCYTE AND ERYTHROCYTE GHOST STUDIES

1. Hot-Cold Haemolytic Spectrum of α-Toxin.

The spectrum of sensitivity of electrofocused α-toxin mirrored that previously reported by Macfarlane (1950). In addition the haemolytic spectrum of α_{Urea} was identical to that of α_{A} preparations indicating that these forms of α-toxin observed by isoelectric focusing in the presence and absence of urea respectively possessed in addition to common physical properties similar biological properties.

2. Quantitative Changes in the Phospholipid Composition of α-Toxin Treated Erythrocyte Ghosts.

The observed overall decrease in the phospholipid content of rabbit erythrocyte ghosts caused by treatment with α-toxin accords with the findings of Coleman et al., (1970) for rat and human erythrocyte ghosts, viz. 70% hydrolysis of phospholipid. However, my studies require to be extended to a quantitative estimation of the extent of hydrolysis of individual phospholipid components. This information might explain why some erythrocytes undergo cold haemolysis more readily than others after α-toxin treatment. Because of the heterogeneity that has been shown to exist within one species of phospholipid within one species of membrane it may prove possible to determine the importance on non-polar substituents in the interaction of α-toxin with membranes. Therein may lie the solution to the plethora of contradictions regarding the substrate specificity of α-toxin.

3. Electron Microscopic Observations on the Effect of α-Toxin on Erythrocyte Membranes.

The characteristic observation of the formation of droplets or globules after treatment of erythrocyte ghosts with α-toxin agrees with the earlier work of Habermann and Pohlmann (1959), Finean and Coleman (1965) and the more recent studies of Finean and Coleman (1970) and Coleman et al., (1970). The structures observed by these authors were however electron dense in their preparations because of fixation with OsO_4 with or without subsequent staining with uranyl acetate. Although Habermann and Pohlmann failed to reveal their composition (see page 70), the droplets released from muscle microsomes (Finean

and Coleman, 1965) and from rat and human erythrocyte ghosts by sonication (Coleman et al., 1970) were shown by X-ray diffraction and thin layer chromatography to contain diglyceride. These droplets also possessed a fine structure comprising regular layers of periodicity 6 nm and in some instances a hexagonal distribution of dense spots at about 7 nm centre-to-centre separation. Finean and Coleman (1970) reported that these lipid droplets also contained cholesterol.

Although I have not attempted to isolate the electron transparent structures seen in negatively stained preparations, they possessed dimensions and a distribution similar to that described by Coleman et al., (1970). However in the case of sheep erythrocytes it must be assumed at present that these droplets contain ceramide, rather than diglyceride, due to the hydrolysis of sphingomyelin.

Further evidence for their containing diglyceride (ceramide) has come from the recent studies of Drs. J.P. Arbuthnott and J.H. Freer (unpublished results) of this department. Treatment of liposomes (Bangham and Horne, 1964; Freer et al., 1967) or lecithin emulsion with electrofocused α-toxin resulted in the release of droplets, similar to those described above. Furthermore, diglyceride isolated from α-toxin treated lecithin and emulsified by sonication formed identical structures.

Finean and Coleman (1965), Coleman et al., (1970) and Finean et al., (1971) have reported that the surface area of membranes was decreased by some 45 - 55% by α-toxin treatment as calculated from diameter measurements on the spherical ghosts using phase contrast

microscopy, electron microscopy and ghost volume measurement by exclusion of ^{14}C-sucrose. I have been unable to either confirm or dispute these observations from my electron micrographs of ghost preparations.

4. Kinetics of α-Toxin Haemolysis.

The kinetic studies demonstrated that α-toxin caused hot lysis of sheep erythrocytes. This observation has not yet been extended to determine whether it is concentration dependent. It was shown by the use of staphylococcus α-toxin that during the prelytic phase of the haemolysis curve, changes occurred in the membrane which affected its sensitivity to staphylococcus α-toxin. As has been discussed Cl. perfringens α-toxin caused the formation of neutral lipid droplets which, in the case of sheep erythrocyte ghosts, were principally associated with the residual ghost. Recent studies by Arbuthnott and Freer (unpublished results) have shown that staphylococcus α-toxin interacts with diglyceride isolated from egg-yolk lecithin. This is consistent with its proposed hydrophobic mechanism of action (Arbuthnott, 1970). Flash lysis was thus probably associated with the interaction of staphylococcus α-toxin with neutral lipid droplets in association with the intact erythrocyte membrane formed as a result of phospholipase activity. This theory would also accommodate the observation that Cl. perfringens α-toxin did not sensitise horse erythrocytes to the action of staphylococcus α-toxin, as in this case most of the neutral lipid droplets formed did not remain associated with the membrane (see Plate 12).

One other observation requires comment. Addition of α-toxin to sheep erythrocytes caused a rise in OD during the pre-lytic phase of hot haemolysis. This could possibly be accounted for in terms of membrane shrinkage through phospholipid hydrolysis as a decreased cell size would lead to an increase in cellular opacity. Moreover, it seems likely that cell shrinkage is possibly one cause of stress leading to haemolysis.

Finally, the diglyceride or neutral lipid droplets formed by α-toxin activity may provide information about the mechanism of hot and hot-cold haemolysis. It would be interesting to determine whether the neutral lipid droplets underwent a change from a lamellar phase structure to a hexagonal phase structure on cooling from 37°C to 4°C. Here the observations of Coleman et al., (1970) may be important. The fine structure in the droplets was more pronounced in the rat erythrocyte ghost preparation than in the human. Gas chromatographic studies revealed that the lipid from the rat ghosts contained appreciably less polyunsaturated fatty acid residues, notably of the C18:2 type. They proposed that rat neutral lipids were more ordered at room temperature because of the resultant lower solidification temperature. It may therefore be that the temperature at which the phase change to the hexagonal phase occurs could determine whether the erythrocytes lyse principally in the hot phase of the incubation or in the cold phase (see page 61).

5. Electron Microscopic Observations on the Interaction of θ-Toxin with Erythrocyte Ghosts and Cholesterol.

By treatment of human erythrocyte ghosts with θ-toxin ring-like

structures were formed possessing similar dimensions to those described by earlier authors who treated erythrocyte ghosts with crude α-toxin either in the presence or absence of Ca^{2+}. Moreover arc-like structures identical with those formed on natural membranes were obtained when a cholesterol emulsion was treated with θ-toxin. Thus it seems likely that the observations of Simpson and Hauser (1966b) were due to θ-toxin contaminating their α-toxin preparations.

The immediate question raised by these observations is the composition of these structures. Three possibilities exist :-

1. They are polymers of θ-toxin.
2. They are complexes of cholesterol.
3. They consist of cholesterol and θ-toxin.

It is also possible that the structures formed on natural membranes could contain other components such as phospholipid or neutral lipid.

In the case of SLO two cysteine residues have been identified as being sensitive to cholesterol and being involved in fixation of the SLO molecule to the erythrocyte membrane (Alouf and Raynaud, 1968c). The cholesterol droplets in the cholesterol emulsion had the same diameter as the arcs and rings. In addition, using the same molecules of known molecular dimensions as used by Alouf and Raynaud (1968c) and assuming a spherical shape, θ-toxin could have a diameter around 6.0 - 6.5 nm. That polymerised toxin can be seen in the electron microscope has been clearly demonstrated in the case of staphylococcus α-toxin (Arbuthnott et al., 1967). Thus all three possibilities are viable at present.

Finally what relation could these structures have to membrane permeability? Dourmashkin and Rosse (1966) proposed that the ring structures in the plane of the membrane caused by SLO and crude α-toxin were in fact 50 nm holes. If this is the case, how are they formed? Alouf and Raynaud (1968a, 1968b) have presented evidence in favour of the interaction of SLO with membranes being a two stage process involving two 'topologically distinct sites'. The first stage involved adsorption onto the cell surface and binding, a step that was temperature independent. It was proposed that the subsequent stage involved accurate orientation of the SLO so that it could exert its disrupting action on the membrane, and was shown to possess certain enzymic characteristics. What possible relationship does the formation of ring or arc-like structures have in relation to these proposed stages?

The finding that θ-toxin is clearly involved in the formation of these structures presents many avenues of research which could lead to a better understanding of the interaction of these oxygen-labile haemolysins at the electron-microscopic and molecular level.

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ADDENDUM

HARDEGREE, M.C. (1965). Separation of Neurotoxin and Haemolysin of Clostridium tetani. Proceedings of the Society for Experimental Biology and Medicine, 119, 405-408.

SUMMARY

α-Toxin is the major extracellular product produced by type A strains of Clostridium perfringens. The primary aim of this thesis was its purification and characterisation. Using the technique of isoelectric focusing it has been possible to achieve high resolution of this factor from the other products elaborated by this organism. A rapid method providing quantitative yields of α-toxin possessing the high purity necessary for biological studies has been devised.

α-Toxin was most conveniently harvested for preparative scale purification by fractional ammonium sulphate precipitation of culture supernatant fluids of strains S107 and BP6K; 35-50% saturation gave optimal yields. A pH gradient from 4 - 6 gave optimal separation of the collagenase, hyaluronidase, α-toxin, θ-toxin and neuraminidase, and with high protein loads of up to 3.9 g, 60-80% of the α-toxin was recovered with some 20-30% of the recovered activity in a single peak fraction.

The major peak of α-toxin had a pI of 5.50 and was designated α_A. In addition to α_A, a shoulder or a minor peak of activity was observed with a pI of 5.25. Lyophilised α-toxin preparations provided by Wellcome Research Laboratories gave two distinct peaks of α-toxin activity. Moreover refocusing of α-toxin from preparative scale electrofocusing columns yielded identical results. Using the Wellcome preparations it was shown that 2 peaks of activity were obtained whether the α-toxin was assayed by hot-cold haemolysis of sheep erythrocytes, opacification of a saline extract of egg yolk, release of phosphocholine from a lecithin or a sphingomyelin emulsion and lethality in mice.

These observations confirm and extend the findings of other workers reported during the current investigation. However, yields in this investigation were much improved. Moreover, an attempt was made to assess the relationship of α_B to α_A. The association of α_B with extensively purified toxin or lyophilised preparations suggested that it might be a conformer or an aggregated form of α_A or an artefact created in the isoelectric focusing. To investigate these possibilities Wellcome toxin was electrofocused in the presence of 6M urea in narrow pH gradients. Only one peak of activity was detected by each of the α-toxin assays employed having a pI of 5.50. Thus α_{Urea} corresponded to α_A, a fact further corroborated by their similar haemolytic spectra for mammalian erythrocytes. Removal of urea led to the reappearance of α_B.

Possible causes of multiple forms of proteins were reviewed and it was concluded that the two forms of α-toxin were related other than as artefacts. However the evidence presented did not distinguish between conformer forms and aggregation. By SDS polyacrylamide disc gel electrophoresis the purified α_A had a molecular weight of 53,800 and contained two minor protein contaminants although it appeared serologically homogeneous. Moreover it was free from detectable collagenase activity (pI 4.54), hyaluronidase activity (pI 4.73) θ-toxin (pI 6.56) and neuraminidase (pI 5.27). Evidence was presented suggesting that α-toxin and neuraminidase can act synergistically in causing haemolysis of sheep erythrocytes.

The yields of θ-toxin were high on electrofocusing in broad pH gradients (72%) and it could be refocused in broad pH gradients with good recovery, but not in narrow pH gradients. It had a MW of 61,500,

was serologically homogeneous, but contained an additional protein band on SDS-disc gel electrophoresis. In many respects it resembled other oxygen labile haemolysins that have been characterised using isoelectric focusing.

Although serologically homogeneous on immunoelectrophoresis, SDS disc gel electrophoresis of collagenase preparations revealed 6 or 7 protein bands ranging in MW from 107,000 - 130,000. It was proposed that these could have resulted from autodigestion as the electrofocusing studies themselves revealed no distinct heterogeneity of collagenase. Identical elution profiles were obtained using rat tail collagen or azocoll as substrate. Yields were of the order of 60%. The initial concentration procedure for α-toxin precluded quantitative yields of hyaluronidase allowing an assessment of purity.

The effects of α- and θ-toxin on the respiration of ascites tumour cells were examined. Whereas pretreatment of the cells with α-toxin in the presence of Ca^{2+} abolished respiration on succinate, it had no effect in their absence. θ-Toxin on the other hand caused a pronounced stimulation in respiration on succinate. These experiments represent the first investigation of the effect of these toxins on the respiration on intact cells.

It was shown that α-toxin caused hot haemolysis in the presence of Ca^{2+} and that this phenomenon was associated with a prelytic phase during which changes in the phospholipid of the membrane were associated with an increased sensitivity of the membrane to staphylococcus α-toxin. Treatment of erythrocyte ghosts from rabbit, sheep, horse and

human erythrocytes resulted in the formation of electron transparent structures in association with the membranes. Evidence from other investigations suggested that these were neutral lipid droplets released as a result of the hydrolysis of lecithin, sphingomyelin and phosphatidylethanolamine.

When human erythrocyte ghosts were treated with electrofocused θ-toxin, ring-like structures were formed on the ghost surface which possessed a distinct border. Moreover identical structures were formed when a cholesterol emulsion was treated with θ-toxin. These structures were similar to those attributed by other workers to the action of α-toxin on membranes or membrane-bound virus particles treated with commercial phospholipase C preparations. These observations may provide a useful system aiding the understanding of the mechanism of action of oxygen-labile haemolysins.

APPENDICES

Appendix I

Media

M-I : Medium of Adams et al., (1947) as modified by Bangham and Dawson (1962).

(i) Basal Salts Solution

$Na_2HPO_4.12H_2O$	11.5 g
KH_2PO_4 (anhydrous)	1.0 g
$MgSO_4.7H_2O$	0.4 g
L-Cystine hydrochloride	40 mg
DL Trytophan	50 mg
Growth Factor Mixture (vide infra)	2 ml
Distilled water	1000 ml
Adjust pH to 7.6	

(ii) Growth Factor Mixture

Calcium pantothenate	150 mg
Nicotinic acid	100 mg
Pyridoxamine hydrochloride	100 mg
Thiamine (aneurine hydrochloride)	50 mg
Riboflavin	25 mg
Biotin	25 µg
Distilled water	100 ml

Biotin was added to the growth factor mixture as 25 µl of a 1 mg/ml solution of the vitamin. The growth factor mixture was stored at -20°C in 10 ml aliquots and thawed as required to make up the Basal salt solution (vide supra).

(iii) Basal Medium

Basal salts solution	225 ml
Tryptone (Oxoid Ltd.)	15 g
Ferrous sulphate (0.01%)	10 ml
Distilled water	250 ml

The basal medium (485 ml) was dispensed into 500 ml screw-capped bottles and autoclaved at 15 lb/in^2 for 15 min.

(iv) Dextrin Suspension

Dextrin (Hopkins and Williams)	20 g
Distilled water	100 ml

Autoclaved at 15 lb/in^2 for 15 min.

(v) 1% Thioglycollic acid

Thioglycollic acid	1 ml
Distilled water	50 ml

The pH was adjusted to 7.0 with 1N NaOH and distilled water added to a final volume of 100 ml. The solution was Seitz filtered. This solution was always prepared immediately prior to use.

(vi) Antifoam

Silcolapse 5000 (I.C.I.)	1 ml
Distilled water	9 ml

Autoclaved at 15 lb/in^2 for 15 min.

(vii) Complete Medium

Basal Medium	485 ml
20% Sterile aqueous suspension of dextrin	10 ml
1% Thioglycollic acid	5 ml
Silcolapse 5000 (1/10)	2 ml

(viii) Starter Culture

Basal Medium	20 ml
20% Dextrin	0.4 ml
1% Thioglycollic acid	0.2 ml
Antifoam	0.1 ml

M-II : Medium of Murata et al., (1956) as modified by Murata et al., (1965).

(a) Growth Factor Complex

Calcium pantothenate	20 mg
Nicotinic acid	20 mg
Thiamine	20 mg
Pyridoxamine	10 mg
Uracil	250 mg
Adenine	250 mg
Biotin	100 µg
Distilled water	100 ml

(b) Fructose

50% solution 'Millipore' filtered

(c) Thioglycollic Acid and Antifoam

Prepared as described for M-I

(d) Basal Medium

Na_2HPO_4 (anhydrous)	5.7 g
KH_2PO_4	1.4 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$ZnSO_4 \cdot 4H_2O$	6 mg
$MnCl_2 \cdot 4H_2O$	4 mg
$FeSO_4 \cdot 7H_2O$	7 mg
Proteose peptone (Difco)	50 g
Growth Factor Complex	5 ml
Distilled water	1000 ml

Adjust pH to 7.5. Dispense as for M-I.

Autoclave at 15 lb/in^2 for 15 min.

(e) Complete Medium

Basal Medium	490 ml
10% Fructose	10 ml
1% Thioglycollic acid	5 ml
Silcolapse 5000 (1/10)	2 ml

(f) Starter Culture

Basal Medium	20 ml
10% Fructose	0.4 ml
1% Thioglycollic acid	0.2 ml
Antifoam	0.1 ml

Appendix II

Assay of Toxins and Enzymes

Buffers

DICaB - α-Toxin Diluent

0.02M 3,3 (β,β) Dimethylglutaric acid (Koch-Light-Puriss)	3.204 g
0.005M Calcium acetate (M & B)	0.882 g
Bovine serum albumin Fraction V (Armour)	1.000 g
0.145M Sodium chloride	8.500 g
1N Sodium hydroxide	c. 19 ml

Distilled water to 1 litre

pH adjusted to 7.2 prior to making volume up to 1 litre

θ-Toxin Diluent (after Roth and Pillemer, 1955)

0.038M $Na_2HPO_4 \cdot 12H_2O$	13.609 g
0.032M $NaH_2PO_4 \cdot 2H_2O$	4.992 g
0.078M NaCl	4.558 g

Adjust pH to 6.8 if necessary with 1N NaOH or 1N HCl

Distilled water to 1 litre

Hyaluronidase Diluent

0.11M $Na_2HPO_4 \cdot 12H_2O$	3.940 g
0.09M $NaH_2PO_4 \cdot 2H_2O$	1.404 g
.077M NaCl	4.500 g
Bovine serum albumin Fraction V	0.100 g

Adjust pH to 6.90 if necessary with 1N NaOH or 1N HCl

Distilled water to 1 litre

Collagenase Diluent A

0.05M Tris (tris-(hydroxymethyl)-methylamine) Puriss A.R. Koch-Light	6.05 g
0.05M Maleic Acid	5.80 g
1N NaOH	0.48 ml
Adjust pH to 7.0 with 1N NaOH	
Distilled water to 1 litre	

Collagenase Diluent B

0.05M Tris	6.05 g
0.05M Maleic Acid	5.80 g
0.145 M Sodium chloride	8.50 g
0.001M Calcium acetate	0.176 g
1N NaOH	c.48 ml
Adjust pH to 6.5 with 1N NaOH	
Distilled water to 1 litre	

Neuraminidase Diluent A

0.1M Tris	12.11 g
0.1M Maleic Acid	11.61 g
1N NaOH	6 ml
Adjust pH to 5.1 with 1N NaOH	
Distilled water to 1 litre	

Neuraminidase Diluent B

0.025M Acetic Acid	1.524 g
0.075M Sodium acetate (anhydrous)	6.04 g
Distilled water to 1 litre	
pH 5.1	

Other Solutions and Buffers required for Assays

Hyaluronidase Assay:

0.3M Sodium Phosphate Buffer, pH 5.40

0.034M $Na_2HPO_4 \cdot 12H_2O$	0.537 g
0.276M $NaH_2PO_4 \cdot 2H_2O$	4.446 g
Distilled water to 1 litre	

Acid Albumin Solution

Glacial Acetic Acid	4.56 ml
Sodium acetate	3.26 g
Bovine Serum Albumin (Armour)	1.00 g
Distilled water to 1 litre	
Adjust pH to 3.75 with glacial acetic acid	

θ-Toxin Assay:

Activator

0.1M Cysteine hydrochloride	1.571 g

a) Dissolve in 40 ml distilled water

b) Add 1N NaOH to adjust pH to neutrality

c) Adjust volume to 100 ml with distilled water

d) Check pH

Appendix III

Enzyme List

Chymotrypsin	:	Sigma, London - Type II from bovine pancreas; 3 x crystallised from 4X crystallised chymotrypsinogen; dialysed, salt-free and lyophilised; batch no. 86B-0470.
Collagenase	:	Koch-Light Laboratories, Colnbrook - Form II ex Clostridium histolyticum; clostridiopeptidase A; salt-free and lyophilised; batch no. 43348.
Ficin	:	Sigma - from fig tree latex; 2x crystallised; cysteine suspension; batch no. 119B-4750.
Hyaluronidase	:	Miles-Seravac Ltd., Maidenhead - Grade 1A ovine hyaluronidase from ovine testes; salt-free and lyophilised.
Lysozyme	:	Sigma - Grade I from egg white; 3x crystallised; dialysed and lyophilised; batch no. 88B-8050.
Papain	:	Sigma - ex Papaya latex; 2x crystallised; sodium acetate suspension; batch no. 79B-0030.
Phospholipase C	:	Koch-Light - ex Clostridium perfringens, as partially purified preparation; lyophilised.
Pronase	:	Koch-Light - ex Streptomyces griseus as a powder; batch no. 0489850.
Protease	:	Sigma - Type VII subtilisin from Bacillus subtilis crystallised and lyophilised; batch no. 59B-0020.
Proteinase	:	Koch-Light - ex Bacillus subtilis as a powder; crystallised.

Appendix IV

Electrofocusing Solution

Sucrose Columns:

Dense Electrode Solution (Anode)	LKB 8101	LKB 8102
	(110 ml)	(440 ml)
Sucrose 'Analar'	12 g	48 g
Distilled water	14 ml	56 ml
Sulphuric Acid (Hopkins and Williams - 99% (v/v) - Nitrogen free)	0.2 ml	0.8 ml

The sulphuric acid is added after the sucrose has dissolved. The solution must be stirred to avoid carmelisation of the sucrose.

Dense Solution for Gradient	LKB 8101	LKB 8102
Sucrose 'Analar'	25 g	100 g
Toxin	X ml	X ml
Distilled water	38 - Xml	152 - Xml
Ampholine (10% w/v)	7.5 ml	30 ml
Total Volume	55 ml	220 ml

Light Solution for Gradient	LKB 8101	LKB 8102
Distilled water	52.5 - X ml	210 - X ml
Toxin	X ml	X ml
Ampholine (10% w/v)	2.5 ml	10 ml
Total Volume	55 ml	220 ml

Light Electrode Solution (Cathode)	LKB 8101	LKB 8102
Distilled water	20 ml	80 ml
Sodium hydroxide	0.2 g	0.8 g

Sucrose + Urea Columns: (LKB 8101, 110 ml)

Dense Electrode Solution (Anode)

Sucrose 'Analar'	12 g
Urea 'Analar'	7.93 g
Distilled water to 22 ml	
Add 0.2 ml H_2SO_4 (99% v/v) with stirring.	

Dense Solution for Gradient

Sucrose 'Analar'	25.0 g
Urea 'Analar'	19.82 g
Distilled water	19 - X ml
Toxin	X ml
Ampholine (10% w/v)	7.5 ml
Total volume	55 ml

Light Solution for Gradient

Urea 'Analar'	19.82 g
Ampholine (10% w/v)	2.5 ml
Toxin	X ml
Distilled water to 55 ml	

Light Electrode Solution (Cathode)

Urea 'Analar'	7.21 g
Sodium hydroxide	0.2 g
Distilled water to 20 ml	

All solutions were made up just prior to electrofocusing. Care was taken not to raise the temperature of the urea solutions above 20°C to prevent the formation of cyanate.

Appendix V

Immunoelectrophoresis

Electrophoresis Tank Buffer

0.05M Barbitone Buffer, pH 8.4	
Sodium barbitone	10.3 g
Barbitone	1.84 g
Distilled water	1000 ml

Electrophoresis Agar

Ionagar No. 2 (Oxoid)	10.0 g
Merthiolate (1% w/v)	10.0 ml
Barbitone buffer to 1000 ml	

The agar is boiled until completely dissolved and the merthiolate then added. The agar can then be stored in 100 ml volumes at 4°C. It should only be melted once for use.

Dye Marker

1% bromothymol blue in distilled water.

Appendix VI

SDS Disc-gel Electrophoresis

Stock Solutions:

A. Separation gel buffer

1N HCl	48 ml
Tris	36.6 g
TEMED	0.23 ml
Distilled water to 100 ml	
pH 8.3	

B. Stacking gel buffer

1N HCl	48 ml
Tris	5.98 g
TEMED	0.46 ml
Distilled water to 100 ml	
pH 6.7	

C. Separation gel

Acrylamide	46.67 g
BIS	0.612 g
Distilled water to 100 ml	

D. Stacking gel

Acrylamide	16 g
BIS	4 g
Distilled water to 100 ml	

E. Stacking gel catalyst

Riboflavin	4 mg
Distilled water to 100 ml	

F. Separation gel catalyst

Ammonium persulphate	0.14 g
Distilled water to 100 ml	

G. 40% Sucrose in distilled water.

H. 20% Sodium dodecyl sulphate (SDS) (Koch-Light) in distilled water.

Preparation of separating gel

Solution A	2.5 ml
Solution C	5.0 ml
Distilled water	2.4 ml
Solution F	10 ml
Solution H	0.1 ml

Mix well. Pipette 1.0 ml volumes into disc-gel tubes which have one end covered with parafilm. Carefully layer each column with water. Place gels in dark to set (approximately 30 min).

Preparation of stacking gel

Solution B	1 ml
Solution D	2 ml
Solution E	1 ml
Solution G	4 ml
Solution H	0.05 ml

Mix well. Pipette 0.2 ml onto the top of each separating gel. Carefully layer each with water to provide a flat surface at the top of the gel on setting. Photopolymerise the gels for 15 min. Stacking gels are milky in colour when set.

Electrophoresis Buffer for Tanks

0.05M Tris/glycine buffer, pH 8.3

Tris	6 g
Glycine	28.8 g
Distilled water	1000 ml

Dilute 1 in 10 for use, making it 0.1% with respect to SDS.

Fixative and Stain

Amido Black (Gurr, London)	1 g
Acetic acid 'Analar'	10 ml
Methanol 'Analar'	50 ml
Distilled water	40 ml

The solution was filtered and stored in a capped bottle.

Destaining gel

As for stacking gel with the omission of SDS. Layer 0.5 ml of gel in the bottom of destaining tubes and photopolymerise.

Application of Samples

Samples are made 0.2% with respect to SDS. They are made more dense by the addition of 3 drops of glycerol per ml of sample. Layering of the samples onto the top of the stacking gel is most conveniently done with the buffer reservoir filled. Disposable polypropylene syringes (1 ml) are suitable for this purpose.

Tracking dye

Electrophoresis is followed by the addition of 5 drops of 1% bromothymol blue to the upper reservoir.

Electrodes

In all cases the anode is placed in the bottom reservoir and the cathode in the upper reservoir.

Standard Proteins for Molecular Weight Determination:

Chymotrypsinogen A : Miles-Seravac Ltd. ex Bovine pancreas. 6 x crystallised Grade 1A. Batch No. 448. MW. = 25,000.

Ovalbumin : Sigma. Crystallised and lyophilised. Electrophoretic purity = 99%. Grade V. Batch No. 108B-8090. MW. = 45,000.

Bovine Serum Albumin : Sigma. Type F. Essentially fatty acid free. Batch No. 89B-7220. MW. = 67,000.

Myoglobin : Koch-Light. ex Sperm whale. Crystallised, salt-free lyophilised. Fe = 0.3%. Batch No. 47158. MW. = 17,800.

Hexokinase : Sigma. ex Yeast. Type III. Batch No. 37B-7210. MW. = 45,000. Tetramer MW = 96,000.