THE IMMUNOCHEMISTRY AND METABOLISM OF CLOSTRIDIUM TETANI

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I N D E X

CHAPTER 3

THE NEUROTOXIN OF CL.TETANI

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ABSTRACT

The introductory chapter provides a historical background to the Clostridia and includes specific details of the epidemiology, clinical manifestations and prevention of tetanus together with a description of the causative organism, Clostridium tetani and its toxins. The proposed areas of study are listed.

The first of these areas, the haemolysin of Cl.tetani, is described in the second chapter. Details of kinetic studies, effects of different physical conditions and methodologies on haemolysin production, detection and stability, its production by different strains and its properties after purification are given.

The neurotoxin is the subject of Chapter 3. Methods of neurotoxin assay, its production by different strains, its enzymatic breakdown and toxin binding sites in brain tissue are described.

Chapter 4 concentrated on attempts to isolate the nonspasmogenic toxin and investigate its binding sites in brain tissue.

In Chapter 5 the biochemical reactions of Cl.tetani were investigated. These included established conventional tests plus many never previously applied to this organism. Both commercially available and non-commercial tests were used and further investigations were made where variable or inconclusive results were obtained. The effect of antitoxin on positive reactions were also studied.

A study of the metabolism of Cl.tetani formed the basis for Chapter 6, with growth requirements, specific amino acid utilisation and production of volatile and non-volatile fatty acids being investigated.

Chapter 7 concentrated on other morphological and cultural characteristics of Cl.tetani including spores, motility, plasmids, bacteriophages, antibiotic susceptibilities, Polyacrylamide gel electrophoresis (PAGE) and Isoelectric focussing (IEF) protein patterns and compared all these properties with other known properties of the

organism to determine whether any definite relationships existed.

The antigenic structure of Cl.tetani was studied by a number of immunological methods including agglutination, double immunodiffusion (DID), two-dimensional immunoelectrophoresis and immunofluorescent assay techniques (IFAT) in Chapter 8 and qualitative differences between the nine serotypes were sought.

Chapter 9 attempts to draw the findings from the whole study together and draw overall conclusions from them. Support for established views are discussed as are areas of disagreement and novel or unexpected findings.

CHAPTER 1

INTRODUCTION

1.1 THE CLOSTRIDIA

1.1.1. Historical background

The earth is now thought to be some 4,500 million years old. What began as a white-hot cloud of gas gradually cooled to form a ball of bare rock and mud surrounded by an atmosphere composed mainly of steam. As the earth cooled further, the steam began to change to water and it began to rain. It rained for millions of years, and during this time volcanic activity added other gases such as ammonia and carbon dioxide to the atmosphere. There was no oxygen present - a condition known as anaerobiosis - therefore the development of primeval living matter due to the power of lightning and the strong sunlight heralded the advent of the first anaerobic life. Even when oxygen appeared in the atmosphere around 1 billion years later these organisms persisted and became the ancestors of modern anaerobic bacteria.[1]

It is only in the last few decades that the anaerobic bacteria have been classified properly and their relative importance as causative agents in human disease fully appreciated. For instance, at one time anaerobic infections were considered to be almost solely the realm of the clostridia and little thought was given to nonclostridial anaerobes. Recently, more attention has been paid to these nonclostridial anaerobes, both bacterial and protozoal and their true role in the disease process is emerging. This however, is beyond the scope of this work which will concentrate on the clostridia.

Members of the genus Clostridium are defined as anaerobic or microaerophilic bacilli, usually staining gram-positively and producing spores that commonly distend the organism. Some species decompose protein or ferment carbohydrates, or have both activities. Some produce exotoxins and are pathogenic to man.[2] Their natural habitat is the soil and the intestinal tracts of animals and man.[3]

The genus is composed of a variety of organisms broadly conforming to the above characteristics but differing markedly in others. Their tolerance to oxygen, nutritional requirements and optimum temperatures for growth vary greatly. For instance, Clostridium butyricum, the type species of the genus and the organism to which Louis Pasteur's observation that butyric fermentation could occur in the absence of oxygen is usually attributed[4] can grow with ammonia as the only nitrogen source and biotin as the only vitamin whereas others such as Cl.perfringens require complex growth media containing more that twenty amino-acids and vitamins[5]. Most pathogenic clostridia are heterotrophs, requiring a diversity of amino acids, carbohydrates and vitamins for growth in artificial media[5]. Although originally clostridia were believed to be incapable of growth in the presence of any oxygen at all it was later found that some strains, for example Cl.histolyticum, Cl.tertium and Cl.carnis are aerotolerant and can grow to a limited extent when incubated aerobically[5,6] whereas others such as Cl.novyi type D are extremely strict anaerobes and will not grow at all if the oxygen tension in the ambient atmosphere is greater than 0.05 per cent.[5]

Most pathogenic clostridia grow best at about 37°C, however, there are many capable of growing at temperatures of 20°C or even lower[6]. Some, known as psychrophiles, are incapable of growth above 30°C and others are thermophiles, with optimum growth temperatures about 50°-60°C and which may not grow below 30°C[5].

The clostridia are generally described as gram-positive but this is a property that is extremely variable, especially in cultures more than a day or two old where great irregularity in the depth of staining is noticeable[6], with some strains appearing uniformly gram negative[5].

The shape of the cell and the size and position of the spores are also sub ject to great variation as the morphology of a strain may alter both from culture to culture and within a culture[3].

This variation from strain to strain and even within a strain on different cultures, extends to almost all of the morphological, cultural and metabolic properties of the clostridia and may have accounted for some of the earlier problems of nomenclature and classification [4,7]. Indeed, much of the early work with clostridia is very confusing partly for the above reasons, partly because earlier bacteriologists may have studied impure cultures, or pure cultures that alternated between two or more phases and partly because many of the tests they performed were poorly understood or inappropriately performed. The same organism frequently received many names and conversely many different organisms were given the same name[6,8]. It was not until the introduction of McIntosh and Fildes jar in 1916 made the intensive study of anaerobes possible, and the advent of war made it necessary, that the clostridia began to be understood on a scientific basis[6].

Early work placed much importance on their pathogenicity, but little on their general biology.[6] Obviously the most pathogenic species were the most studied and most frequently recognised in clinical situations, with the less frequently encountered mainly non-pathogenic species being overlooked to a large degree.

The main clinical manifestations of clostridial disease are: botulism, an often fatal form of food poisoning caused by Cl.botulinum[3]; tetanus or lockjaw, where the infection with Cl.tetani produces persistent muscle spasms often leading to death by cardiac arrest or pulmonary insufficiency[11] and gangrene or anaerobic myonecrosis, where infection of a wound leads to invasion of fascial planes by the organisms and eventually to invasion of healthy muscle tissue and subsequent necrosis which may be fulminating and eventually fatal[12]. The clostridia mainly involved in gangrene are Cl.perfringens, Cl.septicum and Cl.novyi although Cl.histolyticum, Cl.sporogenes, Cl.sordellii, Cl.bifermentans

and Cl.fallax may also cause the disease.

Cl.perfringens may also cause food poisoning following ingestion with food[12], and Cl.difficile has recently been implicated as the causative agent of antibiotic-associated colitis and pseudomembranous colitis[14]. As it became known that the pathogenicity of clostridia depended in most cases on the release of highly destructive enzymes and powerful exotoxins[3], most of which were both antigenic and soluble[4], it is perhaps understandable that these toxins came to play a large part in determining relationships among clostridia.

Most species produce at least one toxin and many produce a range that can be used to subtype them. Perhaps the most studied are the toxins of Cl.botulinum, of which there are 8 types and which can be used to subdivide the species into 8 subspecies[9] and the neurotoxin of Cl.tetani[9]. These toxins are amongst the most potent toxins found in nature and this single fact probably explains the fascination they have held for researchers since their discovery. Cl.perfringens may also be subdivided into 5 types on the basis of production of four major and at least 8 minor toxins [4,5] and Cl.novyi into four types depending on the presence of up to 8 different toxins. Cl.sordellii and Cl.bifermentans may be differentiated on some occasions by the production of a lethal toxin by the former, Cl.septicum and Cl.chauvoei may be differentiated by the production of different toxins[2] and Cl.difficile produces at least 2 distinct toxins[10].

In addition to toxic soluble antigens, most clostridia also possess antigens associated with flagella , capsules or the bacterial bodies themselves, thus Cl.perfringens type A can be serologically subdivided into 76 different types, Cl.tetani into 10, Cl.septicum into 4 and Cl.botulinum types A and B into 8[6].

The value of some of these distinctions may be uncertain however. Oakley[13] feels that a characteristic which is to be useful in classification ought to be consistent, ought to be easy to demonstrate and ought to have a well studied incidence in other organisms. The production of a particular soluble bacterial antigen is felt to fail on all three points, as its production may be inconsistent in a particular species, or even in a particular strain, its demonstration may only be accomplished by tedious or complex methods and its distribution may be little studied. Oakley states "the degree to which the soluble antigen of bacteria have been examined depends very largely on their real or imagined importance in human or veterinary medicine and on the personal interests and opportunities of those who have examined them."[13]

Cl.tetani is possibly the simplest of all pathogenic clostridia with regard to its soluble antigenic composition as it is reported to have only 2 definite soluble antigen toxins, the neurotoxin responsible for the clinical manifestations of tetanus and a haemolysin[2], therefore it should be the simplest of all clostridia to study from this aspect, as most of the other pathogenic clostridia produce multiple toxins. However, even here, there are difficulties. Some workers have suggested the presence of a third 'non-spasmogenic' toxin[12,15,16], while the neurotoxin itself may be composed of as few as two[17], or as many as four[18] distinct antigenic components.

Cl.tetani also appears to be one of the simplest of all clostridia in its metabolic activities. It is described as non-saccharolytic and non-proteolytic by many workers[2,3,7], but others claim that both sugars and proteins may be metabolised by some strains.[5,19].

Much of the work on both the antigenic composition and the metabolic activities of Cl.tetani was done some time ago[5,7] and the recent advent of new techniques such as Gas-liquid Chromatography for volatile fatty

acid production[20,21], strips for testing preformed enzyme activities in bacteria[22], two-dimensional crossed immunoelectrophoresis and its many variations[23], polyacrylamide gel electrophoresis, isoelectric focussing[24] and plasmid analysis[25], which have, until recently been almost completely confined to the research laboratory, have not been comprehensively usedto study this species. Also, the availability of a wide range of commercially prepared, well defined and controlled media, reagents and biochemical test kits[22,26,27,28,29,30] along with commercially prepared antibiotic susceptibility testing and minimum inhibitory concentration kits[26] which were unavailable to may early workers might help to clarify some of the discrepancies reported.

It was felt therefore, that a comprehensive reappraisal and study of all aspects of the metabolism and immunochemistry of Cl.tetani would be profitable. As Cl.tetani is perhaps the simplest of the clostridia in this respect it is not unrealistic to expect it to be the species most likely to provide clear-cut answers and results and it is probable that the knowledge gained in the study of Cl.tetani may then be used to facilitate study of the rest of the pathogenic clostridia.

1.2. CLOSTRIDIUM TETANI

Tetanus is a dramatic medical problem which was recognised and described early in medical history. Hippocrates, Arataeus and others were aware of its existance [11] but knew relatively little about the exact nature of the disease [31]. Indeed, until relatively recently the infective nature of the disease was unproven, although it was suspected by some workers[32], such as the British surgeon Thomas Spencer Wells, who suggested that a wound containing some 'abnormal ferment' originated a nerve poison that was responsible for the clinical manifestations of tetanus[11]. It was not until Carle and Rattone in 1884 successfully transmitted and induced the disease in rabbits following the injection of pus taken from an acne pustule that was the primary lesion of a patient who had died of tetanus, that this theory was proven [32]. Eleven of the twelve animals inoculated rapidly developed symptoms of tetanus and other rabbits inoculated with a sciatic nerve emulsion from the original tetanic rabbits also developed tetanus[11].

This serial transmission of the disease suggested that some living agent was responsible and that there was a specific involvement of the nervous system in some way. At around the same time, Nicolaier found that samples of soil inoculated into experimental animals could also produce tetanus and although he did not isolate an organism in pure culture, he noted the presence of a slender bacillus which was present at the site of infection in all cases[11]. Isolation of the pure organism was accomplished by Kitasato in 1889 [31] by allowing spores to form in mixed cultures and then heating them to 80°C to destroy nonsporing organisms.

Final confirmation of the hypothesis of Wells was provided in the next year when von Behring and Kitasato succeeded in demonstrating the presence of the neurotoxin in culture filtrates of the organism and found that if many small doses of the toxin were given, immunity to it was induced and an antitoxin against it was produced in the serum[11].

Faber in 1890 succeeded in separating the highly lethal neurotoxin, termed 'tetanospasmin', by filtration and showed that it's administration to experimental animals produced the characteristic tetanus symptoms observed in naturally occurring tetanus in man [32].

1.2.1. Epidemiology

The organism now known to be responsible for causing tetanus is Clostridium tetani, a motile, gram-positive anaerobic nonencapsulated, spore-forming bacillus[31]. This organism is widespread in nature, both in the faeces of man and animals and in the soil and environment[31]. The distribution of Cl.tetani in the soil may be influenced by factors such as soil fertility, climate and human populations. Thus warmer, more fertile and overcrowded areas tend to have the highest numbers of Cl.tetani in the soil [11].

It has been found that, although the organism is ubiquitous, the incidence of the disease itself can be related in general to the social environment and habits of the population[12]. So tetanus is frequently seen in underdeveloped and overcrowded countries[31]. The habit of going barefoot in agricultural areas increases the risk of tetanus, and neonatal tetanus resulting from contamination of the umbilical cord is common in many underdeveloped countries, for example, Nigeria, where the cord may be cut with wood or broken grass and 'dressed' with native medicines containing, amongst other things, cow dung[12]. Tetanus has been implicated as the leading cause of neonatal death in disadvantaged countries such as Sierra Leone, Thailand and New Guinea[12].

Immunisation is totally effective in preventing tetanus[31] therefore the immunisation status of the population is paramount in determining the incidence of clinical tetanus. As the underdeveloped countries are those least likely to have an effective immunisation policy it is not surprising that the incidence of tetanus is so much higher than it is in developed countries. This effect is also seen in ethnic groups with incomplete immunity in developed countries. For example, in the

United States, tetanus is seen more frequently in blacks in the rural south, where the immunisation status of the group is lower than the surrounding population[31]. Herd immunity does not protect the individual and so any individual with impaired or inadequate immunity is potentially at risk.

Although in 1973 tetanus remained a major worldwide problem with an estimated 350,000 cases occurring annually[3], due to the widespread immunity in the United States only 253 cases of tetanus were reported from 1982 to 1984[33] (88 in 1982, 91 in 1983 and 74 in 1984). Forty states reported at least one case and the 10 states reporting no cases were all located in the western and northeastern United States, whereas the states reporting the majority of cases were in the south. The average estimated annual incidence rate for whites was 0.033/100,000 and for blacks was 0.059/100,000. Approximately 95% of patients had not received a primary series of tetanus immunisations. In England and Wales there were 52 cases from 1981 - 1983 [34] (23 in 1981, 19 in 1982 and 10 in 1983).

Tetanus is essentially an infective disease usually resulting from the contamination of a wound or cut by Cl.tetani[12]. Indeed, in the United States in 1982-1084, 72% of cases occurred after an identified acute injury[33], the most frequent being puncture wounds (37%) and lacerations (35%). Injuries incurred indoors accounted for 41% of these wounds and outdoor activities such as gardening accounted for 38%. In England, 42% of injuries were incurred whilst gardening[34].

The wound may be small and appear trivial on the surface as Cl.tetani is not a histotoxic organism, and in 5 to 10% of cases the original injury may have been so trivial that it has been subsequently forgotten by the patient[3]. Because of this no medical intervention will be sought and the mortality of tetanus may be high (30 to 70%)[11]. The mortality is at its highest in the elderly and the very young. In patients over 70 years of age the mortality may be above 60% and in infants may be

of a similar order whilst mortality in young adults may be between 15 and 30%[31].

The incubation period is variable depending on the size of the dose and the site of the inoculum[11]. It ranges from a few days to several weeks between inoculation of the spores of Cl.tetani into the wound and the appearance of symptoms[31], although spores may occasionally remain dormant in healed wounds for many months, or even years, before a fresh wound or other trauma in the same area may induce them to germinate and then go on to produce tetanus[3]. The longest latent period recorded is 10 years.[3]

The more peripheral the injury, the longer the incubation period, whilst wounds nearer the central nervous system tend to have shorter incubation periods [11,12,31] and generally the cases with short incubation periods of less than 1 week tend to produce more severe disease with higher mortality[35].

However, although the contamination of a wound with spores of Cl.tetani is necessary for the development of tetanus, the presence of Cl.tetani in a wound does not necessarily result in the disease, as Cl.tetani may often be cultured from the wounds of patients without tetanus[3]. The local conditions which are necessary for the development of tetanus are rarely found when the wound is clean, has a good blood supply and the oxygen tension is high, therefore the spores rarely germinate and the disease cannot progress.

Fildes[36,37] found that tetanus spores could only germinate when the oxidation-reduction potential (Eh) of their surroundings had fallen to 10mV or less at the usual pH of tissues[32]. The presence of dirt, or other organisms constituting a mixed infection, may produce a more marked inflammation of the wound which may lower the Eh and facilitate the growth of Cl.tetani[3]. Also, traumatised tissues usually become acidified due to anoxia and ischaemia of the affected area and at acid pH Cl.tetani

can multiply at much higher oxidation-reduction potentials. At pH6.5, a pH readily obtainable in traumatised tissue, Cl.tetani can grow when the Eh is 85 mV[32].

The presence of soil in the wound greatly enhances the ability of Cl.tetani to grow, even in the absence of any tissue necrosis. Calcium chloride has the ability to produce the tissue necrosis necessary for anaerobic infections to establish themselves and this compound is present in most soils, especially cultivated soil. Tetanus is therefore much more likely to develop from soiled wounds and war has been a prime producer of severe, soiled wounds[12]. Cl.tetani was isolated from 19% of World War 1 wounds expressly examined for it, although the disease itself only developed in around 1%[22]. Gardening wounds are also likely to be soiled and this may help explain their prominence in cases of clinical tetanus. Severe wounds however, are generally followed by the initiation of protective measures these days and tetanus may be more likely to develop from slight wounds such as those produced by wood splinters, rusty nails, thorns or even a dirty scratch where protective measures are not sought[12].

In the Punjab in 1902, 19 of 107 patients inoculated with contaminated plague vaccine developed fatal tetanus and there were 20 cases of tetanus reported in Bombay due to contaminated smallpox vaccination and a further 32 cases where the portal of entry seems to have been the site of injection of various drugs[12]. Tetanus following self-injection of morphine or heroin by drug addicts has recently been observed with increasing regularity and in some studies the mortality rate approaches 90%[12]. Street heroin may contain spores of Cl.tetani and anaerobic conditions may be helped by substances used to dilute it [31].

Tetanus also follows such diverse injuries as lacerations, puncture wounds to the foot, varicose ulcers, thorns in the fingers, cracks on the hands, plaster sores, boils, paronychia, chronic ulcers of

the leg, epistaxis, ear piercing, burns, infection of the umbilibal cord (neonatal tetanus or tetanus neonatorum) and infection of the female genital tract (postabortal or puerperal tetanus)[12]. Recently several cases of postoperative tetanus following surgery have been reported[34]. Several of these cases were after cholecystectomy.

1.2.2. Clinical Manifestations

Tetanus can be differentiated into three separate types on the basis of the clinical manifestations observed. These are local, cephalic and generalised[31], although many authors prefer to recognise just 2 forms, local and generalised[3,12], regarding cephalic as merely an unusual precursor of these types.

Local tetanus is characterised bythe persistant contraction of muscles (spasms), generally around the site of infection, or in the same limb or anatomical area as the infection[31,32]. The spasms may continue for a period of weeks before subsiding, or the disease may progress to generalised tetanus.

Cephalic tetanus is a rare form of tetanus, usually associated with injuries to the head or chronic otitis media caused by Cl.tetani. The cranial nerves are generally involved either singly or combined[31]. As before this may remain localised or may progress to generalised tetanus.

Generalised tetanus usually follows an ill-defined prodromal stage of mild intermittent muscular contractions[32]. After some days trismus ('lockjaw'), which is a spasm of the masseter muscles, develops. This may be accompanied by generalised rigidity, particularly of the abdominal muscles, high temperature, stiffness of the neck and difficulty in swallowing[3,12,31]. These symptoms increase slowly or rapidly depending on the severity of the attack until all the body muscles may be involved resulting in violent spasms of the trunk and limbs[3,12]. In severe cases the trismus produces a characteristic facial expression termed risus sardonicus[31]. Death usually occurs from respiratory failure due to interference with the mechanics of breathing[3].

The clinical manifestations of tetanus are wholly due to the production of toxin by Cl.tetani [2,15]. The toxin involved is the neurotoxin, although two other toxins, the haemolysin and a non-spasmogenic toxin have been reported [11,38].

1.2.3. The Neurotoxin

The Neurotoxin (tetanospasmin) was the second bacterial toxin to be discovered, the first being diphtheria toxin[15]. The word toxin is derived from the Greek Toxicon which means 'bow poison' and refers to poison placed on the arrowheads of their warriors [39]. This alludes to the theory that the bacteria produced a poisonous molecule which was subsequently released or 'shot out' to kill cells at a distance, rather like a poisoned arrow.

Tetanospasmin has been the subject of intensive sudty in many countries for many years and Habermann and Dreyer stated that 'no other group of toxins has aroused so much interest as the clostridial neurotoxins'[40]. Thus a great deal has been learned over the years about the production and action of tetanospasmin but there are still many areas of uncertainty. It is one of the three most poisonous substances known to man, yet why the bacterium should produce it is still totally unknown[15].

Not all strains that are morphologically and culturally characteristic of Cl.tetani produce toxin and those that do cannot always be trusted to retain the property[11]. Also, the medium used to grow the organism has a great effect on its toxigenicity, as not all media will support toxin production[15].

Tetanospasmin production is thought to be governed by the presence or absence of a 75kb (kilobase) plasmid. Strains possessing the plasmid were toxigenic and strains without it or with a 22kb deletion were nontoxic, although a great variety of different plasmids were found in different strains and some nontoxic strains carried plasmids[40]. It is a

protein, having a molecular weight of around 150,000[11,15], although molecular weights ranging from 66,000 to 150,000 have been reported by different authors[11]. The sedimentation value is 6.8 - 7.0[11] and the isoelectric point is 5.1[38]. It has been suggested that the toxin molecule is composed of two antigenically active fragments with molecular weights of 107,000 and 53,000 respectively[43], although other workers have found fragments of 40,000 molecular weight[44]. Later it has been suggested that one of these fragments can be further subfragmented into two antigenic components and another 'conformation dependent' antigen can be found in the whole molecule[18]. Toxin is produced inside the cell during the first few days of growth and released into the surrounding medium on autolysis[15]. This then binds to receptors on peripheral nerve endings and passes into the cytoplasm of the nerve cells. The receptors are thought to be gangliosides[41], but the exact nature of the binding site is as yet unknown. There are no histological demonstrations of toxin binding to nerve terminals, although a number of attempts have been made to clarify the situation with brain tissue[42]. The toxin then travels to the spinal cord and brain stem between the fibres of the peripheral nerves by retrograde axonal flow[3,39,42]. Its primary action is to block acetylcholine release from the neuromuscular junction[42] which allows ungoverned propagation of nerve impulses through the connections of the motor neurones in the central nervous system leading to spasmodic contractions of the muscles[11].

Sometimes the terms Ascending and Descending tetanus are used instead of local and generalised tetanus. This follows the observation that the toxin can apparently travel by two routes, through the lymph and blood, or via the tissue spaces of peripheral nerves[11]. Thus inoculation of small quantities of toxin in areas well supplied with nerves will result in these nerves being affected and, through them the spinal cord and the central nervous system producing ascending or local tetanus. If however, a quantity of toxin too large to be absorbed by local nerves is

inoculated, the excess is taken into the lymphatic system and is subsequently able to involve all nerves in the body producing descending or generalised tetanus[11].

1.2.4. The Haemolysin

The second Cl.tetani toxin, the haemolysin is called tetanolysin and has been little studied as it is claimed that it contributes little or nothing to the toxicity of Cl.tetani[15]. It is an oxygen-labile haemolysin serologically related to other clostridial haemolysins and some streptococcal haemolysins[11,12]. It is produced during the period of active growth but is rapidly destroyed[12]. It can lyse the red cells of many animals including the sheep, rabbit, man and horse[11,12] and is reported to be necrotising and cardiotoxic[38]. It has been suggested that tetanolysin may be an enzyme but no proof of this hypothesis has yet been found[11]. Whether the possession of this toxin is governed by plasmids has not been investigated.

1.2.5 The Nonspasmogenic toxin

The third toxin is a nonspasmogenic peripherally active neurotoxin that appears to be little understood[11,15,38]. It is antigenic and neutralised by antitoxin. It may be the cause of the paralytic peripheral action of tetanus toxin reported by several workers as almost all preparations of tetanospasmin seem to contain some of it, however, further work is needed to clarify this[22].

1.2.6 Prevention

As the clinical manifestations of tetanus are all due to the neuro-toxin, the possession of antibodies to this toxin can protect almost totally from the disease. Tetanus neurotoxin is antigenic but is too toxic for administration, however treatment with formalin[15] produces a non-toxic 'toxoid' which is still an excellent immunogen[3].

World War I with its massive involvement of humanity stimulated a programme of immunisation to tetanus that established a

large population of immune people[39]. The effectiveness of this immunity can be seen by the fact that the incidence of tetanus in World War 1 wounded was 1.47 per 1000 while in World War 11 wounded this dropped to 0.12 per 1000[12, 22] (0.147% and 0.012% respectively)

For full protective immunity a series of primary inoculations with tetanus toxoid, from age 6 weeks upwards is given followed by boosters at 5 or 10 years [12,31]. This active immunity establishes basal immunity before the risk of tetanus and is the prophylactic measure of choice[12]. Passive immunisation at the time of injury, using either horse antitoxin or human antitoxin has been successfully used, particularly in many of the less affluent countries [31], however, horse antitoxin in particular has been shown to cause several allergic reactions such as anaphylactic shock, serum sickness, local reactions and even neurological complications[12].

The treatment of established tetanus with antitoxin is of doubtful value however. By the time symptoms appear the toxin is already binding to nerve tissue and cannot be neutralised by antitoxin, although if toxin production is still continuing, a high concentration of antitoxin will neutralise this as it is produced and before it can bind to nerve tissues[12].

The effects of antibiotic prophylaxis on clinical tetanus are minimal[31]. Although many antibiotics are effective against Cl.tetani. they are without effect on the toxin and can only be effective in the course of the disease if they can reach the inoculation site and kill the organism before it can produce sufficient toxin to cause tetanus. This may be virtually impossible to do in a traumatized region with necrosis and impaired blood supply. Sometimes the growth of the organism and continuing production of toxin can only be interrupted bysurgical intervention, debridement of the wound and removal of all necrotic tissues.

A patient who has had tetanus and recovered will not necessarily

be immune, because tetanospasmin is so powerful that tetanus may develop with quantities of toxin too small to immunise[31].

Injection of a booster dose of toxoid into previously immunised individuals at the time of injury may be effective even 10 to 20 years after primarly immunisation and protective levels of antibody may be produced in 1 to 2 weeks[3].

1.2.7 Proposals

There are many areas therefore, where our understanding of Cl.tetani is incomplete or faulty. There has to date never been a comprehensive study of the metabolism and immunochemistry of the organism, much of our present knowledge is outdated and many areas have been severely neglected. This study was designed to try to rectify this situation as much as possible by initially investigating each aspect separately, but then reviewing all the individual findings together as it is entirely possible that different metabolic, cultural and toxic properties may show hitherto unrecognised relationships.

The particular areas of study proposed are as follows:

1. The haemolysin
2. The neurotoxin (including the receptor)
3. The nonspasmogenic toxin
4. The biochemical reactions of Cl.tetani
5. The metabolism of Cl.tetani
6. Morphological and cultural characteristics of Cl.tetani
7. The antigenic structure of Cl.tetani

CHAPTER 2

THE HAEMOLYSIN OF CL.TETANI

2.1. SURVEY OF THE LITERATURE

It has been known for many years that Cl.tetani produces, in addition to tetanospasmin, a haemolysin called tetanolysin[45], which is responsible for the production of zones of α- or β-haemolysis under growth on horse blood agar plates[2]. Tetanolysin can lyse the erythrocytes of many animals. The red blood cells of sheep are quite susceptible whilst those of the rabbit, man and the horse are moderately susceptible and those of mice and cattle are comparatively resistant[5].

It has been shown that tetanolysin is subject to reversible oxidation and reduction in the same way as the oxygen labile haemolysin of Lancefield group A streptococci[46] and that the oxygen labile haemolysins of streptococci, pneumococci, Cl.perfringens and Cl.tetani are all serologically related[47]. It has been suggested that tetanolysin is an enzyme, hydrolysing some component of susceptible red cell surfaces, although no substrate has been identified[5]; however, Bhakdi et al[48] have shown that Streptolysin-O binds to cholestrol molecules on target membranes and damages the bilayer through a mechanism as yet unknown, with the production of large C and ring structures representing the primary lesion of the toxin and therefore they suggest that membrane damage by Streptolysin-O is analogous to that of complement and that of Staph.aureus-toxin, and is most probably representative for the whole group of -SH activated, cholesterol binding bacterial toxins - the so-called oxygen labile haemolysins.

Whether either of these theories are true or not is debatable. Certainly cholesterol appears to prevent the action of tetanolysin when added to haemolytic extracts[45] and tetanolysin can be inactivated when exposed to air and can be then reactivated by the addition of -SH containing compounds such as thioglycollate[49] and cysteine[50], but the antihaemolytic effect of cholesterol is apparently not exerted against

streptococcal and Cl.perfringens haemolysins[45] and there appears to be no serological relationship between tetanolysin and Staphylococcal haemolysins[47]. The situation therefore is far from clear.

In addition to the above points, there appears to be some disagreement over the antigenicity of tetanolysin. Todd[47] states that tetanolysin was not neutralised by tetanus antitoxin, although he does not state whether human or equine antitoxin was used and discusses the possibility that the use of formol-toxoid in the preparation of the antitoxin may account for the apparent non-antigenicity of tetanolysin, as it has been found that the haemolytic activity and the antigenicity of streptolysin are both destroyed by formalin. However, other workers have found tetanolysin to be antigenic and the antibody produced in immunised animals to inhibit the haemolytic effect of the toxin[2,5].

Tetanolysin is produced during the period of active growth[2] but many of the studies on the properties of the toxin have either not defined the incubation time or growth-rate of the cultures[46,47,49] or have used 48 hour[45] or 96 hour cultures[50]. This is important as Willis [2] states that tetanolysin is not present in old cultures, owing to its rapid inactivation. It is not clear how the inactivation occurs, or at what stage of growth but clearly the use of post-exponential growth supernatants in experiments designed to investigate the properties of the haemolysin would be less than ideal if this were true.

Kerrin[45] found that both neurotoxin producing and non-neurotoxin producing strains of Cl.tetani produced apparently identical haemolysins using 48 hour culture supernatants, although the possibility that some of their non-neurotoxic strains were in reality neurotoxic is extremely likely, as it has been shown that the type of proteose peptone broth used by Kerrin does not necessarily induce all potentially neurotoxigenic strains to produce neurotoxin[51]. Also, it has been suggested that the structural gene for neurotoxin production is on a plasmid and

that loss of neurotoxicity is associated with loss of this plasmid[40,52]. Neurotoxigenicity and sporulating potential of Cl.tetani strains appear to be inversely proportional and non toxic strains may be selected by heat treatment[51]. Whether this correlates with loss of a plasmid and whether an analegous situation occurs with regard to the haemolysin is not known.

Although several studies have investigated the activities and kinetics of tetanolysin[45,46,47,49,50,53], a variety of techniques have been used both to produce the haemolysin and to test its activity. Most workers have added thioglycollate[5] or hydrogen sulphite[47] to the culture supernatants after growth has ceased, using a number of different media. The use of a commercially available medium containing cysteine, thereby maintaining standard reduced conditions both during and after growth might add both reproducibility and reliability to investigations.

Several different methods are available for testing the haemolytic activity of bacterial culture supernatants and cells, the most widely used being the tube haemolysin test such as is used for soluble streptococcal haemolysins[54]. Modifications of this, with measurements of released haemoglobin on a spectrophotometer have been utilised in the study of Staphylococcal α toxin[55] and the study of tetanolysin[50,53]. However, these tests are generally laborious to perform, require relatively large quantities of reactants (1ml minimum) and may take up a great deal of room if multiple assays are necessary. Other haemolysin assay methods such as the microtitre plate method[56] or the radial haemolysis plate assay[57] are available, both of which are much more convenient in use although they appear not to have been used to study tetanolysin previously.

None of the previous investigations into tetanolysin activity investigated the effects of storage conditions on deterioration of the haemolysin and the use of stabilising agents, although Bernheimer and Schwartz have used bovine serum albumin to stabilize Staphylococcal toxin[55].

Although the neurotoxin of Cl.tetani has been purified from

bacterial extracts and both its chemical composition[11,58] and its molecular weight[11,15,59] determined, no such information is available on the haemolysin, although it has been separated from and is serologically unrelated to the neurotoxin[60] and the fact that it elutes from the Sephadex gel column after the neurotoxin suggests it has a molecular weight somewhat lower than that recorded for the neurotoxin.

It was felt that a study of the kinetics of tetanolysin activity should be undertaken first, followed by a full investigation of the properties of the toxin in order to properly assess the validity of some of the suggestions made above and to confirm or negate the conclusions of other workers. The following investigations were therefore undertaken.

1) A study of the kinetics of the haemolysin reaction at different concentrations of tetanolysin.
2) A study of the kinetics of the haemolysin reaction at different temperatures.
3) A comparison of methods to determine haemolysin activity.
4) A study of the optimum red blood cell concentration for haemolysin determination.
5) A study of the effect of different incubation temperatures, atmospheres and buffers on the haemolysin reaction.
6) A study of the effect of different growth temperatures on the production of tetanolysin.
7) A study of the inactivation of tetanolysin at different temperatures for different times.
8) A study of the effect of different growth media on tetanolysin production.
9) A study of the effect of storage on the stability of tetanolysin.
10) A study of the effects of storage under different conditions and with different additives on the stability of tetanolysin.
11) A study of the effect of sucrose and glucose on the kinetics

of the haemolysin reaction.

12) A study of the effect of antitoxins on the haemolysin reaction.

13) A study of the growth curve and haemolysin curve of Cl.tetani.

14) A study of the effects of different pHs on tetanolysin activity.

15) A study of the production of tetanolysin by different strains of Cl.tetani.

16) A study of the effect of heat treatment of cultures of Cl.tetani on tetanolysin production.

17) A study of the properties of tetanolysin after purification.

The strains of Cl.tetani used in these studies were obtained mainly from established culture collections (Appendices 1 and 2) and it is therefore impossible to say how many times they have been subcultured since they were isolated or even if their characteristics now accurately reflect those at the time of initial isolation. However, strains were stored in freeze-dried ampoules during the course of this study and before each set of experiments the required organisms were recovered from the freeze-dried state and subcultured at least 3 times on Columbia blood agar before use. This allows the organisms to re-establish their normal metabolic patterns[162]. Whilst experiments were in progress, strains were maintained by weekly subcultures on Columbia blood agar.

2.2. EXPERIMENTAL TECHNIQUES

2.2.1. Kinetics of the haemolysin reaction at different concentrations

A study of the kinetics of the haemolysin reaction at different concentrations of tetanolysin over a period of time was undertaken to investigate whether the rate of haemolysis was proportional to the concentration of lysin and to determine whether the results, when presented graphically, would be analagous to the rates obtained in kinetic studies of reactions catalysed by enzymes[53]. Also, a knowledge of the kinetics of the reaction would enable further experiments to be planned to exploit the maximum degree of haemolysis in the shortest possible time.

The course of haemolysis was followed by measuring the rate of appearance of extracellular haemoglobin (Appendix 3). The information concerning the rate of appearance of extracellular haemoglobin permits the haemolytic reaction to be treated in a quantitative manner similar to that of classical biochemical reaction kinetics.

Experimental

Cl.tetani NCTC 279 was grown for 24 hours at 37°C in 200ml of FAB[61] medium before being centrifuged for 30 minutes at 1,500 x g. The supernatant was tested by the tube haemolysin method (Appendix 4) to determine both the 50% endpoint and the dilution showing 90% haemolysis after 1 hour. These dilutions proved to be 1:2048 and 1:1024 respectively.

Five different concentrations of tetanolysin were chosen for this investigation to provide a range on either side of the 50% endpoint. This was done because concentrated tetanolysin can lyse horse RBCs in a matter of minutes, which is too fast to do kinetic studies and it was felt that concentrations around the 50% endpoint would produce slow enough haemolysis to make kinetic studies possible.

Five dilutions of the culture supernatant were made; 1:256, 1:512, 1:1024, 1:2048 and 1:4096. These dilutions were made up to a final volume of 20ml each in ASO buffer in plastic 60ml containers[62].

To each of the 5 containers, 20ml of standard RBC suspension (Appendix 3) was added with mixing and the containers placed in a 37°C waterbath. Samples (2.5ml) of each were taken at 0, 5, 10, 15, 20, 30, 40, 50, 60, 120 and 180 minutes into glass 3 x $\frac{1}{2}$" test tubes. These were centrifuged briefly to deposit any intact RBCs and the haemoglobin content of the supernatant measured at 545u on an EEL spectrophotometer (Appendix 4).

The percentage haemolysis was determined by taking the reading given by the positive control as 100% haemolysis and calculating the other readings as relative percentages of this figure by the following formula:

$$\frac{R \quad x \quad 100}{P}$$

where P is the reading of the Positive control (100% haemolysis) and R is the reading of the test.

The percentage haemolysis was plotted against time for each concentration of lysin and a series of curves obtained.

The slope of the linear part of each curve represents the maximum rate of liberation of haemoglobin induced by each concentration of the lysin, and is obtained taking (X_1,Y_1) and (X_2,Y_2) as any two points on that part of the curve, by using the following formula:[252]

$$M = \frac{Y_2 - Y_1}{X_2 - X_1}$$

where M is the slope of the curve, X represents time and Y represents the percent haemolysis observed.

The slopes of the linear parts of each curve (i.e. the maximum reaction rates) can be plotted against each concentration in order to determine whether the rate of haemolysis is directly proportional to the concentration or not.[53]

2.2.2 Kinetics of the haemolysin reaction at different temperatures

The manner in which biochemical reactions are affected by temperature is extremely important and a knowledge of the optimum temperature for the haemolysin reaction is necessary when developing a study of tetanolysin kinetics. A comprehensive study of the effect of temperature upon haemolysis by tetanolysin appears not to have been made with the exception of a study by Bernheimer[53] who only studied temperatures between 0 and 31°C. It was necessary therefore to investigate the kinetics of the haemolysin reaction at different temperatures to determine whether the rate of lysis was dependent on the temperature of incubation and whether the shape of the haemolysis rate curve would be altered.

Experimental

The same culture supernatant as used in 2.2.1 was used in this experiment.

20ml of a 1:1024 dilution of the haemolysin was pipetted into each of four plastic 60ml containers and mixed with an equal amount of the standard RBC suspension (Appendix 3).

Each of the four containers was incubated at a different temperature (4°C, 20°C, 30°C and 37°C) and 2.5ml samples were taken from each at 5 minute intervals. These were centrifuged and the amoun t of haemoglobin in the supernatant was estimated as in 2.2.1. The percentage haemolysis was then calculated as before.

All reagents were brought to the appropriate temperatures before the experiment was begun.

Higher temperatures than 37°C were not used as the RBC suspension began to lyse spontaneously above 40°C.

2.2.3 Comparison of methods to measure haemolytic activity

Three different culture supernatants of Cl.tetani NCTC 279 were used. One was grown in 10ml FAB for 12 hours at 37°C, one was

grown in 10ml FAB for 18 hours at 37°C and one was grown in 200ml FAB for 24 hours at 37°C.

After incubation the cultures were centrifuged at 1,500 x g to remove the cells and the supernatants tested as follows:

Triplicate tests were set up using each of the three methods i.e. the tube haemolysin test (Appendix 4), the microtitre plate haemolysin test (Appendix 5) and the radial haemolysis plate test (Appendix 6).

The results of each were compared after incubation.

As an absolute measure of the amount of haemolytic activity present in the original culture supernatant, the number of haemolytic units /ml (HU) was calculated. A unit of haemolysin is defined as that amount which liberates half the haemoglobin in the test red cell suspension under the conditions stated. In other words the dilution which causes 50% lysis (the 50% endpoint) contains 1 HU. It is simple therefore to calculate the number of HUs per ml of the original supernatant by multiplying the dilution factor by the inoculum divided into 1ml[55].

2.2.4 Optimum red blood cell concentration for Microtitre plate assay

The possibility that the lower haemolysin titres observed with the microtitre plate assay could be due to an inappropriate choice of RBC concentration was considered and several different concentrations of RBC were tested to see if the haemolysin titre could be raised by lower RBC concentrations.

Experimental

Five different RBC suspensions were prepared in the same way as the standard RBC suspension (Appendix 3) except that they were adjusted to give an OD_{545} on an EEL spectrophotometer of 20, 40, 60, 80 and 100 respectively.

A 24 hour FAB culture supernatant of Cl.tetani NCTC 279 was tested by the microtitre plate assay (Appendix 5) in triplicate against each of the 5 RBC concentrations.

The results were compared for titre, clarity of endpoint and ease of reading.

2.2.5 The effect of different incubation temperatures, atmospheres and buffers on haemolysin titres using the Microtitre plate assay

Although an incubation temperature of 37°C proved optimum in the tube haemolysin kinetics experiment (Fig.2), the effects in the microtitre plate assay have not been investigated. Also previous experiments have been performed using a non-reducing buffer and in an aerobic atmosphere. It was necessary therefore to investigate the effects on the haemolysin titre of different temperatures, aerobic and anaerobic atmospheres and both reduced (FAB) and non-reduced (Physiological saline and ASObuffer) diluents as well as one containing gelatin as a stabilizer.

Experimental

Microtitre plate assays were set up using a 24 hour culture supernatant of Cl.tetani NCTC 279. Four different microtitre plates were used and on each duplicate assays were performed in four different diluents (Appendix 7) (1) physiological saline (2) ASO buffer (3) Physiological saline plus 1% (w/v) gelatin (4) FAB broth.

One plate was incubated at 37°C anaerobically, one at 20°C anaerobically, one at 37°C aerobically and one at 20°C aerobically for 1 hour. The titres were then determined and compared.

2.2.6 The effect of different growth temperatures on production of haemolysin by Cl.tetani

It has been shown that the optimum activity of tetanolysin is expressed at 37°C, but the optimum temperature for production of the haemolysin has not been established. It was necessary therefore to grow Cl.tetani at several different temperatures and compare the haemolysin titres produced.

Experimental

Cl.tetani NCTC 279 was inoculated into 120ml fresh FAB medium. This was split into 10ml aliquots and incubated in duplicate at six

different temperatures (56,44,37,30,20 and 4°C) for 24 hours. Culture supernatants were tested for haemolysin titres by the microtitre plate method (Appendix 5).

In addition, all cultures had the amount of growth estimated by reading the OD_{600} on a spectrophotometer as described by Mellanby[94] as there is a linear relationship between dry weight of organisms and extinction at this wavelength (Appendix 8).

2.2.7 The effect of Heat treatment on Cl.tetani haemolysin

Several workers have studied the heat lability of tetanolysin[46, 49,50], but there appears to be some disagreement over the results. Thus Schrek[50] states that the haemolysin is inactivated almost totally by heating at 60°C for 10 minutes and Fleming[49] claims similar inactivation at 60°C for 5 minutes and total inactivation at 10 minutes, but virtually no inactivation at 55°C for 10 minutes; although Neill[46] states that only 50% inactivation occurred following heating at 60°C for 10 minutes or 65°C for 2½ minutes and it required 10 minutes at 65°C for total inactivation.

The methods used by these workers, involving addition of different volumes of culture supernatant, treated with $Na_2S_2O_4$ in some cases and under a vaseline seal in other cases, appear rather cumbersome and difficult to reproduce.

Perhaps the different results obtained by these workers may be due to the use of these methods and the reinvestigation of heat lability of tetanolysin using the microtitre plate assay and the prereduced FAB medium seemed worthwhile.

Experimental

Cl.tetani NCTC 279 culture supernatant grown for 24 hours in 250ml of fresh FAB was used for this experiment.

The culture supernatant was divided into 12 pairs of 10 ml aliquots. These were overlain with liquid paraffin and one pair heated at each of the following temperatures: 42, 56, 60, 65 and 70°C.

Each aliquot was tested for haemolysin activity by the microtitre plate assay method at 0, 5, 10, 15, 20, 30 and 60 minutes and 2 hours.

2.2.8 The effect of different growth media on haemolysin production by Cl.tetani

All the experiments so far have utilised FAB medium as primary growth medium. This is in contrast to other workers who used neopeptone broth[53], glucose broth or plain broth[46], proteose peptone broth[45] or unspecified fluid media[47,49,50].

It was therefore necessary to grow Cl.tetani in several different media to compare the haemolysin production and the bacterial density to determine whether FAB was the best medium to use in further experiments.

Experimental

Cl.tetani NCTC 279 was emulsified in 1ml FAB medium to make a suspension equivalent to a MacFarland No.10 opacity standard[29] to produce a standard inoculum. 0.1ml amounts of this were inoculated in duplicate into five different growth media. These were: FAB, Cooked Meat Medium[61], Wilkins-Chalgren anaerobe broth[28], Serum broth[63], Isosensitest broth[28] and Massachussetts medium (Appendix 9). These were incubated at 37°C for 24 hours before being centrifuged and the supernatants tested for haemolysin activity by the Microtitre plate assay.

2.2.9 The effect of storage of haemolysin at different temperatures

In all the previous experiments the haemolysin has been used immediately following centrifugation of the cultures in order to avoid

any deterioration on storage. This may not be convenient, however, if many different experiments are to be performed, or if the results of one experiment need to be known before the next can proceed. It was necessary therefore to investigate the stability of the haemolysin at different temperatures in order to gain knowledge about the optimum storage temperature and the rate of deterioration at this and other temperatures.

Neill[46] has shown that tetanolysin is relatively heat-labile, being inactivated by 10 minutes at 65°C and partly inactivated by 10 minutes at 55°C and that in a non-reduced medium, is oxidised almost completely after 11 hours at 37°C, but the effect of lower temperatures and reduced medium was not studied.

Experimental

Cl.tetani NCTC 279 was grown in FAB medium for 24 hours at 37°C before being centrifuged to deposit the cells. The culture supernatant was divided into 2ml aliquots and stored under the following conditions: -40°C, -18°C, 4°C, 20°C, 37°C, 44°C and 56°C. One aliquot was tested for haemolysin activity by the microtitre plate assay immediately. The other aliquots were tested after 1 day, 2 days, 3 days, 5 days and 7 days.

Duplicate aliquots were tested for all temperatures above freezing, but 5 aliquots were used for - 18°C and -40°C, one from each temperature being thawed for testing on the appropriate days and then discarded. This avoided false deterioration due to repeated freezing and thawing.

2.2.10 The effects of storage with different additives and under different conditions on the stability of the haemolysin

Hardegree et al[64] added chloroform to tetanolysin preparations

as a preservative to try to prevent denaturation on storage.

Other substances, such as sodium azide[65], gelatin[66], bovine serum albumin[54,67], serum and peptone[32] and phenol[54] have been used as preservatives or stabilisers in other biologically active substances and it seemed advantageous to evaluate the effects of these on tetanolysin.

It has been stated that normal serum and cholesterol prevent the action of tetanolysin while lecithin had no effect[45]. Formalin has been used to inactivate other biological substances by toxoiding[68] and it also seemed advisable to study these as well.

As it is possible that any inactivation on storage was due to oxidation of the fluid, different volumes of haemolysin in reduced FAB containing an oxidation-reduction indicator (Reazurin) were also investigated to see if loss of haemolytic activity could be related to oxidation of the medium. The smaller quantities of medium with their larger surface area: volume ratio would be oxidised more rapidly than the larger quantities and thus might be expected to show lower haemolysin titres on storage.

The incubation temperature for this test was chosen as 37°C because at this temperature untreated tetanolysin undergoes rapid, but not total, inactivation so any modification in action would be easily seen.

Experimental

A 24 hour FAB culture supernatant of Cl.tetani NCTC 279 was used throughout the study. 12 duplicate 5ml aliquots were prepared. To each of a pair of aliquots were added the following:

1) 25ul of Chloroform[69]
2) 0.5ml of 1% sodium azide[27]
3) 0.5ml of 10% gelatin[29]
4) 0.5ml of 10% bovine serum albumin[27]
5) 0.1ml of Horse serum[28]
6) 0.5ml of 10% Proteose peptone No.3[29]
7) 50ul of 80% phenol[69]
8) 0.5ml of 10% Cholesterol[69]

9) 0.5ml of 10% lecithin [69]

10) 0.5ml of 1% formalin [69]

11) 0.5ml of PBS

The final two aliquots were covered with a thick layer of liquid paraffin [69] to exclude any oxygen whatsoever.

From the same culture supernatant, aliquots were placed in glass universal bottles in the following volumes, 1, 2, 3, 5, 10 and 20 mls and in glass medical flats, in 50 and 100ml volumes. These were all incubated at 37°C. Samples were removed and tested by the Microtitre plate haemolysin assay at 2 hours, 6 hours and 24 hours.

2.2.11 The effects of addition of sucrose and glucose on the kinetics of the haemolysin reaction

The action of some haemolytic agents is known to be inhibited by sugars and Bernheimer has shown that the addition of sucrose to Cl.septicum haemolysin interrupts lysis when added after 40 minutes and the initiation of lysis wasdelayed when added after 30 minutes [53]. It was felt necessary to investigate whether a similar situation occurs with tetanolysin, but it was not possible to use the Microtitre plate assay on this occasion. To study haemolysin kinetics it is necessary to use the tube haemolysin assay, measuring haemoglobin liberation over a period of time.

Experimental

A 24 hour FAB culture supernatant of Cl.tetani NCTC 279 was used in this experiment. Six 15ml aliquots were dispensed into 60ml plastic containers [62] at a dilution of 1:1024 in ASO buffer. An equal amount of standard RBC suspension was added and the aliquots were incubated at 37°C. 1.5ml of 3M sucrosewas added to aliquots at 0, 10, 20 and 30 minutes and 1.5ml of 3M glucose added at 30 minutes, the remaining aliquot being a control (no sugar). 2ml samples were removed for analysis at OD_{579} at 5, 10, 15, 20, 25, 30, 35, 40, 50 and 60 minutes.

2.2.12 The effects of addition of antitoxins on the haemolysin reaction

Although the oxygen-labile haemolysins, Streptolysin O, tetanolysin, pneumococcal haemolysin and Cl.perfringens toxin are said to be antigenically related[47] there are several contradictory statements to be found in the literature. Willis[2] states that tetanolysin is inhibited both by homologous antitoxin and by antisera to the other oxygen labile haemolysins and by normal serum, but Neill[46] states that tetanolysin is not neutralised by the anti-haemotoxin produced against pneumococcal haemolysin and the anti-haemotoxin produced against Staphylococcal haemolysin and the haemolysins of other anaerobic bacteria. Todd[47] states that tetanolysin was not neutralised by tetanus antitoxin or normal horse serum although it was neutralised by horse antistreptolysin O, while Smith[5] states that the haemolysins produced by Cl.tetani, Cl.perfringens, Cl.novyi, Cl.sporogenes, streptococci and pneumococci are all serologically related and that normal serum can inactivate tetanolysin. The haemolysins of Cl.novyi and Cl.septicum are oxygen-labile and are said to be serologically related to the other oxygen-labile haemolysins[70]. They are not however, reactivated by reduction and are said to not be neutralised by the antibodies of the other haemolysins. There appeared to be a need to investigate and clarify these points. Also, much of the earlier work in this area utilised the relatively inaccurate method of adding different amounts of culture fluids to red blood cell solutions and examining for complete or partial haemolysis[46, 47]. This study investigated the effect of addition of different antisera and normal

sera on the haemolysin titre of Cl.tetani culture supernatant using the Microtitre plate haemolysis assay. Also, as the cholesterol present in normal serum may exert an antihaemolytic effect on tetanolysin[45], the cholesterol levels in each of the sera were estimated.

Also, many of the earlier workers have used antisera produced in their own laboratories and the commercial antitoxins available now appear never to have been investigated from this point of view.

During the course of this project several commercial Cl.tetani antitoxins were used and it seemed advisable to investigate them all to see if the antihaemolysin activities were the same. Several other commercial Clostridial antitoxins were available which were included in the study. These included Cl.perfringens, Cl.novyi, Cl.septicum and Cl.chauvoei antitoxins.

Experimental

A 24 hour FAB culture supernatant of Cl.tetani NCTC 279 was used throughout this experiment.

Serial dilutions of three different horse tetanus antitoxins, normal horse serum, Cl.perfringens type A antitoxin, Cl.novyi type A and type B antitoxins, Cl.septicum and Cl.chauvoei antitoxins[71], normal horse serum[28], human tetanus antitoxin[72], normal human serum, Antistreptolysin O[29], Pneumococcal omniserum[73] and normal rabbit plasma[29] were made in ASO buffer in 25ul volumes in Microtitre plates. 25ul of the culture supernatant was added to each well, shaken and incubated for 15 minutes at 37°C. Following this 50ul of standard RBC suspension were added to each well, shaken again and reincubated for a further hour at 37°C.

The plates were examined to see which dilution of antiserum would inhibit the haemolysin (original titre 1:256) reaction sufficiently to produce a 50% endpoint. This dilution was termed the haemolysin inhibition titre.

Aliquots ofeach of the sera tested were assayed for cholesterol

content in the Clinical Chemistry Department, Stafford General Infirmary.

In addition aliquots of Cl.tetani horse antitoxin 4 were absorbed for 18 hours at 37°C followed by 24 hours at 4°C with two different culture supernatants of Cl.tetani. (Appendix 18)

1) an 18 hour culture supernatant of Cl.tetani NCTC 279 - a haemolytic strain.

2) an 18 hour culture supernatant of Cl.tetani NCTC 9596 - a non-haemolytic strain.

Following absorption, the antitoxins were centrifuged at 1,500 x g for 40 minutes and the supernatants tested for haemolysin inhibition activity as before.

2.2.13. Comparison of the growth rate and haemolysin production rate of Cl.tetani

It has been stated that tetanolysin is liberated during the period of activegrowth of Cl.tetani[49] although whether the relationship between the growth rate and haemolysin production rate are directly proportional appears to be unknown, as is the ideal time to take samples of culture supernatants for maximum haemolytic activity. This could be important as, without this knowledge, it is impossible to say whether small deviations in time of sampling could produce widely different haemolysin titres or not. Also, it is not clear whether tetanolysin is released from Cl.tetani cells as soon as it is produced, or whether it is stored in large quantities inside the cells and only released when sufficient has built up. It was necessary therefore to compare growth rates and the haemolysin production rates over a period of time with the storage of haemolysin inside cells.

Experimental

Cl.tetani NCTC 279 was inoculated into 250ml of fresh FAB medium in a glass 250ml bottle[74] at 37°C and 10ml aliquots were removed every hour for 18 hours, at 24 hours and thereafter at 2, 3, 4,

5 and 7 days. Aliquots were centrifuged at 1,500 x g for 30 minutes and both supernatant and deposit were stored at - 40°C until needed.

After thawing, all supernatants were tested by the Microtitre plate assay method.

The deposits were resuspended in cold distilled water to the original volume and the optical density of each measured on an EEL spectrophotometer at 600um. This was taken as a measure of the degree of growth (Appendix 8). The deposits were then centrifuged again and washed in cold ASO buffer before being recentrifuged. The supernatant was carefully removed and the cells resuspended in 0.1ml of cold ASO buffer.

The resuspended cells were tested for haemolytic activity by the Microtitre plate method.

The remainder of the cells were incubated at 4°C overnight to allow any haemolysin inside the cells to diffuse out. They were subsequently centrifuged and the supernatants tested by the Microtitre plate assay.

2.2.14 The effects of different pHs on the haemolysin of Cl.tetani

It has been shown[50] that acidification decreased and alkalization increased the rate of inactivation of tetanolysin by heat, and that acidification of lysin up to pH4 decreases the rate of oxidation and acidification below pH4 increases the rate of oxidation of tetanolysin when exposed to air. Also, it has been claimed that acidification and alkalization partially inactivate tetanolysin, the maximum inactivation occurring at pH4[50]. However, these latter experiments were performed after 22 hours incubation at 43°C, a temperature which, in itself can cause inactiviation of haemolysin to a great degree (this study, Table 6), therefore the results cannot be read as simple pH inactivation, but must be interpreted as pH modified heat inactivation. For this reason it was decided to investigate the effects of storage of haemolysin at different

pHs under liquid paraffin (to avoid oxidation effects) and at 4°C (to avoid heat effects).

Experimental

A 24 hour culture supernatant of Cl.tetani NCTC 279 was used in this experiment.

24 5ml aliquots of this culture supernatant were prepared in glass universal bottles. Duplicate samples were adjusted to pH1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 using conc. hydrochloric acid[69] or 6M NaOH[69] as appropriate. The pH readings were made using a Camlab stick pH meter[75]. The samples were then overlaid with liquid paraffin and incubated at 4°C for 24 hours, after which the haemolysin was transferred from below the paraffin to fresh bottles, neutralised with 6M NaOH or conc. HCL as appropriate and tested by the Microtitre plate assay method.

2.2.15 The production of tetanolysin by different strains of Cl.tetani

The haemolysins studied by earlier workers have been isolated from a number of different strains of Cl.tetani[45,46,47,49,50,53,60,64] and therefore the comparability of the results is uncertain. Also, although Smith[5] claims that on blood agar each colony of most strains is surrounded by a narrow zone of haemolysin and Kerrin[45] found that all the ten strains he tested, which included neurotoxin producing and non-neurotoxin producing strains, produced an equally powerful haemolysin, it seems unclear whether all strains of Cl.tetani produce haemolysin and if so, whether there are any quantitative differences in the haemolysin produced. It seemed advisable to test all the different serotypes of Cl.tetani available and a selection of other strains for haemolysin in an attempt to clarify these questions.

Experimental

The nine serotype strains of Cl.tetani (Appendix 1) and 18 other strains (Appendix 2) were inoculated into fresh FAB medium and incubated for 6 hours at 37°C. 1ml of each culture was transferred to a fresh FAB

medium and incubated for 24 hours at 37°C. Following incubation, cultures were centrifuged at 1500 x g for 30 minutes. The supernatants were removed and tested for haemolysin activity by the Microtitre plate assay.

The deposits were washed in distilled water once and then resuspended in distilled water to the original volume before having their optical density measured at 600um on an EEL spectrophotometer (Appendix 8) as a measure of the degree of growth.

2.2.16. The effect of heat treatment on production of haemolysin by Cl.tetani

Nishida et al[51] have suggested that there is an inverse relationship between the ability of a strain of Cl.tetani to sporulate and the degree of neurotoxigenicity and it has been reported that the higher the temperature used in heating soil samples for the isolation of Cl.tetani, the less toxigenic were the strains recovered[76]. Hara et al[77] have isolated various nontoxigenic derivaties from toxigenic parent strains using different treatments including Acridine orange, rifampicin and ultra-violet light and Nishida[51] used direct heat treatment of cultures to the same ends. None of these workers have investigated the effects of heating on tetanolysin production however and it seemed desirable to investigate this aspect here.

Heating to 80°C for 10 minutes may produce sporulating substrains of Cl.tetani[51] but it is reported that this treatment may also result in the destruction of Cl.tetani despite microscopic evidence of spores[2] therefore a temperature of 80°C and a time of 5 minutes was used in these studies.

Experimental

Heavy suspensions of 28 strains of Cl.tetani, the nine serotype strains of Cl.tetani (Appendix 1), 18 other strains (Appendix 2) and a heat treated variant of NCTC 279 (Appendix 10) were made in 1ml of sterile saline, from Columbia Blood Agar plates incubated at 37°C for 24 hours followed by 30°C for 6 days in an anaerobic atmosphere. The suspensions

were approximately equivalent to MacFarland opacity standard No.10[29].

0.5ml of each suspension was inoculated into fresh FAB medium in 10ml amounts which were incubated at 37°C for 24 hours.

The other 0.5mls were inoculated into identical fresh FAB medium which were subsequently heated at 80°C in a water bath for 5 minutes before being incubated at 37°C for 24 hours as before.

The original suspensions were examined for spore production by Gram's stain (Appendix 11) before use,and both heated and non-heated 24 hour cultures were examined for spores in a similar manner. The degree of growth was estimated by measuring the Optical Density at 600um (Appendix 8) and the haemolysin titres of both heated and non-heated culture supernatants were determined by the Microtitre plate haemolysin assay method. All heated cultures were subcultured onto fresh Columbia blood agar plates following incubation to check for viable organisms. These plates were incubated at 37°C for 3 days anaerobically before being examined.

2.2.17. Purification of tetanolysin

Most laboratory studies of the haemolysin have been made using crude culture filtrates of Cl.tetani [45, 46, 47, 48, 49, 50] although Hardegree[60] has separated haemolysin and neurotoxin by gel-filtration and suggested that the haemolysis may be due to the action of esterase or protease activity. As 24 hour culture supernatants of Cl.tetani may contain a number of different substances, further examination of these possibilities would be best carried out using purified haemolysin. It is also suggested that since the haemolysin is eluted from gel filtration columns after the neurotoxin, that the haemolysin molecule is smaller than the neurotoxin molecule[60] but this does not appear to have been further investigated. Examination of the purified haemolysin by SDS - Polyacrylamide gel electrophoresis (PAGE) might help to elucidate the situation. Also there are several antigenic components in crude 24 hour culture supernatants

of Cl.tetani and it is not clear which corresponds to the haemolysin. Indeed it has been suggested[47] that in tetanus antitoxin there is no antihaemolysin due to the use of formol-toxoid in antitoxin preparation. It may be difficult to tell whether the antihaemolytic activity seen with some sera is due to specific antibody or the cholesterol present in the sera, but a study of the precipitating antibodies present in tetanus antitoxin using purified haemolysin might prove enlightening.

As it is possible that precipitating antibodies might not be present in the antitoxin, although non-precipitating antibodies might be, the preparation of a latex agglutination reagent sensitized with tetanus antitoxin and the study of the crude and purified haemolysins might also prove useful.

Other physical properties of the haemolysin, such as its isoelectric point and the amino-acid content might also be determined.

Experimental

Cl.tetani NCTC 279 was incubated in 600ml of fresh FAB medium for 18 hours before being centrifuged. The deposit was discarded and the supernatant split into three 200 ml portions. Each portion was then subjected to a different purification procedure.

(1) 200mls of culture supernatant was brought to 50% saturation by adding 75 grams of solid ammonium sulphate[69]. This was stored at $4^{\circ}C$ overnight and the precipitate was recovered by centrifugation, dissolved in 4mls sterile distilled water and freeze dried. This was then reconstituted in 0.2ml ASO buffer and 0.1ml was applied to a 25 x 2.5cm Sephadex G-200[78] column equilibrated in ASO buffer. 2ml fractions were collected and the protein content of each determined by the method of Peterson[79] (Appendix 12).

The antigenicity of each fraction was determined by the double-immunodiffusion (DD) technique[80] (Appendix 13) and by Rocket Immuno-electrophoresis[81] (Appendix 14). Each fraction was investigated by SDS Polyacrylamide gel electrophoresis (PAGE) in 10% gels by the method

of Laemmli[82] as modified by Byrne[4] (Appendix 15). A technical handbook from Pharmacia was also useful[83].

The fractions showing maximum haemolytic activity with minumum protein content were freeze died, reconstituted to one tenth volume in distilled water and re-analysed by PAGE.

These fractions were also investigated by two-dimensional immunoelectrophoresis[84] (Appendix 14) and Isoelectric focusing [104] (Appendix 20).

(2) 200 mls of culture supernatant were adjusted to pH 5.3 with phosphoric acid (5M) and chloroform was added to a final concentration of 1.5% (v/v)[85]. This mixture was left overnight at 4°C with occasional mixing after which the precipitate was collected by centrifuging at 1,500 x g for 45 minutes.

The precipitate was resuspended in 50ml chloroform/methanol (2:1 by vol) and left overnight at 4°C. The precipitate was again collected bycentrifuging and the supernatant was filtered through Whatman No.1 paper. To this supernatant was added 10mls of 0.1M phosphate buffer pH 6.8 with thorough mixing. The phases were left to separate for 1 hour at room temperature. The lower layer (expected to contain the toxin[85]) was removed and extracted with 4mls of acidified chloroform/methanol/water (3:48:47 by vol) containing 20ul of 1M phosphoric acid. The precipitate was removed by centrifugation and freeze dried. Before use this was reconstituted in 0.2ml of distilled water[85].

The final product was examined as before for haemolysin activity, precipitating antigens by two dimensional immunoelectrophoresis and for SDS PAGE bands. Insufficient material was available for protein determinations.

(3) 200mls of culture supernatant was saturated by addition of 152g solid ammonium sulphate and allowed to stand overnight at 4°C. The precipitate was recovered by centrifugation and resuspended in 20mls cold ASO buffer. This was centrifuged and any insoluble material discarded. The supernatant was made 60% saturated by the addition of 8.4g solid ammonium sulphate and was stirred for 25 minutes at 4°C before being centrifuged. The supernatant was discarded and the precipitate stored overnight at 4°C. 10mls of cold 40% saturated ammonium sulphate was then added and stored at 4°C for 25 minutes with occasional stirring. This was then centrifuged and the clear supernatant discarded. The precipitate was dissolved in 2ml of ASO buffer and dialysed overnight against distilled water. The small amount of precipitate which formed during dialysis was removed by centrifugation and the supernatant was freeze dried. This was reconstituted in 0.1ml ASO buffer and applied to a 25 x 2.5cm Sephadex G-200 column equilibrated with ASO buffer. 4ml fractions were collected.

The fractions were examined as before for antigenicity, haemolysin content and PAGE bands. Protein content was determined by absorption at 280nm in a SP 1800 Pye Unicam spectrophotometer[86] using quartz cuvettes and bovine serum albumin as the standard.

The fractions showing maximum haemolysin activity were pooled, freeze dried and reconstituted in 0.1ml ASO buffer. This was applied to a 25 x 2.5cm column of Sepharose 6B[27] equilibrated with ASO buffer and 2ml fractions were collected. These were analysed as before for antigenicity, haemolysin activity and PAGE bands.

The addition of 1 drop of 50nm dithiothreitol[27] to each fraction was necessary before haemolysin activity could be seen. The fractions showing maximum haemolysin activity were concentrated by freeze drying to 0.2ml.

All the Sephadex gel fractions plus the original haemolysin (neat and concentrated) and the concentrated fractions with maximum haemolysin activity from method 1), the final product from method 2) and the concentrated Sepharose 6B fractions from method 3) were examined for antigen activity by the latex agglutination test[87] using latex particles sensitized with Wellcome tetanus antitoxin (Appendix 16) and also with latex particles sensitized with tetanus antitoxin absorbed with Cl.tetani NCTC 9596 (serotype VI - a non-haemolytic strain) (Appendix 18) which should remove all antibodies to cell wall and exoproteins with the exception of tetanolysin.

These investigations were repeated using the Staphylococcal coagglutination test (Appendix 17)[88].

The concentrated fractions from methods 1) and 2) showing maximum haemolysin activity and the product from method 3) were examined by two-dimensional paper chromatography for amino-acids[89] (Appendix 19).

2.3 RESULTS

2.3.1 Kinetics of the haemolysin reaction: concentration of lysin

When the 5 different concentrations of haemolysin were studied, it became obvious that the rate of lysis was dependent on concentration. When the percentage haemolysis was plotted against time (Fig.1) it could be seen that the most concentrated haemolysin (1:256 dilution) produced 100% haemolysis in just 15 minutes, whereas the next most concentrated (1:512) took 50 minutes to produce 100% lysis and the other three dilutions (1:1024, 1:2048 and 1:4096) produced only 90%, 70% and 55% haemolysis after one hour. With these latter 3 concentrations the percentage haemolysis did continue to rise slowly over the next 2 hours but only by a small amount, the haemolysin reaction being essentially over after 1 hour.

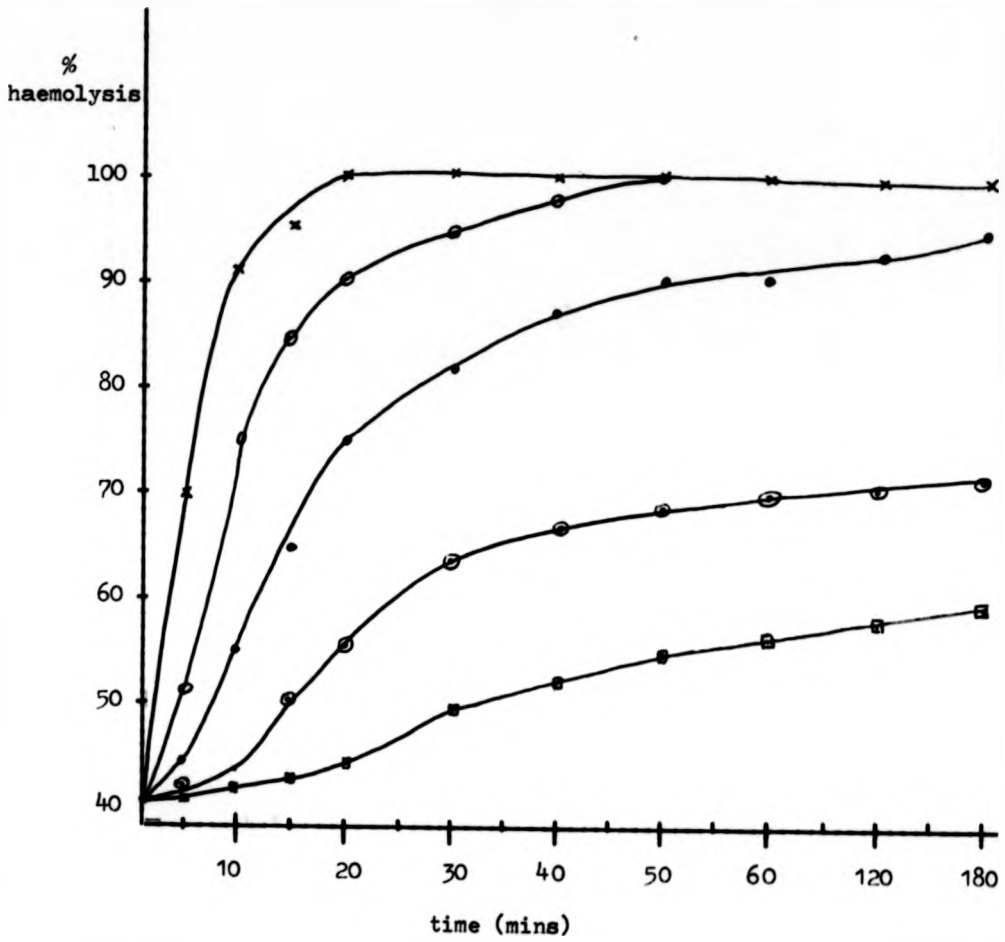
When the slopes of the linear parts of each curve (i.e. the maximum reaction rates) were calculated for the 5 different concentrations they were as follows:

4, 2.3, 1.8, 1 and 0.33 per cent haemolysis per minute respectively in increasing order of dilution.

When these reaction rates were plotted against dilution (as a measure of concentration of haemolysin) they were seen to be roughly linear. (Fig.2).

FIG. 1

Comparison of lysis rates of different concentrations of haemolysin

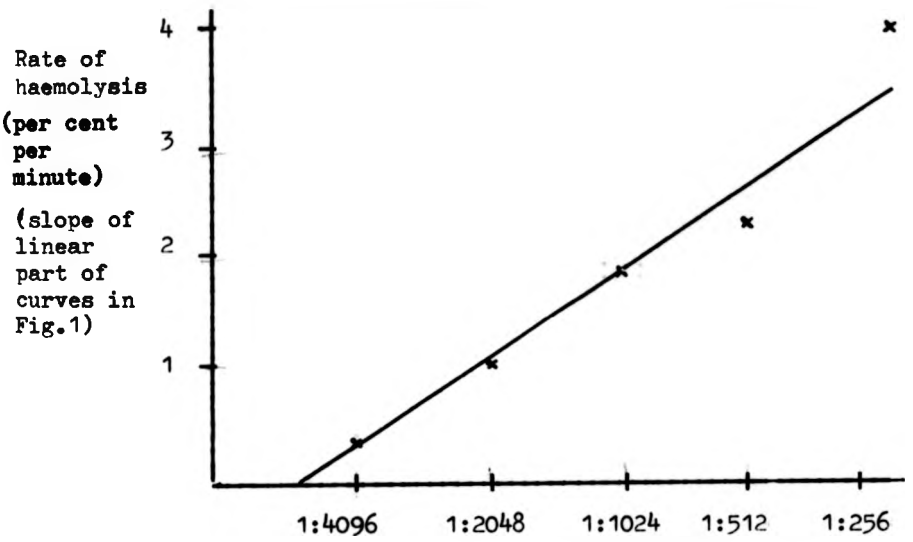


KEY:

x	1:256 dilution of haemolysin	
0	1:512	"
•	1:1024	"
●	1:2048	"
▫	1:4096	"

FIG.2

Rate of haemolysis as a function of haemolysin concentration



2.3.2 Kinetics of the haemolysin reaction: temperature incubation

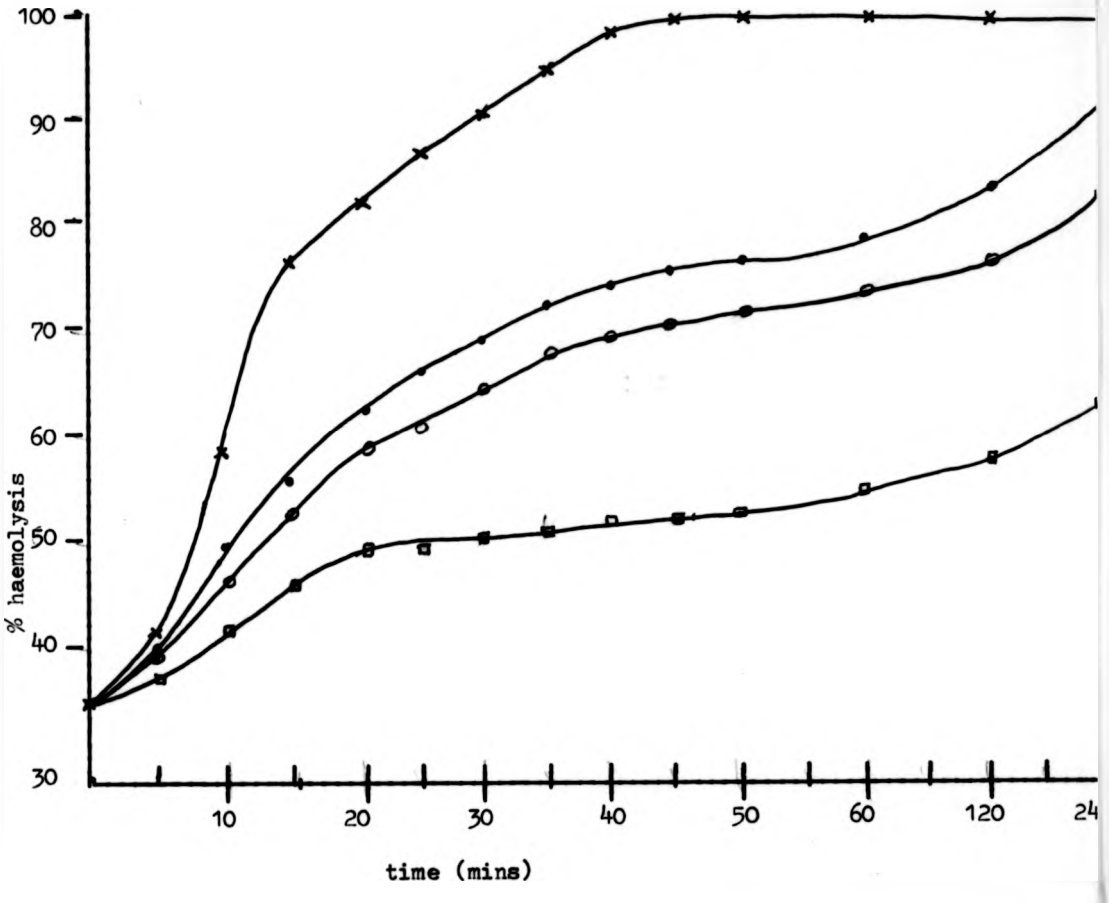
When four different temperatures were used to incubate haemolysin reactions, the maximum rate of haemolysis was seen at 37°C, with less at 30°C, 20°C and 4°C respectively (Fig.3). It can be seen that at 37°C maximum haemolysis is achieved in 40 - 50 minutes, following which no further lysis occurs; however at 30°C, 20°C and 4°C haemolysis continued gradually over the next 3 hours.

When the slopes of the linear parts of each curve (the maximum reaction rates) were calculated for the 4 different temperatures they were found to be 0.45, 1.0, 1.6 and 3.5 per cent haemolysis per minute for 4, 20, 30 and 37°C respectively.

When these reaction rates were plotted against temperature the curve produced showed a sharp rise towards 37°C (Fig.4).

FIG.3

Comparison of lysis rates at different incubation temperatures

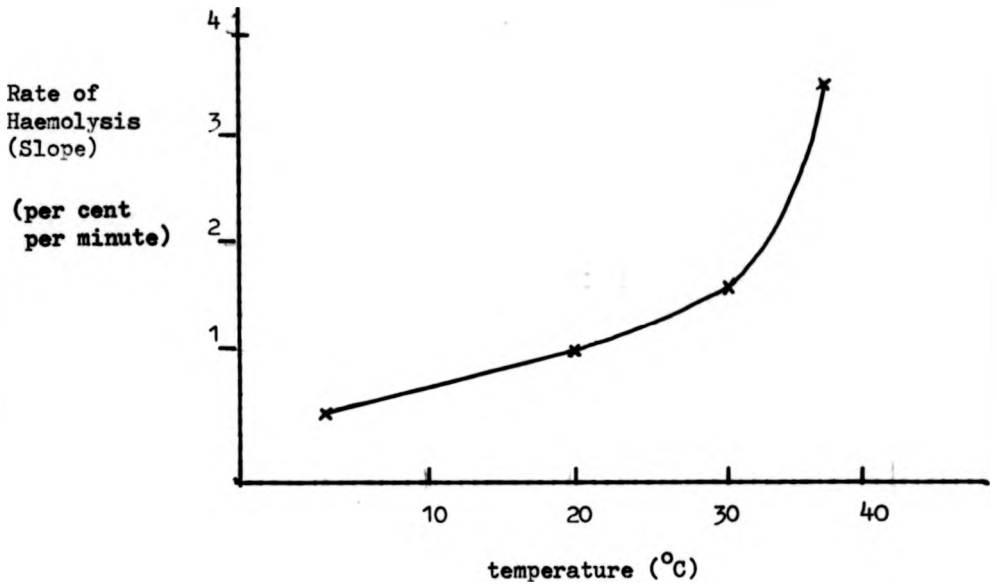


KEY:

x 37°C
• 30°C
o 20°C
□ 4°C

FIG.4

Rate of haemolysis as a function of incubation temperature



2.3.3. Comparison of methods to measure haemolytic activity

Using a 12 hour culture supernatant of Cl.tetani the tube haemolysin technique produced a haemolysin titre of 1:256. The Microtitre plate technique produced a titre of 1:64 and the radial haemolysis plate produced a titre of 1:2 both in wells and on filter paper strips.

Using an 18 hour culture supernatant the tube, Microtitre plate and radial haemolysis plate titres were 1:1024, 1:128 and 1:2 respectively and using the 24 hour culture supernatant these titres were 1:2048, 1:256 and 1:2 respectively (Table 1).

There were no major discrepancies between any of the triplicate tests by any method, the only variation being an occasional one dilution difference.

The above titres are therefore the average of the triplicate tests.

Table 1 shows the volume of culture supernatant required to perform each test and comments on the procedures as well as the haemolysin titres observed.

The tube dulution technique produced endpoints that were difficult to interpret visually, appearing to occur over several tubes and requiring spectrophotometric analysis to determine the 50% endpoint (Fig.5).

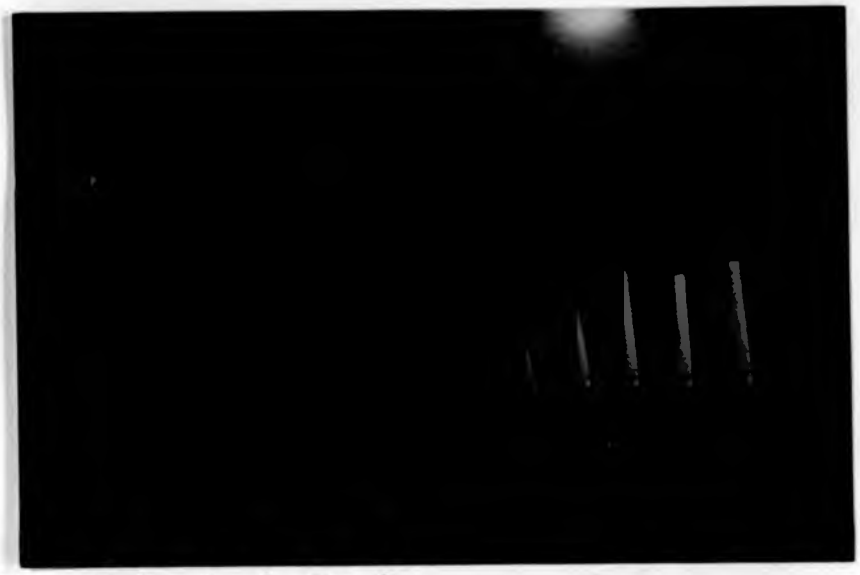
In contrast, endpoints were sharp with the microtitre plate assay and were easily interpreted visually (Fig.6). The method was extremely easy and allowed many tests to be done simultaneously, although it produced haemolysin titres two or three twofold dilutions below the tube haemolysin method. This was, however, not a sufficient reason not to use the test and it was decided that the Microtitre plate method would be used in future investigations unless spectrophotometric analysis was necessary.

The radial haemolysis plate method produced very low titres and haemolysis zones were extremely narrow around the sample well or disc (Fig.7). Although very quick and convenient, the method was not readily applicable to the present study.

When the haemolysin titres obtained by each of the 3 methods for 24 hour culture supernatants were used to calculate the number of haemolytic units per ml of the original supernatant, widely differing levels were obtained. It was calculated that, from the tube haemolysin test there were 2040 HU/ml, from the microtitre plate assay there were 10240 HU/ml and from the radial haemolysis method there were 20 HU/ml in wells and 80 HU/ml in discs.

FIG. 5

The Tube Haemolysin Test



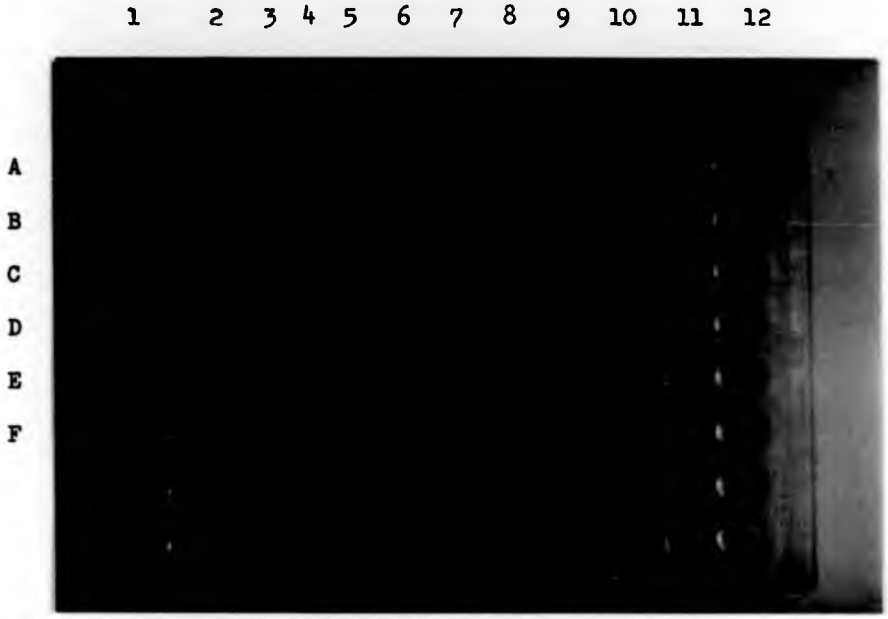
Tube No. 3 4 5 6 7 8 9 10 11 12 13 14 15

Dilutions of Cl.tetani haemolysin from 1:4 to 1:1638

50% endpoint (1:1024) is in tube 11 (arrow)

FIG.6

The Microtitre plate haemolysin Assay Test



Serial dilutions of Cl.tetani haemolysin from neat to 1:2048 (wells 1 to 12 respectively) tested using different suspensions of RBCs.

Row A : RBC suspension giving absorbance of 100 at 545u

Row B : " 80

Row C : " 60

Row D : " 40

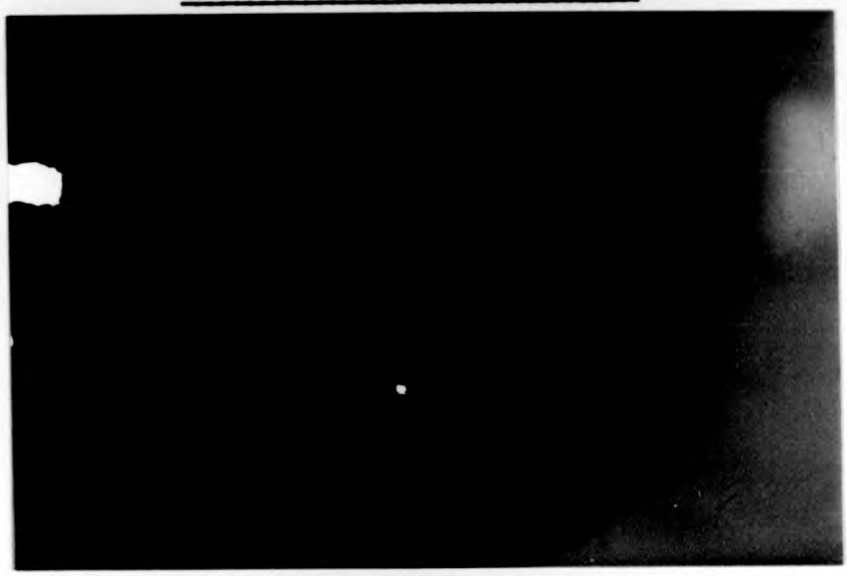
Row E : " 20

Row F : Positive and negative controls

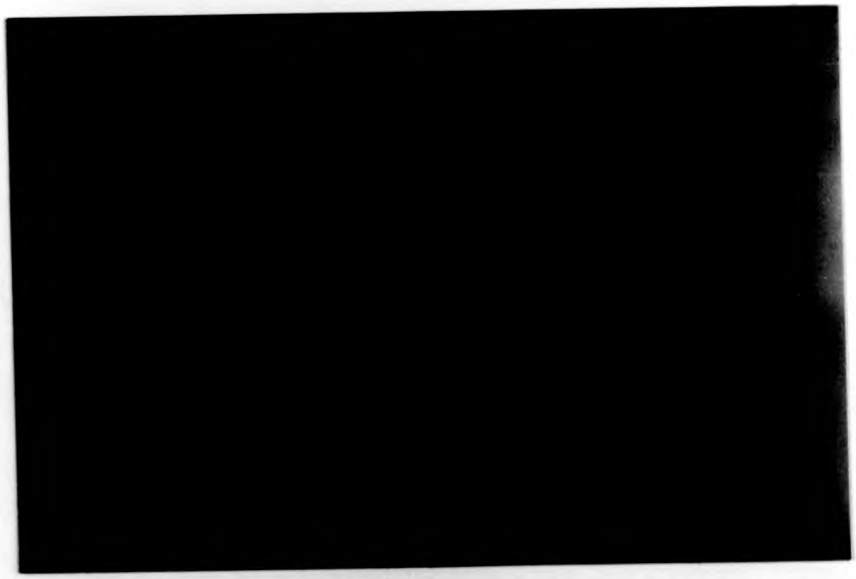
Note: 50% endpoints (arrowed) indicate haemolysin titres of 1:64 (Rows A and B), 1:256 (Row C), 1:512 (Row D), and 1:1024 (Row E.)

FIG. 7

The Radial Haemolysis Plate Method



a) using wells 1) Neat 2) 1:2 3) 1:4 4) 1:8 5) 1:16



b) Using discs 1) Neat 2) 1:2 3) 1:4 4) 1:8 5) 1:16

2.3.4. Optimum red blood cell concentration for Microtitre plate assay

Results are shown in Table 2. RBC suspensions giving OD_{545} of 100 and 80 produced haemolysin titres lf 1:64 each. A suspension giving OD_{545} of 60 produced a haemolysin titre of 1:256 and suspensions giving OD_{545} of 40 and 20 produced titres of 1:512 and 1:1024 respectively (Fig.6). The tube haemolysin test titre using the OD_{545}60 RBC suspension was 1:1024.

The results using RBC suspensions 1 and 2 showed very large buttons of cells in the wells and incomplete haemolysis in several wells below the 50% endpoint whilst suspensions 4 and 5 produced very faint buttons which were so pale that the actual endpoints were difficult to read. The RBC suspension with an OD_{545} of 60 gave a clear endpoint and appeared the optimum suspension for use in this test.

2.3.5. The effect of different incubation temperatures, atmospheres and buffers on haemolysin titres using the Microtitre plate assay

Results are shown in Table 3. The physiological saline dilutions showed lower haemolysin titres than the other diluents regardless of incubation temperature or atmosphere. Adding 0.1% gelatin to saline increased the results by one twofold dilution in most cases. ASO buffer and FAB produced identical results.

All tests incubated at 37°C produced higher haemolysin titres than those incubated at 20°C regardless of atmosphere and with ASO buffer and FAB incubation aerobically showed little difference to incubation anaerobically. Anaerobic incubation made endpoints slightly more difficult to interpret and was considerably less convenient. Using ASO buffer as diluent and incubating plates for 1 hour at 37°C aerobically appeared to combine convenience with maximum haemolysin titres.

2.3.6 The effects of different growth temperatures on the production of haemolysin by Cl.tetani

Results are shown in Table 4. It can be seen that no haemolysin was present in cultures incubated at 56°C, 44°C, 20°C or 4°C. Also the OD_{600}'s at these temperatures were extremely low, indicating that little or no growth had taken place.

At 30°C a haemolysin titre of 1:64 and an OD_{600} of 20 indicated that growth had taken place with the production of some haemolysin, but the maximum growth (OD_{600} of 34) and maximum haemolysin production (haemolysin titre of 1:256) took place at 37°C.

2.3.7 The effect of heat treatment on Cl.tetani haemolysin

Results obtained when Cl.tetani haemolysin was held at different temperatures for different periods are shown in Fig. 8. It can be seen that rapid inactivation of the haemolysin occurred at 75 and 70°C. At 65 and 60°C inactivation was less rapid but was still complete inside 1 hour. Inactivation at 56°C and 42°C was even less rapid, requiring 2 hours at 56°C for total inactivation. Total inactivation was not observed inside 2 hours at 42°C. The slopes of the linear part of each temperature curve shown in Fig.8 were calculated by taking two points on this curve (X_1, Y_1) and X_2 and Y_2) and using the following formula:

$$M = \frac{Y_2 - Y_1}{X_2 - X_1}$$

where M is the slope of the curve, X represents time and Y represents the number of twofold decreases in haemolysin titre observed.

These slopes, whilst not analogous to true reaction rates, do give an indication of the degree at which inactivation occurs at each temperature and were calculated to be - 0.03, -0.07, -0.13, -0.28, -0.46 and -0.7 twofold decreases in haemolysin titre per minute at 42, 56, 60, 65, 70 and 75°C respectively. These slopes are shown in Fig.9 plotted

against each temperature. The relationship between degree of inactivation and temperature shows a sharp rise as the temperature increases.

Fig.10 shows the results of the initial haemolysin tests, haemolysin after 30 minutes at different temperatures and after subsequent incubation at 4°C for 24 hours.

It can be seen that haemolysin heated to 75, 70, 65 and 60°C showed either complete inactivation or a large degree of inactivation after 30 minutes treatment (haemolysin titres of 0, 0, neat and 1:4 respectively), but considerable reactivation after incubation at 4°C (haemolysin titres of 1:32, 1:32, 1:64 and 1:64 respectively). The haemolysin treated at 56°C showed some inactivation after 30 minutes but no subsequent reactivation at 4°C and the haemolysin treated at 42°C showed no inactivation after 30 minutes but some inactivation after subsequent incubation at 4°C.

The slopes of the reactivation portion of Fig.10 (the increase in haemolysin titre observed on incubation at 4°C following heating at each temperature) could be calculated as before and these give an indication of the degree of reactivation observed following heating. These slopes were calculated to be -0.04,0, 0.125, 0.208, 0.25 and 0.25. twofold increases in haemolysin titre per hour for haemolysin heated at 42, 56, 60, 65, 70 and 75°C respectively. The slopes are shown in Fig.11 plotted against each temperature. The relationship between degree of haemolysin reactivation and the original temperature used is linear and appears directly proportional, up to 70°C above which no increase in reactivation was observed.

FIG.8

Haemolysin inactivation at different temperatures over a two hour period

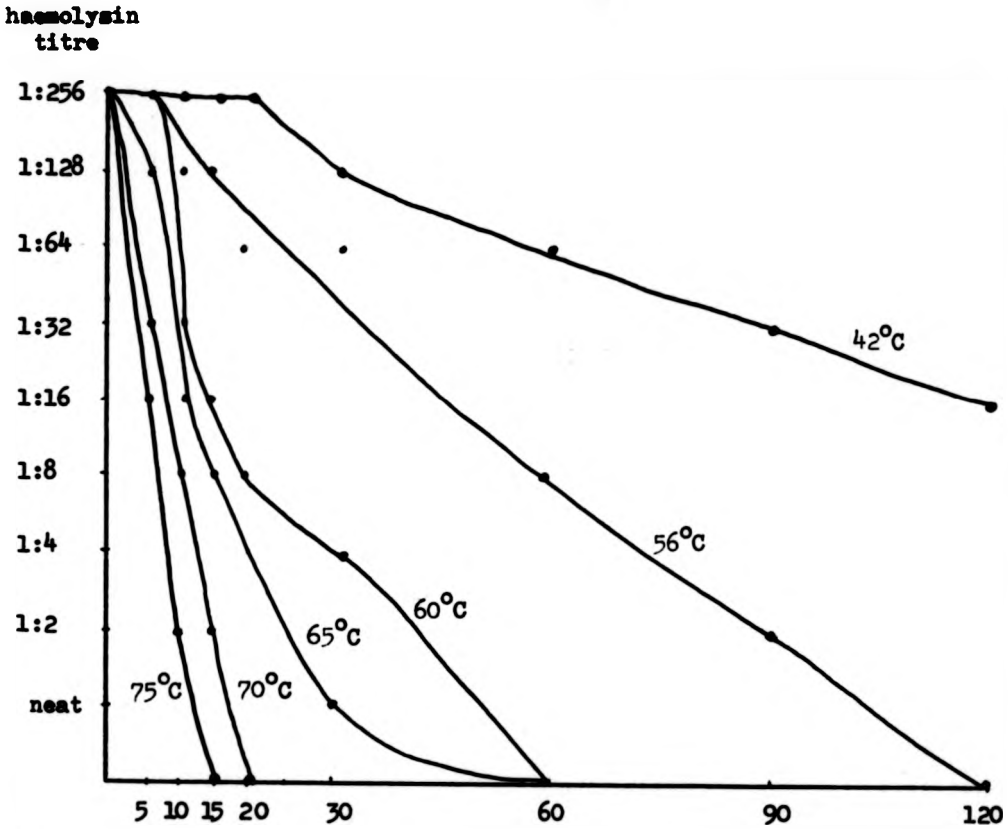


FIG.9

Comparison of degrees of inactivation of tetanolysin at different temperatures

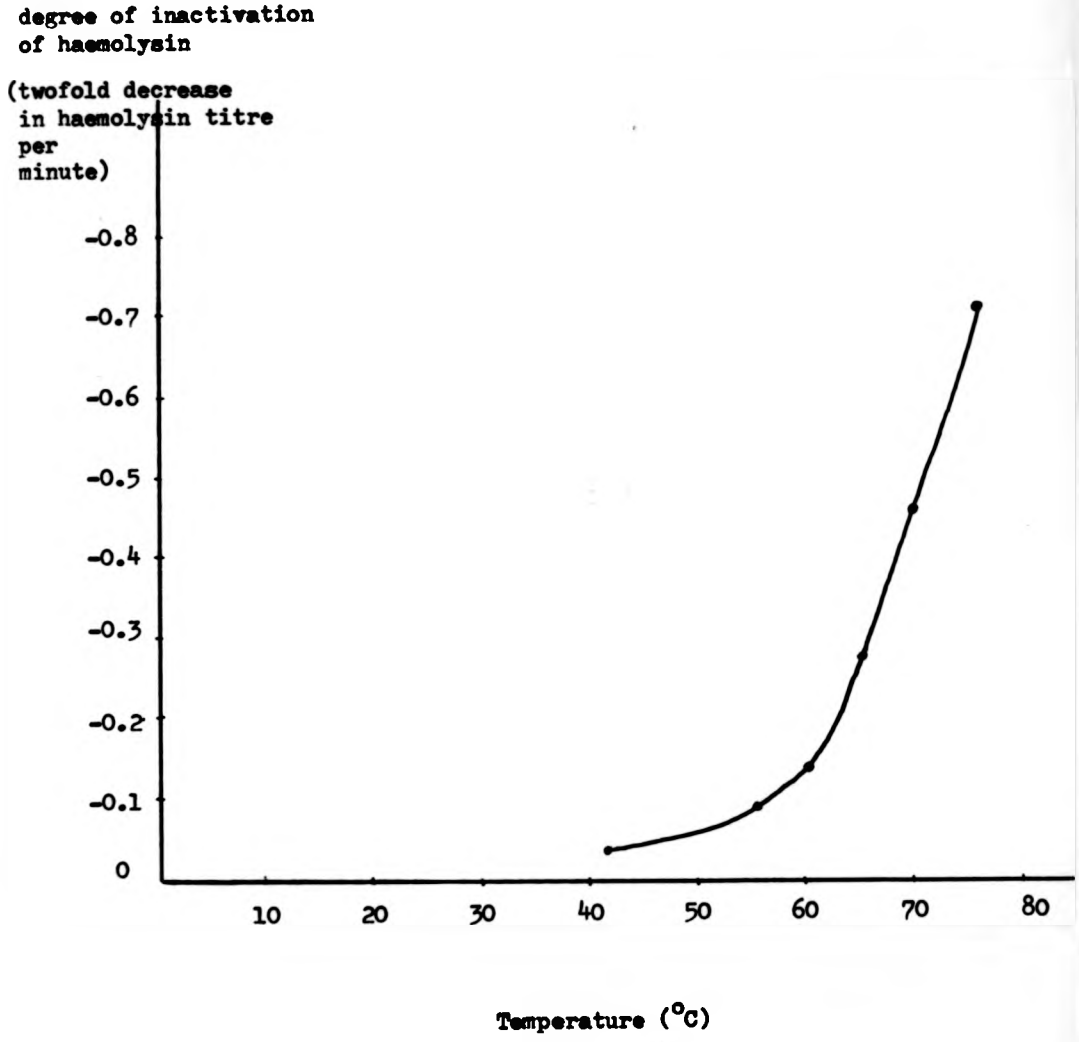


FIG. 10

Comparison of haemolysin titres obtained after heating at different temperatures and subsequent incubation at 4°C overnight

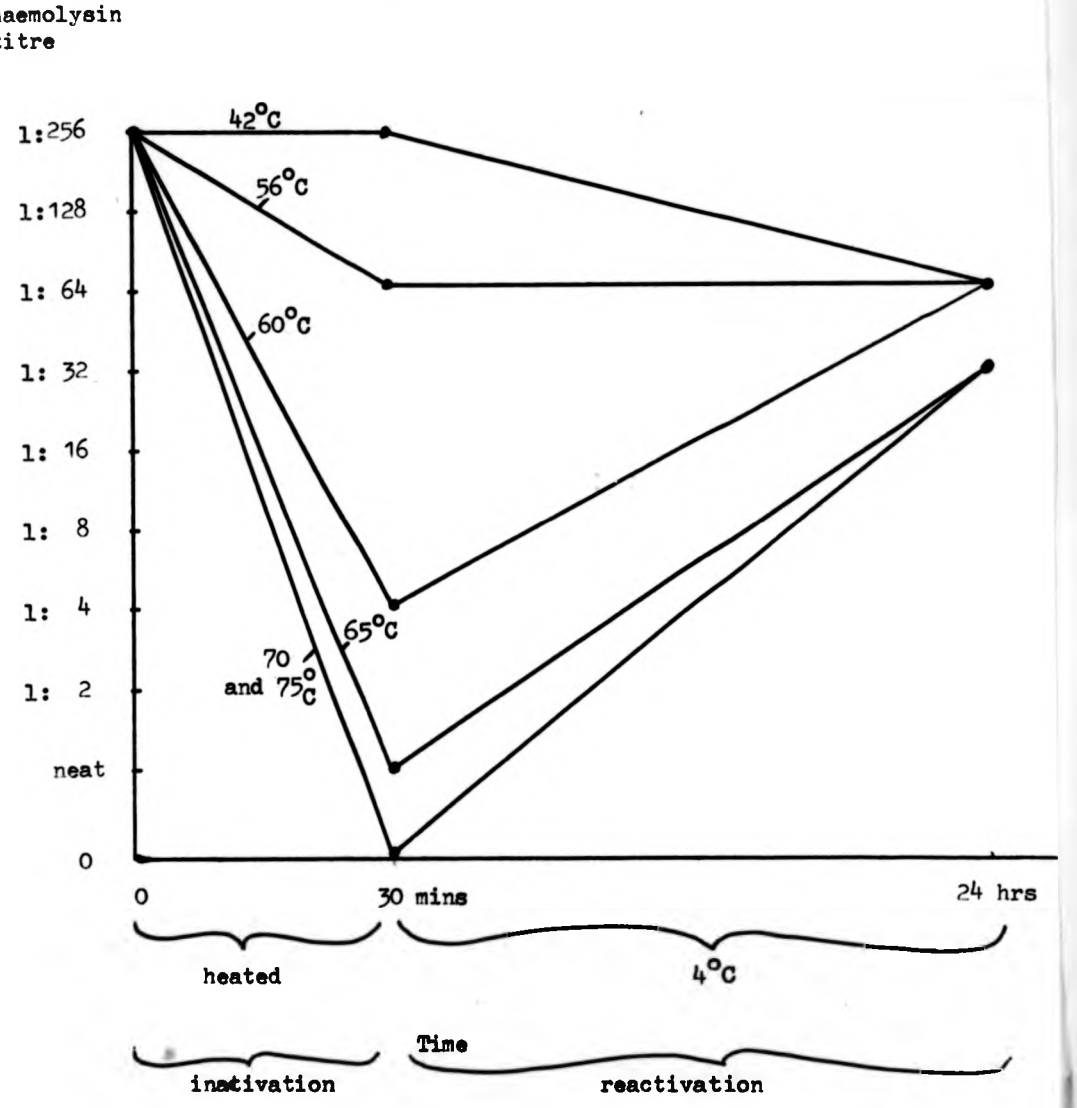
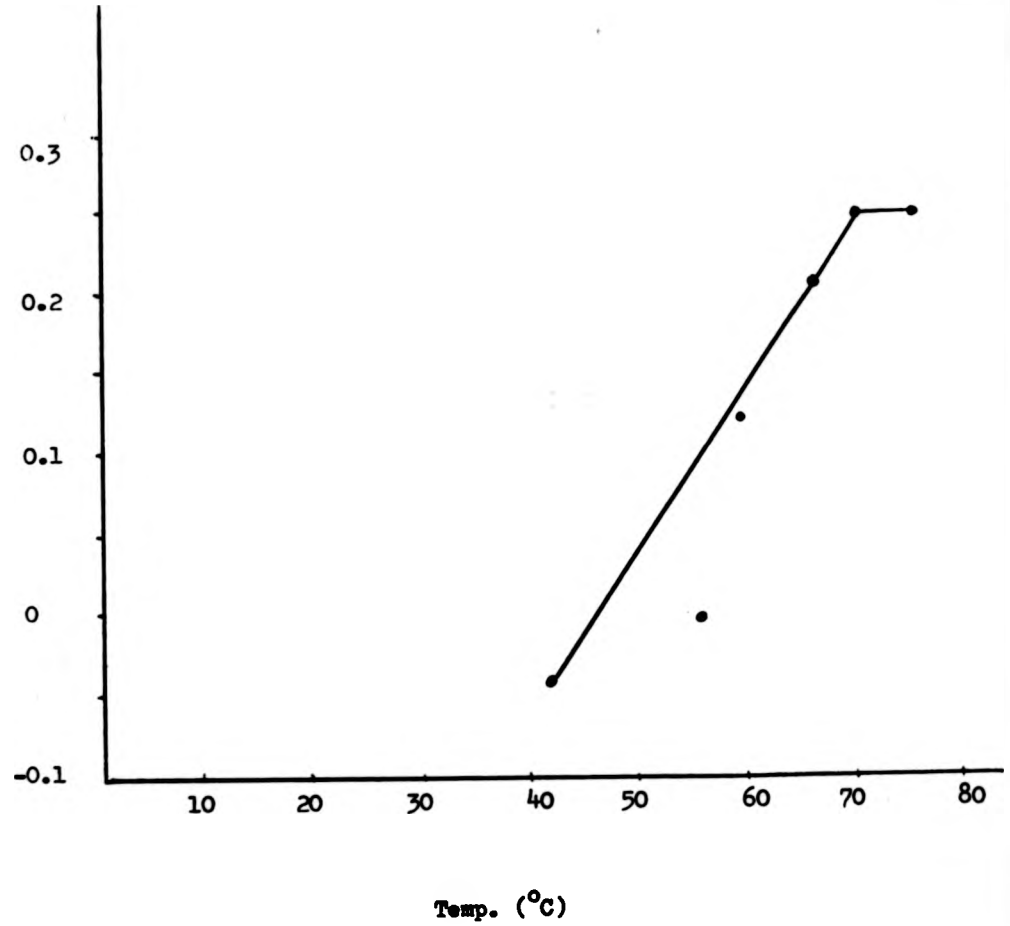


FIG.11

Comparison of degrees of reactivation of tetanolysin after heating at different temperatures and incubation at 4°C



2.3.8 The effect of different growth media on haemolysin production by Cl.tetani

Results are shown in Table 5. It can be seen that FAB, Wilkins-Chalgren broth and Massachusetts medium all produced identical haemolysin titres of 1:256 and very similar amounts of growth as measured by OD_{600}. The cooked meat medium produced growth almost equivalent to FAB and greater than Wilkins Chalgren broth, but the haemolysin titre was only 1:2. There appeared to be no or very little growth produced in serum broth or IST broth and no haemolysin produced after 24 hours.

2.3.9 The effects of storage of haemolysin at different temperatures

Results are shown in Table 6. It can be seen that, even in the reduced medium FAB, inactivation proceeds rapidly at most temperatures above freezing. Total inactivation occurs in less than 1 day at 56°C, in 2 days at 44°C, in less than 5 days at 37, 30 and 20°C and in less than 7 days at 4°C. Inactivation proceeds less rapidly at temperatures below freezing, but even at -18°C some deterioration was seen after 5 days. No change was observed with haemolysin stored at -40°C, but this necessitates storage in small aliquots to avoid having to thaw and re-freeze samples.

2.3.10 The effects of storage with different additives and under different conditions on the stability of the haemolysin

Results are shown in Tables 7 and 8. It can be seen that none of the additives could prevent some degree of inactivation of tetanolysin. Sodium azide, formalin and horse serum all hastened inactivation, no haemolytic activity being observed after 2 hours incubation with these agents. The haemolysin titres of the PBS, gelatin, peptone and lecithin treated preparations were reduced to 1:64 after 24 hours, although the major inactivation appeared to take place within the first 2 hours. The titres of the chloroform and BSA treated samples were reduced to 1:32 and the titres of the phenol treated sample was reduced to 1:16. The titre of the cooked

meat treated sample was reduced to 1:8 although the titre of the sample stored under liquid paraffin remained the same as the original titre, i.e. 1:256. All these samples, with the exception of the one stored under paraffin were pink, indicating some degree of oxidation of the oxidation-reduction indicator, Resazurin, contained in the FAB medium.

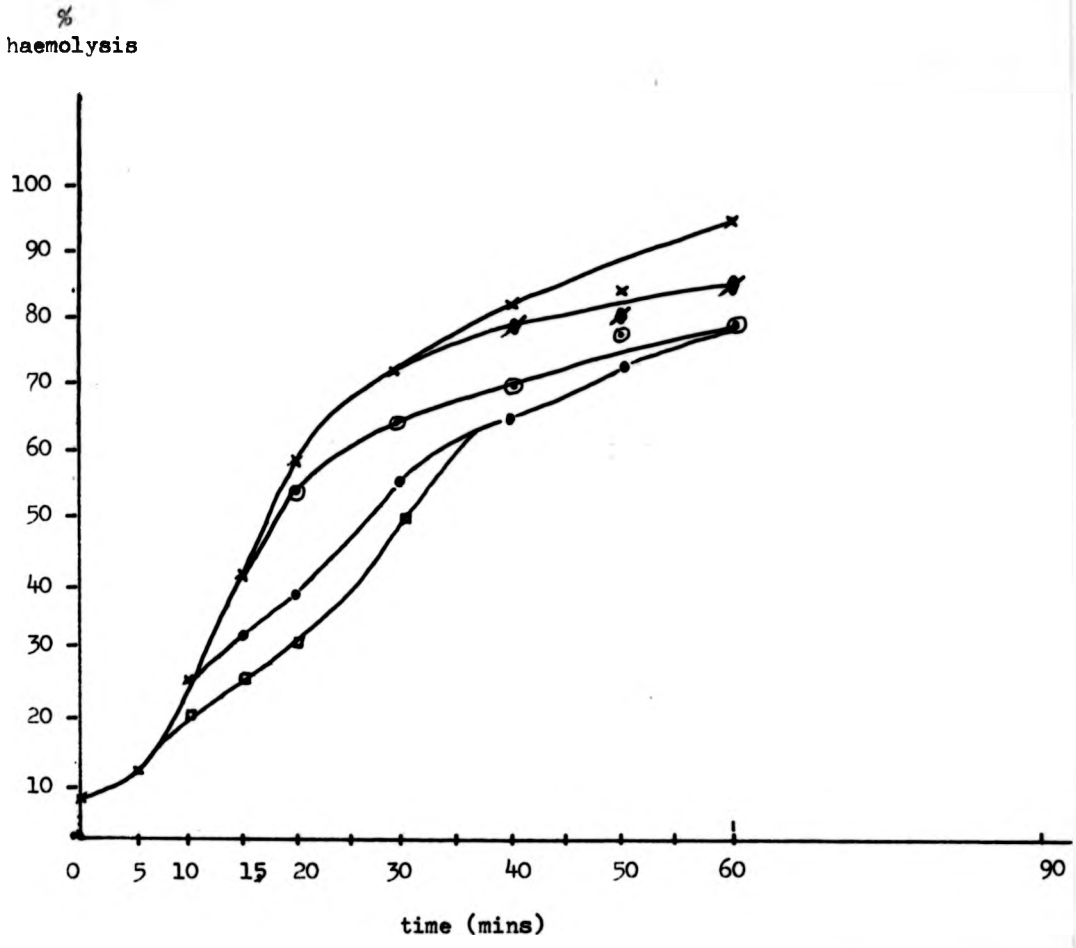
Haemolysin stored in volumes of 1, 2, 3, 5, 10 and 20mls had titres of 1:2, 1:4, 1:16, 1:64 and 1:64 respectively and all showed some degree of oxidation. Sample volumes of 50 and 100mls showed haemolysin titres of 1:128 and 1:256 respectively and showed no evidence of oxidation, being yellow in colour.

2.3.11. The effect of addition of Sucrose and Glucose on the kinetics of the haemolysin reaction

Results are shown in Fig.12. It can be seen that the control curve shows a similar pattern to that seen in Fig.1 for a 1:1024 dilution of haemolysin. The addition of sucrose or glucose at 30 minutes did not arrest haemolysis, but reduced the rate slightly, the final haemolysis being 85% rather than 95% as seen in the control. The addition of sucrose at 20 minutes reduced the haemolysis rate even further with the final haemolysis being 82%. Adding sucrose at 10 minutes and 0 minutes also reduced the initial rate of haemolysis although eventually it climbed to the same rate as when sucrose was added at 20 minutes, the final haemolysis being 82%.

FIG.12

Comparison of haemolysis rates before and after addition of Sucrose and Glucose to tetanolysin



x control (no Sucrose)

Sucrose/Glucose added at 30 minutes

Sucrose added at 20 minutes

Sucrose added at 10 minutes

Sucrose added at 0 minutes

2.3.12. The effects of addition of antitoxins on the haemolysin reaction

The effects of the different antisera are shown in Table 9. Cl.perfringens type A antitoxin had the highest haemolysin inhibition activity, closely followed by Normal horse serum. Cl.novyi type A and type B had the next highest inhibition activity, followed by Cl.septicum, Cl.chauvoei and human antitetanus toxin sera.

The three horse antitoxins had the lowest observable haemolysin inhibition titres; the remaining sera showing no inhibition.

Tetanus antitoxin 4 absorbed with the haemolytic culture supernatant (NCTC 279) had its haemolysin inhibition titre reduced from 1:2 to 0, whereas when absorbed with the non-haemolytic supernatant (NCTC 9596) had no observable decrease in haemolysin inhibition titre.

However, Normal human serum, Normal horse serum and Human tetanus antitoxin all had very high cholesterol levels and the other Clostridial antitoxins had raised cholesterol levels when compared to Cl.tetani antitoxin, Pneumococcal omniserum, Antistreptolysin O and Rabbit plasma which had no detectable cholesterol.

2.3.13. Comparison of the growth rate and haemolysin production rate of Cl.tetani

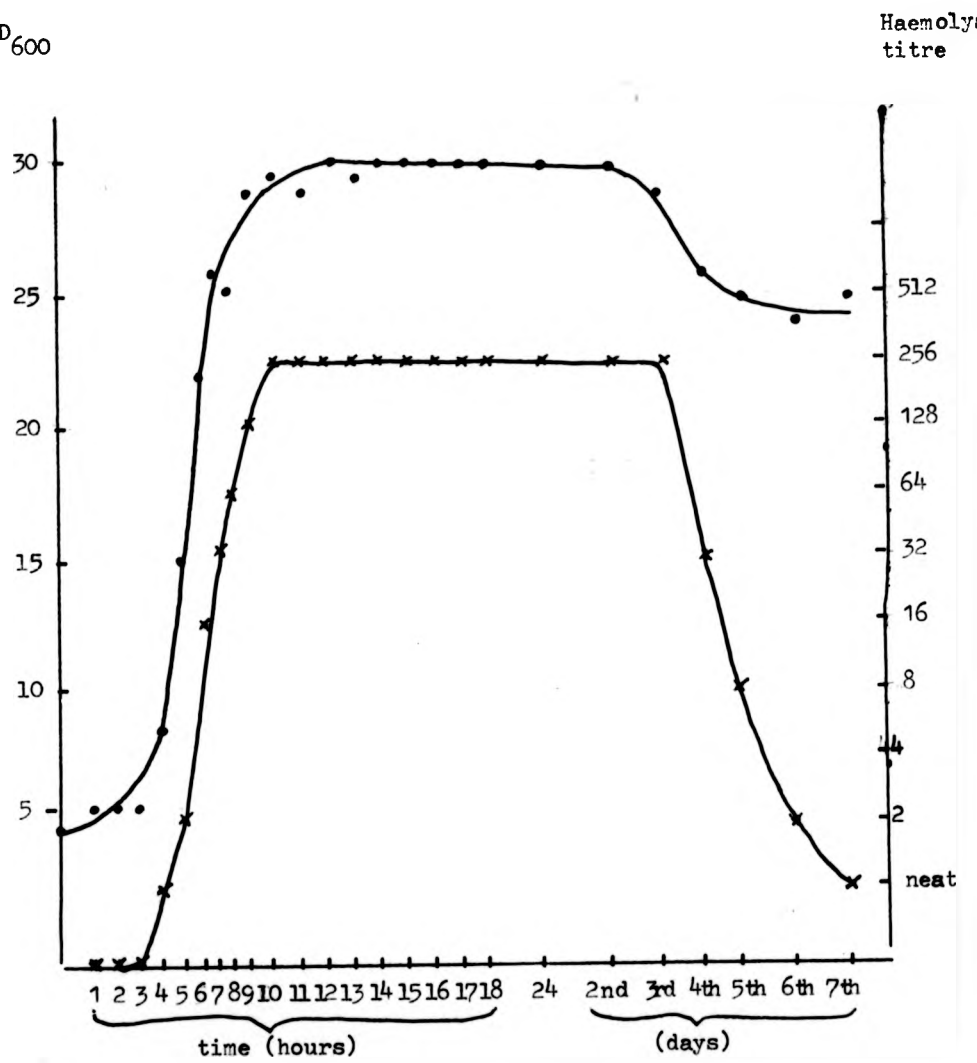
It can be clearly seen from Fig.13 that there is a direct relationship between the growth rate and the haemolysin production rate of Cl.tetani when both were monitored over a period of 7 days. Maximum growth was achieved about 10 hours after the start of the experiment and maximum haemolysin production was achieved virtually at the same time. The haemolysin titre remained at this level (1:256) until between 2 and 3 days when it began to fall rapidly to a level that varied between negative and 2. The growth rate showed a similar pattern, a lag phase of around 3 hours, a log phase lasting until around 12 hours, a stationary phase until about 48 hours and then a gradual decline until 7 days after inoculation.

When the amounts of haemolysin obtained in culture supernatants, washed cells tested immediately and washed cells extracted overnight at 4°C were compared (Fig.14), it can be seen that the presence or absence of haemolysin is roughly comparable although the titres obtained were substantially lower with washed cells and even lower after incubation overnight.

The appearance of haemolysin in the cell preparations was some 2 to 3 hours after the appearance in the supernatant and the haemolysin in the cell preparations began to reduce and eventually disappear some 2 to 3 days before that in the supernatant.

FIG.13

Comparison of growth rate and haemolysin production in Cl.tetani

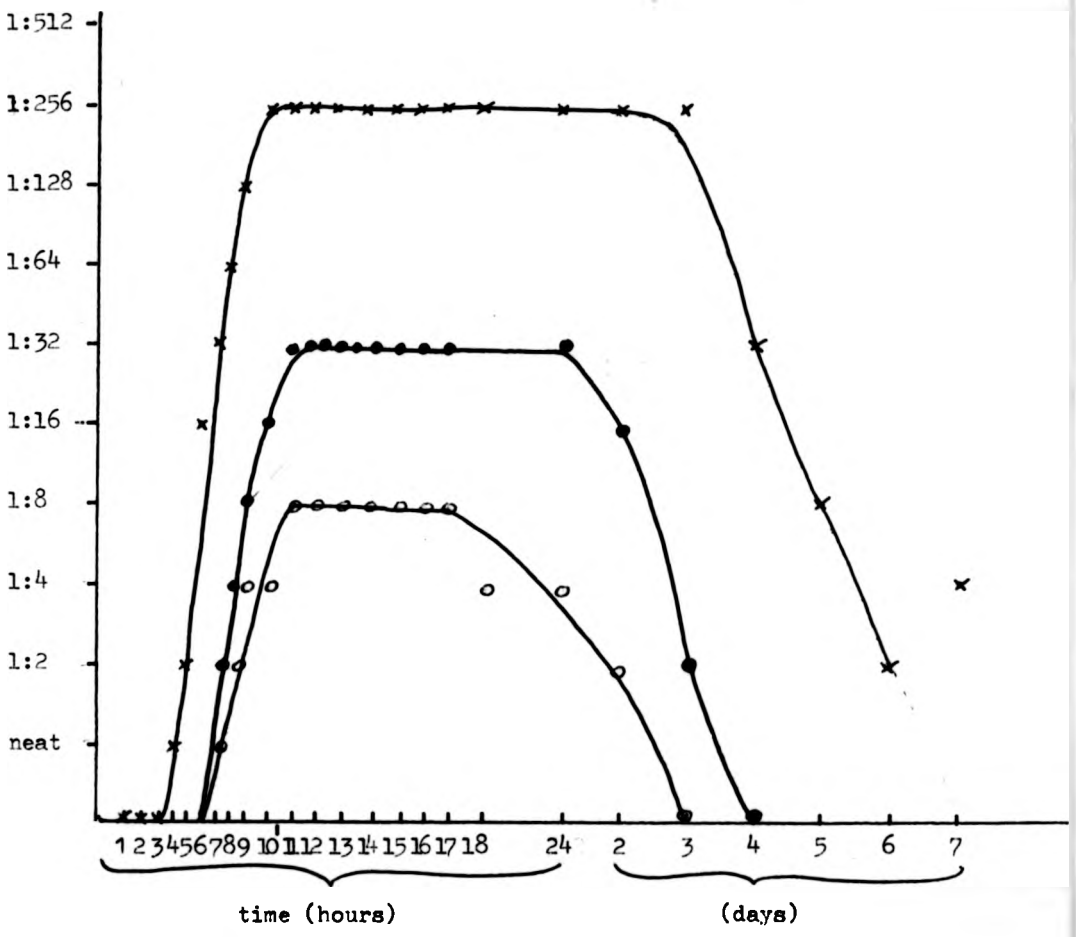


Growth rate •

Haemolysin titre x

FIG.14

Comparisons of haemolysin in culture supernatant and in washed cells of Cl.tetani with period of incubation



× haemolysin in supernatant

o haemolysin in washed cells

● haemolysin in supernatant of washed cells incubated overnight

2.3.14. The effects of different pH on the haemolysin of Cl.tetani

It can be seen from Table 10 that tetanolysin stored at pH 5, 6, 7, 8 and 9 underwent little inactivation, each producing haemolysin titres of 1:64, compared to the original titre of 1:128. Storage at pH 10 reduced the titre to 1:32 and at pH 11 the titre was reduced to 1:2. It was not until pH 12 that the haemolysin was totally inactivated. In contrast, at pH 1, 2, 3 and 4 the haemolysin was totally inactivated.

2.3.15. The production of tetanolysin by different strains of Cl.tetani

Results are shown in Table 11. All strains tested produced haemolysin under the conditions stated with the exception of NCTC 9569 (serotype VI). The amount of growth was very similar in all cases, with the exception of NCTC 9569 which is a non motile strain and grows poorly in broth. Therefore the haemolysin titres could be compared directly to each other. Titres ranged from 1:64 to 1:256 with the different strains, although individual strains rarely showed more than one dilution difference in different tests.

2.3.16. The effect of heat treatment on production of haemolysin by Cl.tetani

Results are shown in Table 12. It can be seen that the haemolysin titres produced by the unheated strains were all in the range 1:64 - 1:128 and were very similar to those obtained earlier (Table 11), with the exception of strain VI which produced no haemolysin as before. The growths were of the same order in general. However, 21/28 of the heated strains produced no haemolysin at all and produced very little growth (although all except serotype 1 grew well on subculture to blood agar plates). These 21 strains were almost exclusively the ones with few or no spores in the original suspension. The other strains produced less haemolysin than the

parent strains, even though the amount of growth appeared greater. The heat treated variant of strain I (1-H-80) produced a similar growth to the parent strain but a substantially reduced haemolysin titre (1:8 compared to 1:64 with the parent strain) before heating and after heating no haemolysin at all was observed.

The number of spores seen in the original suspensions varied from 0 to 40%, although many had < 1% and the majority of the rest < 10% (Table 12).

Of the unheated cultures only seven strains produced more than 1% spores (strains 1-H-80, L5500, LQ1, LQ2, LQ931, LQ914 and LQ730, producing 6, 4, 10, 8, 10, 8 and 6% spores respectively). The heated strains had four strains producing spores (strains 1-H-80, Harvard, 761 and 947 producing 50, 12, 16 and 2% respectively).

2.3.17 Purification of tetanolysin

The haemolysin titre and protein content of the fractions eluted from the Sephadex G-200 column in Method 1 are illustrated in Fig.15. The fractions containing maximum haemolytic activity (Nos. 7, 8 and 9) eluted between the first protein peak (fraction No.5) and the major protein peak (fractions 9, 10, 11, 12 and 13). The first protein peak corresponded to the Antigenic activity observed by both DD and Rocket IEP (fractions 4, 5 and 6) (Table 13). A single precipitin line was observed by DD (Fig 16) and single peaks by Rocket IEP (Fig.17).

PAGE analysis showed 5 protein bands between fractions 4 and 14 (Table 13), with 4 high molecular weight bands (141.25, 136, 125 and 116 KD respectively) and one low molecular weight band (68.75KD). This low molecular weight band appeared on some gels as a doublet band of 66 and 67.75 KD respectively. The high molecular weight bands were strongest in fraction 5 (Figs.18, 19) and were also seen in fractions 4 and 6 corresponding to antigenic activity. The low molecular weight doublet was present in fractions 4, 5, 6, 7, 8, 9, 10, 11 and 12. All

FIG.15

Haemolytic activity and protein concentrations of Method 1 Sephadex G-200 fractions of concentrated culture filtrate of Cl.tetani NCTC 279

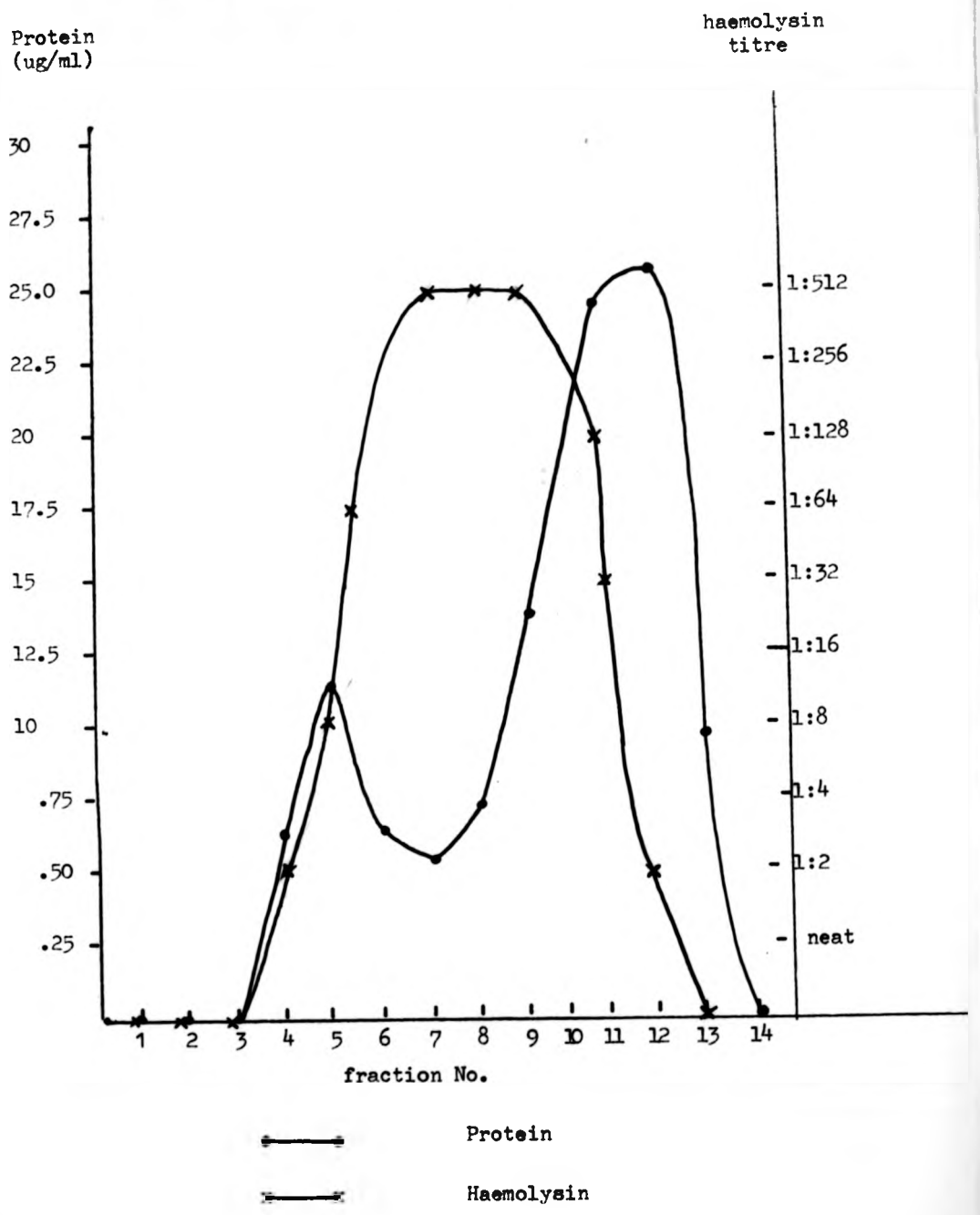
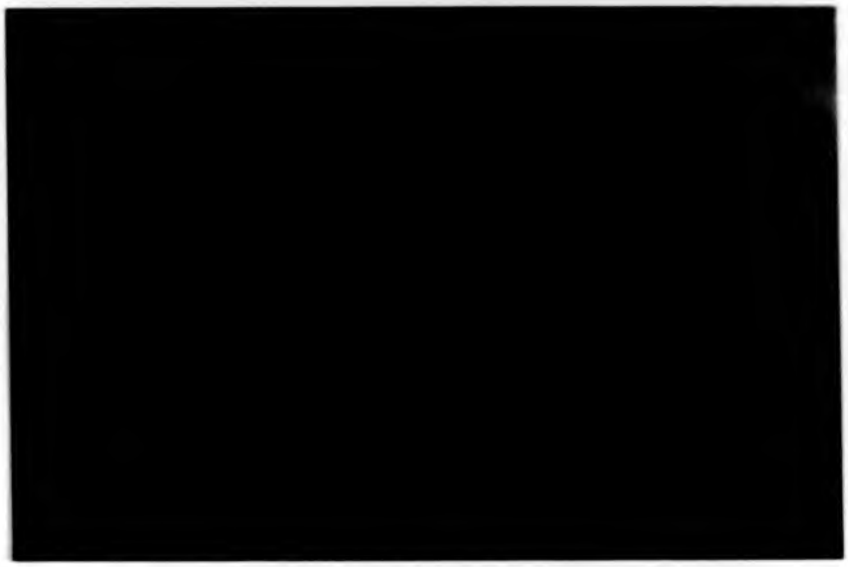


FIG.16

Antigenic activity of Method 1 Sephadex G-200 fractions of concentrated culture filtrate of Cl.tetani NCTC 279 examined by DD



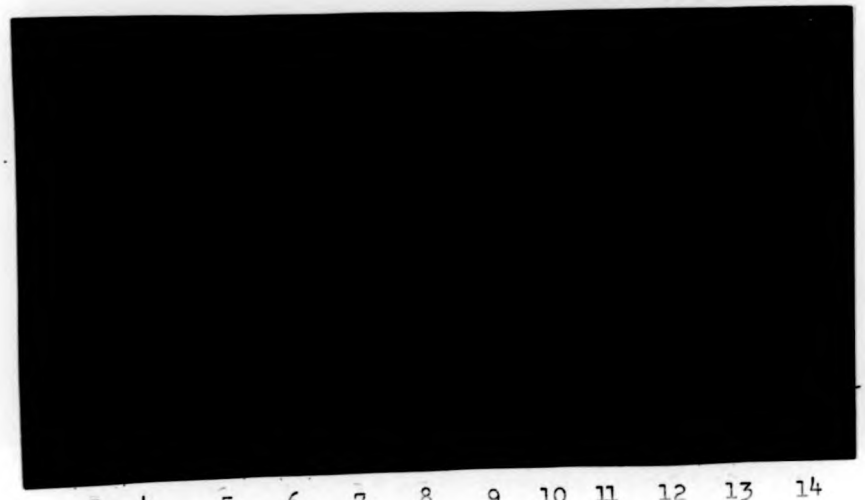
Central well Cl.tetani antitoxin neat

Wells 1) Sephadex G-200 fraction 3
2) fraction 4
3) fraction 5
4) fraction 6
5) fraction 7
6) fraction 8
7) fraction 9
8) fraction 10

Note: precipitin line produced by fractions 4, 5, 6 and 7 (arrow)

FIG.17

Rocket Immunoelectrophoretic analysis of Sephadex G-200 fractions of concentrated 24 hour FAB broth supernatant of Cl.tetani NCTC 279



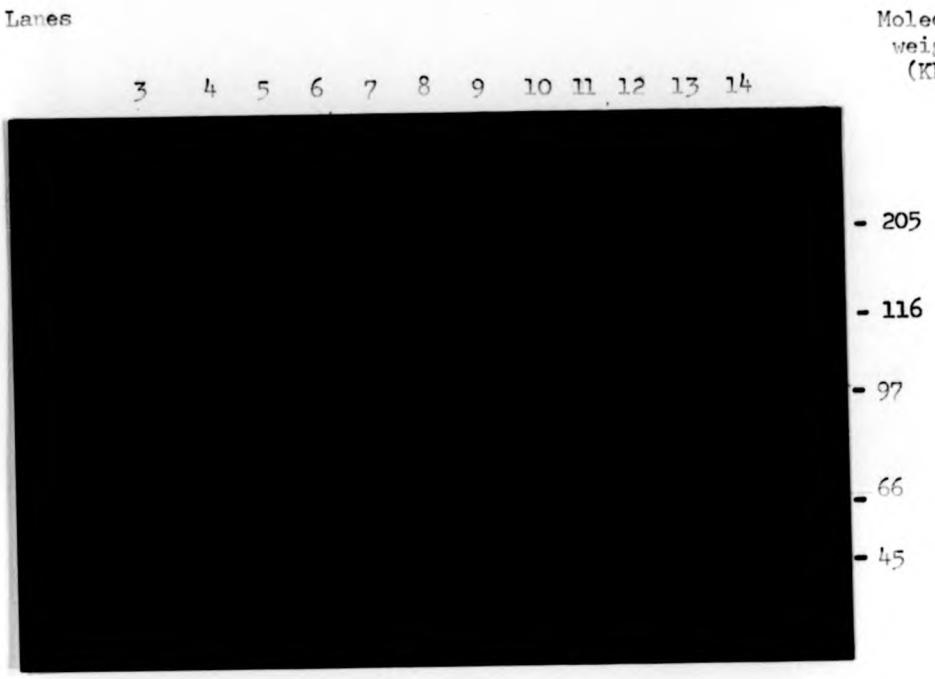
3 4 5 6 7 8 9 10 11 12 13 14

Sephadex fraction No.

Note: precipitin peak in fractions 4, 5 and 6 (arrow)

FIG.18

PAGE analysis of Sephadex G-200 fractions of concentrated culture supernatant of Cl.tetani NCTC 279



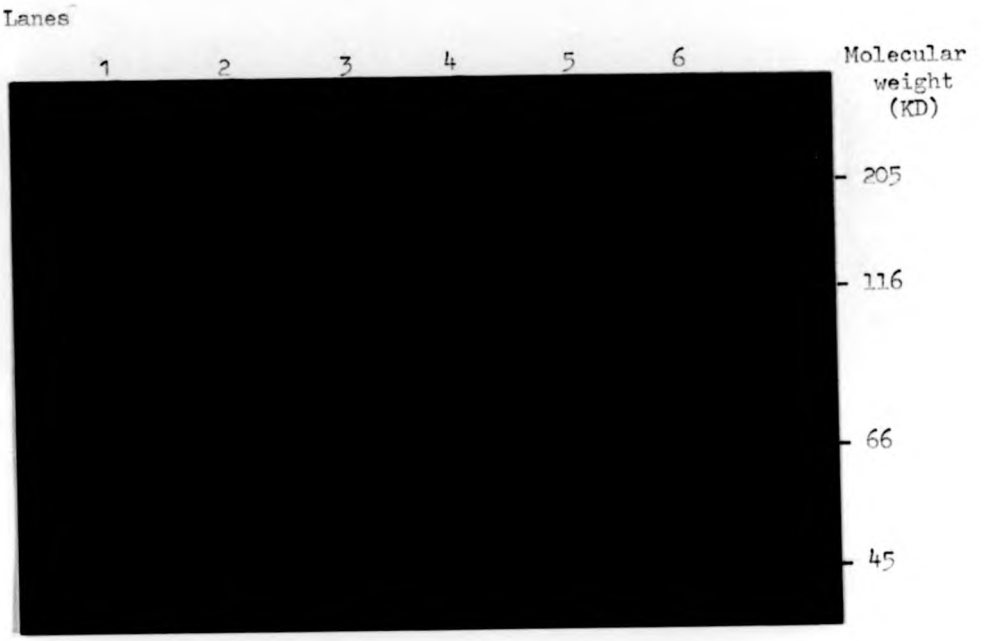
Lanes:

3 Sephadex G-200 fraction 3
4 " " 4
5 " " 5
6 " " 6
7 " " 7
8 " " 8
9 " " 9
10 " " 10
11 Original ammonium sulphate precipitate
12 " " " "
13 -
14 Molecular weight standard

The 66 - 68 KD band is apparent in fractions 4 - 10 and the original ammonium sulphate precipitate (arrow)

FIG.19

PAGE analysis of Sephadex G-200 fractions of concentrated culture supernatant of Cl.tetani NCTC 279



Lanes:

1 Concentrated Sephadex G-200 fraction 11
2 Original 24 hour culture supernatant
3 Concentrated Sephadex G-200 fraction 11
4 " " " " 5
5 " " " " 8
6 " " " " 8

these fractions had haemolysin present. Fraction 8 appeared to be almost pure haemolysin with a haemolysin titre of 1:512 and a protein content of 0.75ug/ml.

Concentrated fraction 8 showed no antigenicity by DD or Rocket IEP and possessed the low molecular weight PAGE band.

The original starting culture supernatant showed 6 precipitin peaks by 2 dimensional IEP, but concentrated fraction 8 showed no peaks at all when tested similarly (Fig.20).

The final product from method 2 showed very limited haemolysin activity, a titre of 1:16 only and no antigenicity by DD or Rocket IEP.

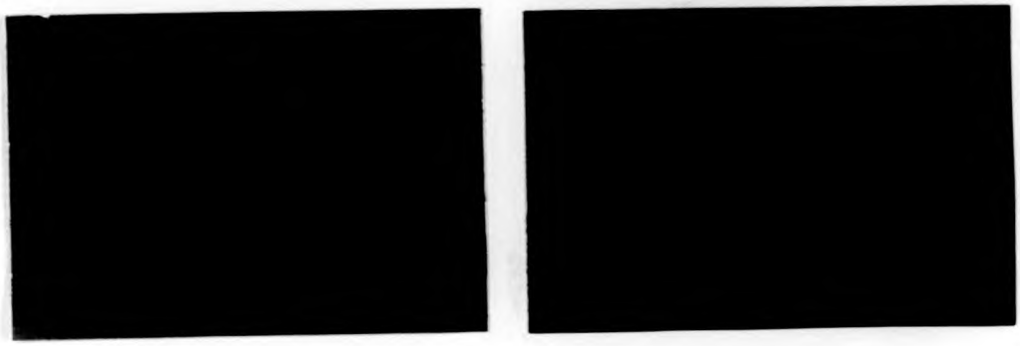
There was insufficient material for protein determination but a 68.75 kd PAGE band was observed by SDS - PAGE.

The haemolysin titres and protein content of the Sephadex G-200 fractions obtained in method 3 are illustrated in Fig.21. The fractions containing maximum haemolytic activity were No.2, 3, 4 and 5 and eluted with a major protein peak. However, these fractions also possessed the high molecular weight PAGE bands and antigenic activity and were therefore relatively impure. The haemolytic activity and protein concentrations of Sepharose 6B fractions of the pooled, concentrated Sephadex fractions 2, 3, 4 and 5 are shown in Fig.22. There were four protein peaks observed, but only one (fractions 11, 12 and 13) showed haemolytic activity. These fractions showed no antigenic activity by Double Immunodiffusion or Rocket Immunoelectrophoresis and were virtually free of the high molecular weight bands by SDS - PAGE. The 68.75 kd band was strongly present in all these fractions however. Fraction 13 appeared to be pure haemolysin with a protein content of 92 ug/ml.

The agglutination reactions of the different Sephadex fractions from method 1, the final product from method 2 and the concentrated culture supernatant and fraction 8 are shown in Table 14. The Staphylococcal coagglutination reagents autoagglutinated in all cases. Although concentrated culture supernatant produced strong agglutination with

FIG.20

Two-dimensional Immunoelectrophoretic analysis of Starting material from 24 hour culture supernatant and Sephadex fraction showing maximum haemolysin activity



a) 24 hour culture supernatant (25ul) (FAB) of Cl.tetani NCTC 279

(6 peaks)

b) Sephadex fraction 8 of original material (maximum haemolysin activity) (25ul)

(no peaks)

FIG.21

Haemolytic activity and Protein concentration of Method 3 Sephadex G-200 fractions of concentrated cultrate filtrate of Cl.tetani NCTC279

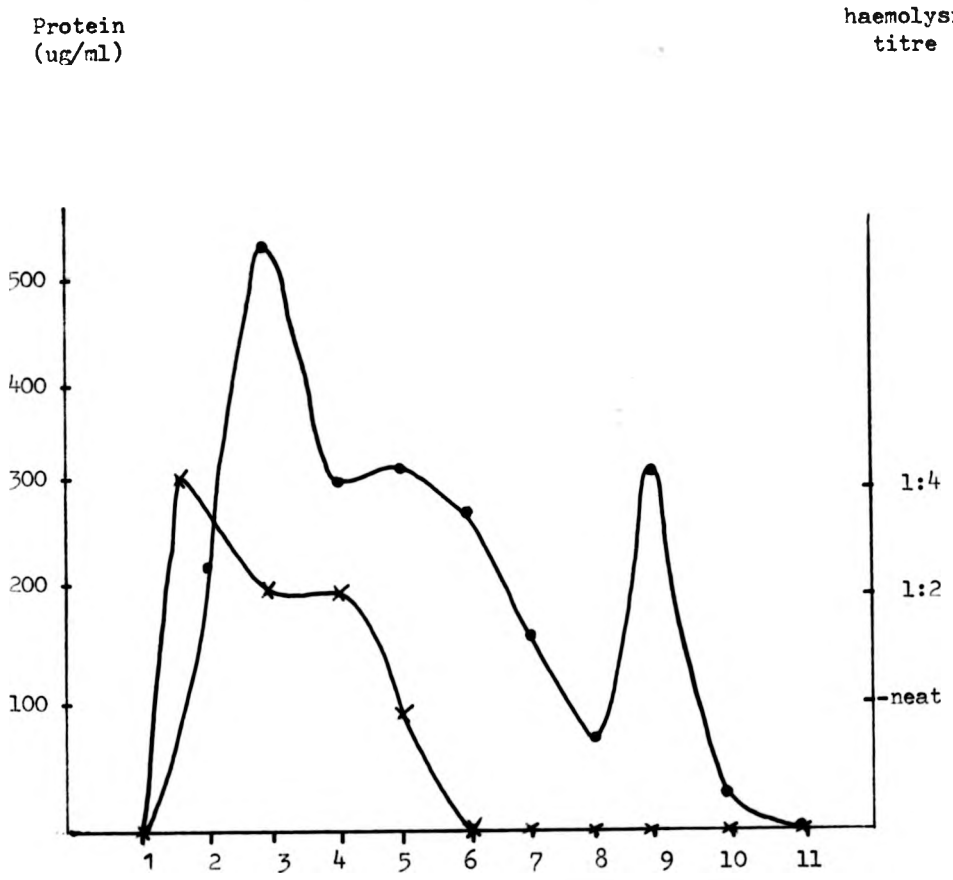
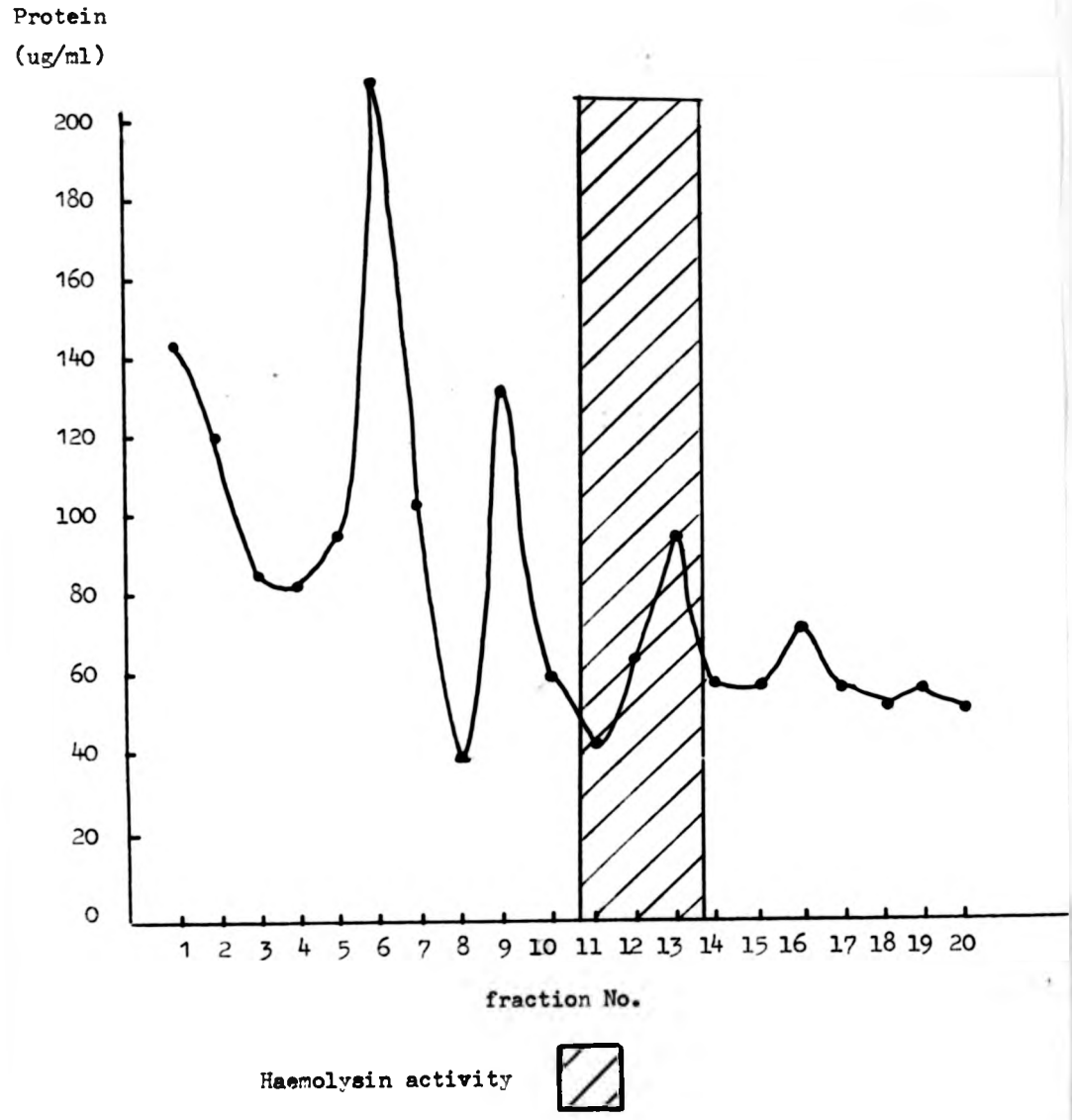


FIG.22

Haemolytic activity and Protein concentration of Method 3 Sepharose 6B fractions of concentrated pooled Sephadex fractions 2, 3, 4 and 5



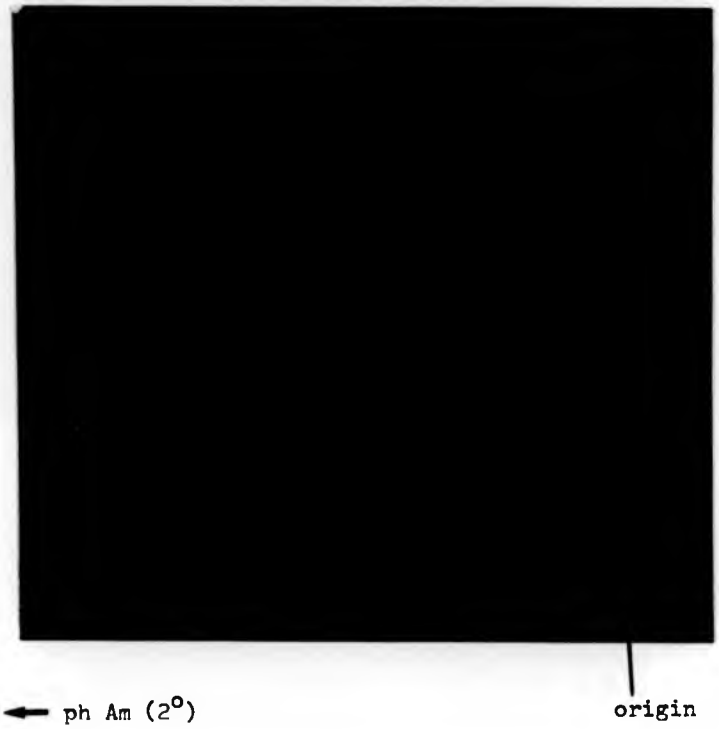
both absorbed and unabsorbed latex reagents, no agglutination was observed with any of the Sephadex G-200 fractions, the concentrated fraction 8 or the method 2 product using the unabsorbed latex reagent and trace or very weak reactions only with the absorbed reagent. When the concentrated method 1 haemolytic fraction and the method 2 product were examined by Two dimensional paper chromatography, no ninhydrin active spots were observed. With conc. Sepharose fraction 13 from method 3 however, anumber of ninhydrin active spots appeared (Fig.23). By comparison with the standard patterns (Fig.24) these spots were identified as isoleucine, alanine, lysine, threonine, glutamine, glutamic acid and serine.

When concentrated method 1 Sephadex fraction 8 and concentrated method 3 Sepharose fraction 13 were examined by isoelectric focusing a single major band was seen with both (Fig.25). This band had an isoelectric point of 5.0.

FIG.23

Products of Sephadex G-200 filtration (Method 1) and Chloroform/methanol extraction (Method 2) of concentrated Cl.tetani culture supernatants examined by two-dimensional paper chromatography

Sepharose fraction 13 from Method 3 40 ul



KEY

a - isoleucine
b - alanine
c - lysine
d - glutamine
e - threonine
f - glutamic acid
g - serine

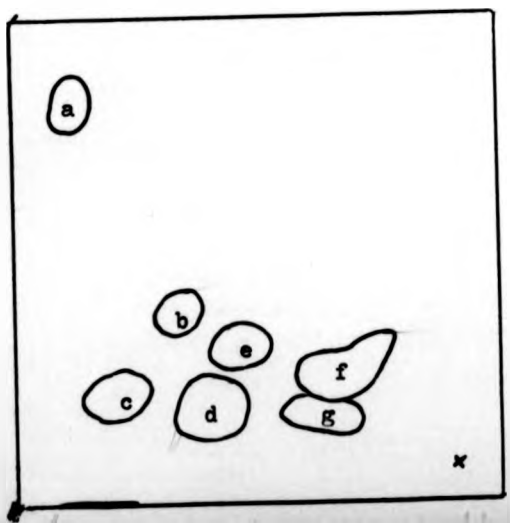
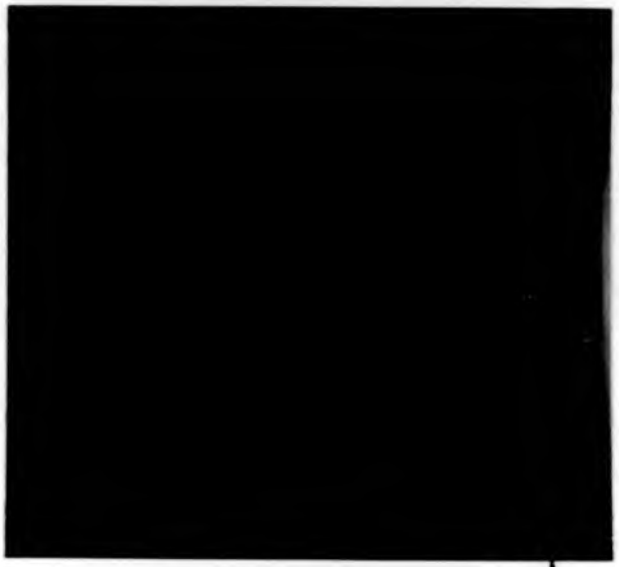


FIG.24

Amino acid standards examined by two-dimensional paper chromatography

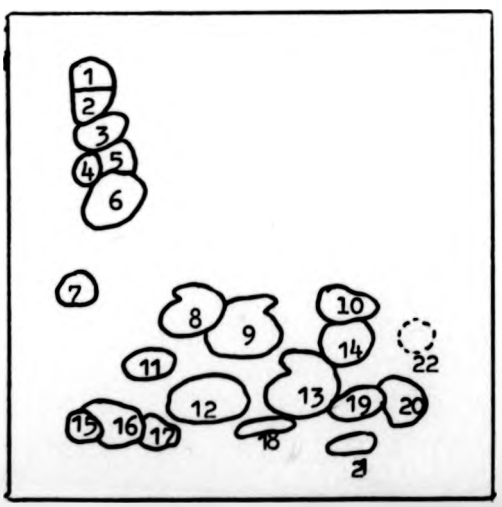


BuA (1^{o})

Ph Am (2^{o})

origin

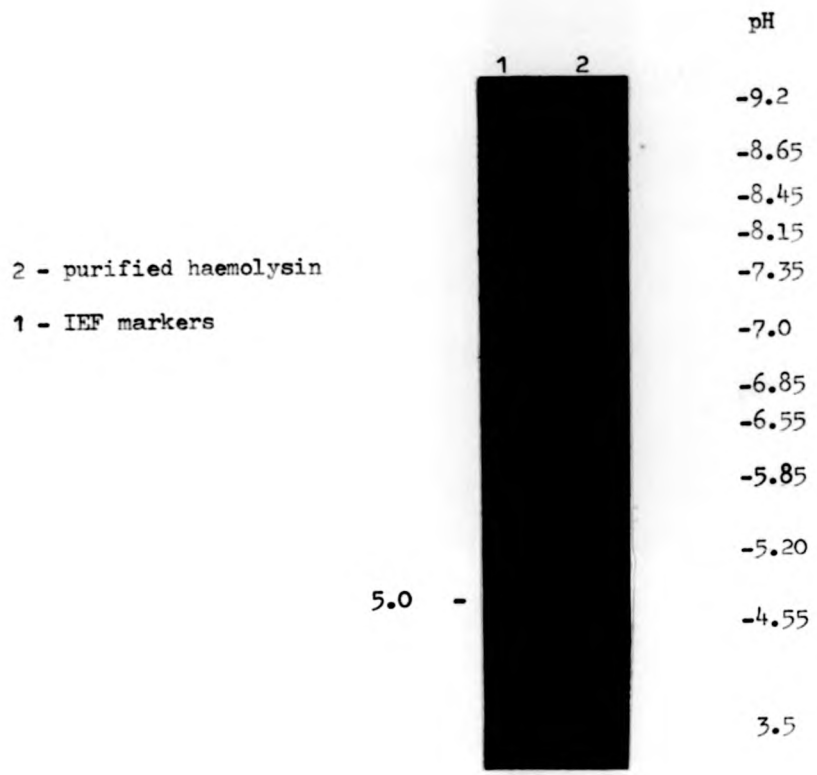
KEY



1. Leucine
2. isoleucine
3. phenylalanine
4. methionine
5. valine
6. tryptophan
7. proline
8. alanine
9. threonine
10. glutamic acid
11. hydroxy proline
12. glutamine
13. glycine
14. serine
15. arginine
16. Lysine
17. histamine
18. asparagine
19. cysteine
20. cysteic acid
21. cystine
22. aspartic acid

FIG.25

Isoelectric focusing of Cl.tetani haemolysin



2.4 DISCUSSION

The haemolysis curves obtained when per cent haemolysis was plotted against time for several different concentrations of tetanolysin were found to be more or less sigmoid (Fig.1). This is in agreement with the findings of Bernheimer[55]. Inspection of the curves shows that part of each is linear. The slope of the linear part is the maximum rate of haemoglobin liberation induced by each concentration of tetanolysin and although the curves in Fig.1 show clearly that the rate of lysis is dependent on concentration, when the rate of maximum haemolysis is plotted against the dilution of haemolysin used (Fig.2) it can clearly be seen that the relationship is linear and directly proportional over the concentrations tested. It is also c lear that, regardless of concentration, the major part of the haemolysin reaction is complete after 1 hour.

The haemolysis rates and curves obtained here are analogous to the rates obtained in studies of reactions catalyzed by enzymes and are extremely similar to those obtained earlier[55].

When the rate of haemolysis was considered as a function of temperature (Fig.3) very similar sigmoid curves to the concentration experiments were obtained. However, when the slopes for each temperature were plotted against the actual temperature (Fig.4) it could be seen that the relationship between the two was not linear and was not directly proportional, a logarithmic increase in rate being seen with increasing temperature up to 37°C This of course, is only to be expected, as reactions of this type usually have a single optimum temperature, with reaction rates falling steeply on either side of it. It is likely

that, had it been possible to test higher temperatures than 37°C, a corresponding sharp fall in reaction rate would have been found, with 37°C appearing as the optimum temperature for the reaction.

In earlier investigations of tetanolysin varying amounts of culture filtrate were added to constant amounts of RBCs, the mixtures incubated for a time and the amount of haemoglobin in the supernatant measured to determine the dilution causing total haemolysis or 50% haemolysis[45,46,47,49,50]. It has been shown that the use of the 50% endpoint gives more accurate results[50] and in this study 50% endpoints were used throughout.

When the tube haemolysin test, the microtitre plate haemolysin test and the radial haemolysis plate test were compared for ease of use, reliability and ease of determining endpoints, the microtitre plate assay was judged to be superior. This method has not been used in studies of tetanolysin before, but the ease in which multiple assays can be made and the clarity of the 50% endpoint made it a very good choice for the rest of the study.

Although the tube method produced higher titres than the microtitre plate method (Table 1), when the number of haemolytic units/ml (HU) of original culture supernatant were calculated, the levels found by the microtitre plate method were 5 times greater.

This is unexpected, as different methods of haemolysin determination would be expected to show roughly comparable levels of haemolytic units per ml in the same supernatant. Even a misreading of one of the tests by one dilution would only produce a twofold

difference. The reason for such a gross difference and the difference between these levels and the very low levels produced by the radial haemolysis plate method are unexplained, but are presumably inherent in the methods themselves. Although the same concentration of standard RBC suspension was used in each method, the different total volumes used may have affected the reaction sufficiently to cause the observed discrepancies. However, many definitions of a haemolytic unit define it as 'the amount that liberates half the haemoglobin in the test red cell suspension under the conditions stated'[55], so perhaps the variability of HU estimations using different techniques has been encountered before. Indeed, some workers feel it necessary to titrate a standard haemolysin with each test and correct the results according to the deviation from the fixed value of the standard to make all titrations comparable.[55]

Therefore, although both HU and percentage of total haemolysis have been used previously to express the haemolytic potential of tetanolysin[50,53,64], it was felt that for the purpose of the present work haemolytic activity would be best expressed simply as the titre, or highest dilution showing 50% lysis.

The concentration of RBCs used in this experiments was originally arbitrarily chosen to give OD_{545} of 60 as this was close to the concentration routinely used beforehand, but this may not have been the optimum concentration for the microtitre plate assay under the conditions of the test. In actual fact, however, when 5 different concentrations of RBCs were investigated, the one giving an OD_{545} of 60 proved to give the clearest 50% endpoint.

The haemolysins used in several of the earlier studies were filtered before use[49,50,60] and even though the studies of Fleming[49] have shown that there is no loss of haemolytic activity on filtration, it was felt advisable to use simple culture supernatants obtained by centrifugation in this study. It was also quicker and more convenient than filtration when large numbers of cultures were to be tested.

Several authors have discussed the effects of oxidation on the haemolysin reaction[46,47,49,50] and some have taken the precaution of incubating the reactants under a vaseline seal, or in the presence of sodium thioglycollate to try to prevent undue destruction of the lysin and to reactivate some of the lysin that may have been reversibly inactivated by oxidation. Whether significant destruction of the lysin would take place in one hour seemed doubtful and this was proven correct when several different incubation conditions for the haemolysin reaction were examined (Table 3). The presence or absence of reducing agents (cysteine hydrochloride), incubation in an aerobic or an anaerobic atmosphere and the type of buffer all made very little difference, in general, to the test results. Saline alone produced lower haemolysin titres than saline plus gelatin, buffered saline (ASO buffer) and FAB when incubated aerobically, but not anaerobically. The fact that the reduced medium (FAB) produced exactly the same haemolysin titres as

the non-reduced ASO buffer indicates that not sufficient oxidation of the haemolysin takes place during the performance of the test to interfere significantly with the results (Table 3). Todd[47] stated that 'the relationship between the haemolytic titre and the combining power of streptolysin is therefore dependent on the degree of reduction attained in the filtrate' and indicated that this held true for other oxygen-labile haemolysins. He obtained this reduction by adding sodium hydrosulphite to the medium before testing: however, it has been suggested that if hydrosulphite treated fluids are exposed to air for even a few minutes some of the lysin is destroyed due to agents formed from hydrosulphite in the presence of air[46], therefore the addition of reducing agents after production of haemolysin seemed to be undesirable, particularly as the degree of reduction (and therefore presumably the amount of haemolysin reactivated) would be dependant on the length of the reduction period.

Mixtures of lysin plus phosphate solution are not rapidly destroyed when exposed to air according to Neill[46] therefore the ideal system for maximum haemolysin production and activity would, theoretically, be to grow the cultures in a closed system in the presence of a reducing agent and to test dilutions of the lysin in a non-reducing buffer. This system was used in this study with great success. Cultures were grown in FAB medium containing cysteine hydrochloride (which has been shown to be capable of augmenting the activity of tetanolysin[53]) plus resazurin as an oxidation reduction indicator, and dilutions of haemolysin were made in ASO buffer. It must be noted, however, that even when dilutions of haemolysin were made in FAB, there were no differences in the haemolysin titres observed (Table 3) even though the FAB was oxidised almost immediately on exposure to air. It is possible that cysteine does not form the same destructive agents as sodium thiosulphite on exposure to air and it is therefore suggested that, if a reducing agent

is to be added to tetanolysin, it should be cysteine rather than thioglycollate.

In all the previous investigations of tetanolysin, the organisms were either cultured at 37°C[46,60] or the exact growth temperature was not specified[47,49,50,53,64]. Although the optimum reaction temperature for the haemolysin reaction has been observed to be 37°C, the effect of growth temperatures on haemolysin production has not previously been investigated.

This study has shown that 37°C is the optimum temperature for tetanolysin production and incidentally, for growth of Cl.tetani as well (Table 4).

The discrepancies observed regarding the heat lability of tetanolysin[46,49,50] were to some extent explained by this study. Tetanolysin was rapidly inactivated by temperatures of 75°C and 70°C and inactivated slightly less quickly at temperatures of 65°C and 60°C (Fig.8). Inactivation was complete in 1 hour or less. Inactivation at 56°C was slower, taking 2 hours and inactivation at 42°C even slower. All these rates of inactivation are slower than those observed previously[46,49,50], however, these workersused comparatively crude haemolysin detection methods and all used the rather variable technique of adding sodium thiosulphite to the lysin before testing. It is possible therefore that some of the lysin has already been inactivated by oxidation and is subsequently reactivated by the thiosulphite[47] and that this portion is more susceptible to heat inactivation than the lysin in this study, which has been produced under reducing conditions and has presumably never been inactivated before. When the rates of inactivation of the haemolysin at each temperature were calculated and plotted against temperature (Fig.9) it could clearly be seen that heat inactivation is not a linear phenomenon, but shows a sharp rise with the higher temperatures.

This could be another reason for the discrepant results in earlier studies of heat inactivation of tetanolysin. With an inactivation rate so high above 65°C, any slight variations or experimental errors would tend to be magnified much more than errors at the cooler end of the scale, where the rate is not so great.

An interesting and apparently unknown fact emerged when heat-treated haemolysin samples were incubated at 4°C overnight and then retested. There was significant re-activation of the most inactivated samples, i.e. those treated at the highest temperatures (Fig.10).

When the rates of reactivation of haemolysin at 4°C were plotted against the original temperature used to heat the lysin, a clearly linear relationship was observed up to 70°C above which no increase in reactivation was observed (Fig.11). Thus it appears that the greater the degree of heat inactivation of a sample of haemolysin, the greater its potential for reactivation.

Schrek[50] has suggested that heat inactivation is predominantly a monomolecular or pseudomonomolecular reaction as shown by the fact that his experiments produced a heat inactivation curve that was a straight line for the most part. This conclusion can be supported by the results of this study which also show heat inactivation curves which are predominantly straight or almost straight (Fig.8).

The nature of heat inactivation has only been speculated on so far. Thus Neill[46] suggests that the mechanism involved is protein denaturization and that it is irreversible and cites experiments dealing with treatment of heat inactivated haemolysin by sodium thiosulphite and subsequent failure to show any reactivation. Schrek[50] also feels that heat inactivation is irreversible and that the mechanism involved is protein denaturation or coagulation, but also states that preliminary experiments seemed to indicate that a secondary reaction occurs in which the products of heat inactivation were reverted to the original

lysin and that recent work on protein denaturation by heat lends support to the hypothesis that heat inactivation is reversible.

This study confirms this theory, although total reactivation was not seen in any case and where the inactivation was very slow i.e.at 42°C, there was no reactivation.

The mechanism of reactivation remains unknown, but it is certainly not analogous to the reactivation of haemolysin oxidised by exposure to air, which can be simply accomplished by reduction of the lysin with sodium thiosulphite or other suitable reducing agents.

Other workers in this field have used a variety of media to grow the organisms and produce the lysin, but the preferred option in this study was to use FAB containing cysteine for haemolysin production. When the haemolysin producing potential of several common media were compared (Table 5), it could be seen that serum broth and iso-sensitest broth produced insufficient growth in 24 hours for any haemolysin to appear. In fact, serum broth did begin to show growth on incubation for several more days, thereby exhibiting a long lag phase before initiating growth, but iso-sensitest broth appeared incapable of supporting growth of Cl.tetani. The other media all produced growth of a similar order when measured by $OD_{600}$and three of the media (FAB, Wilkins-Chalgren broth and Massachussetts medium) produced the same haemolysin titre whilst the fourth (cooked meat medium) produced a much lower titre. This may be explained by the finding that cooked meat granules in themselves appear to inactivate tetanolysin (Table 7), possibly by a non-specific absorption mechanism.

It is interesting to note that although both FAB and Massachussetts medium contain cysteine as reducing agent, Wilkins-Chalgren broth has no reducing agent added[106], therefore the presence or absence of reducing agents during growth appears to have little effect on haemolysin production.

However, all the media used here was freshly made wherever

possible and the effect of media which was not fresh, in the absence of reducing agents, is debatable.

For this reason, Wilkins-Chalgren broth was not considered suitable for the study of tetanolysin.

When the haemolysin was stored at different temperatures without a liquid paraffin seal, inactivation proceeded relatively rapidly at all temperatures above freezing, being essentially complete in 7 days in all cases (Table 6). The difference in the rate of inactivation at 42°C in this experiment (from 1:256 to 1:16 in 24 hours) and the rate of 42°C in the last experiment (Fig.10) (from 1:256 to 1:128 in 24 hours) was due to the fact that in this experiment there was no liquid paraffin seal and in the previous experiment the samples were sealed under liquid paraffin thus ensuring that only heat inactivation was studied and precluding any inactivation due to oxidation.

In these experiments without seals, the inactivation is due to a combination of heat inactivation and oxidation and naturally proceeds at an enhanced rate.

At temperatures below freezing there was a very slight degree of inactivation observed and it was not until a temperature of -40°C was reached that haemolysin could be stored for periods of up to 7 days without any inactivation at all being observed. One sample of haemolysin stored at -40°C for 1 month showed no observable inactivation (results not shown).

It is suggested therefore, that when haemolysin samples must be stored for a period of time, they should be stored in small aliquots at -40°C. Once thawed, an aliquot should be used and discarded as it is probable that repeated freezing and thawing would have a deleterious effect on the haemolysin.

The possibility that tetanolysin could be stored at temperatures above freezing with the addition of some preserving substance which would arrest the inactivation of the lysin was studied (Table 7) but

unfortunately none of the additives investigated proved successful. Indeed formalin, sodium azide, cholesterol and horse serum all had the opposite effect and increased the degree of inactivation. This is understandable, as formalin is a well known toxoiding agent[68] which has the property of rendering proteins biologically inert whilst retaining their basic structure and antigenicity. Sodium azide probably works in a similar manner. Cholesterol and serum have been shown to prevent the action of tetanolysin[45] probably inactivating the lysin by binding to its active sites in the case of cholesterol[48]. It is suggested that the inactivating effect of serum is also due to its cholesterol content[45]. Therefore, the only method of storing tetanolysin successfully at temperatures above freezing without significant inactivation was to use a liquid paraffin seal. This is satisfactory for up to 24 hours, but the method is too messy and inconvenient for routine use unless there is no alternative.

Another interesting observation was the fact that the volume of haemolysin had a distinct effect on its inactivation when incubated at 37°C for 24 hours (Table 8). This is almost certainly due to the rapid oxidation of the smaller volumes of medium and subsequent inactivation by this means. The larger volumes of medium, with a much greater depth are impossible to fully oxidise by surface contact with air and therefore no significant oxidation of the haemolysin occurs.

When Bernheimer[53] added sucrose to Cl.septicum haemolysin initiation of lysis or completion of lysis was completely interrupted, depending on the timing of the addition.

With tetanolysin the picture is slightly different. Although addition of sucrose to a final concentration of 0.3M inhibited haemolysis to a degree, this was very slight and at maximum only produced a reduction of around 10% of the maximum haemolysis (Fig.12).

The addition of glucose appeared to produce similar results and this may have abearing on future studies because glucose may be

added to some of the growth media commonly used for anaerobic organisms and it is possible that haemolysin titres obtained using such media may not be the maximum possible, as up to 10% may have already been inactivated.

The slightly confused situation regarding the antihaemolytic effects of different sera on tetanolysin was investigated in a rather different way to the approaches of other authors[5,46,47] by investigating the cholesterol level of the sera as well as the degree of inhibition of the haemolysin (Table 9).

Normal horse serum had a very high cholesterol level and arguably no antibodies to Cl.tetani haemolysin, yet it produces significant inhibition of the haemolysin reaction.

Similarly, Cl.perfringens, Cl.novyi. Cl.septicum and Cl.chavoei antisera, all raised in horses, show relatively high cholesterol levels and marked haemolysin inhibition, less in most cases than normal horse serum, although the cholesterol levels are also lower. Interestingly, the one organism which most authors agree has a serologically related haemolysin, namely Cl.perfringens shows greater haemolysin inhibition with its antitoxin than the other clostridia, which most authors suggest do not have related haemolysins. Does this mean that part of the antihaemolytic effect with this antitoxin is due to the serological relationship between Cl.perfringens haemolysin and tetanolysin, while the rest is due to non-specific binding of cholesterol?

If so, why does normal human serum, with the highest cholesterol level show no inhibition at all? Does this mean that the partially purified human antitetanus globulins, with cholesterol levels reduced to zero owes all its haemolysin inhibition activity to specific antibodies?

If part of the inhibition of haemolysis produced by Cl.perfringens antitoxin is due to specific antibodies, why does Antistreptolysin O produce no inhibition as it is generally agreed that the two haemolysins

are related to tetanolysin?

Pneumococcal omniserum is not an antitoxin but an anti-capsular antibody and would not be expected to show any haemolysin inhibition effect. Neither would normal rabbit plasma.

The tetanus antitoxins tested are highly refined and purified horse antibodies and subsequently show no cholesterol at all. They do however show a low level of haemolysin inhibition. That this is genuinely due to an antihaemolysin antibody is shown by the fact that this inhibition effect can be removed by absorbing the antitoxin with a culture supernatant from a haemolysin producing strain of Cl.tetani, but not by absorbing with a culture supernatant from a non-haemolytic strain.

These results suggest that,contrary to the suggestion of Todd[47], tetanolysin can be neutralised by tetanus antitoxin, however, the relationships between tetanolysin and other haemolysins produced by Cl.novyi, Cl.septicum, Cl.chauvoei and Antistreptolysin O may require reinvestigation as it is clear that the amount of cholesterol present in the antitoxin can contribute to a greater or lesser extent to the inhibition effect. This is probably due to competitive binding of the active sites on the haemolysin by both free cholesterol in the serum and specific antitoxin before the haemolysin can bind to the red cells. It has been shown that Streptolysin O and probably all oxygen-labile haemolysins, bind to cholesterol molecules on target membranes in order to damage the cell by producing large transmembrane channels in a manner analogous to that of complement[48], therefore it is not inconceivable that free cholesterol can compete with cholesterol on RBC membranes for the active sites on the haemolysin.

These findings are in direct contrast to the findings of Todd[47] who found that tetanolysin was inhibited by Antistreptolysin O but not by tetanus antitoxin or normal horse serum and suggested that the failure of tetanus antitoxin to neutralise tetanolysin may be due to

the use of formol toxoid in the preparation of antitoxin, as formalin destroys haemolysin. This study shows that the production of antitoxin using formol-toxoid does not produce antitoxin devoid of antihaemolytic activity and confirms the findings of Hardegree et al[64] who also found antihaemolysin in tetanus antitoxin, however, the reason for the discrepancies in the other findings remain unknown.

Previous workers have used samples of tetanolysin from cultures grown for varying times from 24 hours[53], 48 hours[60], 4 days[50] to 7 days[49], with little concern for the relationship between growth rate and haemolysin production, even though Willis[2] has stated that haemolysin is rapidly destroyed once formed.

This study shows that there is a direct relationship between the growth curve and haemolysin curve of Cl.tetani, with maximum growth and maximum haemolysin production being attained at virtually the same time, some 10 hours after initiation of growth. This is in agreement with the findings of Fleming[49] and Hardegree[60] who both found that maximum haemolysin production was during the period of active growth.

Maximum haemolysin titres remained for up to 3 days before a rapid fall in the titres occurred (Fig.13).

This confirmed that the use of 24 hour cultures of Cl.tetani in the preceding experiments of this study was the best choice for all practical purposes, combining the greatest convenience with the maximum haemolysin production, and the least degree of destruction or inactivation.

Although tetanolysin is produced and set free during active growth, there is still a substantial amount left inside the cells, which diffuses out on standing (Fig.14).

The presence of detectable haemolysin inside the cells does not appear until 2 to 3 hours after haemolysin appears in the supernatant. This suggests that there is an active transport of haemolysin out of the cell as soon as it is produced for the first few hours and it is only when haemolysin production inside the cell exceeds the capability

of the transport system to remove it that the level of haemolysin inside the cell begins to build up. The fact that the intracellular haemolysin level falls significantly before the extracellular haemolysin would support this hypothesis, indicating that, once haemolysin production ceases inside the cell, the transport system quickly removes the built up intracellular haemolysin into the surrounding culture fluid.

Schrek[50] has shown that acidification decreases the rate of inactivation of tetanolysin by heat and that alkalinization increases the rate of inactivation, with maximum inactivation being seen at pH 7 and maximum resistance to inactivation being seen at pH 1, 2 and 3. He also states however, that acid inactivation has been observed to occur at pH1 to 6, and that it seems reasonable to assume that the same reaction takes place to a lesser extent at pH 7. If this is so, spontaneous deterioration of lysin is at least partly due to acid inactivation. There is no mention of alkaline inactivation however, and as Schrek did not study pH inactivation of tetanolysin per se, but rather the modifying effect of pH on heat inactivation over a period of only 10 to 15 minutes, the exact degree of pH inactivation of tetanolysin remained unclear.

This study clearly shows that acid inactivation does occur, particularly below pH 5 and that alkaline inactivation occurs at pH 10 and above (Table 10). Otherwise the haemolysin appears remarkably stable over the pH range 5 to 9, showing only a very minor decrease in haemolysin titre from the original. The lysin appears to be rather more resistant to alkaline inactivation as it was not until the pH reached 12 that all the haemolysin was inactivated, compared to acid inactivation where complete inactivation was observed at all pHs below 4. This would indicate that, at pH 7, spontaneous deterioration due to pH as suggested by Schrek[50] does not occur, or occurs to a very

small degree. This is, of course, in sealed containers where other factors such as oxidation inactivation of the haemolysin are not involved, because it has been shown that pH also has a modifying effect on this[50] and if oxygen is not excluded from these experiments any inactivation observed may be due to a combination of factors.

Other investigators of tetanolysin have generally used only one strain of Cl.tetani throughout their work,[46,47,49,50,53,60,64] and the only comparative study of the haemolysin producing capabilities of different strains of Cl.tetani utilised a rather clumsy technique involving the addition of different volumes of culture filtrates to RBC solutions and the determination of complete or partial haemolysis[45]. This has the disadvantage that minor experimental errors may be magnified and the results are highly objective. The microtitre plate haemolysin assay used in this study was thought to minimise these effects and the results obtained confirmed that the vast majority of Cl.tetani strains tested produced tetanolysin in very similar quantities under identical conditions (Table 11). The only exception to this was NCTC 9569, a serotype VI strain which is non-haemolytic. The haemolysin titres produced by each strain were remarkably consistant for that strain, rarely varying more than one dilution on repeated testing, when all other conditions were equal. From these findings it can be assumed that similar growth/haemolysin curves would exist as those observed earlier in this study for Cl.tetani NCTC 279 (Fig.13).

The suggestion that there is an inverse relationship between the ability of a strain of Cl.tetani to spore and its ability to produce toxin[51] was investigated and a definite correlation was observed (Table 12). The original suspensions from a number of strains of Cl.tetani had been grown at 37°C overnight followed by room temperature for 6 days to produce the maximum number of spores from each strain. The % of the total cells containing spores was shown to vary from 0 to 40 in these suspensions. However, when these suspensions were inoculated into fresh

media and incubated overnight, the majority of stains showed <1% spores. This is expected as generally sporulation is a post-exponential phase event[107]. The haemolysin titres obtained were generally in accordance with those found earlier in this study and there seemed no obvious relationship between the haemolysin titres produced and the number of spores in the original suspensions. Nor did there appear to be any less haemolysin produced bythe 6 'normal' strains of Cl.tetani that did produce spores after 24 hours incubation (L5500, LQ1, LQ2, LQ931, LQ914 and LQ730). There was however, a visible decrease in the amount of haemolysin produced by the seventh strain that produced spores after 24 hours. This strain was an unstable, heat-induced variant of NCTC279 (serotype I) and when grown under identical conditions to the parent strain (which is virtually asporogenous) produced a similar level of growth but a markedly reduced haemolysin titre. This suggests that there is indeed an inverse relationship between sporulation and toxin production. It is suggested that, as haemolysin production is an 'active growth' phenomenon, the presence of a substantial number of cells which are beginning to sporulate during this period, rather than post exponentially, could lead to competition for the biosynthetic activity of the cell, resulting in suppression of exoprotein formation as suggested by Coleman et al[108].

When the original cell suspensions were heated to 80°C for 5 minutes prior to incubation, the vast majority of strains produced very low levels of growth after 24 hours and no haemolysin whatsoever was detected. The exceptions to this were 6 strains which had 5% spores or greater in the original suspensions. These produced growth levels equivalent to, or in some cases greater than, those observed in the unheated controls. This is not unexpected as it has been shown that 'heat-shocking' of some strains of Cl.botulinum may lead to rapid growth immediately afterwards[107] and it is not unreasonable to expect the same mechanism to work with some strains of Cl.tetani. These six

strains, however, produced markedly lower haemolysin titres than the unheated cultures, comfirming that sporulating potential is linked to toxin production.

Interestingly, 1-H-80 and Harvard, the strains with the greatest number of spores in the original suspensions (40 and 24% respectively) produced little growth and no haemolysin on subsequent incubation, in a manner similar to the rest of the strains apart from the six mentioned earlier. These strains were all viable with the exception of serotype I, as shown by growth on subculture to blood agar, therefore it is suggested that the reason for very little growth in the first 24 hours is due to a long lag phase due to slow germination of the spores that survived the heat treatment. Of course, there will be fewer spores in the majority of these cultures than the six that grew well, as there were fewer in the original suspensions, therefore perhaps these comparisons are invalid where similar growth indices (OD_{600}) do not exist, as different parts of the growth curve are being measured.

When purification of the tetanolysin was attempted by the chloroform; methanol method (Method 2), very little haemolytic activity was observed in the final product, no bands were seen by PAGE or IEF, no precipitating antigens were seen by DD or Rocket IEP and no amino-acids were detected by two dimensional paper chromatography. Although this technique has been used to purify the delta haemolysin of Staph.aureus[85] it appeared unsuitable for the purification of Cl.tetani haemolysin. This is possibly due to the fact that the procedure takes a full day to perform and for much of the time the haemolysin is exposed to the inactivating effect of atmospheric oxidation, or alternatively, the method may not be appropriate to tetanolysin purification.

Ammonium sulphate precipitation followed by Sephadex G-200 gel filtration (Method 1), however, seems to produce a highly active haemolysin,apparently free from other extracellular proteins (Table 13).

The fraction showing maximum haemolytic activity shows no precipitating activity and apparently no agglutinating activity (Table 14).

This suggests that the haemolysin is incapable of raising either precipitating or agglutinating antibodies in vaccinated animals, or that formalin treatment has denatured the haemolysin in such a way that, although neutralising antibodies are produced, neither precipitating nor agglutinating antibodies were. The autoagglutination observed with the staphylococcal coagglutination reagent is probably due to the difficulties inherent in growing, harvesting and preparing the Cowan 1 Staphylococci for sensitisation.

he purified haemolysin produced by method 3 also appeared relatively free of contaminating proteins, and no precipitating or agglutinating activity was seen. When these purified haemolysins were examined by SDS-PAGE one common band was observed. This band was the only one present in the fractions showing maximum haemolytic activity and was present as a single rather diffuse band with a molecular weight of 68.75 kilodaltons, or occasionally as a doublet of bands at 66 and 68.75 kilodaltons. It is suggested that this is the pure haemolysin being observed and this is supported by the observation that these samples gave a single major band with an isolectric point of 5.0 when investigated by isoelectric focusing.

The finding that the haemolysin has a lower molecular weight than the neurotoxin ($\simeq$150,000 kilodaltons) is not unexpected, as Hardegree found it to elute from Sephadex columns after the neurotoxin[60], an observation consistant with this situation.

The amino acid chromatography results were disappointing as only the method 3 haemolysin produced any spots at all. It appears that the haemolysin is a very active substance, because even when subjected to exhaustive purification methods such as those used in this study, there appears to be only a very small amount of material isolated with a high degree of haemolytic activity. For example the

protein content of the method 1 haemolysin was 0.75 ug/ml and the haemolysin titre was 1:512. Even concentrating this ten times was apparently insufficient to produce enough protein to make a distinguishable pattern by paper chromatography. When the product from method 3 was examined, the presence of isoleucine, alanine, lysine, threonine, glutamine, glutamic acid and serine was detected. It is arguable that more amino acids than these might have been detected had there been sufficient haemolysin to enable it to be concentrated further before chromatography, but this was not possible.

2.5 CONCLUSION

Tetanolysin was found to produce rapid haemolysis of red blood cells. The rate of lysis is dependent on the concentration of the haemolysin and the incubation temperature, the optimum being apparently 37°C. The microtitre plate haemolysin test was found to be superior to tube haemolysin and radial haemolysis techniques, and an RBC concentration giving an OD_{545} of 60 was found to be the optimum for this method.

The haemolysin was produced during the period of active growth, with maximum growth and maximum haemolysin production occuring at virtually the same time, some 10 hours after initiation of growth. Maximum haemolysin titres were demonstrated when the organism was grown at 37°C in medium containing a reducing agent (such as FAB).

The majority of the haemolysin was liberated outside the cell but a substantial amount remained intracellularly. After around 3 days when the organisms began to autolyse the haemolysin levels, both intracellular and extracellular, fell rapidly.

The haemolysin was rapidly inactivated byatmospheric oxygen and by temperatures above freezing. The rate of inactivation increased with the temperature. Heat inactivated haemolysin could be reactivated to a degree by incubation at 4°C under a liquid paraffin seal. The greater the original inactivation, the greater was the potential for reactivation.

None of the additives tested were proved capable of halting the inactivation of tetanolysin. The only methods to do that successfully were storing under liquid paraffin, or storing at -40°C. The greater the volume stored, the less inactivation occurred. This is almost certainly due to the smaller vessels having a larger surface area to volume ratio and thus a greater potential for oxygen inactivation.

The addition of glucose or sucrose to the haemolysin produced a slight inhibition of its action (up to 10% maximum).

The haemolysin was specifically inhibited by antitoxin raised against tetanus toxins, but it was also affected by cholesterol and so was non-specifically inhibited by antitoxins raised against other organisms and normal sera with high cholesterol contents. The haemolysin was inactivated by acid pH below 5 and alkaline pH above 10.

The majority of Cl.tetani strains tested produced haemolysin in similar quantities, the exception being the non-haemolytic serotype VI strain.

There was an inverse relationship between the sporulating potential of a strain and its haemolysin production, probably due to competition for the biosynthetic pathways of the cell.

Purification of the haemolysin was accomplished by ammonium sulphate precipitations followed by Sephadex and Sepharose gel filtrations. The purified haemolysin was a protein with molecular weight between 66 and 68.75 kilodaltons, had an isoelectric point of 5.0 and contained isoleucine, alanine, lysine, threonine, glutamine, glutamic acid and serine.

No precipitating activity could be detected in the haemolysin by Immunoelectrophoretic techniques and no agglutinating activity could be detected by either latex agglutination or Staphylococcal coagglutination techniques.

CHAPTER 3

THE NEUROTOXIN OF CL. TETANI

3.1. SURVEY OF THE LITERATURE

Because the neurotoxin of Cl.tetani, tetanospasmin, has long been categorised as a well-studied toxin with a clear role as a major effector in the disease pathogenesis of Tetanus[39], there are numerous reviews of the pharmacodynamics, synthesis, immunological properties and action of the toxin[40]. Most of these reviews mirror not only the state of the art at the time of their publication but also the particular interests of their authors, and many of the findings of earlier workers have been overturned by those coming later. Indeed, several articles and reviews published since the beginning of this work have significantly clarified areas of dispute that were under investigation here[40,42].

Most of the work on sythesis and purification of the neurotoxin has been done since the Second World War using a highly toxigenic strain of Cl.tetani called the 'Harvard' strain[15] and with the major impetus towards large scale production for vaccine manufacture. The toxin so produced may lose activity, but not antigenicity, on standing or storage[15] or by treatment with formaldehyde[11]. Indeed, as the toxin itself is far too poisonous to be given to patients as a vaccine artificial denaturing with formaldehyde, or 'toxoiding', is performed commercially to produce a non-toxic but still highly antigenic toxoid for use as a vaccine[11,15]. The Harvard strain is described as a variant strain which had developed over a period of some six years by a gradual alteration[111]. The strain is rather unstable however, having apparently lost the ability to sporulate and being difficult to store without losing to a considerable degree the ability to produce high levels of toxin[11,111]. Also this strain needs to be grown in a particular medium devised specifically for neurotoxin production by the Massachussetts Department of Public Health[95] and the production of

neurotoxin by other strains of Cl.tetani in this medium or in other medium does not appear to have been much studied. It has been stated that in ordinary media (4% Proteose peptone medium, chopped meat broth and liver broth) most of a library of 57 strains of Cl.tetani were nontoxigenic whereas using special media half of them proved toxigenic[51] and it may be concluded that earlier problems of differentiating toxigenic Cl.tetani from non-toxigenic strains that were identical in many ways may be due to the use of inappropriate media for toxin production which might make toxigenic strains appear as non-toxigenic[76].

Toxin production does not always parallel the degree of growth, because there are a number of factors affecting toxigenesis which do not affect growth. To some extent these are inhibitory factors such as the production of hydrogen sulphide[11] or unknown inhibitors in casein or meat[110,111] and in some case there is a requirement for certain essential substances such as thiamine, calcium pantothenate, nicotinic acid and uracil [111], and also free arginine lysine and histidine in peptide linkages[113,114].

One of the major drawbacks to comprehensive studies on the production and purification of tetanus toxin is the fact that no simple in vitro assay of its action has been developed as there is no readily observable in vitro property of the molecule to base such as assay on[15].

The assay of tetanus toxin is therefore generally carried out by observing its lethal effect in experimental animals[11,15]. However, apart from the strong ethical objections that may be raised to the use of a large number of animals for research purposes[115], there are other problems inherent in this method. Firstly, not all animals are equally susceptible. The horse, man and goat are most susceptible, followed by the mouse, rat, rabbit, monkey and guinea pig in that order. The dog, cat, pigeon and hen are increasingly resistant whilst birds and cold blooded animals are most resistant[11].

Secondly, the lethality of the toxin depends on its route of

administration, the size of the dose and its purity,[11,15], therefore small variations in these can bring about differing results.

Thirdly, although most authors use the term Minimum Lethal Dose (MLD) to quantitate the lethality of the toxins, many do not state what they actually mean by the term, which, although usually defined as the last dose that will kill the animals injected, this could mean all, some or just one of the injected animals, and as the doses required to show these effects have been calculated to be significantly different[15] any results given without defining the MLD are meaningless. In practice, the LD_{50} (the least dose that will kill 50% of the injected animals) is commonly used, although the dose-response curve of tetanus toxin is so steep that this may be difficult to determine[15]. The definition of the LD_{50} must include the time within which the animals die.

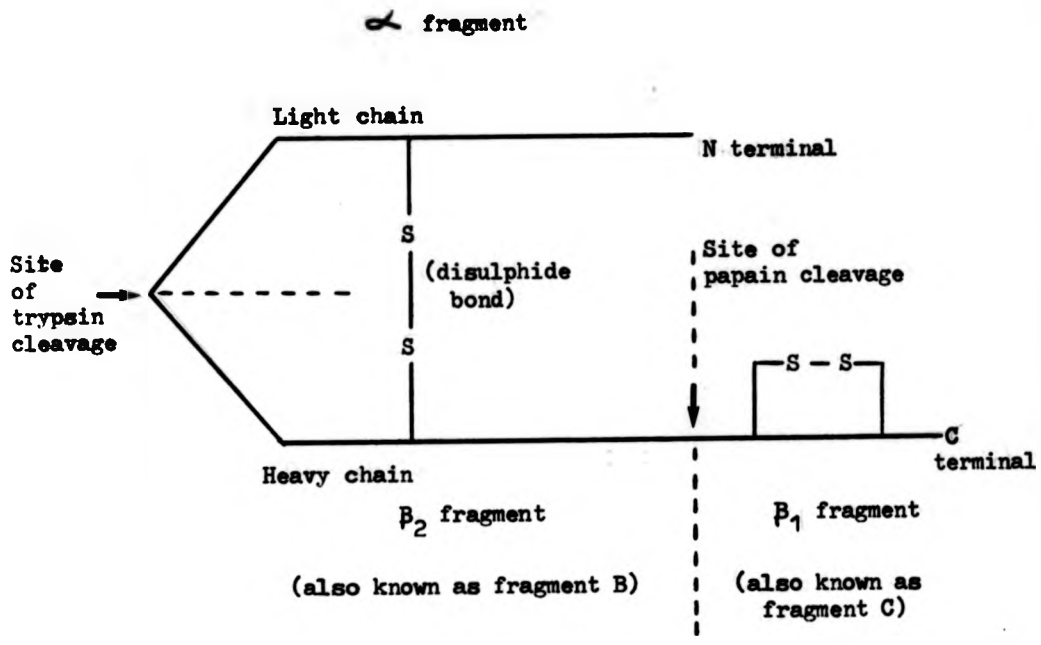
Animal experiments are the only direct method of assessing the activity of tetanus toxin[11] however there are other techniques available which can determine the amount of toxin in culture filtrates although they can not make any estimates as to its actual toxicity. These methods include the flocculation test[116,117] and haemagglutination inhibition test[118,119,120,121]. The flocculation test suffers from nonspecific zones of flocculation[116,119] and it has been suggested that it may therefore show an incorrect estimate of the toxoid content of a preparation[119]. It is also laborious and requires relatively large quantities of reactants and constant observation[116,117]. The haemagglutination inhibition test appears to be sensitive and readily repeatable whilst giving good agreement with other available tests[119]. Newer immunological tests such as single and double immunodiffusion, latex agglutination and Rocket immunoelectrophoresis appear not to have been investigated from this standpoint.

Several different approaches to the production and purification of tetanus toxin have resulted in some confusion over the nature of the molecule and its physical structure. Thus workers who grew the organism for 4 or 5 days and recovered the toxin from culture supernatents of autolysed cells produced different values for diffusion coefficients, sedimentation constants and molecular weights, from workers who extracted the toxin from young (1 - 3 days old) cultures of cells by suspending them in hypotonic saline[15]. The reason for many of these differences is that tetanus toxin is initially produced inside the cells as an inactive, single chain polypeptide with a molecular weight around 150,000 daltons. This is converted to an active dichain molecule with molecular weights of around 100,000 and 50,000 daltons by intrinsic proteases and is released into the culture medium on autolysis of the cell[40,42]. Thus 'extracellular' toxin in older cultures will be in the active monomeric form with lower molecular weights (estimated at around 68,000 daltons), sedimentation constants (3.9-4.5) and a diffusion coefficient of 5.5, and the 'intracellular' toxin extracted from young cells will be in the dimeric form with molecular weight around 150,000, sedimentation constants around 6.1-7.1 and a diffusion coefficient of 4.4[15].

The conversion of the dimeric form to the monomeric form ('nicking') can also be accomplished by mild trypsinisation under reducing conditions[40,42,43] and a further breakdown product with molecular weight around 40,000 daltons may be obtained by papain treatment[17]. These fragments and their nomenclature are shown in Fig.25. Although they have been studied to some extent[18,43] there is still a need to define their exact isoelectric points and molecular weights. Until recently, little was known about the mode of action of the toxin at a molecular level. Suggestions have been made that the toxin produces toxic substances in host tissues or that it affects cholinesterase or choline acetylase activity but these have not been substantiated[15]. It

FIG.25A

General structure of Cl.tetani toxin and nomenclature of their fragments



now appears that tetanus toxin blocks acetylcholine release from the neuromuscular junction following nerve stimulus and thus allows ungoverned propagation of nerve impulses which lead to spasmodic contractions of the muscles[11,42].

It has been suggested that the action of tetanus toxin in causing disease follows a series of steps[40,42]. The molecule must first bind to receptors on nerve tissue (the extracellular binding step), it must then be transported across the target cell membrane to the inside of the cell (the internalization step), and finally it must exert its action in the appropriate place (the intracellular poisoning step)[42].

It has further been suggested that the binding step is mediated by the carboxyterminus of the heavy chain (Fig.25 fragment β_1 or fragment C), the internalization step is mediated by the aminoterminus of the heavy chain (fragment β_2 or fragment B) and the light chain (fragment α) is assumed to be responsible for the poisoning step[42].

The actual receptor to which tetanus toxin binds in nervous tissue has not been unequivocally identified[42], although it has long been known that brain tissue could selectively bind tetanus toxin[15] and it has been tentatively suggested that the receptor is a ganglioside[41], although further work is needed to isolate and definitely characterise the receptor[42]. Much of the work with receptor sites has been done by observing the decrease in toxicity of preparations of toxin following incubation with the proposed receptor[40,41], a situation vulnerable to the effects of many external phenomena such as non-specific binding. Recently two dimensional immunoelectrophoresis was used to visualise tetanus toxin amongst other culture filtrate exoproteins[77,122] but this does not seem to have been used to study either the breakdown fragments of tetanus toxin, or the specific absorption of any of these fragments by specific extracts of brain tissue or by individual gangliosides.

Since the beginning of this work it has been suggested that tetanus toxin may interact with many gangliosides with different affinities,

depending on their nature and their endowment with other lipids particularly GT_{16} and GD_{16}[39.40.125]

The localisation of the receptor in nervous tissues has been attempted using fluorescence[40] and radioimmunoassay[42]. Fluorescence microscopy has shown that toxin decorates the whole surface of cultured nerve cells[40], but comparable experiments using histological sections of brain tissue appear not to have been done, even though brain has been shown to be a plentiful source of receptor sites[40,41,42].

It would appear that there are still areas where our understanding of the neurotoxin of Cl.tetani requires clarification, and the following investigations were therefore undertaken.

1) A study of the different in vitro methods of neurotoxin assay

2) A study of the toxigenicity of other strains of Cl.tetani and other growth media.

3) A study of the properties of breakdown fragments of purified toxin by 2 dimensional Immunoelectrophoresis, Isoelectric focusing and Polyacrylamide gel electrophoresis.

4) A study of the binding of whole tetanus toxin and specific fragments by different extracts of brain tissue using immunoelectrophoresis.

5) A study of the binding of toxin to histological sections of whole brain by Immunofluorescent antibody techniques.

3.2 EXPERIMENTAL TECHNIQUES

3.2.1. In vitro methods of Tetanus neurotoxin assay

Two different toxin preparations were available for this part of the study. The first was kindly supplied by Dr. R.O. Thomson[67] and contained purified tetanus toxin (1.5mg/ml) with 740 Lf/ml and 4×10^6 MLD per ml. This preparation was designated TOX. The second preparation was Wellcome tetanus vaccine in simple solution[71] and contained 28 Lf/ml. This preparation was designated VAC.

i) The tube flocculation test (Appendix 21)

Only VAC was examined by this method as insufficient TOX was available. A 3 day FAB supernatant of NCTC 279 which should contain no appreciable toxin was also tested as a negative control.

ii) The Haemagglutination Inhibition (HAI) test (Appendix 22)

Tanned RBCs were sensitised with VAC, as insufficient TOX was available. The amount of toxin in TOX was calculated with VAC as the reference standard and vice versa. A 3 day FAB supernatant (NCTC 279) was also tested.

iii) The Latex agglutination test (Appendix 16)

Dilutions of TOX and VAC and a 3 day FAB supernatant ofNCTC 279 in PBS were tested for visible agglutination using Wellcome horse tetanus antitoxin conjugated to latex particles.

iv) Single radial Immunodiffusion (SRID) (Appendix 13)

Standard curves of VAC and TOX were prepared by using suitable dilutions of each in PBS. The Lf value of a dilution of TOX was calculated from the VAC standard curve and vice versa. A 3 day FAB supernatant (NCTC 279) was also tested.

SRID was performed using Wellcome horse tetanus antitoxin both unabsorbed and after absorption with a young Cl.tetani culture to remove all antibodies to cellular and early growth phase antigens (Appendix 18), and leave only antibodies to the neurotoxin.

v) Double Immunodiffusion (DID) (Appendix 13)

Dilutions of TOX, VAC and NCTC 279 3 day FAB supernatant were tested by DID to find the endpoint dilution of each. Also suitable dilutions were placed in adjacent wells to observe lines of antigenic identity between the three.

vi) Rocket Immunoelectrophoresis (Appendix 14)

Standard curves of TOX and VAC were prepared using suitable volumes or dilutions in PBS. The Lf value of a TOX dilution was calculated from the VAC curve and vice versa. A 3 day FAB supernatant (NCTC 279) was also tested. Both absorbed and unabsorbed antitoxin was used as before (section 3.2.1.iv).

vii) Rocket-line IEP (Appendix 14)

This was performed as above with the modification that an intermediate gel containing either TOX or VAC was included. The peak heights were measured from tip to where they met the intermediate gel lines and reactions of identity at this point were sought.

3.2.2. Assay of other Cl.tetani strains and other growth media for toxin production.

All the 27 strains of Cl.tetani (Appendices 1 and 2) were grown in 50 ml volumes of Massachussetts medium (Appendix 9) and in 100ml volumes of FAB broth (Appendix 7) for 7 days at 34°C. At this stage the cultures were centrifuged and the supernatants tested for toxin by the haemagglutination inhibition test (Appendix 22), single radial immunodiffusion (Appendix 13) using both absorbed and non-absorbed antitoxin and Rocket immunoelectrophoresis (Appendix 14) using both absorbed and unabsorbed antitoxin.

A 3 day old FAB supernatant of NCTC 279 was also tested.

In addition, strain E88, a noted toxin producer was grown for 7 days at 34°C in Wilkins Chalgren broth[28], Cooked meat medium[61], Isosensitest broth[28] or [29], serum broth[63] and thiol broth[29] before being

centrifuged and the supernatants tested as before.

NCTC 279(I) was grown in Massachussetts medium for 2 days at 34°C before being centrifuged and the deposited cells extracted by the method of Raynaud (Appendix 23). This extract was then tested as before.

3.2.3. Investigation of enzymatic breakdown fragments of tetanus toxin

Wellcome purified toxin (TOX) was used for this study. Earlier studies[18,43,44,126,127,128] have concentrated on fragments separated by gel filtration. It is not clear what effects this might have on the individual fragments, and as the immunoelectrophoretic techniques used here should have sufficient resolving power to visualise each fragment in the mixture simultaneously, it was felt that wherever possible the enzymatically degraded toxin mixture should be analysed whole, without any attempts at purification.

Experimental

Toxin breakdown.

TOX was divided into 5x100 ul aliquots and these were treated as follows:

Aliquot (1) 300 ul of buffer (50mM Tris-0.6 M glycine, ImM EDTA pH 8.5)[69] was added, shaken and the mixture incubated at room temperature for 1 hour.

Aliquot (2) 200 ul of buffer and 100 ul of 100mM dithiothreitol[27] (DTT) were added, shaken and incubated at room temperature for 1 hour.

Aliquot (3) 100 ul of buffer and 100 ul of Trypsin (1 ug/ml)[27] were added and incubated at room temperature for 1 hour before 100 ul of Soybean Trypsin Inhibitor (1.5 ug/ml)[27] was added.

Aliquot (4) 100 ul DTT and 100 ul Trypsin were added and incubated at room temperature for 1 hour before 100 ul Soybean Trypsin Inhibitor was added.

Aliquot (5) 200 ul buffer and 100 ul Papain (10mg/ml)[129] were added and incubated at 55°C for 4 hours.

Each aliquot was then stored at -40°C until required.

Examination of toxin breakdown fragments

Each aliquot was tested by two dimensional IEP (Appendix 14) using Wellcome tetanus antitoxin[71], by SDS PAGE (Appendix 15) and by Isoelectric focussing (Appendix 20).

The relative mobilities of each of the breakdown fragments was estimated by two dimensional IEP (Appendix 14).

3.2.4. Investigations of the binding of tetanus toxin to brain tissue extracts

A fresh whole cow's brain was obtained from the Public Health Laboratory, Stoke-on-Trent. This was stored at -40°C until required.

The brain was extracted by the method of Folch[130] (Appendix 24) and the different fractions stored at -40°C until required.

10 ul of TOX was mixed with 10 ul of buffer (50mM Tris - 0.6M glycine, ImM EDTA, pH 8.5) and 10 ul of each extract A, C, F, H, J, Y and Z.in narrow tubes and incubated at room temperature for 1 hour before being centrifuged. The supernatent fluids of each were investigated by 2 dimensional IEP (Appendix 14) using Wellcome tetanus antitoxin[71] to determine if any extract could absorb significant amounts of toxin. Absorption of toxin resulted in a lower precipitin peak compared to non-absorbed toxin.

Each extract was similarly incubated with 10 ul of buffer and 10 ul of toxin aliquot 2 containing fragments α and β before 2 dimensional IEP to determine which fragment was absorbed by the extracts.

3.2.5. Investigation of the site of toxin binding in histological sections of brain by Immunofluorescent assay techniques (IFAT)

Sections of fresh cow's brain were kindly made by the Histopathology Dept., Stafford General Infirmary.

The sections were cut at 16 um in a cryostat and air dried.

Tetanus toxin (TOX) was labelled with fluorescein isothiocyanate

(FITC)[69] by the method of Scheff et al [131] (Appendix 25).

i)Direct Immunofluorescent assay technique (DFAT)

A 3mm circle was inscribed on triplicate brain sections using a diamond, and 10 ul of Fluorescein labelled TOX (F-TOX) was added to each circle. The slides were incubated at 37°C for 30 minutes in a humidity chamber before being washed twice for 15 minutes in PBS. After a final rinse in distilled water the slides were dried and examined by epi-illumination on a Leitz SM-LUX microscope fitted with a 'Ploem-pak' FITC ultra-violet system and a mercury vapour lamp.[251]

ii)Indirect Immunofluorescent assay technique (IFACT)[133]

Ten 3mm circles were inscribed on triplicate brain sections as before. Into each circle 10 ul of doubling dilutions of TOX in PBS was pipetted so each slide had dilutions of TOX ranging from neat to 1:512. The slides were incubated at 37°C for 30 minutes in a humidity chamber before being washed twice for 15 minutes in PBS, rinsed in distilled water and dried. 10 ul of Wellcome Horse tetanus antitoxin[71] was added to each well and the slides reincubated at 37°C for 30 minutes. The slides were subsequently washed twice in PBS for 15 minutes, rinsed in distilled water and dried. 10 ul of Rabbit anti-horse immunoglobulin Fluorescent Conjugate[71] at a use-dilution of 1:40 was then added to each well and the slides were incubated at 37°C for 30 minutes, washed, rinsed and dried as before, then examined on the fluorescence microscope. Duplicate sections were stained by Haematoxylin and Eosin for comparison.

3.3 RESULTS

3.3.1. In vitro methods of Tetanus neurotoxin assay

i) The tube flocculation test

This required relatively large quantities of reagents (0.5ml minimum) and was cumbersome and time-consuming to perform. Endpoints were very hard to determine due to the faintness of the flocculation. The amount of toxin in the original VAC used in the test was 14 Lf /ml, however the amount calculated from observing the results of the test was between 5 and 7.4 Lf /ml (Table 15). In addition, the FAB supernatent also gave faint flocculation in several tubes when it was tested.

ii) The Haemagglutination Inhibition (HAI) test

This was much easier and simpler to perform than tube flocculation, volumes were much less and endpoints were much clearer (Table 15) (Fig.26).

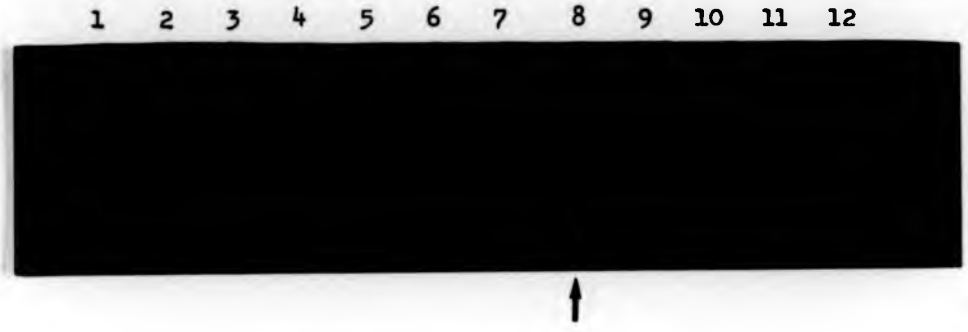
The amount of VAC calculated from the test results (28 Lf /ml) corresponded exactly with the amount present while the amount of TOX calculated (70 Lf/ml) was in close agreement with the amount present (74 Lf/ml).(Table 15)

The FAB supernatent showed no toxin present when tested.

FIG.26

The Haemagglutination Inhibition (HAI) test for tetanus toxin and toxoid

Well No.



Note:

Complete inhibition of haemagglutination is shown in the first 6 wells of each row. Haemagglutination with no inhibition is shown best in well 12, row F, and 50% haemagglutination inhibition (the endpoint) is shown in well 8 in each row. **(arrow)**

1 - 12 = doubling dilutions of toxin.

Sensitised red cells at a working dilution of 1:5 (Row F), 1:7 (Row G) and 1:10 (Row H).

iii) The Latex agglutination test

Trace agglutination was visible in all dilutions of TOX, VAC and the FAB supernatent tested, and also in the negative control (diluent plus latex reagent). There was no visible strong agglutination with any dilution of either toxin preparation (Table 15).

iv) Single radial immunodiffusion (SRID)

With unabsorbed antitoxin dilutions of VAC and TOX produced small sharp precipitin zones (Fig.27). These were hard to measure accurately as there was little difference in size with the result that the value for TOX calculated from the results was significantly different from the amount present (50 and 74 Lf/ml respectively)(Table 15). The value calculated for VAC was in close agreement with the amount present (25 and 28 Lf /ml respectively). The test was rather insensitive however, as toxin preparations with less than around 5 Lf /ml did not produce precipitin zones. Altering the amount of antitoxin present did not improve this.

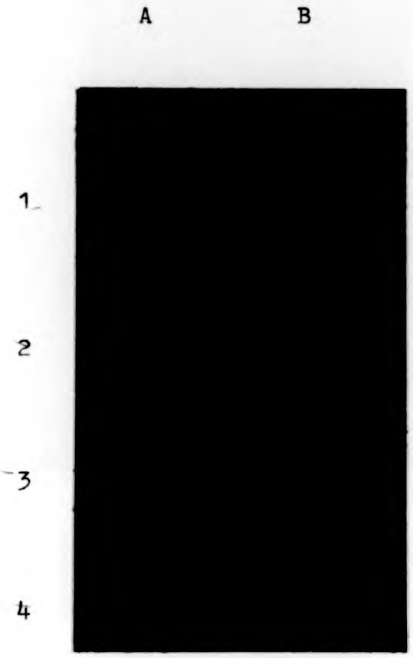
Also, the FAB supernatent produced a double precipitin ring indicating that antigens other than the toxin were being detected. When absorbed antitoxin was used these non-specific precipitin rings were removed but the specific toxin-antitoxin precipitin ring was not, although it became rather diffuse and even more difficult to measure (Fig.27) The test was relatively easy to perform and large numbers of toxin preparations could be tested simultaneously.

v) Double Immunodiffusion (DID)

The endpoints observed by this method (i.e. the limits of detection of VAC and TOX were 3.75 and 8 Lf /ml respectively. When VAC and TOX were placed in adjacent wells there was a joining of the precipitin lines, indicating antigenic identity

FIG.27

Single Radial Immunodiffusion test for detection of tetanus toxin and toxoid



A - using unabsorbed antitoxin

B - using absorbed antitoxin

Wells: 1 - VAC 28 /ml

2 - VAC 14 /ml

3 - VAC 7 /ml

4 - 3 day FAB supernatent

Note the similarity of zone sizes and the diffuse zones produced usi ng absorbed antitoxin.

however the lines produced spread a great deal making interpretation difficult. Also the FAB supernatant produced 2 to 3 non-specific precipitin lines itself which interfered with the toxin lines making determination of lines of identity almost impossible. (Fig.28).

Double Immunodiffusion under these circumstances was not quantitative (Table 15).

vi) Rocket Immunoelectrophoresis

With both absorbed and unabsorbed antitoxin VAC produced a standard curve with very vague peaks to the rocket precipitins (Fig.29) making measurement difficult. This resulted in a calculated value for TOX significantly different to the actual value when read off the VAC standard curve (60 and 74 Lf/ml respectively). (Table 15). The TOX rocket peaks were much sharper however, and as a result, VAC values calculated from them were much closer to the actual value. (30 and 28 Lf /ml respectively).

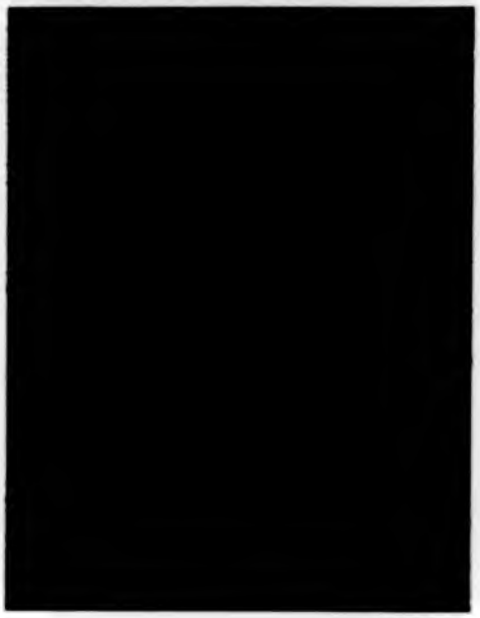
When the FAB supernatant was tested against unabsorbed antitoxin, three precipitin rockets were seen. These were not present with the absorbed antitoxin, although the remaining specific toxin rockets were slightly more diffuse than before (Fig.29b).

vii) Rocket-line Immunoelectrophoresis

The results here were similar to those obtained with Rocket IEP except that with unabsorbed antitoxin, the non-specific precipitin rockets produced by the FAB supernatant produced no lines of identity with the horizontal toxin precipitate and so could be easily identified as non-toxin rockets, whereas toxin in the wells produced a rocket showing lines of antigenic identity with the horizontal toxin line. (Fig.30a). Again however the VAC standard curve produced hard to measure rockets

FIG.28

Double Immunodiffusion test for detection of tetanus toxin and toxoid



Wells: 1 FAB supernatant

2 VAC

3 FAB supernatant

4 empty

5 VAC

6 FAB supernatant

7 empty

8 TOX

Central: Wellcome tetanus antitoxin

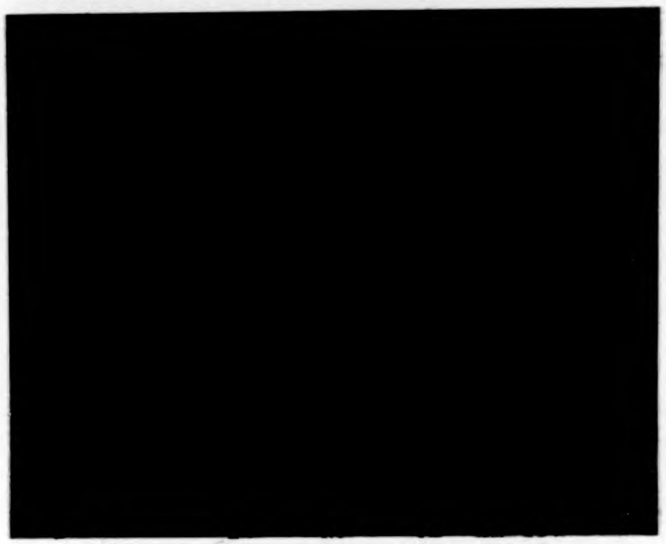
Volumes: 25 ul Incubation: 48 hours at room temperature.

Note: TOX and VAC produce a single strong line (wells 2, 5 and 8) but this shows marked spreading and curving even where the adjoining well is empty(well4) and especially where the FAB supernatant is in an adjoining well (i.e.wells 3 and 6). Where the FAB supernatant is between TOX and VAC wells, in addition to the 2 or three lines produced by FAB, the TOX/VAC line appears to join completely and it would appear as though FAB produces a strong toxin line as well. (arrow)

FIG.29 a)

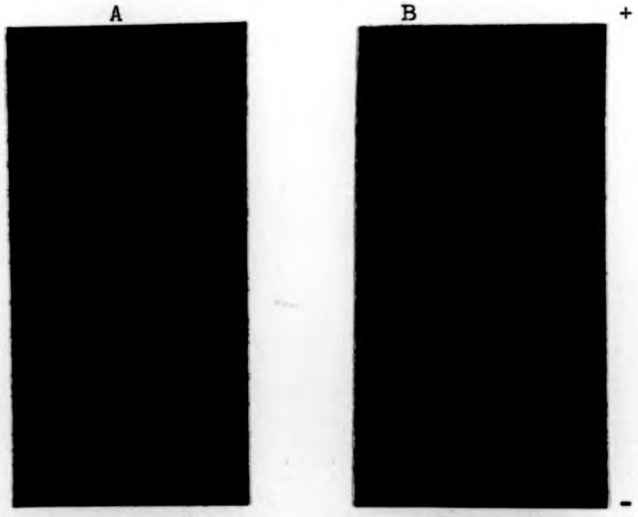
Rocket Immunoelectrophoresis

The VAC standard curve



Wells	1	2	3	4	5
	6.6 Lf/ml	14 Lf/ml	28 Lf/ml	56 Lf/ml	112 Lf/ml

b)



A - absorbed antitoxin

B - Unabsorbed antitoxin

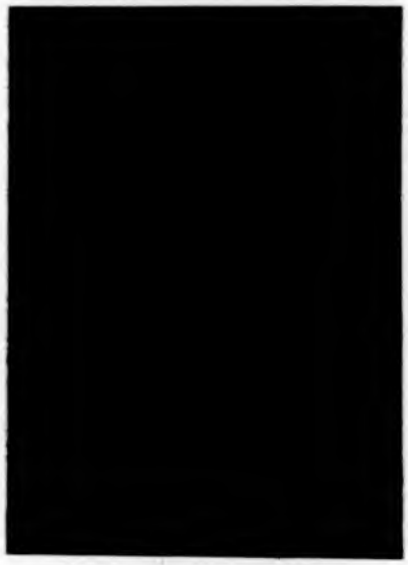
Wells: 1 - TOX

2 - 3 day FAB supernatant (note: 3 nonspecific peaks with unabsorbed toxin - arrows)

resulting in poor correlation between calculated and actual valuesfor TOX (85 and 74 Lf /ml)(Table 15) whereas the sharper TOX rockets allowed calculation of VAC values to come much closer to the actual value (30 and 28 Lf/ml respectively). It was noted that there was only partial antigenic identity seen between a horizontal toxin line produced by TOX and a rocket produced by VAC (Fig.30b) and vice versa, although homologous toxin lines and rockets always showed complete identity (Fig.30b).

FIG. 30 a)

Rocket-Line Immunoelectrophoresis of TOX and FAB supernatent with TOX forming the horizontal line

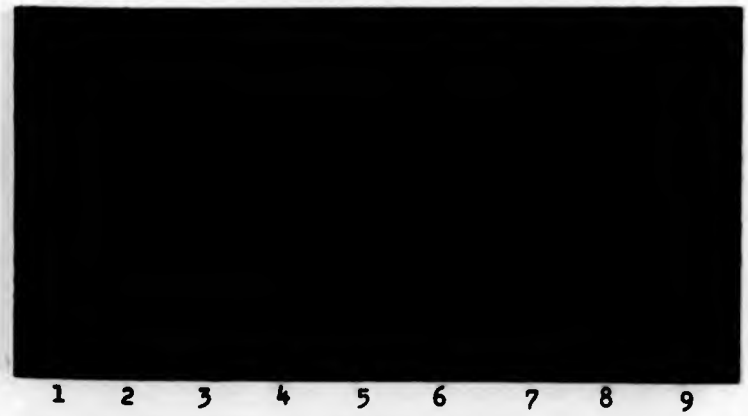


Wells: 1 - 3 day FAB supernatent

2 - TOX

Note: the reaction of identity between the rocket formed by TOX in the well and the horizontal precipitin line formed by TOX, and the lack of identity between the non-specific rockets produced by FAB (arrow)

FIG.30 b) Rocket-Line Immunoelectrophoresis of TOX and VAC with TOX forming the horizontal line



wells
1 TOX 5 Lf/ml
2 " 10 "
3 " 15 "
4 " 25 "
5 " 50 "
6 " 75 "
7 ←
8 VAC 56 Lf/ml
9 " 28 "

Note the reactions of identity between the rockets formed by TOX in the standard curve and the TOX precipitin line, and the partial antigenic identity between the rockets produced by VAC. (arrow)

3.3.2 Assay of other Cl.tetani strains and other growth media for toxin production

The results of testing all 27 Cl.tetani strains grown both in Massachussetts medium and in FAB broth by HAI are shown in Table 16. The 3 day FAB was negative for the presence of toxin when tested at both the LA/10 and LA/100 levels of sensitivity, which can detect as little as 0.095 Lf/ml toxin. It can be seen that only 3 strains were non-toxigenic in Massachussetts medium (CN 1342, LQ914 and LQ 931) whilst 3 others were very weak toxin producers (LQ1, LQ2 and L109). The other strains produced toxin in excess of 14 Lf/ml, the greatest amount (224 Lf/ml) being produced by strain E88. When grown for 7 days in FAB broth, the same 3 strains as before were non-toxigenic, with the other 24 producing toxin, although in most cases in lesser quantities than when grown in Massachussetts broth.

The amount of growth appeared to bear little relationship to the amount of toxin produced, as the same amount of toxin could be produced by strains with low levels of growth (i.e. NCTC IV and VI) as by strains with high levels of growth (CN 761, NCTC VIII).

Both SRID and Rocket IEP were uninterpretable using unabsorbed antitoxin as two or three different precipitin rings or peaks could be seen with most strains whether grown in FAB or Massachussetts medium Fig.31).

When absorbed antitoxin was used, these multiple precipitin reactions were removed leaving only specific toxin-antitoxin reactions but these were in the form of very small precipitin rings by SRID and very diffuse, faint peaks by Rocket IEP (Fig.32). None of the strains tested were shown to produce toxin when grown in FAB broth by either method (Table 17), and only three strains by SRID and four strains by Rocket IEP in Massachussetts medium. The Lf/ml values were very low in all cases.

When strain E88 was grown in a variety of media and tested by

FIG.31a

Assay of Cl.tetani strains for tetanus toxin by Single Radial Immunodiffusion using unabsorbed antitoxin

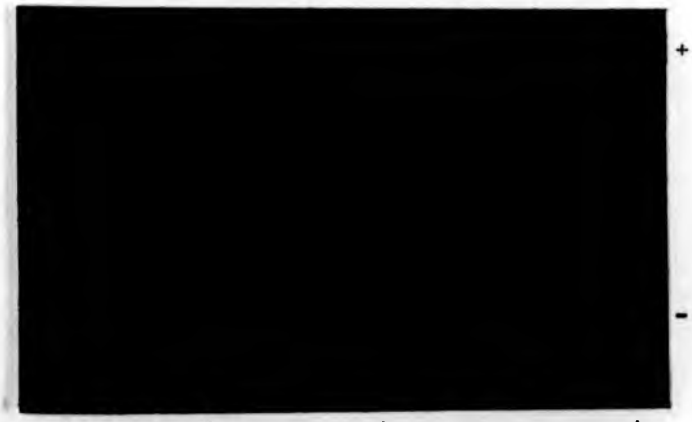
Wells

1	NCTC 540	(Massachussetts)	15	CN 4878	(Massachussetts
2	NCTC 539	"	16	CN 780	"
3	NCTC 5410	"	17	CN 761	"
4	NCTC 5411	"	18	CN 361	"
5	NCTC 9569	"	19	CN 1445	"
6	NCTC 9568	"	20	CN 3973	"
7	NCTC 9574	"	21	CN 947	"
8	NCTC 540	(FAB)	22	CN 4878	(FAB)
9	NCTC 539	"	23	CN 780	"
10	NCTC 5410	"	24	CN 761	"
11	NCTC 5411	"	25	CN 361	"
12	NCTC 9569	"	26	CN 1445	"
13	NCTC 9568	"	27	CN 3973	"
14	NCTC 9574	"	28	CN 947	"

Note: The presence of 2 or 3 precipitin rings around some wells (e.g. well No. 20) (arrow)

FIG.31b

Assay of Cl.tetani strain for tetanus toxin by Rocket Immunoelectrophoresis using unabsorobed antitoxin



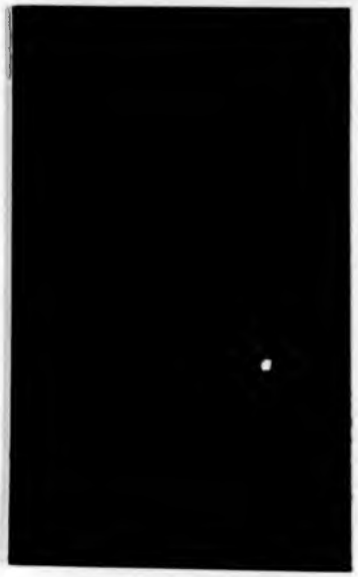
Note: the presence of 2 or 3 precipitin peaks above some wells (e.g. well No.11) (arrow)

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Wells 1) L109 2) L5500 3) CN 947 4) CN 3973 5) CN1445
6) CN761 7) CN 361 8) CN 1342 9) LQ1 10) LQ2
11) LQ 914 (FAB growths)

FIG. 32a

Assay of Cl.tetani strains for tetanus toxin by Single Radial Immunodiffusion using absorbed antitoxin

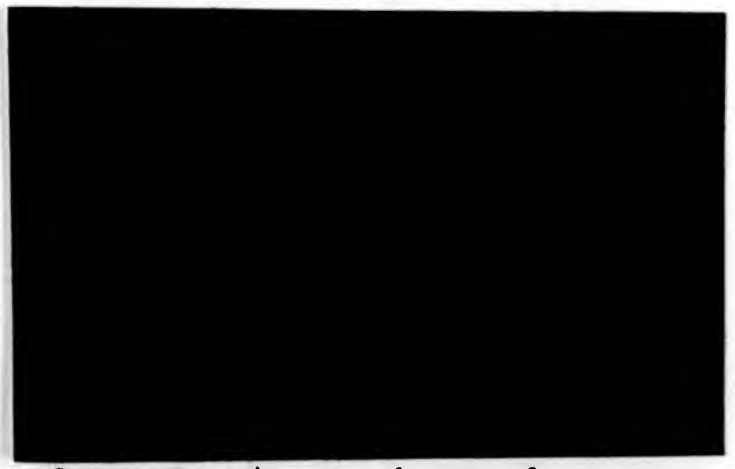


Wells

1	NCTC 540	(Massachussetts)	15	CN 4878	(Massachussetts)
2	NCTC 539	"	16	CN 780	"
3	NCTC 5410	"	17	CN 761	"
4	NCTC 5411	"	18	CN 361	"
5	NCTC 9569	"	19	CN 1445	"
6	NCTC 9568	"	20	CN 3973	"
7	NCTC 9574	"	21	CN 947	"
8	NCTC 540	(FAB)	22	CN 4878	(FAB)
9	NCTC 539	"	23	CN 780	"
10	NCTC 5410	"	24	CN 761	"
11	NCTC5411	"	25	CN 361	"
12	NCTC 9569	"	26	CN 1445	"
13	NCTC 9568	"	27	CN 3973	"
14	NCTC 9574	"	28	CN 947	"

FIG.32b

Assay of Cl.tetani strains for tetanus toxin by Rocket Immunoelectrophoresis using absorbed antitoxin



1 2 3 4 5 6 7 8 9 10 11

Wells: 1 - L109 2 - L5500 3 - CN 947 4 - CN 3973
5 - CN 1445 6 - CN 761 7 - CN 361 8 - CN 1342
9 - LQ1 10 - LQ2 11 - LQ914

Note: very faint diffuse precipitin peaks above wells 5, 6 and 8 (arrows)

the three methods, only Cooked meat medium cultures, in addition to Massachussets and FAB media produced toxin when measured by HAI, although this was not detected by SRID or Rocket IEP (Table 18). Isosensitest broth did not support growth of the strain, and serum broth exhibited only minimal growth, however Wilkins Chalgren broth and thiol broth gave good growth but no toxin production.

Raynaud extraction of 2 day old cells of strain 279 (serotype I) gave a toxin Lf value of 448 Lf/ml when measured by HAI. This is a 16- fold increase on theamount produced in a 7 day Massachussetts broth supernatent (Table 18). SRID and Rocket IEP could detect toxin in the Raynaud extract but produced Lf values of 8 and 12 Lf/ml respectively.

3.3.3. Investigation of enzymatic breakdown fragments of tetanus toxin

When examined by two dimensional IEP, native toxin (aliquot 1) showed a single precipitin peak, although close examination indicated the possibility of two superimposed peaks (Fig.33a). Reduced toxin totally degraded to 2 antigenic components α and β (Fig.33b) while trypsinised toxin showed the native toxin peak with another peak emerging (Fig.33c). Trypsinised, reduced toxin showed a smaller native toxin peak and also the two breakdown fragments (Fig.33d) whilst papain digested toxin showed only one peak.(Fig.33c). This is known to be fragment $\beta_1^{40,44}$. This is in the same position as the slower moving fragment (Figs.33b and 33d) which must be the whole β fragment. The faster moving fragment is therefore α (Fig.33f).

The relative mobilities of the native toxin and its two breakdown fragments were calculated to be 0.236 for the whole toxin, 0.264 for the slower moving fragment and 0.441 for the faster moving fragment (Fig.34), when compared to bovine serum albumin.

When the aliquots were tested by isoelectric focussing, a single line corresponding to approximately pH 5.2 was observed with each of them and no obvious differences could be seen (Fig.35).

When the aliquots were tested by SDS-PAGE, three bands corresponding to molecular weights of approximately 150,100 and 50 kilodaltons were produced by aliquot 3 and two bands corresponding to approximately 100 and 50 kilodaltons were produced by aliquots 2 and 4. (Fig.36) A single band corresponding to approximately 40 kilodaltons was produced by aliquot 5.

To visualise the native toxin as a single band by PAGE it was necessary to omit the addition of sample buffer (as this contains reducing agents) to the toxin and simply add a small quantity of sucrose to increase the density before electrophoresis. This produced a single band of approximately 150 kd.

The results obtained indicate that unreduced native toxin is

FIG.33

Two dimensional IEP of enzymatic breakdown fragments of tetanus toxin

a) Native toxin (aliquot 1)

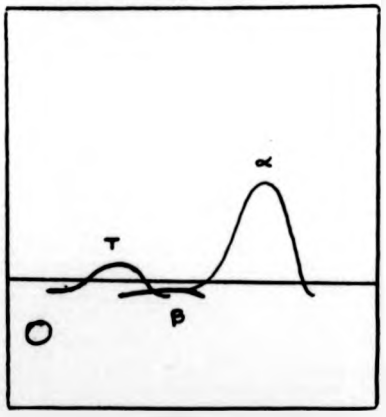
b) Reduced toxin (aliquot 2)

c) Trypsinised toxin (aliquot 3)

d) Trypsinised and reduced toxin (aliquot 4)

e) Papain digested toxin (aliquot 5)

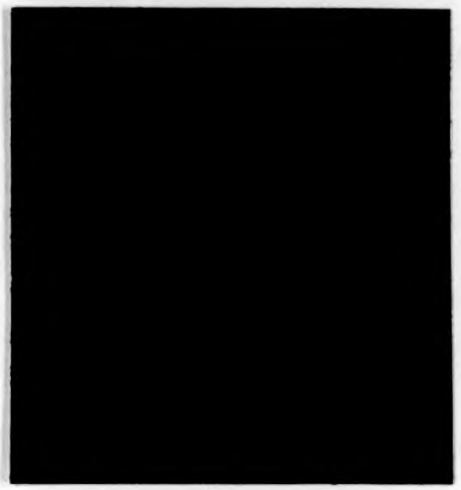
KEY



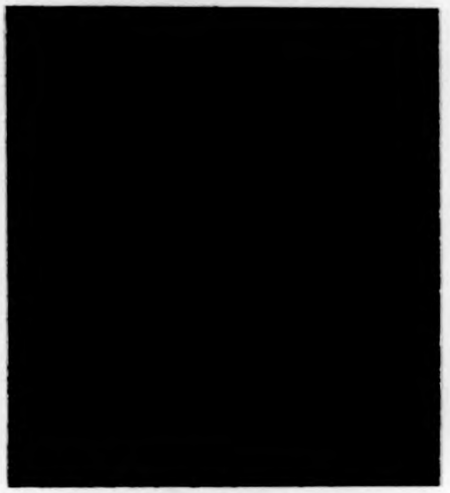
f) fragments of tetanus toxin
T=native toxin
β=fragment β. α=fragment α

FIG.34

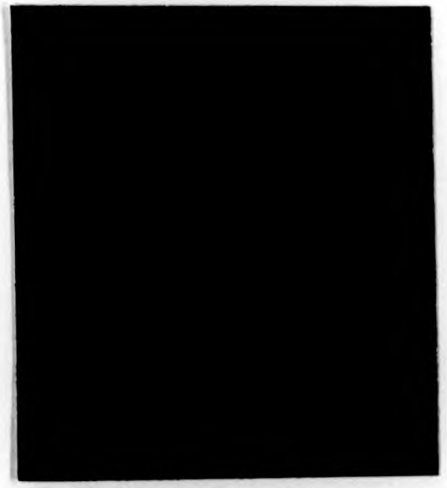
Relative mobilities of tetanus toxin and its breakdown fragments



a) BSA - Anti BSA reference peak



b) Native toxin and reference peak

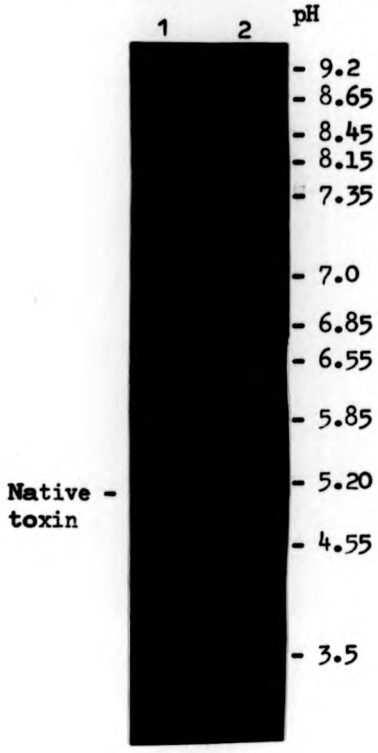


c) Breakdown fragments and reference peak

a single protein of molecular weight 150 kd, but that this can be dissociated by reduction or mild trypsin treatment to two polypeptide fragments of 100 and 50 kd respectively. Papain digestion of the toxin results in further digestion until a single fragment of 40 kd is left.

FIG.35a

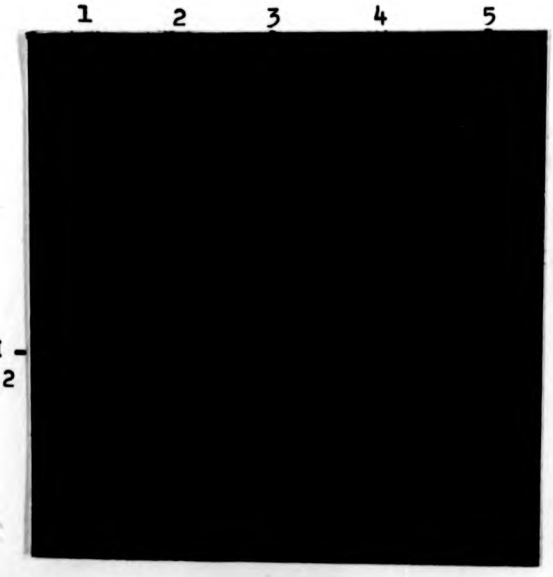
Isoelectric focussing of tetanus toxin and enzymatic breakdown products



1: Native toxin

2: IEF markers

FIG.35b



tracks:-

1 Native toxin (aliquot 1)

2 reduced toxin (aliquot 2)

3 trypsinised toxin (aliquot 3)

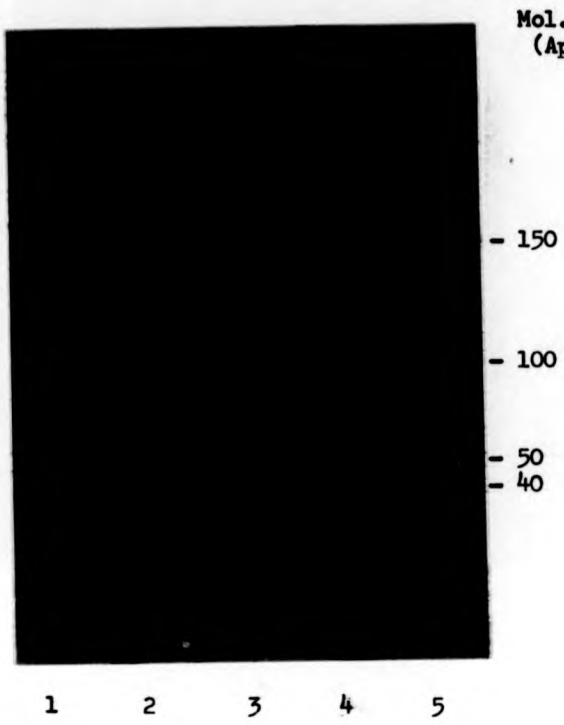
4 trypsinised reduced toxin (aliquot 4)

5 papain digested toxin (aliquot 5)

Note: All preparations give one band at around pH 5.2

FIG.36

Polyacrylamide gel electrophoresis of tetanus toxin and enzymatic breakdown products



Lane 1) Native toxin (aliquot 1)(in sucrose)
2) Trypsinised toxin (aliquot 3)
3) Reduced toxin (aliquot 2)
4) Trypsinised and reduced toxin (aliquot 4)
5) Papain digested toxin (aliquot 5)

3.3.4. Investigation of the binding of tetanus toxin to brain tissue extracts

When TOX was mixed with various brain extracts prior totwo dimensional IEP, the height of the precipitin peak was reduced to some extent with extract A and markedly with all the other extracts with the exception of extracts P and Y which did not noticeably affect the peak (Fig.37)(Table 19). A curious heterogeneity was observed with most of the reduced peaks where, in addition to the central main peak, a sharp pre-peak and a diffuse post-peak became visible (Fig.37). These are considered to be artifacts due to contamination from slowly migrating antigen (? bound to brain tissue) remaining in the well after the first dimension electrophoresis and then being spilled or spread along the gel by the transfer of gel to second dimension plate[135].

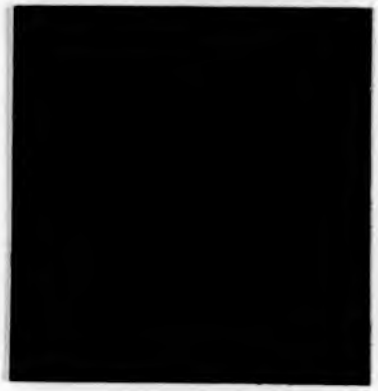
When toxin aliquot 2 (containing 2 antigenic fragments α and β) was mixed with the extracts, the height of the fragment α peak was reduced slightly by extract **Z**, and to some extent by all the other extracts with the exception of extracts P and Y which had no effect and J which removed the peak totally.

The fragment β peak was reduced by extract A, unaffected by extract Y, totally removed by extracts J and Z and markedly reduced by the other extracts (Table 19)(Fig. 38).

Refer to Appendix 24 for identification of brain extracts.

FIGURE 37

Absorption of tetanus toxin by brain tissue extracts



a) TOX unabsorbed

b) TOX absorbed with Protagon (extract Z)

c) TOX absorbed with acetone extract (extract A)

d) TOX absorbed with alcoholic extract (extract C)

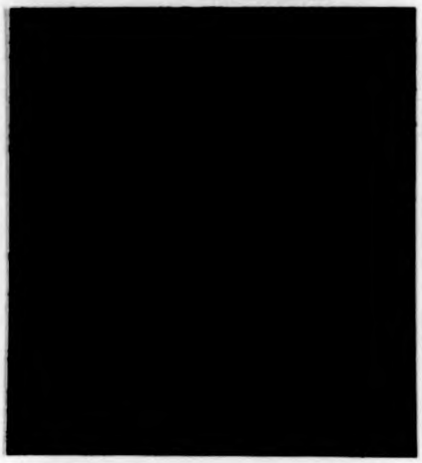
e) TOX absorbed with aqueous extract (extract Y)

f) TOX absorbed with cephalin (extract J)

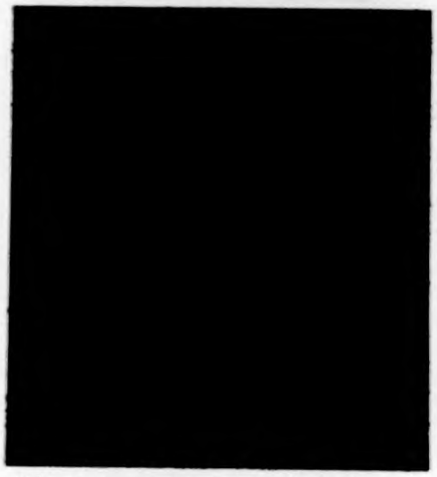
Note: sharp pre-peak and diffuse post-peak in absorbed TOX preparations e.g. d) (arrows)

FIG. 38

Absorption of tetanus toxin fragments by brain tissue extracts

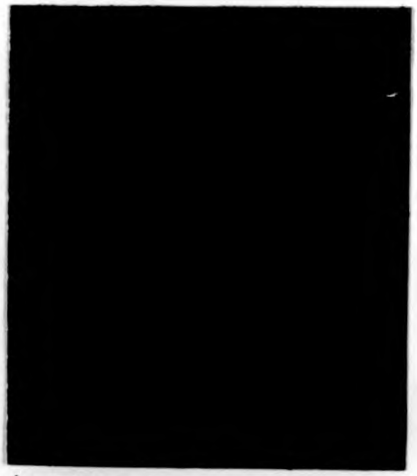


a) fragments ∝ and β unabsorbed



b) fragments ∝ and β absorbed with Protagon (extract Z)

note: fragment ∝ peak reduced and fragment β peak removed



c) fragments ∝ and β absorbed with cephalin (extract J)

note: both fragment ∝ and fragment β peaks totally removed

3.3.5. Investigation of the site of toxin binding in histological sections of brain by Immunofluorescent assay techniques IFAT)

When sections of cow brain stained with fluorescein labelled TOX were examined, diffuse green fluorescence was noted all over the stained area. There were no visible foci of fluorescence.

When slides stained by the indirect IFAT were examined only a very vague overall green fluorescence was seen. Again no obvious foci were visible.

Haematoxylin and Eosin sections showed normal brain tissue when examined.

3.4. DISCUSSION

The studies on in-vitro methods of tetanus neurotoxin assay showed conclusively that the best technique amongst those investigated was the Haemagglutination inhibition (HAI) technique. This method gave clear, reproducible endpoints and was quick and easy to perform. Although it has previously been used mainly for the assay of antitoxin [118,120] it has also been used for identity testing of tetanus toxoid[121] and quantitation of toxoid[119] and has been found to give good agreement with in vivo tests[119]. It must be stressed, however, that no in-vitro test can assess the toxicity of a sample, only in-vivo tests can do that. The best that in-vitro tests can do is to provide an estimate of the quantity of toxin or toxoid present.

Making the sensitised red cells for the HAI test is relatively complex, and the method must be followed exactly to produce a workable reagent. Once this has been done however, the reagent is stable in the refrigerator for several months. The reaction appears to be extremely specific, and shows no interference from other antigen-antibody reactions that might be proceeding simultaneously. This is not true of most of the other techniques investigated. The flocculation test has been shown to give false flocculating zones in addition to the true one[116,119,121, 136,137] and a special 'one zone serum' which is difficult to prepare[121] is required to circumvent this problem. False flocculation with a non-toxin containing culture was noted in this study and this, combined with the large quantities of reactants required, the time required to perform the test and the lack of correlation observed between the estimated value and the true value of toxins under test make the technique unreliable for routine use. The possibility that the technique gives an incorrect estimate of the toxoid content of preparations has been raised before[119] and this study supports that suggestion.

Single radial Immunodiffusion and Rocket Immunoelectrophoresis

are techniques that also suffer interference from non-specific antigen-antibody reactions. Antitoxins must be absorbed to remove all antibodies to antigens other than the toxin otherwise the tests became impossible to interpret because it was impossible to determine which of the various antigen-antibody precipitin rings or peaks corresponded to the actual toxin-antitoxin reaction. However, when absorbed antitoxins were used, the precipitin bands or peaks became quite diffuse and difficult to measure properly. This resulted in incorrect estimates of toxin content in some cases. The techniques were also rather insensitive as, due to the small precipitin zones produced, SRID had a lower detection limit of around 5 **Lf**/ml and Rocket IEP had a lower detection limit of around 6 **Lf**/ml.

Double Immunodiffusion is not a quantitative technique although it can be made semi quantitative by dilution of reactants until the precipitin line disappears. In this way the lower limits of detection were found to be between 3.75 and 8 **Lf**/ml using unabsorbed antitoxin. Again, this means the technique is relatively insensitive and, although reactions of identity could be observed between toxin preparations in adjacent wells, this could not always be relied on to establish the identity of an unknown precipitin line as non-specific precipitin lines tended to mask specific reactions by running into them, over them or beside them. Thus the technique proved unsatisfactory for either identification or quantitation of toxin preparations.

It had been hoped that the problems of determining which of the multiple Rocket IEP precipitin peaks produced by broth cultures of Cl.tetani corresponded to the toxin, when using unabsorbed antitoxin, could be solved by using the Rocket-line IEP technique where a reaction of identity would be produced between toxin in the line precipitate and toxin in the toxin precipitin peak, thereby avoiding the need to absorb antitoxin. However, this proved not to be the case. Although precipitin peaks due to VAC showed complete reactions of identity with

a precipitin line due to VAC, as did lines and peaks due to TOX, peaks due to TOX showed only partial identity with lines due to VAC and vice versa (Fig.30b). This was also noted with toxin containing culture supernatants. The reason behind this phenomenon is not clear, although as VAC is a formalin treated toxoid, and formalin treatment has been shown to denature the toxin to some extent[119] and cause the antigenic molecules to undergo polymerization[15] and become less susceptible to precise fractionation[11] it is perhpas not surprising that it does not show total antigenic identity with TOX, which is a purified culture filtrate of a toxigenic strain. It has been shown that the amino-acid composition of toxin and formalin treated toxoid is not identical[58], but this does not explain why only partial antigenic identity was observed between precipitin lines due to TOX and peaks due to toxin containing culture supernatents, which should theoretically show full identity. It is possible that the purification procedure employed to produce TOX (which is a commercial process and therefore details were not available) has caused some antigenic changes which are reflected in the incomplete reactions of identity formed between it and fresh toxin preparations.

Latex agglutination proved singularly unsuitable for the detection or quantitation of tetanus toxin as no agglutinations stronger than trace were seen. There are several possible reasons for this failure to work. For instance, as different substances absorb to latex particles in different ways, the optimal quantity of reactant giving the highest end-point titre but not leading to nonspecific reactivity must be determined experimentally[138]. Although this was attempted in this study, it is possible that the optimum quantity of antitoxin was not absorbed onto the latex, thus rendering the test highly unsatisfactory. Also, it has been shown that proteins undergo alterations in absorbing to the surface of latex particles[138] and the antitoxin may therefore have been altered to an inactive form. It is likely that, had a working

latex agglutination test been developed, it would have been troubled by non-specific reactions caused by other antibodies in the antitoxin reacting with other clostridial antigens, which could have masked or interfered with specific toxin-antitoxin reactions and would have made the test difficult to interpret.

When the 27 strains of Cl.tetani were tested for toxin production by HAI, SRID and Rocket IEP, it was obvious that SRID and Rocket IEP were of little value. Both tests were non-interpretable with unabsorbed antitoxin due to the multiplicity of non-specific reactions and the low sensitivity of both techniques with absorbed antitoxin resulted in few strains being positive,and those at very low levels. HAI on the other hand proved extremely useful for the quantitation of toxin in culture supernatants and showed that the majority of strains tested were toxigenic although some only weakly so. It has been stated that toxin production does not always parallel growth[11] and the findings of this study would tend to support that (Table 16). It should be noted that strains NCTC 5410 and NCTC 5411 are listed in the National Collection of Type Cultures Catalogue[167] as non-toxigenic. This is in direct opposition to the findings here, which indicated that both strains were capable of producing toxin. The original work was done on these strains in 1925 [152], before the advent of specific toxigenic media and, as the constitution of the medium has been shown to have a major effect on toxigenicity it is possible that these strains were genuinely toxigenic but simply failed to produce toxin in the medium used in that study. This is certainly possible as at least one of the strains was isolated from a case of human tetanus and so must have been toxigenic at that stage[152]. The other strain is impossible to identify precisely.

All the strains capable of producing toxin in Massachussetts medium were also toxigenic when grown in FAB. This finding was unexpected as Massachussetts medium is specially developed to promote

toxin production[95] as it has been shown that many strains of Cl.tetani appear nontoxigenic on ordinary medium[51] and the removal of inhibitors, the concentration of iron and the type of peptone used may all be critical[15,77,110,111]. It appears that the formulation of FAB is sufficiently near the optimum for these parameters to allow toxin to be produced, although it is worth noting that in most cases the amount produced was around half that produced in Massachussetts medium. It is also noteworthy that the three strains that were only weakly toxigenic in Massachussetts medium produced more toxin in FAB, although the amount of growth was not greatly different. It is possible that different strains produce toxin under slightly different optimum conditions and perhaps these particular strains favoured the conditions present in FAB to those in Massachussetts. The amount of toxin produced in Massachussetts medium is of the same order as found by other workers [110,111,113,114] and, as expected, the 'Harvard' strain(E88) of Cl.tetani proved to be the most prodigious toxin producer.

Cooked meat medium is used to produce toxins from other clostridia[2,139] therefore it is not surprising that it is capable of supporting toxin production by Cl.tetani, however it was expected that none of the other media would support toxigenesis and this supposition was borne out in practice.

The finding that hypertonic saline extracts of young cells[58] show increased toxin content compared to culture supernatents (a 16-fold increase in this study) is in agreement with other workers' findings[11,15].

The findings in this study that native (untreated) tetanus toxin had an isoelectric point (pI) of approximately 5.2 and migrated as a single band with molecular weight approximately 150 kd by PAGE is in complete accordance with previous finding[15,43,127,128] although others have put the molecular weight as low as 140 kd[44] or as high as 160 kd[126]. Using PAGE in this way only an approximate value can be obtained and this may be varied due to slight errors of measurement

therefore it is probable that these all represent the same value for tetanus toxin modified by experimental error rather than 10 or 20 kd deletions from the molecule.

The native toxin has been immunologically visualised as a single precipitin line by DID in the past, and its breakdown fragments identified by partial or lack of antigenic activity with the parent toxin[18,122,126,128,140]. In this study for the first time the toxin and its breakdown fragments have been visualised by two-dimensional IEP. The native toxin appears as a single peak or perhaps two superimposed peaks. This is possible as it has been shown that tetanus neurotoxin contains intrinsic proteases[40,141,142], which 'nick' the molecule and this double peak may be an early sign of the toxin dissociating.

The toxin used in these experiments is a mixture of 'intracellular' and 'extracellular' toxins[67] or whole and nicked toxins[43,127,128] which appeared as a single precipitin line in the native state but dissociated into two polypeptide fragments (α and β) when reduced. These have been shown in the past to be antigenically distinct[43.127,128] and this study has visualised them as two separate precipitin peaks. The identity of each of these peaks was confirmed by examining papain digested toxin which is known to degrade to a single component, β_1,[44] which is a breakdown fragment of fragment β. Fragment β_1, produced a small precipitin peak with the same relative mobility as the slower moving peak in the whole reduced toxin and this must therefore correspond to the parent fragment β, whilst the faster moving component is the α fragment.

Isoelectric focussing of the whole toxin and fragments α, β and β_1 showed that all had an isoelectric point of approximately 5.2. However, it has been shown that the individual polypeptide fragments can reconstitute to form the native toxin under certain conditions[127] and it is possible, although unlikely, that the individual fragments

may be recombining under the experimental conditions used here and thus producing a false estimate of their isoelectric points.

Previous work has shown that unreduced native toxin has a molecular weight of approximately 150 kd, whilst fragment α, fragment β and fragment β_1 have molecular weights of approximately 50 kd, 100 kd and 40 kd respectively[43,44,126,127]. This study produced approximate values of 50 kd, 100kd and 40 kd respectively for each fragment by SDS-PAGE. Although one other study has obtained a figure of 50 kd[128] for fragment β_1, it is likely that this is due to experimental variation and 40 kd is closer to its true molecular weight.

Having obtained a model for direct visualisation of the toxin and its breakdown products using two-dimensional IEP, attempts were made to determine which of the fragments were responsible for the binding of toxin to nervous tissues, and which components of nervous tissue bound the most toxin. This has mainly been attempted previously using absorption <u>in-vitro</u> followed by animal inoculation to determine the amount of toxicity absorbed and removed by the substance under test [41,66,142,143]. This method has the disadvantage that the toxicity of samples may reduce spontaneously by natural toxoiding, and measurements of toxicity cannot be made with any great precision[144], therefore errors may have been inadvertanly introduced. It has been shown that 'Protagon' - an extract of brain containing a mixture of sphingolipids particularly cerebrosides and sphingomyelins - was very effective at binding tetanus toxin[66]. It has also later been suggested that the binding is due to gangliosides[41] particularly the GT series[39] and the GD and GM series[40]. However, the question remained, are there any other binding sites in brain tissue other than gangliosides? It has been suggested that gangliosides represent only a low-affinity site, and that in physiological systems tetanus toxin binds with high affinity to a protein receptor[145].

Protagon, - containing gangliosides (acidic glycosphingolipids) - is insoluble in water[66], therefore the use of an aqueous extract of brain tissue in the two dimensional IEP system would show the presence of any non-ganglioside, water soluble binding sites by lowering the precipitin peak produced by the toxin and the use of protein extracts using Triton X-100 would indicate the presence of any protein binding sites inthe same way. This was not the case therefore there can be no water soluble binding sites or prdein binding sites present. The toxin peak was lowered substantially by absorption with protagon, confirming the previous work[66] and lowered to a degree by acetone, alcohol and ether extracts of brain, all of which contain gangliosides to some extent. When fragments α and β were absorbed by acetone, alcohol and ether extracts of brain, both were absorbed to some extent although fragment β generally showed the most absorption. The aqueous extract had no effect on either fragment. Cephalin, a mixture of phosphatides, primarily phosphatidyl ethanolamine[130,146] absorbed both fragments completely, however this is less likely to be due to any of these phosphatides being a specific toxin binding compound than to the preparation containing substantial amounts of gangliosides, as these are acidic glycosphingolipids[147] and are extracted from nervous tissues in a similar solvent system[41]. The fact that protagon absorbs all the fragment β, whilst very little of fragment α would tend to support previous suggestions that the binding site is the carboxyterminus of the heavy chain of the toxin, fragment B_1[40,42]. Unfortunatley, insufficient fragment β_1 was available to test this hypothesis directly. The use of this two-dimensional IEP model for visualisation of toxin binding capacity with specific purified gangliosides was not possible with the context of this study, but such experiments should be able to show directly the extent of binding by these compounds.

It had been hoped that direct visualisation of the toxin

binding site in brain sections by immunofluorescent assay techniques would lead to further information of the nature of the binding site by the use of specific histochemical stains and an examination of different nervous tissues and isolated nerve fibres,but the failure to produce definite fluorescent areas in the brain sections led to this approach being abandoned. Other workers have reported that fluorescent staining demonstrates that toxin binds to the whole surface of nerve cells[40], and the diffuse fluorescence observed over the brain cells in this study may have been a manifestation of that phenomenon. If so, then the reaction was far too vague to provide any meaningful results.

3.5. CONCLUSION

The Haemagglutination Inhibition technique proved to be the best in-vitro method of tetanus neurotoxin assay. Latex agglutination did not work and Double immunodiffusion was not quantitative. Rocket IEP and Single radial immunodiffusion suffered from interference from non-specific reactions and were very insensitive when absorbed antitoxin was used. Rocket-line IEP did not resolve the problem with non-specific reactions as only partial antigenic identity was observed with different toxin preparations.

When 27 different strains of Cl.tetani were tested for toxigenicity by the Haemagglutination inhibition technique, 24 strains proved to be toxigenic when grown in Massachussetts medium although 3 of them were only weakly so. The same strains were also toxigenic when grown in FAB mediumalthough generally toxin levels were lower. Cooked meat medium appeared to be capable of supporting toxigenesis although Wilkins-Chalgren broth, Isosensitest broth, Serum broth and Thiol broth did not. A hypertonic saline extract of 2 day old cells grown in toxigenic medium produced a 16-fold increase in the amount of toxin present when compared to a 7 day culture supernatant.

Native tetanus toxin appears as two superimposed precipitin peaks by two dimensional IEP, has a molecular weight of approximately 150 kd and an isoelectric point of 5.2. It can be separated into two polypeptide fragments by reduction or mild trypsinisation. These fragments can be visualised as two distinct peaks, α and β by two dimensional IEP, have molecular weights of approximately 50 kd and 100 kd respectively and appear to have isoelectric points of 5.2. A further breakdown product can be obtained by papain treatment which gives a single peak, β_1, by two dimensional IEP, has a molecular weight of approximately 40 kd and an isoelectric point of 5.2.

Using two dimensional IEP, the absorption of whole toxin by ganglioside containing brain extracts, but not by aqueous or protein

extracts can be directly visualised. This supports the theory that gangliosides are the major toxin receptor sites in brain tissue. In the same way, the preferrential absorption of fragment β by ganglioside containing extracts was seen, supporting the theory that a subcomponent of fragment β, fragment β_1, is responsible for tetanus toxin binding in-vivo.

Immunofluorescent assay techniques proved incapable of visualising toxin binding sites in histological brain sections.

CHAPTER 4

THE NONSPASMOGENIC TOXIN

4.1 SURVEY OF THE LITERATURE

The main features of clinical tetanus are convulsions and spasticity of the muscles[15,39] and these are caused by a direct action of the neurotoxin on the mammalian spinal cord and central nervous system[15]. Toxin is taken up at myoneural junctions and transported by retrograde axonal flow to the central nervous system where its specific action is primarily the prevention or blocking of the transmitter substance, acetylcholine in the central inhibitory synapses in the spinal cord[11,40,42]. However, it is believed that this is not the only action of tetanus toxin as there is evidence for a peripheral action of the toxin also[15], primarily a blocking of neromuscular cholinergic transmission[15,40]. Although it was at one time suggested that tetanus toxin has a dual action, acting on both the central nervous system and the muscular nerve endings[15], it was later found that preparations of crude tetanus toxin contained a distinct fraction which appeared to have a direct effect on the peripheral nerve endings[11,16].

This fraction was called the Nonspasmogenic toxin of Cl.tetani and was shown to be distinct from the spasmogenic neurotoxin (tetanospasmin) by absorbing crude toxin preparations with protagon, a crude extract of brain tissue[16]. As protagon contains gangliosides which are known binding sites for the spasmogenic toxin and absorption with this material removed the greater part of the centrally acting fraction, the remaining fraction, which retained the majority of the peripheral action, was regarded as mainly the nonspasmogenic toxin. Further work indicated that this toxin had a molecular weight of 35-40 kd and contained 17 amino acids [148].

The nonspasmogenic toxin increased the frequency of miniature end plate potentials (MEPPs) in neuromuscular junctions and it has been suggested that this would eventually produce depletion of available

acetylcholine leaving less available for nerve impulse transmission. This situation would then lead to an apparent neuromuscular blocking action[15,149]. Whether this fraction causes the peripheral paralysis in tetanus is unclear[11,15].

The nonspasmogenic toxin is antigenic and, as almost all preparations of tetanus toxin that have been used to date probably contained some nonspasmogenic toxin as well as the spasmogenic toxin[11], most tetanus antitoxins contain antibodies to it.

Since the original reports however, little appears to have been published on this toxin. It is not known whether tetanus vaccine preparations contain it, and whether immunised humans produce antibodies to it. If its specific receptors are not gangliosides, what are they? The following investigations were undertaken to try to clarify the situation.

1) **Isolation of the nonspasmogenic toxin and examination by electrophoretic techniques.**
2) **A study of the absorption of the nonspasmogenic toxin by brain tissue extracts.**

4.2 EXPERIMENTAL TECHNIQUES

4.2.1 Isolation of the nonspasmogenic toxin

Cl.tetani strain CN 761 (Appendix 2) was grown for 7 days in Massachussetts medium (Appendix 9) before being centrifuged and the cellular deposit removed. The supernatent was absorbed with an equal amount of Protagon (Appendix 24) for 24 hours at room temperature, before being centrifuged for 30 minutes at 1,500 x g following which the supernatant was removed and designated 761 MP-Abs. An aliquot of the original culture supernatent was mixed with an equal amount of sterile PBS and treated identically to serve as an unabsorbed control (761M).

Tetanus toxin (TOX) and vaccine (VAC) were absorbed in a similar manner and designated TOXP-Abs and VACP-Abs. Unabsorbed controls were also made as above.

Each of these preparations was investigated by two dimensional IEP (Appendix 14), SDS-PAGE (Appendix 15) and Isoelectric focussing (Appendix 20).

761M and 761MP-Abs were concentrated 20 times by freeze drying and subsequent dissolving in one-twentieth the original volume of distilled water before being re-examined by SDS-PAGE and isoelectric focussing.

4.2.2 Investigation of the absorption of the nonspasmogenic toxin by brain tissue extracts

761MP-Abs was absorbed with an aqueous extract of brain, a Triton X-100 extract of brain and an acid-heat extract (Appendix 24) (extracts Y, P and S), by mixing 20 ul of 761MP-Abs with 20 ul of each extract and standing at room temperature for 2 hours. Following this, the absorbed culture supernatants were centrifuged for 30 minutes before the supernatents were examined by two dimensional IEP using Wellcome tetanus antitoxin.

4.3 RESULTS

4.3.1. Isolation of the nonspasmogenic toxin

When examined by two dimensional IEP, unabsorbed TOX produced a precipitin peak similar to that observed in Chapter 3, but TOXP-Abs produced no precipitin peak whatsoever (Fig.39 a and b). The findings with VAC and VACP-Abs were identical in that the unabsorbed VAC produced a single precipitin peak while VACP-Abs produced none. 761M produced a double peak corresponding to the neurotoxin and a smaller, faster moving peak while 761MP-Abs produced only the faster moving peak (Fig.39 c and d).

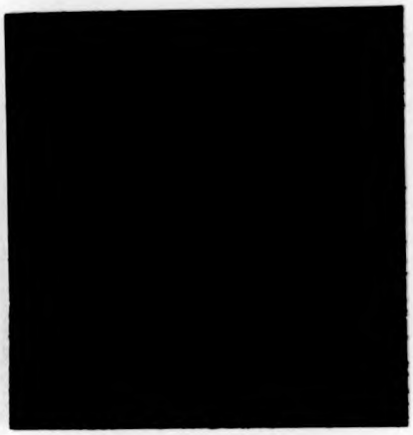
When examined by SDS-PAGE, unabsorbed TOX produced two bands, approximately equivalent to molecularweights of 100 and 50 kd (as seen in Chapter 3) but TOXP-Abs produced no bands. Unabsorbed VAC produced a vague 'smear' instead of a band, whilst VACP-Abs produced no bands.

761M produced 3 faint but distinct bands corresponding approximately to 100, 80 and 50 kd and 2 very faint bands corresponding to 37 and 31 kd but 761MP-Abs produced no bands at all.

However, when the concentrated supernatants were tested 761M produced 15 bands, many faint, with approximate molecular weights ranging from 127 to 23 kd, including one at 38 kd, while 761MP-Abs produced only one very faint band with an approximate molecular weight of 65kd (Fig.40) .

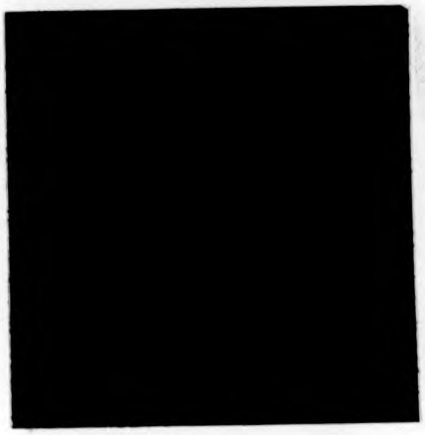
When examined by isoelectric focussing, unabsorbed TOX produced one band at approximately pH 5.2 (as seen in Chapter 3) but TOXP-Abs produced no bands. Again unabsorbed VAC 'smeared' giving no clear bands, whilst VACP-Abs produced no bands at all. 761M produced 4 bands, one moderately strong at approximately pH 5.0 and 3 weak at pH 5.1 , 4.6 and 4.3 respectively. 761MP-Abs produced no bands at all (Fig.41).

FIG.39 Absorption of Tetanus neurotoxin with Protagon in an attempt to demonstrate the nonspasmogenic toxin by two dimensional IEP



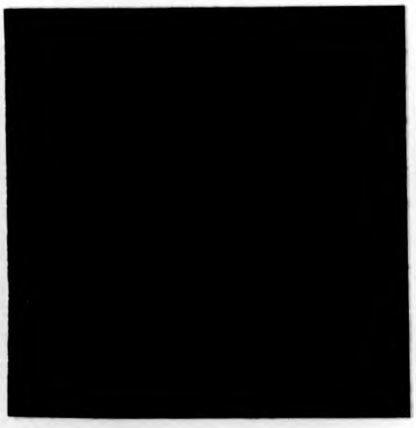
a) TOX

Note: similarity of peak to that seen in Fig.37.



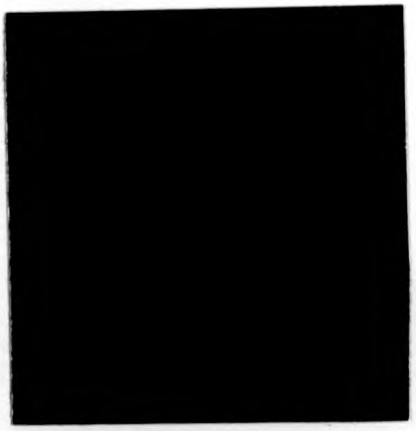
b) TOXP-Abs

Note: absence of peaks following absorption.



c) 761M

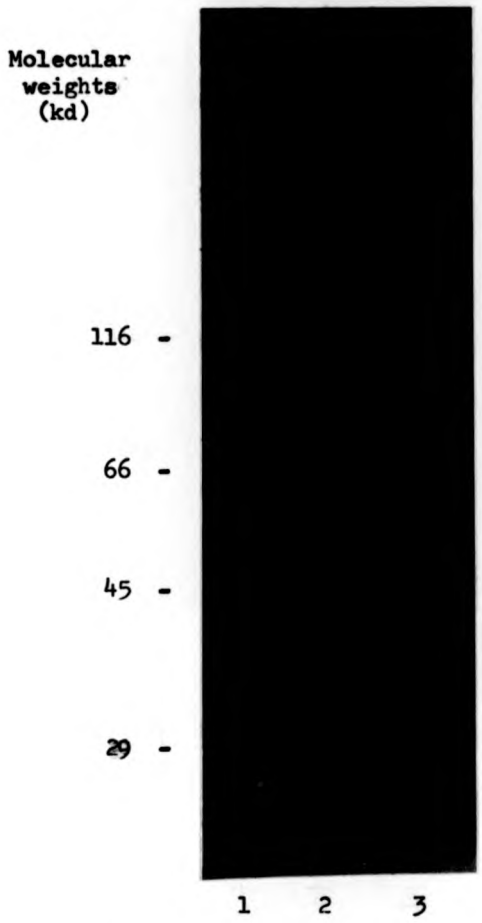
Note: Slower moving peak corresponding to tetanospasmin and a smaller faster moving peak **(arrow)**



d) 761MP-Abs

Note: After absorption with protagon - smaller peak remains **(arrow)**

FIG. 40 SDS-PAGE of Cl.tetani CN761 culture supernatant both unabsorbed and absorbed with Protagon



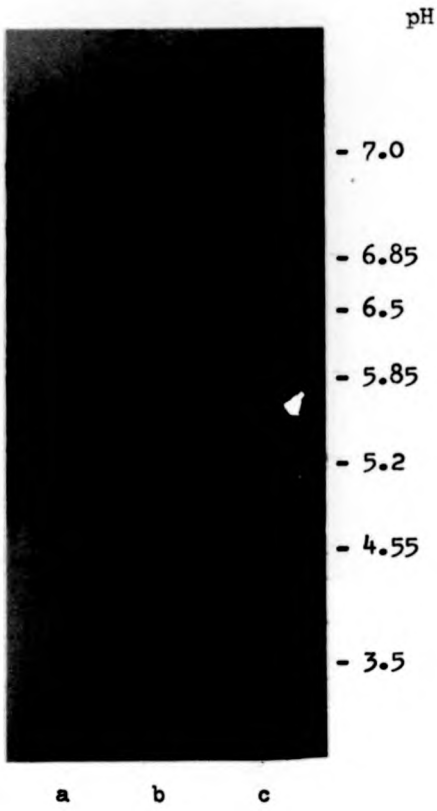
Lanes 1) Molecular weight standards

2) 761M

3) 761 MP-Abs

Note: very faint band at approximately 65 kd in lane 3 (arrow)

FIG.41 Isoelectric focussing of Tetanus Cl.tetani CN761 culture supernatant both unabsorbed and absorbed with Protagon



a) 761M

b) Isoelectric focussing calibrator

c) 761MP-Abs

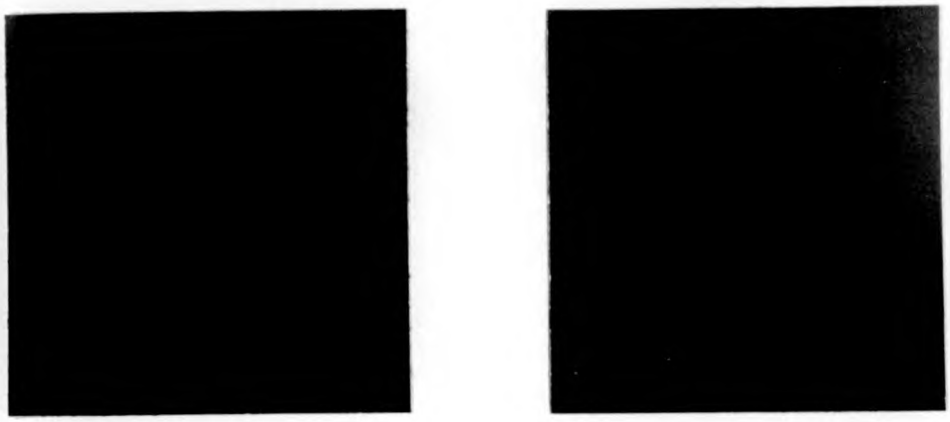
Note: the four bands seen with 761M (arrows). These are not present in 761MP-Abs.

When the concentrated supernatants were examined only smears were seen with no clear bands in either.

4.3.2. Investigation of the absorption of the nonspasmogenic toxin by brain tissue extracts

When 761MP-Abs was further absorbed with aqueous, Triton X-100 and acid-heat extracts of bovine brain tissue, there was no removal of the single small, fast moving precipitin peak which was visualised by two dimensional IEP in any case. The height of the peak was identical to that seen in the original 761MP-Abs indicating that no absorption whatever had taken place (Fig.42).

FIG.42 Absorption of the nonspasmogenic toxin with Triton X-100 and acid-heat brain extracts



a) 761MP-Abs further absorbed with Triton X-100 brain extract

b) 761MP-Abs further absorbed with acid-heat brain extract

Note: The appearance of the single small precipitin peak is identical to that observed in 761MP-Abs (Fig. 39d) (arrows)

4.4 DISCUSSION

Although other workers have apparently demonstrated a nonspasmogenic toxin distinct from the neurotoxin in toxin preparations from Cl.tetani strains,[16,148,149] this study was unsuccessful in clearly demonstrating the presence and nature of such a toxin.

One possible conclusion is that there is, in fact, no nonspasmogenic toxin and the peripheral nonspasmogenic effect seen in tetanus and in animal models is due to the neurotoxin itself, or a subfragment of it. Indeed, the stated molecular weight for the nonspasmogenic toxin is 35-40kd[148] which is close tothe value quoted for fragment β1 (fragment c) obtained by papain cleavage of the neurotoxin (around 40kd)[44]. This is unlikely however, as fragment β_1 is the fragment which mediates binding to gangliosides and so would be expected to be removed entirely by absorption with ganglioside-containing Protagon. Even so, it is still possible that an as yet unknown fragment of the neurotoxin is responsible for the nonspasmogenic peripheral action which has been described.

If the nonspasmogenic toxin does exist as a distinct entity, then the TOX preparation used here should have contained some, and the Wellcome antitoxin ought to have contained antibodies to it. Therefore, when TOX was absorbed with Protagon to remove the neurotoxin, a precipitin peak corresponding to the nonspasmogenic toxin ought to have been visible, but was not. It is possible that this is caused because the antibody to the nonspasmogenic toxin is nonprecipitating, but previous work has shown that it is precipitating, 12 precipitin lines being found by immunoelectrophoresis. This finding must be suspect however because the same report claims that the unabsorbed crude toxin showed 20 lines, whereas most workers today accept that native tetanus toxin produces a single precipitin line, and even when fragmented, a maximum of 3 [40,42,59,126,128], therefore the preparations used in that

work must have been contaminated with a number of other antigens, any of which might have been mistaken for a putative nonspasmogenic toxin. This is probably the origin of the single fast moving precipitin peak seen in the Protagon absorbed culture supernatent of strain 761. This peak was not removable by Protagon,(containing lipids)[66], Triton X-100 extracts of brain,(containing protein)[23], hot acid extracts of brain(containing carbohydrates)[23] or aqueous extracts of brain. If this peak was the nonspasmogenic toxin, it must have binding sites of some kind and so should theoretically be removable by absorption with an extract containing the receptor for these sites. Although it is possible that none of the extracts used contained the receptor it is more likely that the precipitin peak was not due to the nonspasmogenic toxin but to some other antigenic component.

It is also possible that the absorption protocol with Protagon used here, being slightly different to that described earlier[16], may have resulted in complete removal of the nonspasmogenic toxin from all the preparations before investigations began, but this is unlikely as fairly exhaustive absorptions have been used previously without affecting the amount of nonspasmogenic toxin present.

SDS-PAGE was also unsuccessful in demonstraing the presence of a 35=40 kd band in toxin preparations absorbed with Protagon. Neither unabsorbed nor absorbed TOX had a band in this region and although unabsorbed 761M had a band at approximately 37kd, this was removed by Protagon absorption. The concentrated 761M also produced a band at approximately 38 kd, along with 14 others, but all were removed by Protagon absorption with the exception of a faint band at approximately 65 kd. It is highly likely that this corresponds to the antigen causing the small fast precipitin peak seen by two dimensional IEP and is further evidence against this being the nonspasmogenic toxin.

Isoelectric focussing was no more successful that the other

techniques, however it is worthy of note that all of them can only detect relatively large amounts of protein when stained in the manner used in this study [23,83, 104].

It is possible that the nonspasmogenic toxin was present but in too small quantities to be detected. Perhaps more sensitive staining techniques such as silver staining[83] could have made it visible but these techniques are expensive and were not available in this study.

The published descriptions of the nonspasmogenic toxin have all been produced by one group of workers, [16,148.149] and it appears that no other reports of this toxin have been made. The detection of the toxin has been accomplished by observing its effect on the intracellular Miniature end plate potentials (MEPPs) in the thorax of mice[16] or in rat diaphragm-phrenic nerve preparations[149]. These techniques were unavailable in this study and it may be that they were successful in detecting the toxin because they are much more sensitive to small quantities of toxin than the techniques used here.

Because of the failure to isolate the nonspasmogenic toxin,. no further work could be done on its biochemistry, immunology or physiology.

One interesting finding was the 'smeared' appearance of VAC when investigated by SDS-PAGE and Isoelectric focussing. This may perhaps be explained by the fact that the formaldehyde used to produce the toxoid for the vaccine combines with it by means of methyl linkages to sites on the toxin and the nonspecific combination renders the toxoid less susceptible to precise fractionation[11], making it difficult to produce clear bands in electrophoretic techniques. This is probably the reason that Rocket IEP peaks produced by VAC are much more diffuse and unclear than those produced by nonformolised toxin (see Chapter 3).

The 'smeared' bands seen with the concentrated 761M and 761MP-Abs when tested by isoelectric focussing were probably due to either a precipitate appearing in the sample caused by the concentration

process, or a poorly soluble sample[104].

4.5 CONCLUSION

This study was unable to isolate or detect the nonspasmogenic peripherally acting toxin of Cl.tetani by two-dimensional IEP, SDS-PAGE or Isolectric focussing. This failure may be caused by the inability of the relatively insensitive techniques used here to detect small amounts of toxin when the original reports used electrophysiological assay techniques which appear to be extremely sensitive.

It is possible, though unlikely, that the nonspasmogenic toxin does not exist and the peripheral nonspasmogenic activity seen in tetanus and the effect on MEPPs is due to the neurotoxin itself or an active fragment of it.

An antigen detected by two-dimensional IEP following Protagon absorption of a neurotoxin containing culture supernatant was not considered to be the nonspasmogenic toxin as it had an apparent molecular weight significantly different from that described for the toxin and it could not be removed by absorption with various extracts of brain tissue.

CHAPTER 5

THE BIOCHEMICAL REACTIONS OF CL.TETANI

5.1 SURVEY OF THE LITERATURE

The biochemical reactions of Cl.tetani are subject to conflicting views, particularly with regard to the proteolytic and saccharolytic properties of the organism. Thus some workers describe Cl.tetani strains as completely non-proteolytic and non-saccharolytic[2] whilst others state that they are non-saccharolytic but proteolytic[3,6,150,151], some that they are both proteolytic and sometimes saccharolytic[152] and some that they are non-proteolytic but may occasionally be saccharolytic [153]. There appears also to be confusion over the gelatinolytic activity of the species[154], some authors claiming the majority of strains to be gelatinolytic[11,152,154], while others feel that most strains are negative with only a few showing weak or delayed gelatinase activity[151,155]. Likewise, hydrogen sulphide production, indole production, milk proteolysis, casein digestion, fibrinolysin production, growth in the presence of bile, lipase production and phosphatase production all appear subject to confusion with some authors reporting all positive, some all negative and many simply as weak or variable[11,12,150,151,152,153,154,155].

Many of these discrepancies appear to be due to differences in the methodology of the tests when performed bydifferent workers. For instance, all of 71 strains of Cl.tetani were found to produce gelatinase when an agar test was used butonly 17 of these showed activity in the charcoal gelatin test and only 16 in the nutrient gelatin test[11].

In addition, there appears never to have been a systematic study of all of the different possible biochemical reactions and cultural properties of Cl.tetani. This has been done for other organisms such as Haemophilus[156] and Bordetella[157], albeit on a generic basis.

Much of the work on the biochemical reactions of Cl.tetani was done originally several decades ago, and much was done between

World War I and World War II when interest in the organism was at its height[11]. This has led to a very haphazard and confused understanding of the situation. In addition, there are now many newer tests available based on improved understanding of bacterial enzyme actions, which may be used to produce an enzymatic profile of an organism in order to understand better its basic metabolism. Such a system has been used successfully with Pseudomonas maltophila[158]. Many of the tests had never previously been applied to Pseudomonas species and it seems likely that they have not been applied to Cl.tetani either. The enzymes detected by some of these tests have been proposed as potential virulence factors in Pseudomonas aeruginosa and other bacteria and it would be desirable to see if any of them could be virulence factors for Cl.tetani.

Many enzymes can be detected easily and reliably by commercial enzyme systems that have only recently become available. Thus the API ZYM system[22] has been used to study Bacteroides, Carnocytophaga and oral spirochaetes[159], Legionella pneumophila[160] and Gram-negative anaerobes[161], and an extended system is used in-house in the API Laboratories[162].

Commercial systems for biochemical testing of a number of different organisms are also available, which use micro-modifications of conventional biochemical tests such as the Seward AP 60 system[26], the Abbott BID system[163] and The Anaerobe-Tek system[164], or combinations of conventional tests and enzyme tests such as the API Strep system[22]. Also, most conventional biochemical tests can now be purchased commercially in a plate format[30].

A systematic study of all the possible biochemical reactions, enzymes and related properties of Cl.tetani was planned, utilising all the available commercial products where possible, and in-house preparations where not possible. Where tests had been done on Cl.tetani previously by other workers who had arrived at discrepant results, a number of

different protocols for the tests were investigated in an attempt to clarify the situation. A search was made of the literature to find recently developed biochemical tests never previously applied to Cl.tetani, such as the Glutamic acid decarboxylase test[165] and the Nicotinamide adenine dinucleotide glycohydrolase test[166], and these were also included in the study.

It was not possible to test all the strains of Cl.tetani available in this study by all the commercial systems, mainly for reasons of expense, therefore a representative sample were tested. The 9 NCTC strains (Appendix 1) were chosen for this as they are all different serotypes[167], and contain one nonmotile strain (serotype VI). It was felt that these strains were the ones most likely to show any biochemical differences as they had already been shown to posess antigenic differences. As those strains were all toxigenic (Chapter 3) a non-toxigenic strain was also included. The non-commercial tests could be investigated using all 27 strains of Cl.tetani. Some of the tests that appear to be particularly prone to variation such as milk digestion and indole production were investigated further in an attempt to clarify the situation.

The haemolysin of Cl.tetani is known to be antigenic and capable of being neutralised by horse antitoxic sera[11,12] as is the enzyme causing precipitation of milk[154], and the swarming of motile Cl.tetani may also be inhibited by antitoxin[11,154]. The effect of antitoxin on most other biochemical reactions of Cl.tetani appears to be unknown, and it seemed desirable to investigate all the positive biochemical reactions found in this study from that aspect.

5.2 EXPERIMENTAL TECHNIQUES

5.2.1 Investigation of the biochemical reactions of Cl.tetani using commercial systems

Ten strains of Cl.tetani were investigated. They included Cl.tetani serotypes I to IX inclusive (Appendix 1) and strain CN 1342 - a non toxigenic strain (Appendix 2). The ten strains were investigated using the API ZYM system (Appendix 26), the API 20 STREP system (Appendix 27), the API Anaerobic enzyme system (Appendix 28), the Seward AP60 system (Appendix 29), the Abbott BID system (Appendix 30), the Anaerobe-Tek system (Appendix 31) and commercially available glucose, H_2S, gelatin and indole plates (Appendix 32).

Inoculation, incubation, reading and interpretation of these tests were as described by the manufacturers and as shown in the appropriate appendices.

In order to establish whether any of the positive reactions observed were due to extracellular enzymes, a 48 hour FAB cell-free supernatant of NCTC 279 (serotype 1) was also tested by all the above systems.

5.2.2 Investigation of the biochemical reactions of Cl.tetani using non-commercial methods

All 27 strains of Cl.tetani (Appendices 1 and 2) were used in this part of the study, and were investigated using laboratory prepared agar plates to demonstrate the following activities: collagenase, albumin-hydrolysis, chitin hydrolysis, lipolytic activity, chondroitinase, esterase, arbutin hydrolysis, elastase, mucinase, DNase, RNase, gelatinase, casein digestion or precipitation, casein hydrolysate digestion or precipitation, milk digestion, fibrinolysis, desulfoviridin production, glutamic acid decarboxylase, nicotinamide adenine dinucleotide glycohydrolase, hyaluronidase, H_2S activity, indole production, phosphatase activity and tetrazolium reduction (Appendix 33).

Other investigations using the same 27 strains of Cl.tetani using

laboratory prepared media, albeit not in plates were meat digestion and reddening, milk digestion and acid production, ammonia production from peptone and gas production in deep agar stabs (Appendix 34).

5.2.3 Investigation of tests giving variable or inconclusive results

i) Milk digestion

In this study milk digestion was found to be negative using the Anaerobe-Tek system and using whole milk agar plates, although digestion was observed using skimmed milk litmus media and casein hydrolysis was observed in plate tests. It is possible that the type of milk used may have had an effect on the degree of digestion, therefore milk agar plates were prepared as before (Appendix 33) but using whole milk[177], two kinds of powdered milk[178,179] and four kinds of skimmed milk[176,180,181,182].

The 27 strains of Cl.tetani used previously were inoculated onto each plate as before, the plates were incubated anaerobically at 37°C for 48 hours and examined as before for zones of clearing or zones of precipitation around the growth.

ii) indole production

In this study the indole test was negative using the API Anaerobic enzyme system, the Sensitive AP 60 system, the Abbott BID system, the Anaerobe-Tek system and both commercial and non-commercial agar test plates. This is surprising as many reference manuals claim that most or all Cl.tetani strains produce indole[3,6,12,150,151,153,155]. An investigation into the different methods and reagents for the detection ofindole was therefore performed.

Firstly, the same 27 strains of Cl.tetani used before were used in this investigation, and inoculated into both FAB broth (15 mls) in glass universals, cooked meat medium (15 mls) in glass universals, and Columbia blood agar plates. These were

incubated anaerobically at 37°C for 48 hours before being tested by three different indole detecting reagents (Kovacs' reagent, Aqueous Kovacs' reagent and para dimethyl amino-cinnemaldehyde (DMACA)(Appendix 35a) using three different techniques (spot test, direct test and extracted test) Appendix 35b).

iii) H_2S production

Using both commercial plates and SIM agar plates the H_2S reactions, although positive, were weak and faded rapidly. The H_2S tests incorporated in the AP60 system and the Anaerobe-Tek system gave strong positive results. Two further investigations were therefore undertaken. Firstly, SIM agar was prepared as before (Appendix 33) but poured into sterile glass universals[151] in 20 ml amounts before use. The 27 strains of Cl.tetani were inoculated into the centre of each bottle by stabbing with a sterile loop right to the bottom of the cooled media. Incubation was at 37°C for 48 hours. The appearance of a black colour around the inoculum was regarded as positive.

Secondly, saturated lead acetate impregnated strips[168] were dried and then wedged in the top of FAB cultures of the 27 strains and incubated for 48 hours at 37°C. Blackening of the strips was regarded as positive.

5.2.4 Investigation of the effect of tetanus antitoxin on the biochemical reactions of Cl.tetani

A strain of Cl.tetani (NCTC 539 serotype III) which had been shown to give positive results in phosphatase, lipase, gelatinase, DNase, RNase, casein hydrolysis, fluorescence on MacConkey, fibrinolysis, H_2S production, indole, Glutamic acid decarboxylase and skim milk hydrolysis tests was used in this part of the study.

These tests were performed as described earlier (Appen dix 33)

with the modification that two chambered plastic petri dishes[29] were used. 15 ml of each medium was poured into one chamber of each plate and 15 ml of the same medium plus 0.5 ml Wellcome Horse Tetanus Antitoxin [71] was poured into the other chamber. The test strain was inoculated as a streak on both sides of each plate and the plates were incubated at 37°C anaerobically for 48 hours before examination. Where the reactions were equivalent in both chambers there was no neutralising effect by the antitoxin. Where the reaction in the antitoxin-containing chamber was absent or significantly reduced when compared to the non-antitoxin containing chamber, neutralisation of the reaction had occurred.

Indole was tested in 48 hour FAB broths using Kovacs' reagent and the extracted test (Appendix 35) **One broth (15ml) contained 0.5m Wellcome antitoxin[71] whilst the other contained no antitoxin.**

5.3 RESULTS

5.3.1 Investigation of the biochemical reactions of Cl.tetani using commercial systems

i) The API ZYM system (Fig.43a)

With the 10 strains of Cl.tetani tested, 16 of the constitutive enzymes on the strip were consistantly negative and 4 (acid phosphatase, alkaline phosphatase, naphthol-AS-BI-phosphohydrolase and esterase lipase (C8)) were weakly positive in all cases. (Table 20). The FAB cell-free supernatant was negative in all reaction wells.

ii) The API 20 STREP system (Fig.43a)

Nineteen of the 20 biochemical tests were negative with all 10 strains and one (alkaline phosphatase) was positive in all cases (Table 21). The FAB supernatant produced no positive results.

iii) The API Anaerobic enzyme system (Fig.43a)

55 of the 60 tests were negative in all cases, and 5 were positive with all 10 strains. These were alkaline phosphatase, acid phosphatase, esterase C4, naphthol-AS-BI-phosphate and tetrathionate reductase. (Tables 22,23 and 24). The FAB supernatant produced no positive results.

iv) The Sensititre AP60 system (Fig.43b)

With the 10 strains tested, 22 of the 24 tests were totally negative. One test (H_2S production) was positive with all strains and one test (gelatin liquefaction) was positive with 7 out of 10 strains (Table 25). The FAB supernatant produced no positive results.

v) The Abbott BID system (Fig.43b)

With the 10 strains tested, 20 of the 21 tests were negative in all cases. One test (polymyxin B resistance) was positive in all cases (Table 26).

vi) The Anaerobe-Tek system (Fig.43b)

With the 10 strains tested, 11 of the 18 tests were negative in all cases. Of the other tests, 5 were positive in all cases (gram positive reaction, a bacillus, H_2S, bile tolerance and DNase production). One test (spore formation) was positive with 8 out of 10 strains and one test (gelatinase production) was positive with 7 out of 10 strains (Table 27).

The FAB supernatant produced no positive results.

vii) Commercial biochemical test plates (Fig. 43b)

With the 10 strains tested, 3 of the tests (glucose, indole and gelatin) were consistantly negative, while one test (H_2S) was positive in all cases (Table 28). FAB supernatant placed in wells in the agar produced no positive results.

FIG.43 Investigation of the biochemical reactions of Cl.tetani using commercial systems

i) API ZYM system

ii) API 20 STREP system

iii) API Anaerobic system

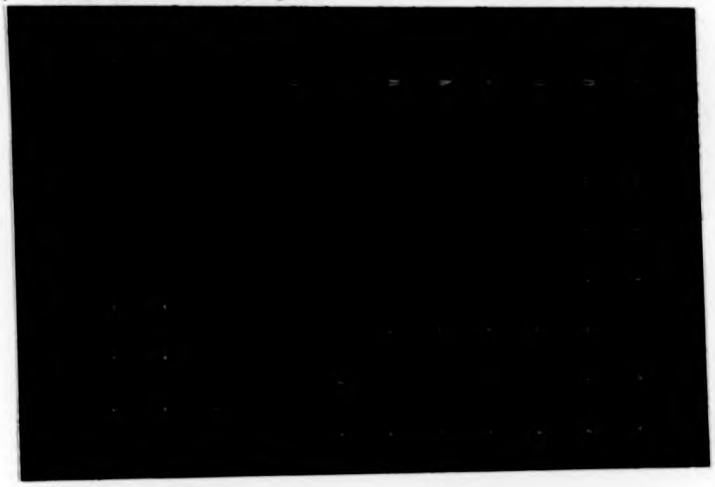
(a)

(b)

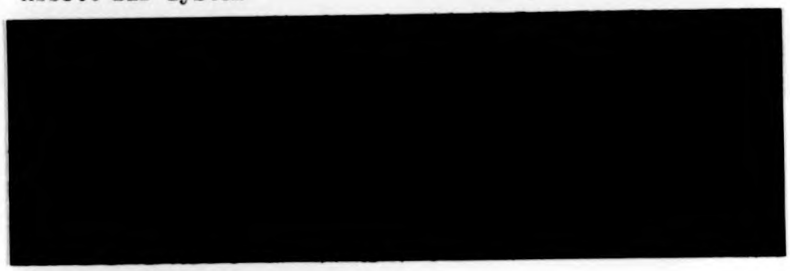
(c)

FIG.43 (continued)

iv) Sensititre AP 60 system



v) Abbott BID system



vi) Anaerobe Tek system

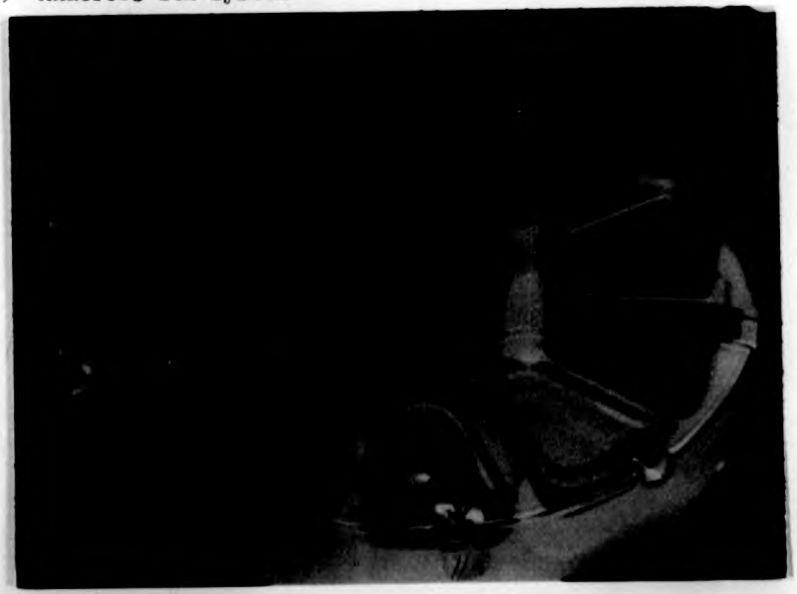
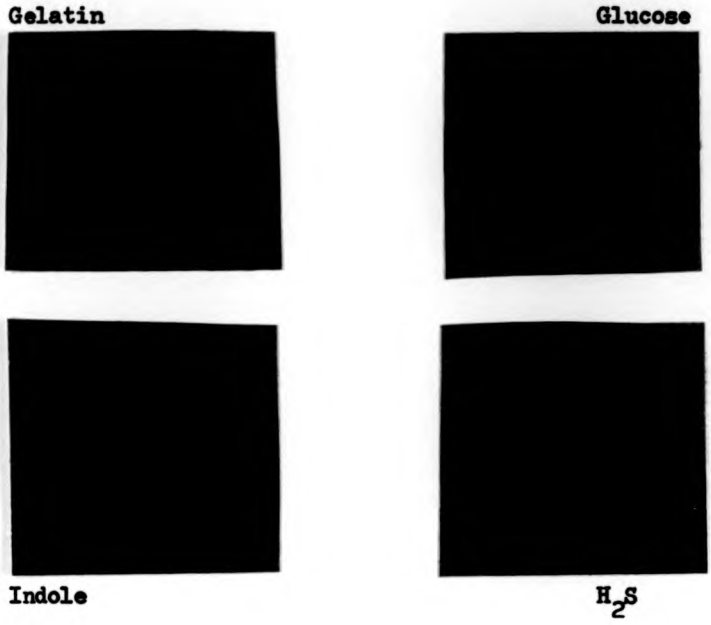


FIG.43 (continued)

vii) Commercial biochemical test plates



Note: No reaction on Gelatin, Glucose or indole plates.

Trace reactions only on H_2S plate.

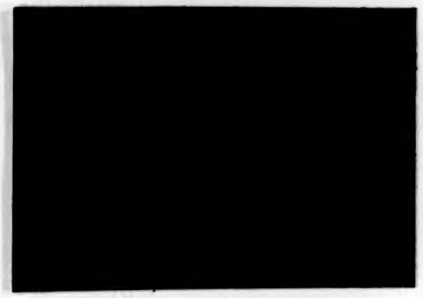
5.3.2 Investigation of the biochemical reactions of Cl.tetani using non-commercial methods

With all 27 strains tested, 15 of the 25 plate tests proved consistantly negative. These tests were collagenase, albumin hydrolysis, chitin hydrolysis, chondroitinase, esterase, arbutin hyrolysis, elastase, mucinase, casein hydrolysate digestion or precipitation, milk digestion or precipitation, desulfoviridin production, nicotinamide adenine dinucleotide glycohydrolase, hyaluronidase, indole production and tetrazolium reduction (Table 29).

All 27 strains showed positive reactions in 4 plate tests. These were lipolysis, glutamic acid decarboxylase, H_2S production and phosphatase activity (Fig.44). In all cases there were variations in the strength of reactions between different strains which seemed independent of the amount of growth produced. 26 of the 27 strains were positive in a further 4 tests, DNase, RNase, Casein hydrolysis and precipitation and fibrin hydrolysis. In all cases the strain giving the negative reaction was the same one(NCTC 947). 25 of the 27 strains showed fluorescence on MacConkey, the negative strains being CN1344 and L109, and 23 of the 27 showed gelatinase activity, the negative strains being NCTC 279, NCTC 9569, NCTC 9574 and NCTC 947.

In the tests using bottled media, none of the 27 strains produced digestion of meat, but all produced slight reddening after 7 days incubation (Table 30). All strains produced visible digestion of the milk medium but no acid production. All strains produced ammonia in FAB broth and all produced gas in Columbia agar deeps (Fig. 45b).

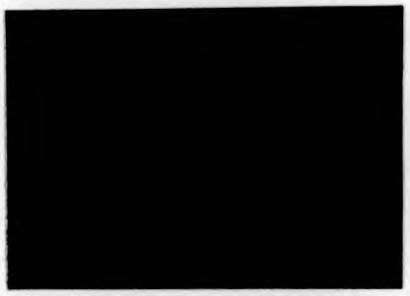
FIG.44 Investigation of the biochemical reactions of Cl.tetani using non-commercial methods



a) lipolytic activity

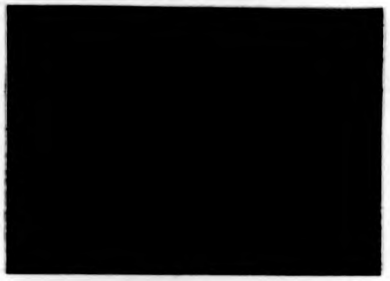
b) DNase activity

c) RNase activity



d) Casein digestion and precipitin

e) Glutamic acid decarboxylase



f) H_2S production

g) phosphatase activity

h) fluoresence on MacConkey (actually yellow)

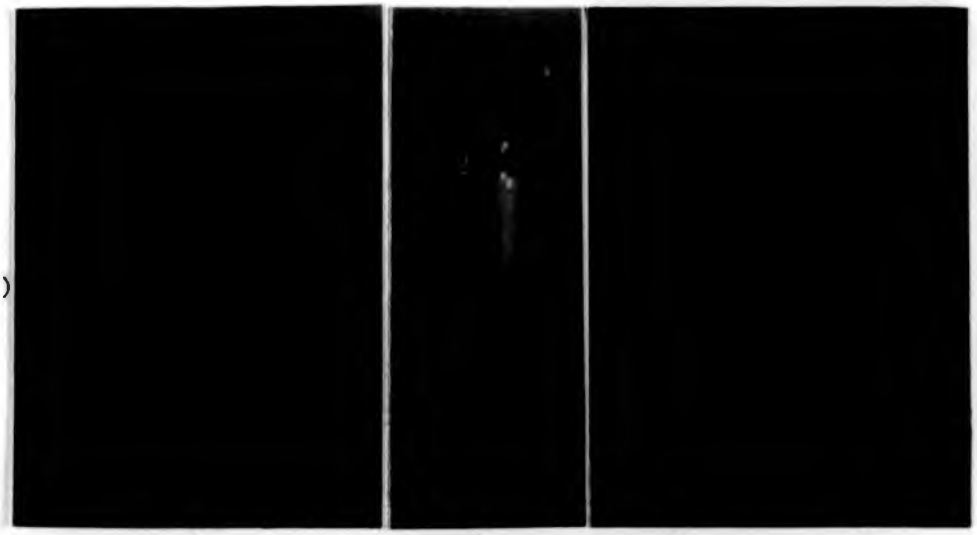
FIG.45 Investigation of the biochemical reactions of Cl.tetani using non-commercial methods

a)

Gelatinase production

Fibrinolysin production

b)



1) Ammonia production from peptone

2) Reddening of cooked meat

3) gas production in agar deeps (note cracking of agar - arrow)

4) Milk digestion

5.3.3 Investigation of tests giving variable or inconclusive results

i) Milk digestion

Whole milk was not digested by any of the 27 strains of Cl.tetani used in this investigation, but 26 of the 27 strains showed zones of digestion on 2 kinds of powdered milk and 4 kinds of skimmed milk (Table 31) Zones of precipitation were not observed with any of these milk products as substrate. (Fig.46)

ii)indole production

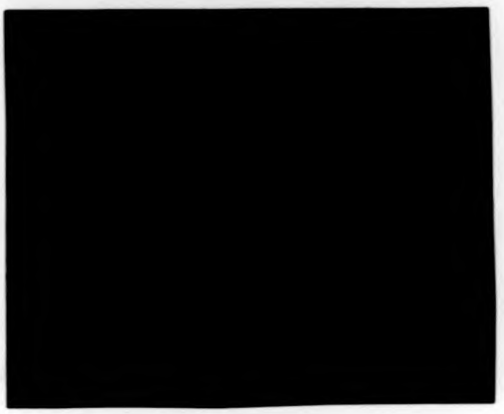
All of the 27 strains of Cl.tetani when grown on Columbia blood agar were indole positive by the spot test using DMACA and aqueous Kovacs' reagents but none were positive using the standard Kovacs' reagent (Table 32) (Fig.47).

The results obtained using FAB and RCM were comparable.

None of the 27 strains were positive by the direct test with any of the 3 reagents with the exception of 3 strains which gave weak positive results with standard Kovacs' reagent, and none were positive by the extracted test using aqueous Kovacs or DMACA. 20 or the 27 strains were positive by the extracted test using standard Kovacs' reagent (Table 32)(Fig.47).

iii) All of the 27 strains tested produced strong positive reactions both in SIM in bottles and lead acetate strips (Fig.48). The black colour of the lead acetate strips faded rapidly in the atmosphere but the colour remained stable in SIM. It was noted that a colourless layer was present at the top of the SIM medium even with the strongest positive strains (Fig.48).

FIG.46 Testing of Cl.tetani for milk hydrolysis using skim milk

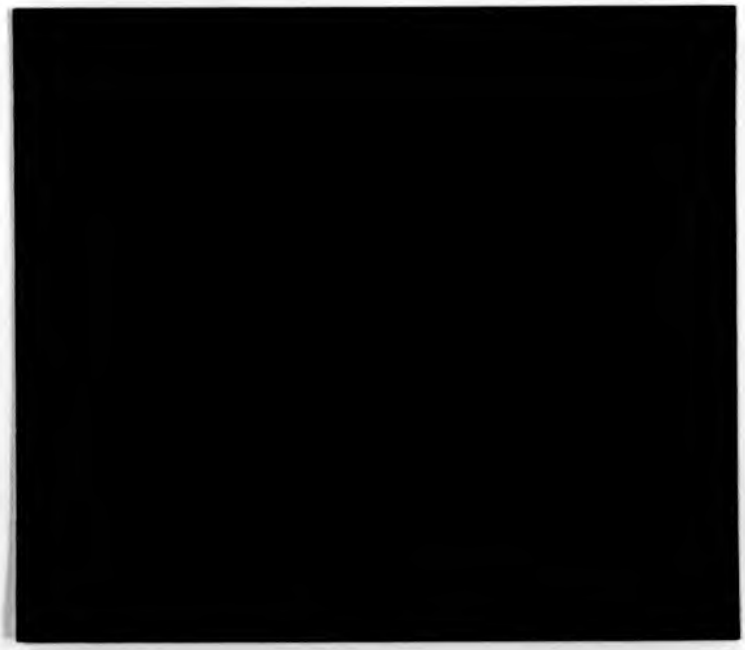


Note: Zones of digestion but no precipitation

FIG.47 Indole testing of Cl.tetani using 3 different reagents and 3 different test protocols

a) 1 Kovacs' reagent - extracted
2 Aqueous Kovacs' - extracted
3 DMACA - extracted
4 Kovacs' reagent - direct
5 Aqueous Kovacs' - direct
6 DMACA - direct

Note the pink/red colour at the interface in tube 1 denoting a positive reaction. The other tubes are negative.



b) Spot tests of 10 Cl.tetani strains.

Clockwise from top: 1) Aqueous Kovacs' 2) DMACA 3) Kovacs' reagent

Note blue colour with DMACA and pink colour with Aqueous Kovacs' denoting positive reactions. The pale straw colour obtained with Kovacs' reacent was considered to be negative.

FIG.48 H_2S testing of Cl.tetani using SIM agar and lead acetate strips

1 2 3

a) SIM agar

1, strong positive 2, moderate positive 3, negative control

Note the depth of colour reaction and the colourless layer at the top of the medium

b) lead acetate strips

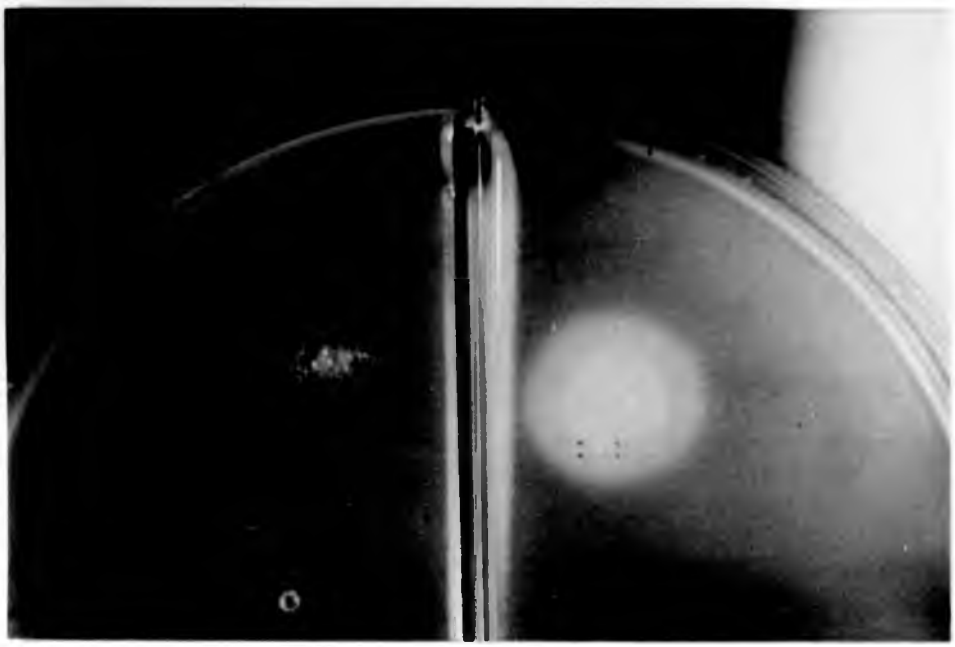
Note blackened strip in top of bottle

5.3.4 Investigations of the effect of tetanus antitoxin on the biochemical reactions of Cl.tetani

8 of the 12 tests studied showed no inhibition by tetanus antitoxin. These were: phosphatase, lipase, DNase, RNase, fluorescence on MacConkey, H_2S and glutamic acid decarboxylase.

The other 4 tests (casein hydrolysis and precipitation, fibrinolysis, indole production and skim milk hyrolysis and precipitation) were inhibited by antitoxin. Growth still occurred on the antitoxin containing side of the plate but the biochemical reactions on that side of the plate were completely neutralised. (Fig.49).

FIG.49 Inhibition of casein hydrolysis and precipitation by tetanus antitoxin



a b

a) Casein agar containing tetanus antitoxin

b) Casein agar without antitoxin

Note: Zone of precipitation around growth which is inhibited by antitoxin. A faint zone of hydrolysis may be seen around the zone of precipitation but this is better visualised after addition of 1N HCl.

5.4 DISCUSSION

The findings in this study would tend to agree with established beliefs that Cl.tetani is relatively biochemically inactive. Thus, out of 120 different tests investigated here, only 25 proved positive, and many of these were related. For instance, the positive alkaline phosphatase, acid phosphatase, Naphthol-AS-B1-phosphohydrolase and the plate phosphatase test are probably due to either one enzyme, or two very closely related ones. It has been shown that membrane extracts of Poa pratensis seeds contain 3 acid phosphatase isoenzymes[186] and that Entamoeba histolytica and Entaboeba coli cells contain several different isoenzymes for malate dehydrogenase, hexokinase, phosphoglucomutase and glucosephosphate isomerase,[187] therefore it is possible that both the phosphatase enzymes and the C4 esterase, the C8 esterase and glycerol tributyrate lipolytic enzymes may have more than one isoenzyme present. Unfortunately it was not feasible to test this hypothesis in this study. Although generally regarded as not producing a lipase on egg-yolk agar[12,150,155] Cl.tetani has been reported to show occasional weak lipolysis on egg yolk[151] and it is suggested that the C_8 esterase/glycerol tributyrate lipase enzyme or enzymes may be responsible. It seems likely that egg yolk is not the best substrate for this enzyme and therefore only when present in large quantities or under ideal conditions would it have any visible effect. Thus the occasional weak reaction reported[11,151].

The enzyme hydrolysing casein is almost certainly the same one that hydrolyses skim milk and causes digestion of milk in bottles, and either this enzyme or the esterase/lipase enzyme may be responsible for the fibrinolytic activity also seen with all strains of Cl.tetani tested. The fact that fibrinolytic activity was neutralised by antitoxin, as was milk hydrolysis and casein hydrolysis but not esterase/lipase activity suggests that if the fibrinolytic enzyme is identical to or related to either, then it is probably the milk and casein hydrolysis

enzyme. The rennin-like enzyme responsible for precipitation of casein[12,154] is also likely to be closely related to this enzyme.

The RNase and DNase enzymes may also be related but it was not possible to investigate this in this study.

The production of ammonia from peptone and the gas produced in agar deeps are almost certainly the same reaction, and glutamic acid decarboxylase, whilst not producing NH_3 directly in itself[165,15] an important step in the chain which eventually does so [11].

It is also possible that the reddening of the meat in RCM cultures, due to reduction, and the reduction of tetrathionate are brought about by the same, or closely related mechanisms.

Several of the other tests investigated are not strictly biochemical tests at all. For instance, the gram reaction, shape of bacterial cell and spore production utilised by the Anaerobe-Tek kit and the Polymyxin B resistance in the Abbott BID system.

In the final examination therefore, Cl.tetani possesses fewer positive reactions than might have appeared earlier when all the idential or related enzymic activities are grouped together. These total 13 and are comprised by: The phosphatase group; the esterase/lipase group; the milk/casein hydrolysis group; the DNA/RNA hydrolysis group; the ammonia producing group; the reduction group; H_2S; gelatinase; indole production; bile tolerance; fluorescence on MacConkey medium; fibrin hydrolysis and casein precipitation (Table 34).

In a study of 71 strains of Cl.tetani, Willis and Williams[154] reported that none produced a phosphatase. The test used was similar to that used here but the fact that this study used only 10 strains placed a question mark by the results. However, a total of 7 other phosphatase tests on differentcommercial test strips were all universally positive, and, as it is unlikely that the 10 strains investigated here were a random sample of genuinely phosphatase

positive strains in a species that is normally photphatase negative, a more likely explanation is that the method used to test the original 71 strains was less sensitive in some way that the ones used here and that had they been tested by these methods, the vast majority would have proved positive.

Esterase/lipase activity is not usually associated with Cl.tetani, although the organism does possess such activity as shown by this study, where 3 different test systems were positive. However, some discrepancies exist. Esterase lipase (C8) was positive by API ZYM (Table 20) and esterase (C4) was negative, wherease esterase (C4) was positive by the API anaerobic enzyme system. The API ZYM system has a lower detection limit for this test of 5 nanomoles[162] and it is unlikely that this cannot detect esterase where the API anaerobic system can. Tests for lipase (C10) and lipase (C14) were negative as were esterase tests using Tween 20,40,60 and 80.

The substrates for esterase (C4) and esterase lipase (C8) are 2-naphthyl butyrate and 2-naphthyl caprylate respectively[162] and the substrate for the plate lipolysis test is glycerol tributyrate. All three appear to be closely related and the findings of this study indicate that the esterase/lipase possessed by Cl.tetani has a fairly narrow range of useable substrates, outside of which no reaction occurs except occasionally perhaps when production of large amounts of enzyme and suitable conditions allow one of the sub-optimal substrates, like egg-yolk to be utilised.

The findings in this study that, although purified milk protein, in the form of casein, could be readily hydrolysed and precipitated in the way described by some workers[154] whole milk could not be, may explain some of the discrepant results observed previously. Whenever a purified or semi-purified milk preparation was used, the hydrolysis became evident. This was enhanced greatly by the addition of hydrochloric acid to precipitate undigested milk, a technique not used

by earlier workers. The skimmed and dried milk preparations used here had all or most of the fat removed during manufacture, and it is suggested that it is the presence of fat that inhibits the digestion of milk by somehow interfering with the enzyme responsible. An early theory that the milk digestion might be due to the esterase/lipase which preferentially utilised the fat as a substrate when it was present and so left no enzyme free to digest the milk was discarded when it was shown that the esterase/lipase was not neutralised by antitoxin whilst the milk digesting enzyme was neutralised, indicating that they were not the same. The protective effect of the fat appears therefore to be of a non-specific nature and the use of different milk preparations with different fat contents may explain why some workers feel that Cl.tetani can not hydrolyse or digest milk[11]. The precipitation of casein seen in this study with casein has been described by other workers using whole milk and assigned to a rennin-like enzyme[154]. Precipitation was not, however, seen here with either whole milk plates or skimmed milk plates. Two possibilities exist to explain this. Firstly, experimental methods may have differed between the two studies causing visible discrepancies in results, or secondly and more likely, the treatment of the whole milk used may have altered significantly in the 20 years between the studies and the composition may now be different, with the component responsible for the precipitation either removed, or altered to the degree that precipitation can no longer occur. The same may be true for skimmed milk preparations.

The digestion of casein and skimmed milk seems likely to be due to a proteinase enzyme although this was not investigated further here. Other workers have suggested that milk digestion would be due to proteinase activity[11,154] and also that gelatin liquefaction would be due to proteinases[188], although the two are unlikely to be the same enzyme as milk hydrolysis is neutralised by tetanus antitoxin whereas gelatin liquefaction is not.

The fibrinolytic enzyme is stated to be a kinase rather than a proteinase[12,154], but even so, it is certainly incorrect to describe Cl.tetani as non-proteolytic when at least 3 different tests (gelatin, milk and fibrin hydrolysis) can be shown to be positive for the great majority of strains. Previous studies have suggested that Cl.tetani is non-fibrinolytic[189] or conversely, that just over half the strains tested may produce fibrinolysin[154]. The suggested reason for the discrepancy was that the original method - a clot lysis method - was less sensitive than the subsequent plate method. In this study 90% of the strains proved to be positive, and it is suggested that perhpas the use of more purified thrombin and fibrinogen available today, or the subsequent staining step with Naphthalene black to enhance weak reactions, a technique not used before, may have increased the sensitivity of the test again thereby picking up even more positive reactions

Gelatin liquefaction was tested by 4 different methods and although all strains were negative by one method, therewas complete concordance with the other 3 techniques, showing 7 of the 10 strains to produce gelatinase. It has been stated that "there is little doubt that the great majority of strains can hydrolyse gelatin when tested under favourable conditions"[11] and the findings here would tend to support that statement and also the further observation that the basal medium is of considerable importance in the test.[11] Thus one medium used here showed no reaction, and in other studies, only 17/71 were positive using charcoal gelatin and 16/71 were positive using nutrient gelatin while 71/71 were positive using a gelatin plate test.[11]

DNase activity has been described previously for some strains of Cl.tetani with one study suggesting that more than half the strains tested produced DNase,[154] however RNase does not appear to have been similarly investigated although the two activies are closely related if not identical. Both RNase and DNase are usually specific

phosphodiesterases which hydrolyse certain phosphoric ester linkages of either RNA or DNA or both[190]. In this study all strains tested were DNase positive by 2 different techniques and RNase positive by one technique, and neither reaction was neutralised by antitoxin. It is possible that the same enzyme, a non-specific nuclease, could be responsible for both reactions. The discrepancy between the number of positive DNase results obtained in the earlier study and here (40/71 and 10/10 respectively) may again be explained by increased sensitivity of the test in the latter investigation, using perhaps a more purified DNA preparation that the sodium salt of thymus gland DNA previously used,[169] although this can not be absolutely certain.

The production of ammonia and gas in growing cultures has been noted before [6,151] and is not entirely unexpected, for an organism that is entirely non-saccharolytic must have some way of obtaining energy, and the breakdown of peptones and subsequent amino-acid metabolism seems a likely alternative. Glutamic acid dehydrogenase probably plays a major part in this pathway as the addition of extra glutamic acid to cultures of Cl.tetani resulted in increased growth over the first 24 hours[191].

The reddening of the meat particles in RCM broth cultures of Cl.tetani is also not really unexpected either, as reducing agents in the cooked meat itself (particularly glutathione) can produce a faint reddish colour on standing due to the continuing reduction of haemin[12]. However, the reddish colour produced by the growing Cl.tetani was deeper and more extensive than in uninoculated bottles and was probably due to further reduction caused by the growing organism lowering the Eh substantially further than the intrinsic reducing substances could. There was no evidence of true digestion or blackening of the meat as has been reported previously[150,151]. The mechanism of the tetrathionate reductase test employed in the API anaerobic enzyme system is not understood, but it may be a simple chemical colour change occurring on

reduction of tetrathionate in the same way that resazurin changes colour.[168]

All strains tested produced hydrogen sulphide using 6 different techniques, although the plate techniques gave weak reactions and faded quickly on exposure to air, as did the lead acetate test. It is suggested that, on exposure to air the H_2S quickly diffuses out and the black colour therefore disappears. It is possible therefore that cultures incubated in a large volume of atmosphere (albeit anaerobic) can be affected in a similar manner, with the H_2S diffusing out as soon as it is produced. This effect could also be caused if small amounts of oxygen are present in the medium, or if the cultures were incubated along with a detoxifier (such as activated charcoal) which would remove H_2S from the atmosphere thereby setting up a diffusion gradient and encouraging more H_2S to diffuse out of the medium. The finding that large stable amounts of H_2S are produced in deep agar cultures with a thin colourless layer at the interface between medium and atmosphere tends to support this theory.

The apparent aptitude for H_2S to diffuse out of test media and produce false-negative results is possibly the reason that many workers claim Cl.tetani to be either variable in its H_2S production[153] or totally negative [6,12,155], but this study would suggest that the great majority of Cl.tetani strains are H_2S positive.

Indole was tested here initially by 5 different techniques and all gave negative results with the 10 strains tested. It was not until further investigations revealed that the spot indole test using the DMACA reagent was the optimum technique that all 10 strains could be shown to be indole positive. The DMACA reagent has been shown to be more sensitive than Kovacs' reagent with Enterobacteriaceae[184] and with anaerobes[192] and thus it seems likely that Cl.tetani strains produce only small quantities of indole that can only be detected using this reagent, or an extraction technique and Kovacs' reagent. It has

been shown however that Kovacs' reaction only detects around 74% of indole positive anaerobic bacteria[192], and this correlates well with the finding in this study that only 20/27 strains were positive with Kovacs' reagent even after xylene extraction. It is not known why the DMACA reagent was negative by both direct and extracted tests when it was positive by the spot test. The false-negative and variable reactions observed by the standard indole techniques using Kovacs' reagent are almost certainly the reason some workers claim most but not all strains of Cl.tetani are indole positive[12], or that indole production is variable[153], when in fact the great majority of, if not all strains are almost certainly indole positive if tested correctly.

Growth on bile has been stated as negative for most strains[151], whereas all 10 strains grew on the bile medium used here. It is possible that the amounts of bile used were different, however both methods quote 2% (w/v) oxgall as the quantity used [151,164], so unless the composition of the oxgalls used were different this finding is not readily explained.

The yellow fluorescence of Cl.tetani strains on MacConkey agar has been noted before (albeit as green fluorescence)6 and is due to reduction of the neutral red in the medium, a characteristic manifested by other clostridia[193]. All the strains tested here were positive to varying degrees, with the exception of two.

Some of the reports on biochemical activities of Cl.tetani suggest that some activity, especially proteolysis, may be observed following incubation for extended periods, often 1 to 3 weeks. Thus blackening of the meat[6,154], gelatin liquefaction[14,154] and digestion of coagulated serum[152] have all been described following extended indubation but there is no clear indication that any of these are due to digestion. Indeed, as the cultures cease active growth and begin to autolyse in 3 - 5 days[191], any reactions that have not taken place by then may be considered as simple degenerative sequelae following

release of autolytic enzymes or other cellular chemicals into the medium. Most of the positive test results obtained in this study gave clear-cut results following 48 hours incubation and so can unequivocally be regarded as genuine reactions of the growing organism. It is possible of couse, that the anaerobic conditions obtained in this study were much better than those obtainable in earlier studies so that growth was proportionally better, and that slower growing organisms in the earlier studies did require much longer to produce positive reactions. This is quite likely, as this study used anaerobic cabinets to produce the required degree of anaerobiosis, and these may be assumed to give better anaerobiosis than methods available 20 years and more ago.

It is worthy of note that, although it has been shown that Cl.tetani cells contain considerable amounts of an enzyme which could hydrolyse histidine peptides when grown on a toxigenic medium[114,194], none of the 3 tests that detect breakdown of histidine peptides in this study were positive, even though one detected glutamyl-histidine arylamidase and glutamyl-histidine is one of the peptides known to be hydrolysed by this enzyme[114]. There are two possible explanation. Firstly, the Columbia blood agar that the Cl.tetani cells in this study were grown on does not support toxin production and hence would not support histidine peptidase production. This was not investigated, but in view of the substantial effect different media can have on toxin production seems quite likely. Secondly, as the enzymes detected by the commercial strips in this study are thought to be present in the cytoplasmic membrane[162], it is possible that enzymes residing at deeper sites in the cell would not be detected. If the histidine peptidase enzyme was situated deep in the cell it might give negative reactions by the tests used here. This is unlikely however, as 'considerable amounts' of enzyme are produced[194] and it would be expected that some would escape and be detected by the test system.

5.5 CONCLUSION

Of 120 biochemical tests investigated, all, or most strains of Cl.tetani gave positive results in 25. However, several of these appear to be related and may be grouped together, and several are not strictly biochemical reactions. Four were classed as not biochemical reactions (gram reaction, shape of bacterial cell, spore production and Polymyxin B resistance), and there were 13 positive true biochemical reactions or groups of related reactions. These were: The phosphatase group (alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and the phosphatase test); The esterase/lipase group (esterase C4, esterase lipase C8 and glycerol tributyrate hydrolysis); The milk hydrolysis group (skim milk hydrolysis, casein hydrolysis, milk digestion); The DNA/RNA hydrolysis group; The ammonia production group (ammonia production from peptone, gas production and glutamic acid decarboxylase); The reduction group (reddening of meat particles and tetrathionate reductase); H_2S production; gelatinase production; indole production; bile tolerance; fluorescence on MacConkey agar; fibrin hydrolysis and casein precipitation.

It has been shown that Cl.tetani is more reactive than has previously been thought, and that both the basal medium and the test protocol used may have a significant effect on the test result.

Of the positive reactions, only casein and milk hydrolysis and precipitation, fibrinolysis and the indole test were antigenic and could be neutralised by tetanus antitoxin.

It is clear that, with evidence of several proteolytic enzymes (casein and milk digestion, fibrinolysis and gelatinase production) Cl.tetani can no longer be regarded as entirely non-proteolytic. There was no evidence of any saccharolytic activity in this study.

CHAPTER 6

THE METABOLISM OF CL.TETANI

6.1 SURVEY OF THE LITERATURE

It has been established that Cl.tetani strains can produce gas or ammonia from peptones during growth,[6,151] and this is generally held to be due to breakdown of amino acids[150] as part of the organism's metabolism. As the organism is non-saccharolytic it is probable that it obtains most of its energy from the breakdown of amino acids, although the actual amino acids fermented seem to be open to question. It has been suggested that Cl.tetani strains do not attack glutamic acid,[195] although this has been disputed by other workers[11,196,197]

Other amino acids utilised include aspartic acid and serine[11,196] although some studies have suggested that histidine is also utilised and that threonine, methionine and tyrosine utilisation varies from strain to strain[197] or that valine, leucine, isoleucine, serine, threonine, methionine, phenylalanine and tryptophan were essential or stimulatory to growth[113].

The fermentation products of glutamic acid are principally carbon dioxide, ammonia and a mixture of acetic and butyric acids.[11,196,197] The small amount of propionic acid that may be produced was thought to be related to the utilisation of threonine.[197]

The methods of determination of amino acid utilisation have differed in the different studies, and this may be one cause of the discrepancies observed. Thus, while the earlier studies used visible turbidity and gas bubbles as evidence of amino acid utilisation[195,198] later studies used manometric methods to measure evolved carbon dioxide and ammonia,[196,199] and these were followed by measurement of growth levels by nephelometry compared to controls and low voltage electrophoresis combined with descending chromatography followed by mass spectrometry to identify amino acids present.[197] The detection of ammonia nitrogen by steam distillation has also been used[198].

Many of these techniques are laborious and difficult to perform, giving results of unknown accuracy, therefore it seemed desirable to reinvestigate the situation using newer and simpler methods such as the deamination technique of Nakamura et al[200] which detects the ammonia produced during amino acid breakdown.

In addition, some studies have used relatively few amino acids,[196] or have not stated whether the amino acids were in D- or L- form,[197] and it seemed possible that a study of all L- and D- amino acids available would clarify the situation greatly.

The metabolic products of growth in peptone-yeast-glucose broth are given as acetic acid, butyric acid and a small amount of propionic acid,[151] similar to the fermentation products of glutamic acid and threonine,[197] but the first study does not state the number of strains investigated and the second investigated only 8 strains. A study of the volatile fatty acids produced by an increased number of Cl.tetani strains would show if all strains produced the same metabolic products and in similar quantities.

The essential vitamin requirements for toxin production have been exhaustively studied,[110,113] and during these investigations some conclusions were reached indicating that thiamin, calcium pantothenate nicotinic acid and uracil were essential togrowth, whilst riboflavin, pyridoxine and adenine were helpful to growth.[110] However, the authors themselves state that their 'procedure clearly is open to criticism and represents merely a convenient and workable compromise' referring to their removal of H_2S formed during growth by acid and heat treatment followed by measuring of turbidity by comparison with standard barium sulphate suspensions. Also, the use of the Harvard strain of Cl.tetani which may have altered growth requirements to normal[113] could have introduced differences. Clearly a reappraisal of the situation using direct nephelometric readings in media not showing significant H_2S

formation would be a valuable adjunct to current knowledge, and the investigation of other, less adapted strains, might also be useful.

The following investigations were therefore undertaken.

1) **A study of the growth requirements of Cl.tetani.**

2) **A study of the amino acids utilised by Cl.tetani.**

3) **A study of the volatile and non-volatile fatty acids produced during metabolism.**

6.2 EXPERIMENTAL TECHNIQUES

6.2.1 Investigation of growth requirements

Cl.tetani serotype III (NCTC 539) was used in this part of the study. This strain is a reasonably rapid grower in fluid media without being particularly outstanding. Serotype I (NCTC 279) is usually quoted as the working type for this species,[167] but did not grow in fluid media very well. Occasionally the strain refused to grow at all and it often demonstrated variable amounts of growth therefore the serotype III strain, which gave more reproducible growth was preferred.

A growth medium based on that described by Latham et al for toxin production,[95] and which should contain all necessary growth requirements was devised (Appendix 36). Although the original medium contained 0.8% glucose, and it has been suggested that glucose was stimulatory to growth of Cl.tetani, it tended to make the medium very dark following growth and was therefore not utilised here. The growth studies in this section all followed the same general protocol. The complete growth medium was made up along with the same medium but with one or more constituents absent. These were then aseptically inoculated with 0.5ml of an 18 hour FAB broth culture of NCTC 539 and incubated for 48 hours at 37°C.

10ml of each culture was removed, centrifuged and the supernatant used as a blank while the OD600 of the uncentrifuged cultures was read on an EEL spectrophotometer.[91] This procedure removed any falsely high readings due to changes in the colour of the media during growth that were not due to bacterial cells.

The OD600 of the complete medium culture was taken to be 100% and the degree of growth of the test cultures compared to the complete medium was calculated as follows:

$$\frac{TR \times 100}{CR}$$

where TR is the OD_{600} of the test culture and CR is the OD_{600} of the

complete medium culture.

This procedure allowed the direct comparison of different batches of media as slight variations in the amount of growth due to physical conditions was compensated for.

All observations were repeated a minimum of two times on separate occasions and the average of the results taken. Where the two observations showed discrepancies the experiments were repeated until the true readings were evident.

6.2.1.i) Casein hydrolysate and yeast extract

Hydrolysed casein is the basis for most media used in growth studies of clostridia[95,110,111,113,114,191,197,201], therefore it seemed logical to investigate the requirements for this compound first. Also many workers use yeast extract as a source of vitamins and other nutrients[197,198] and the effects of this on growth was also studied. Cl.tetani was grown in the following media:

1. The complete medium (with 3% Casein hydrolysate and 1% yeast extract).
2. The complete medium but with only 1% casein hydrolysate.
3. The complete medium but with only 0.1% casein hydrolysate.
4. The complete medium minus casein hydrolysate.
5. The complete medium minus the 8 vitamins.
6. The complete medium minus the 8 vitamins but with only 0.1% yeast extract
7. The complete medium minus the 8 vitamins and minus yeast extract.
8. The complete medium minus the 8 vitamins, minus yeast extract and minus casein hydrolysate.

6.2.1.ii) Other chemicals

The effects on growth of removing the other chemicals one by one from the complete medium was studied in a similar way by

growing Cl.tetani NCTC 539 in the following media:

1. The complete medium
2. The complete medium minus $MgSO_4$
3. The complete medium minus $FeCl_3$
4. The complete medium minus cysteine hydrochloride
5. The complete medium minus NaCl
6. The complete medium minus the 8 vitamins

These investigations were followed up to observe the effects of removing two or more of these chemicals simultaneously using the following media:

7. The complete medium
8. The complete medium minus $MgSO_4$ and $FeCl_3$
9. The complete medium minus NaCl and cysteine hydrochloride
10. The complete medium minus NaCl, cysteine HCl and yeast extract
11. The complete medium minus NaCl, cysteine HCl, $MgSO_4$ and $FeCl_3$
12. The complete medium minus NaCl, cysteine HCl, yeast extract, $MgSO_4$ and $FeCl_3$
13. The complete medium minus NaCl, yeast extract, $MgSO_4$ and $FeCl_3$
14. The complete medium minus NaCl, $MgSO_4$ and $FeCl_3$
15. The complete medium minus 8 vitamins, NaCl, yeast extract, cysteine HCl, $MgSO_4$ and $FeCl_3$

6.2.1.
iii) Vitamins

The effects on growth of removing each of the 8 vitamins individually was studied using the following media:

1. The complete medium with no yeast extract
2. The complete medium with no yeast extract minus uracil

3. The complete medium with no yeast extract minus thiamine

4. The complete medium with no yeast extract minus riboflavin

5. The complete medium with no yeast extract minus pyridoxine

6. The complete medium with no yeast extract minus nicotinic acid

7. The complete medium with no yeast extract minus calcium pantothenate

8. The complete medium with no yeast extract minus biotin

9. The complete medium with no yeast extract minus vitamin B_{12}

The effects on growth of removing all 8 vitamins and adding each one individually to the complete medium was studied using the following media:

10. The complete medium with no yeast extract

11. The complete medium with no yeast extract or vitamins plus uracil

12. The complete medium with no yeast extract or vitamins plus thiamine

13. The complete medium with no yeast extract or vitamins plus riboflavin

14. The complete medium with no yeast extract or vitamins plus pyridoxine

15. The complete medium with no yeast extract or vitamins plus nicotinic acid

16. The complete medium with no yeast extract or vitamins plus calcium pantothenate

17. The complete medium with no yeast extract or vitamins plus biotin

18. The complete medium with no yeast extract or vitamins plus vitamin B_{12}

6.2.2. Amino acid utilisation

6.2.2.i Amino acids necessary for growth

This investigation was similar to the previous investigation

of growth requirements in that 0.5ml amounts of an 18 hour FAB culture of Cl.tetani NCTC 539 were used to inoculate 50 ml amounts of complete medium containing the 21 available individual L-amino acids. The following media were used:

1. The complete medium
2. The complete medium minus casein hydrolysate but plus all 21 amino acids
3. The complete medium minus casein hydrolysate but plus L-phenylalanine
4. The complete medium minus casein hydrolysate but plus L-lysine
5. The complete medium minus casein hydrolysate but plus hydroxy-L-proline
6. The complete medium minus casein hydrolysate but plus L-alanine
7. The complete medium minus casein hydrolysate but plus L-aspartic acid
8. The complete medium minus casein hydrolysate but plus L-valine
9. The complete medium minus casein hydrolysate but plus L-glutamine
10. The complete medium minus casein hydrolysate but plus L-tryptophan
11. The complete medium minus casein hydrolysate but plus L-methionine
12. The complete medium minus casein hydrolysate but plus glycine
13. The complete medium minus casein hydrolysate but plus L-isoleucine
14. The complete medium minus casein hydrolysate but plus L-leucine

15. The complete medium minus casein hydrolysate but plus L-cysteine
16. The complete medium minus casein hydrolysate but plus L-histidine
17. The complete medium minus casein hydrolysate but plus L-arginine
18. The complete medium minus casein hydrolysate but plus L-asparagine
19. The complete medium minus casein hydrolysate but plus L-glutamic acid
20. The complete medium minus casein hydrolysate but plus L-proline
21. The complete medium minus casein hydrolysate but plus L-serine
22. The complete medium minus casein hydrolysate but plus L-threonine
23. The complete medium minus casein hydrolysate but plus L-cystine

All amino acids were made up to 0.1M in sterile distilled water and stored at $4^{o}C$. 2mls of each was added aseptically to each medium before inoculation. All investigations were repeated a minimum of two times on separate occasions and the average of the results taken.

6.2.2.ii Deamination of amino acids

The utilisation of amino acids can be tested directly by detecting the ammonia given off by washed suspensions of organisms incubated with the individual amino acid,[196,200] and this approach was used here in two different ways.

a) Firstly, Cl.tetani NCTC 539 was grown for 48 hours anaerobically at $37^{o}C$ on Columbia blood agar plates, harvested into sterile distilled water with a swab, centrifuged once and resuspended

in distilled water to a turbidity approximately equivalent to a MacFarland no.10 opacity standard.[29] 100 ul of this was pipetted into each of 21 optically identical polystyrene tubes[163] and 100 ul of each amino acid at a concentration of 0.1M in PBS was added to the tubes. These were incubated at 37°C for 4 hours after which 200 ul of Nessler's reagent[69] was added, the tubes shaken and 2ml PBS added. The tubes were centrifuged to remove any precipitate and the absorbance read on an Abbott Quantum II spectrophotometer[163] with a dual beam at wavelengths 492 and 600nm using mode 1.1 on the program module J. The instrument was blanked on distilled water.

b) The deamination test of Nakamura et al[200] is much simpler than that above, and can be reduced to a micromethod to make it even easier to perform. Using this method it is possible to test larger numbers of organisms and this was felt to be necessary in order to establish that NCTC 539 was giving representative results. It was also possible to test a wider variety of amino acids including the commonly available D-forms.

The 21 L-amino acids and 16 D-amino acids (Appendix 37) were made into 0.1M solutions in distilled water and 30 ul of each were pipetted into wells in plastic trays[22] (Fig.50). These were stored at -40°C until required.

The 27 strains of Cl.tetani (Appendices 1 and 2) were grown on Columbia blood agar plates anaerobically at 37°C for 48 hours. The growth from each was harvested into sterile PBS (pH 7.2) to a turbidity equivalent to a No.5 MacFarland opacity standard,[29] and one drop from a pasteur pipette from each strain was placed in each of the 37 amino acid wells. The trays were covered and incubated at 37°C for 4 hours when 1 drop of Nessler's reagent was added to each well. The

development of a deep yellow/orange or dark brown colour within 2 minutes was regarded as a positive deamination test. Nessler's reagent was added to a series of uninoculated amino acid wells as controls and the development of any colour in the test wells not significantly different from the control wells was ignored.

These investigations were repeated a minimum of two times to check reproducibility. When a discrepancy occurred the test was repeated in triplicate until the true result became apparent.

6.2.3 Volatile and non-volatile fatty acid production

Gas-liquid chromatography has been found to be a rapid, convenient and accurate method of identifying and quantitating the volatile fatty acid (VFA) and non-volatile fatty acid (Non-VFA) products of metabolism of anaerobic bacteria[12,151,202] and this technique was used here to determine whether all 27 species of Cl.tetani would produce the same VFAs and Non-VFAs in similar quantities when grown in identical medium.

The 27 strains of Cl.tetani were grown overnight on Columbia blood agar plates and one loopful of bacterial growth from each was transferred to 20 ml of freshly made FAB broth. These were incubated at 37°C for 18 hours. After incubation the cultures were centrifuged and the supernatants extracted with ether (Appendix 38). The extracted supernatants were chromatographed on a Packard model 430 gas liquid chromatograph[203] (Appendix 38).

6.3 RESULTS

6.3.1 Investigation of growth requirements

6.3.1.i Casein hydrolysate and yeast extract

It can be seen from Table 35 that the presence of casein hydrolysate is proportional to the amount of growth produced. No growth was seen when no casein hydrolysate was present, 63.6% growth compared to the complete medium was seen when 0.1% casein hydrolysate was present and 74.9% growth was seen with 1% casein hydrolysate. When there was no yeast extract present, and no vitamins, growth was minimal at only 3.4%. When there was 0.1% yeast extract growth was 40% and when there was 1% yeast extract growth was 109% compared to the growth in the complete medium.

6.3.1.ii Other chemicals

Removing the 8 vitamins, $MgSO_4$ or NaCl from the complete medium individually has little effect on the amount of growth produced (Table 36), however removing cysteine hydrochloride caused the growth level to drop to 56.2% and removing $FeCl_3$ caused it to drop to 12.5% compared to the growth in the complete medium.

Removing the chemicals in various combinations showed that whenever $FeCl_3$ was removed, there was a significant drop in growth level, whatever the combination, and whenever cysteine hydrochloride was removed there was a similar, although not quite so great, drop. When the two chemicals were removed together the growth dropped to an insignificant level, almost no growth at all being measured. When the yeast extract was removed in whatever combination, a further drop in growth level was observed.

6.3.1.iii Vitamins

Removal of each of the 8 vitamins from the complete medium

minus yeast extract led to a 10 - 30% reduction in growth level in the majority of cases. The exceptions were riboflavin, where the growth level remained the same and thiamine where the amount of growth increased. (Table 37)

When the 8 vitamins were all removed and replaced by individual vitamins, 4 (uracil, thiamine, nicotinic acid and vitamin B_{12}) did not support significant growth while riboflavin supported 43.7% growth and pyridoxine, calcium pantothenate and biotin supported high levels of growth (85, 92.3 and 94.8% respectively).

6.3.2 Amino acid utilisation

6.3.2.i Amino acids necessary for growth

When all 21 L-amino acids (Appendix 37) were added to the complete medium in the absence of casein hydrolysate, the growth level obtained was only 25% of that with the complete medium (Table 38). When the individual amino acids were added therefore the growth levels were all very low. The majority showed no growth at all or barely measurable growth and only 7 amino acids showed a growth level above 5% (L-aspartic acid, L-tryptophan, L-methionine, L-asparagine, L-glutamic acid, L-threonine and L-cystine). However, when the growth levels obtained were compared to the growth level obtained with all 21 amino acids, this picture became much clearer, with these 7 amino acids producing comparative growth levels of 22.7, 45.4, 31.8, 22.7, 45.4, 36.3 and 90.9% respectively. Thus, L-cystine was the amino acid most stimulatory to growth followed by L-tryptophan and L-glutamic acid, L-threonine, L-methionine, L-aspartic acid and L-asparagine in order.

6.3.2.ii Deamination of amino acids

a) When the absorbances produced by the 21 amino acids were compared, it was soon evident that there was very little

difference between the results with the exception of L-glutamine which gave a much higher reading than the rest and L-threonine which gave a slightly higher reading than the rest (Table 39).

b) When the 27 strains of Cl.tetani were investigated for their ability to deaminate 37 different amino acids, a general pattern was readily observed. (Table 40). L-aspartic acid, L-glutamine, L-methionine, L-glutamic acid and L-threonine were utilised by every strain while L-cysteine was utilised by 7 strains (although weakly), L-cystine by 6, L-asparagine by 3, L-leucine by 2, L-valine by 1 and L-serine by 1. None of the others showed observable ammonia production. It is worth noting that none of the D-amino acids were deaminated by any strain of Cl.tetani (Fig.50).

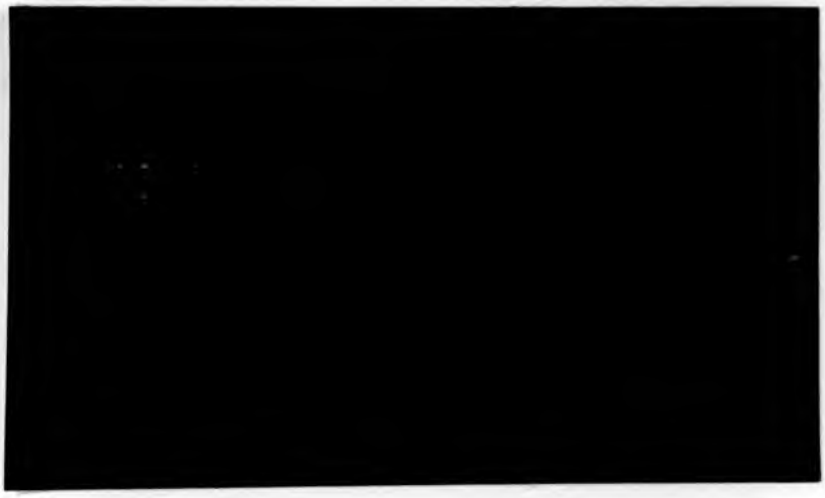
Control (uninoculated) suspensions of amino acids were necessary to avoid false-positive results caused by some of the amino acids producing spontaneous orange colouration. This appeared with L-aspartic acid but the colour produced by the test wells was judged to be deeper by comparison (Fig.50).

6.3.3 Volatile and non-volatile fatty acid production

When the VFAs and Non-VFAs produced by 27 strains of Cl.tetani grown in FAB broth were compared, it was immediately apparent that the majority of strains produced the same fatty acids in very similar quantities. (Table 41)

Acetic, propionic and butyric acids were the only VFAs produced and were produced by all strains and butyric acid was the major product (60-70%) followed usually by acetic (10-25%) and then propionic (6-15%) (Fig. 51). Lactic and succinic acids were the only non-VFAs produced and were produced by all strains with lactic acid always the major product (60-70%).

FIG.50 Deamination of amino acids by different strains of Cl.tetani



Top tray: L-amino acids (Appendix 37) 1 - 21 and control

Upper row LQ2

Middle row LQ730

Bottom row Control (uninoculated)

Lower tray: D-amino acids (Appendix 37) 1-16

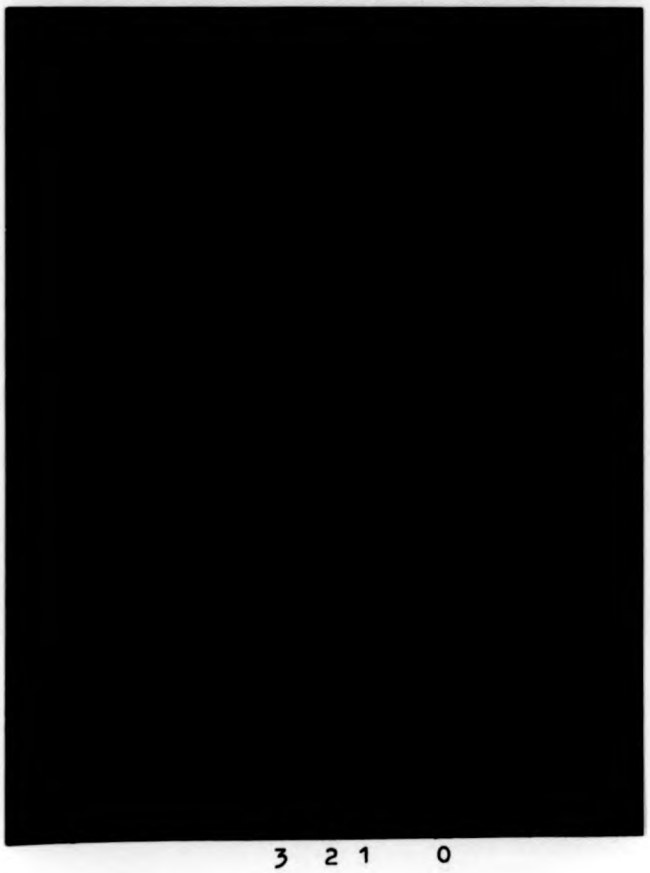
Upper row LQ2

Middle row LQ730

Bottom row Control (uninoculated

Note: the strong colour produced by uninoculated L-aspartic acid (well 5), L-glutamine (well 7) and L-glutamic acid (well 17),after adding Nessler's reagent. However, the colours produced in the inoculated wells were judged to be slightly stronger. L-methionine (well 9), L-threonine (well 20) are also utilised. LQ2 utilises L-valine (well 6) as well. **(arrow)**

FIG.51 Gas-liquid chromatograph trace of volatile fatty acids produced by Cl.tetani



0 = origin

1 = Acetic acid peak

2 = propionic acid peak

3 = butyric acid peak

6.4 DISCUSSION

Casein hydrolysate is a rich source of nutrients, mainly amino acids, peptones and polypeptides which are important for the growth of Cl.tetani. In a complete growth medium containing casein hydrolysate, vitamins, NaCl, $MgSO_4$, $FeCl_3$, yeast extract and cysteine hydrochloride Cl.tetani grows well, but the level of growth is reduced by almost a quarter when the casein hydrolysate content was dropped from 3 to 1% and the growth level reduces to almost 2/3 the original level when the content is dropped to 0.1%. The complete medium cannot support growth of Cl.tetani when casein hydrolysate is completely absent.

Yeast extract is a source of phosphates, nitrogen and vitamins [207] and could support growth as good as the complete medium when the specific vitamins were removed from it. Under these circumstances reducing the yeast extract to 1/10 its original concentration reduced the growth level by over half and removing yeast extract altogether led to only minimal growth.

There is a direct relationship therefore between the amount of casein hydrolysate and yeast extract present in the medium and the level of growth achieved. No attempts were made to see if higher concentrations of the two compounds would lead to higher growth levels, but this is entirely possible.

Other work has suggested that a high level of iron must be provided for toxin production in Cl.tetani[110], and this work indicates that a source of iron is required for optimum growth as well. It has been shown previously that media containing too little iron fail to support growth, and that increasing the iron content led to increased toxin and growth levels. With large amounts of iron, the growth improved still further but the toxin levels dropped sharply[111].

Iron is important in a number of cellular functions including RNA and DNA synthesis, enzyme sythesis and activity, control of metabolic

pathways and control of bacterial morphology, including sporulation, and has been shown to enhance several clostridial infections[208]. Whenever $FeCl_3$, the only source of iron in the complete medium, was removed, the growth level obtained dropped significantly to around 12% of the original level.

Cysteine hydrochloride was added to the medium as a reducing agent[168], to provide appropriate anaerobic conditions in the medium to allow Cl.tetani to initiate growth easily. Removing it from the complete medium resulted in the level ofgrowth dropping to approximately half the original level. This is almost certainly due to the non-reduced medium causing the organism to be extremely slow at initiating growth. It is possible that, once growth has started, it will eventually reach the same level as the complete medium but will just take longer as it got off to a poorer start.

The action of $FeCl_3$ and cysteine hydrochloride are not independent but additive, as the growth level appears to decline even further when the two are removed simultaneously.

The removal of $MgSO_4$, NaCl or the 8 separate vitamins had little effect on the growth level individually, reducing it by less than 10% each, and appeared to have little added effect when removed in combination with each other or other compounds.

One puzzling fact emerged from the investigation. Although the removal of $FeCl_3$ on its own reduced the growth level by almost 90%, removal of $FeCl_3$ with simultaneous removal of $MgSO_4$ only reduced the growth level by around 65%. It seems unlikely that the $MgSO_4$ could stop the organism obtaining its necessary iron from any other source in the absence of $FeCl_3$ (possibly some other chemical contaminated by iron) therefore these findings remain unexplained.

The findings of this study suggest that while biotin, calcium pantothenate and pyridoxine were extremely stimulatory to growth,

giving growth almost as good as the complete medium and riboflavin was moderately stimulatory, racil, thiamine, icotinic acid and itamin B_{12} did not enhance growth at all. Further, it appeared that no single vitamin was essential for growth although the removal of Uracil, pyridoxine, nicotinic acid, calcium pantothenate, biotin or Vitamin B_{12} caused a small decrease in growth. Removing riboflavin had no effect on the growth but removing thiamine appeared to enhance the growth, indicating that it was actually inhibitory.

These findings differ from those of earlier workers who felt that thiamine was essential to growth, as were nicotinic acid and uracil[111,209]. The findings that calcium pantothenate, riboflavin and pyridoxine were stimulatory to growth agree with other workers[110,209] although this study and one other[209] found that biotin was stimulatory whereas the other found it to have no effect[110]. There are three possible explanations for these discrepancies. The first - that the vitamins used by the earlier workers were impure and thus led to discrepant results - may be discounted as it is quite unlikely that such experienced workers in this field would make such an elementary mistake. The second, (and most likely) possibility is that the casein digest contains sufficient of the essential substances to allow growth to continue even in their absence. Fisek et al[110] have shown that removal of nicotinic acid - which they contend is essential for growth - from the medium does not affect growth, and they attribute this to the presence of nicotinic acid or something replacing it, in the casein digest. This is almost certainly the reason that none of the vitamins tested here appeared essential, although their absence caused a decrease in growth level. The third possibility for the discrepancies is that the use of the variant 'Harvard' strain in the earlier experiments[110] may have given a false picture of the nutritional requirements of the majority of Cl.tetani strains. Unfortunately the present study, which also used a single strain, is subject to the same criticisms.

It has been suggested that the precise vitamin requirements are variable from strain to strain and although the majority of strains require biotin, pyridoxine, nicotinic acid, calcium pantothenate and uracil, this may differ for others which may need riboflavin, thiamine, adenine or oleic acid.[209] If this is true it would explain the discrepancies observed by different workers.

The likelihood that the casein digest contained other growth factors than amino acids and peptones was shown by the fact that replacing it with the 21 available L-amino acids reduced the growth level by 75%. With the strain of Cl.tetani used in this study it appeared that 7 of these amino acids were necessary for growth, L-cystine, L-tryptophan, L-glutamic acid, L-threonine, L-methionine, L-aspartic acid and L-asparagine in decreasing order of importance. L-cystine supported growth to a level amost equivalent to that obtained with all 21 amino acids suggesting that it was possibly the major growth factor requirement amongst the amino acids for this strain of Cl.tetani. When the evolution of ammonia from individual amino acids by this strain was investigated quantitatively, only L-glutamine and L-threonine gave readings noticeably higher than the rest; the reason probably being that the assay wasnot sensitive enough to detect deamination products from the rest. Other studies have suggested that arginine, histidine, isoleucine, leucine, tryptophan, valine and tyrosine are required,[209] or that isoleucine, leucine, tryptophan, valine, serine, threonine, methionine and phenylalanine are essential or stimulatory to growth[113], or that glutamic acid, glutamine, serine and histidine were essential or stimulatory to growth[110], or that glutamic and aspartic acids and serine are decomposed[196] or that glutamic acid, serine and histidine were required with aspartic acid, threonine, methionine and tyrosine utilization varying from strain to strain.[197] Although some of the same amino acids seem to crop up repeatedly, none of the studies agree on the requirement for a single amino acid. The terms 'requirement' meaning an essential nutrient and 'utilisation' meaning

a compound that can be used as a nutrient but which may not be absolutely essential seem to be used rather loosely however, and again two of the studies used the variant Harvard strain[110,113] and two others, including the present one used a single strain[196]. When the deamination of amino acids by all 27 strains was investigated it could be seen that, although L-aspartic acid, L-glutamine, L-methionine, L-glutamic acid and L-threonine were utilised by almost every strain, L-cysteine, L-asparagine, L-leucine, L-valine and L-serine were only utilised by some strains. Thus it would appear that there is great heterogeneity amongst Cl.tetani strains with regard to their amino acid utilisation and requirements. This is in accord with the findings of Mead[197] who used 8 strains of Cl.tetani. The use of single, possibly variant strains in studies together with differences over the use of 'requirement' and 'utilisation' may have led to more confusion than is necessary. A surprising finding was that although the growth factor experiments had shown that 7 amino acids were necessary for growth, with L-cystine being the most stimulatory to growth, the actual deamination experiments indicated that only 6 strains utilised L-cystine, and none of these was the strain used in the growth experiments. Also, no strain appeared to utilise L-tryptophan and only 3 utilised asparagine, both of which appeared to be necessary for growth. This seeming paradox must be due to the lack of sensitivity of the assay. Presumably these 3 amino acids are utilised but it may be that only very small quantities are required, leading to the production of only small quantities of ammonia that can not be detected by the system used in this study.

The possibility exists therefore that the apparent heterogeneity of Cl.tetani strains may not be quite so great as it appears, and that more sensitive assay systems would detect a more homogenous pattern of amino acid utilisation. If this is the case, the differences in sensitivity of the assay systems used by different workers may be the reason for the apparent discrepancies in the literature.

Cl.tetani strains all produce three volatile fatty acid metabolic products, acetic, propionic and butyric acids, in fairly similar quantities. Butyric acid was always the major product(usually 60 - 70% of the total) and acetic acid was generally next (13 - 30%) followed by propionic acid (9 - 26%). Although the majority of strains were only tested once, occasional repeat tests suggested that the relative percentages for the three acids could vary by as much as ± 20% each, therefore the concentrations are not static but can vary, presumably with slight variations in medium, growth temperature, inoculum and extraction techniques. All strains produced two non-volatile fatty acids, lactic and succinnic acids, with lactic acid always as the major product (60 - 75% of the total). These findings are in accordance with established facts[11,12,151] and suggest that whatever mixture of amino acids are utilised, the end products are remarkably similar, or, more likely, that one or more amino acids are preferentially utilised and produce the products observed. All the strains investigated here could utilize glutamic acid (indeed it appeared to be a growth requirement) and Gale has shown that in a study of the amino acid decarboxylating powers of a number of different clostridial species, the glutamic acid decarboxylase was the most frequently encountered and, in general, the most active, the product always being butyric acid.[199] Although that investigation did not include Cl.tetani, this organism has also been shown to possess a glutamic acid decarboxylase (this study, Chapter 5) and it seems likely that the same situation applies and that the butyric acid is the product of glutamic acid fermentation. Acetic acid is a common end product which can also be formed from glutamic acid, as seems likely here, or from serine or lysine.[197] Propionic acid production is thought to be related to the utilisation of threonine,[197] and its production by all 27 strains concurrent with the utilisation of theonine by all 27as well would support this suggestion. Lactic acid is reported as being produced

from aspartic acid,[196] and this is probably the source of the lactic acid in this study as all 27 strains utilised aspartic acid. The mechanism of succinic acid production is not known.

6.5 CONCLUSION

The amount of growth of Cl.tetani produced is proportional to the amount of casein hydrolysate in culture media. The complete removal of casein hydrolysate resulted in no growth unless the medium was supplemented by 21 individual L-amino acids when a reduction in growth level of 75% was seen. L-cystine alone could support growth almost at the same level, whereas L-tryptophan, L-glutamic acid, L-threonine, L-methionine, L-aspartic acid and L-asparagine supported lower growth levels. All 7 of these amino acids were essential to growth. None of the other 14 L-amino acids was essential or stimulatory to growth.

In amino acid deamination studies it was found that L-aspartic acid, L-glutamine, L-methionine, L-glutamic acid and L-threonine were utilised by all 27 strains of Cl.tetani used, while L-cysteine was utilised by 7, L-cystine by 6, L-asparagine by 3, L-leucine by 2, L-valine by 1 and L-serine by 1. It is suggested that the assay used was too insensitive to detect all amino acid utilisations present, especially if only small quantities were utilised and may have given a falsely heterogenous view of the species with respect to its amino acid utilisation properties. No D-amino acids were deaminated by any strain.

Acetic, butyric and propionic acids are produced by all the 27 strains of Cl.tetani with butyric acid as the major metabolic product. It is suggested that acetic and butyric acids are produced by glutamic acid decarboxylation and that propionic acid is produced from threonine. Lactic and succinic acids are also produced and it is suggested that the lactic acid is produced from aspartic acid. The source of the succinic acid is not known.

Yeast extract is an essential growth factor the presence of which is proportional to the amount of growth produced. Removal of yeast extract in the absence of individual vitamins resulted in no growth. The yeast extract could be replaced by a number of individual vitamins which supported growth at almost the same level, namely pyridoxine,

calcium pantothenate and biotin, while riboflavin supported less than half as much growth. Uracil, thiamine, nicotinic acid and Vitamin B_{12} could not support growth individually. None of the 8 vitamins appeared to be essential to growth although it is suggested that the presence of unknown growth factors, possibly vitamins or substitutes, in the casein digest may have interfered with these investigation. Thiamine appeared to be inhibitory to growth. **The presence of iron appeared to be necessary for maximal growth.**

CHAPTER 7

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF CL. TETANI

7.1 SURVEY OF THE LITERATURE

In addition to the properties already investigated, Cl.tetani has several morphological and cultural properties of note. It is described as staining gram-positively in young cultures but often gram-negatively in older cultures[212, 210] and showing considerable variation in length[6]. Unless autolysis occurs the organism has a relatively constant width, around 0.5 um [2,6,12,153,210], but the length varies from short rods in fluid media to long rods[2] on solid media with filamentous forms being common[2,6,11,112].

The most notable morphological feature of Cl.tetani cells is the spherical terminal spore produced. These spores may take 2 - 3 days incubation at 37°C before they assume the characteristic highly refractile 'drum-stick' appearance.[212] Prior to this the spores appear as slight subterminal enlargements that stain slightly more gram-positively that the rest of the cell.[2] It has been suggested that the 'drum-stick' appearance in Gram films is an artifact as wet films show the spores to be more wedge-shaped.[2]

The degree of spore production by different strains of Cl.tetani does not appear to have been investigated thoroughly, although it is known that some strains, such as the 'Harvard' strain do not readily form spores[2] and that spore-free forms of Cl.tetani have no distinctive features[12], the relationship of sporulating potential to other biological activities is relatively unknown.

A 'competition' model for the regulation of extracellular protein production has been suggested by Coleman et al[108] which puts cellular protein production (i.e. growth of the cell) in direct competition with extracellular protein production (i.e. toxins). The mechanism for this is felt to be competition for the cells

biosynthetic capacity at the m-RNA level. Exoprotein m-RNA competes unfavourably with cellular protein m-RNA during exponential growth and thus the amount of exoprotein produced is low. However, when cell protein manufacture stops due to nutritional limitations (the end of exponential growth), exoprotein m-RNA takes over the biosynthetic capability of the cell and post exponential growth exoprotein production increases. This has been found to be true for a number of strains of Staphylococcus aureus[211] and with Bacillus amyloliquefacieus[108]. Both exoprotein production and sporulation are post-exponential growth phenomena and might be expected to be under similar controls, thus establishing a 'competition' situation between the two where increased sporulation would lead to decreased exoprotein production and vice versa. Although this has not been established, and in fact, appears not to be the case with B.amyloliquefacieus[108], a direct relationship can apparently be shown between the sporulating potential and the toxin producing capability of Cl.bifermentans and Cl.sordellii[212], Cl.sporogenes[51], Cl.perfringens[213], Cl.novyi[214] and Cl.tetani[51,76]. These findings are not totally conclusive however, as highly sporulating but still relatively toxigenic strains of Cl.novyi have been isolated[214] and toxigenic but otherwise biochemically aberrant sporulating Cl.tetani strains have been isolated by heat treatment.[56]

Most of the work done on clostridial sporulation and toxigenicity has been done using strains isolated by, or artificially modified following, heat treatment and it has been suggested that heat treatment may have further mutagenic effects on the strain involved[214] which may give rise to variations not only in toxigenicity but in other biological properties and antigenic structures.[51]

It has been stated that "The relationship of sporulating potency to toxigenicity and other taxonomic characters needs further Investigation",[76] as both glucose fermentation[76] and gelatin hydrolysis[51] have been observed to occur in strains of Cl.tetani dependent on their

degree of heat treatment. It was felt that a study of these factors in a population of different Cl.tetani strains with a range of different naturally occurring sporulating potentials would perhaps clarify the natural situation. Also, the synthesis of dipicolinic acid has been reported to coincide with the development of heat resistance due to spore formation[215] and this was also investigated.

Cl.tetani is motile[2] with peritrichous flagella[6,12,153,210] although non-motile forms do occur[2,12] which produce discrete colonies with no tendency to swarm, and it is not clear what other biochemical or cultural differences these non-motile strains may exhibit. There are several methods of determining motility including wet preparations,[20] flagellar stains,[151] growth in fluid filled capillary tubes and broth[20]. Electron microscopy has also been shown to be useful for the demonstration of flagella[221]. As some of the strains used in the earlier part of this study appeared to grow as discrete colonies, their motility and the possession of flagella was investigated by several of these different methods. As motility of Cl.tetani is usually characterised by the tendency to spread over the surface of solid media in a fine, delicate film, and this swarming can be inhibited by commercial tetanus antitoxin[2], it is usually assumed that the inhibition is due to the presence of agglutinating antibody since it is known that strains of Cl.tetani possess common somatic O antigens and may have type-specific flagellar H antigens[216,217]. Generally the possession of flagellar antigens is assumed because the type-specific antigens found in Cl.tetani are heat labile, and non-motile strains do not possess them,[217] but this has never been conclusively proved. Other workers have isolated highly purified flagellins from Salmonella[218] and Pseudomonas[219], and it was felt that isolation of such a preparation and attempts to visualise it directly by two dimensional IEP would be advantageous. Motile strains of Proteus species exhibit swarming but when two different strains are inoculated at different points on an agar plate,

the two films of growth never quite meet, a distinct line of demarcation being seen between the two.[220] (Dienes phenomenon). This is thought to be due to the production of mutually inhibitory substances. Whether Cl.tetani swarming growth will exhibit Dienes phenomenon appears never to have been investigated, and was felt to be a further area that should be studied.

Antimicrobial resistance patterns have been used to differentiate strains of Cl.difficile,[222] but has apparently not been investigated with Cl.tetani. Although most strains of Cl.tetani are probably highly sensitive to penicillin, tetracycline,[210] erythromycin and cloxacillin[12], a study of the Minimum Inhibitory Concentrations (MICs) of these and other antibiotics might reveal striking differences between strains.

Clostridium species have been shown to differ in their soluble cellular proteins by Polyacrylamide Gel Electrophoresis (PAGE), and different strains of Cl.tetani have been shown to possess different soluble protein profiles.[223] The use of 35 S-methionine radiolabelled culture supernatants has been similarly used in PAGE systems to differentiate species of Cl.difficile,[224] and it was felt that investigation of the strains available in this study might prove valuable, particularly if differences at this level could be correlated with differences in other physical or biochemical characteristics.

Isoelectric focussing (IEF) is, like PAGE, a highly discriminating technique for separating proteins in mixtures, but one which appears never to have been used in this context with clostridia as a whole, or Cl.tetani in particular, therefore the investigation of whole cell and culture supernatant protein patterns by this technique was also included in this study.

It is now generally believed that the possession of a plasmid by Cl.tetani is necessary for production of neurotoxin.[40,225] The size of this large plasmid appears to be around 75 kilobases (kb),[226]

although plasmids with molecular weights between 25 and 70 kb have also been observed in randomly selected strains.[40] The occurrence of plasmids in non-neurotoxic strains has been observed and spontaneous deletions of 22 kb from the original 75 kb plasmid led to loss of neurotoxin producing capability.[40,226] The functions of the other plasmids have not been established. It is possible that haemolysin production is plasmid controlled, but this appears not to have been investigated, nor has the possibility that other physical or biochemical differences may be under direct genetic control by plasmids been studied, therefore the possession of plasmids by Cl.tetani strains was investigated in this light.

The following investigations were therefore made.

1) **A study of the sporulation process in Cl.tetani.**
2) **A study of the motility of Cl.tetani.**
3) **A study of the antibiotic susceptibility of Cl.tetani.**
4) **A study of soluble protein profiles of Cl.tetani by PAGE and IEF.**
5) **A study of plasmid and bacteriophage carriage by Cl.tetani.**

7.2 EXPERMINENTAL TECHNIQUES

7.2.1 Spores of Cl.tetani

7.2.1.i The physiology of the sporulation process in Cl.tetani

Cl.tetani NCTC 540 (serotype II), a strain which had been observed to produce spores on Columbia blood agar plates was used for this part of the study.

The test strain was brought to a reasonable degree of synchronicity by heavily inoculating a cooked meat broth[61], heating at 80°C for 10 minutes, incubating at 37°C for 10 hours and using this culture to inoculate the medium for sporulation. This technique of heat-shocking is modified from that of Day and Costilow.[215]

Fresh cooked meat broths and fresh FAB in 10ml volumes were each inoculated with 2.5ml of the heat-shocked culture and incubated in triplicate at 30°C and 37°C for 7 days. Smears were made from these cultures on days 1, 2, 3, 4, 5 and 7. The smears were Gram-stained (Appendix 7) and examined. Sporulating potential was taken as the percentage of the total number of cells counted that exhibited spores or forespores. 20 microscopic fields were examined during each estimation. In addition, 1ml of both of the cooked meat broth culture fluids was removed on days 1, 3, 5 and 7 and subjected to GLC analysis for volatile fatty acids (Appendix 39).

7.2.1.ii The sporulating potential of different strains of Cl.tetani

All 27 strains of Cl.tetani (Appendices 1 and 2) were grown for 7 days in Cooked meat broth at 30°C, FAB at 37°C and anaerobically on columbia blood agar plates at 37°C before smears were made of each and Gram stained. These were examined as before and the sporulating potentials of each determined. Tests were done on 3 separate occasions and the sporulating potential for each strain taken as the mean of the 3 individual

observations to the nearest whole number.

7.2.1.iii Dipicolinic acid production in sporulating cultures of Cl.tetani

The 27 strains of Cl.tetani were grown in cooked meat broth for 7 days at 30°C. The culture fluid was aspirated from each and centrifuged at 1,500 x g for 10 minutes to deposit the cells. These were then resuspended in 5ml of sterile distilled water before being assayed for the presence of dipicolinic acid. (Appendix 39)

7.2.2 Motility of Cl.tetani

7.2.2.i The motility of different strains of Cl.tetani

The motility of all 27 strains of Cl.tetani (Appendices 1 and 2) were investigated by four different methods as follows:

1) observation of visible swarming on Columbia blood agar plates incubated anaerobically at 37°C for 24 hours. Plates were inoculated over $\frac{1}{3}$ of the surface with a sterile loop. After incubation they were examined for evidence of a fine spreading film or growth across the uninoculated area.

2) observation of visible motility in Motility test agar 30,183. Motility test agar (Appendix 7) was distributed in 3ml amounts in sterile bijoux bottles and each of the 27 strains was stab-inoculated into the centre of one of these. The bottles were incubated, loosely capped at 37°C anaerobically overnight before being examined for the presence of a diffuse cloud of growth throughout the medium which denoted motility. Non-motile organisms grow as a distinct line along the track of inoculation and do not spread.

3) The wet film method.[151] The 27 strains were grown for 18 hours in fresh FAB at 37°C. One drop from each was placed on a sterile slide, covered with a coverslip and observed immediately under x40 dry magnification. Only distinct random

movement or movement against the flow of liquid when present was counted as positive. Brownian movement and moving with the current were ignored.

4) Flagella staining.[151] The 27 strains were grown in fresh FAB for 18 hours at 37°C before formalin[69] was added to each to a final concentration of around 5%, and were then refrigerated until required. The formolised cultures were mixed gently and then centrifuged at 1,500 x g for 3 minutes. The supernatant was decanted and sterile distilled water added until the resuspended deposit was only slightly turbid. A loopful of each was placed on a flame-cleaned glass slide and allowed to run 1 to 1.5 inches along the slide before being dried in a slanted position.

The slides were stained with Leifsons' flagella stain (Appendix 7), and examined for the possession and arrangement of flagella.

7.2.2.ii Electron microscopy[228]

One known motile strain (NCTC 540, serotype II) and one known non-motile strain (NCTC 9569, serotype VI) were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) at the Electron Microscope Dept., John Radcliffe Hospital, Oxford. The strains were supplied as 1% glutaraldehyde suspensions of 24 hour old cultures grown on Columbia blood agar plates anaerobically at 37°C, before being harvested into the glutaraldehyde with a sterile swab. Preparation, electron microscopy and photography were kindly done by Mr. A. R. Skinner of the above department. Briefly, after glutaraldehyde fixation, the bacterial suspension was post-fixed in 1% osmium tetroxide and embedded in Spurr epoxy resin. Sections were stained with uranyl acetate and lead citrate and examined using a Phillips EM301 electron microscope.[228]

7.2.2.iii Dienes phenomenon in Cl.tetani

To investigate this phenomenon, the 27 strains of Cl.tetani were grown on Columbia blood agar plates at 37°C anaerobically overnight for use as inocula. Fresh Columbia blood agar plates were spot inoculated with four different strains of Cl.tetani, one in each quarter of the plate, about 1cm from the edge, before the plates were incubated at 37°C anaerobically overnight. Following this the plates were observed for areas of demarcation between the spreading films of growth from each strain.

A series of test plates were put up with different combinations of strains until all 27 strains had been tested against each other.

7.2.2.iv Isolation of flagellin

Strain NCTC 540, serotype II, a known motile strain andthe one used in the electron microscopy studies was used in this investigation.

Flagellin was isolated by the method of Ibrahim et al [218] (Appendix 41) and was examined bytwo dimensional immuno-electrophoresis (Appendix 14) against Wellcome tetanus antitoxin.

7.2.3 Antibiotic resistance patterns of Cl.tetani

All 27 strains of Cl.tetani were grown overnight on Columbia blood agar plates anaerobically at 37°C. Organisms were harvested from these into fresh FAB to a turbidity approximately equal to a MacFarland No.1 opacity tube (approx. 10^5CFU/ml). 50 ul of these suspensions were inoculated into the wells of both APOI and APO8 Sensititre MIC determination plates[26]. These plates were covered with a transparent seal with small perforations above each well to allow free exchange of gases, and incubated anaerobically at 37°C for 48 hours in a humidity chamber. **Staphylococcus aureus (NCTC 6571) and Cl.perfringens (NCTC 8237) were used as control organisms.**

The plates were examined following the manufacturers' instructions in a technical product leaflet for Sensititre.[26] Growth appears as turbidity or as a deposit of cells at the bottom of a well. The MIC is recorded as the lowest concentration of antibiotic that inhibits visible growth.

The antibiotics tested were as follows:

In the APOI plate	Penicillin	0.06 - 8 mg/l
	Methicillin	0.12 - 16
	Ampicillin	0.12 - 16
	Cephalothin	0.5 - 64
	Gentamicin	0.12 - 16
	Kanamycin	0.5 - 64
	Erythromycin	0.25 - 32
	Chloramphenicol	0.25 - 32
	Tetracyclines	0.12 - 16
	Vancomycin	0.25 - 32
In the AP08 plate	Penicillin	0.03 - 32 mg/l
	Clindamycin	0.06 - 64
	Metronidazole	0.06 - 64
	Chloramphenicol	0.06 - 64
	Cefoxitin	0.12 - 128
	Erythromycin	0.06 - 64
	Carbenicillin	0.025 - 256
	Cephalexin	0.12 - 128

7.2.4 Polyacrylamide gel electrophoresis (PAGE) of Cl.tetani proteins

7.2.4.i Whole cell proteins

All 27 strains of Cl.tetani were grown for 7 days at 37°C in 20 ml of FAB broth. The cells were harvested by centrifugation at 1,500 x g for 15 minutes and the supernatants removed. The cells were washed once in 5 ml sterile distilled

water before being resuspended in 0.2ml sterile distilled water. 0.1ml of each was removed and placed in a sterile glass bottle and 0.1ml of PAGE sample buffer was added (Appendix 15) and mixed. The bottles were subsequently boiled for 10 minutes, cooled and 50 ul of each was applied to a 10% Polyacrylamide gel with a 4% stacking gel and electrophoresed and stained as before (Appendix 15).

7.2.4.ii Culture supernatant proteins

The supernatants from the 7 day FAB cultures above were used in this investigation. 0.2ml of each was mixed with 0.2ml of PAGE sample buffer and boiled for 5 minutes. 75 ul of each was applied to a 10% Polyacrylamide gel with a 4% stacking gel as before (Appendix 15).

7.2.5 Isoelectic focussing (IEF) of Cl.tetani proteins

The 27 strains were each grown in 20ml FAB for 7 days at 37°C. The cells were deposited by centrifugation and 2 ml of each culture supernatant was lyophilised on a freeze dryer[100]. These were reconstituted in 0.2ml distilled water and 25 ul of each was applied to a wide range pH isoelectric focussing plate (pH 3-10) which was electrophoresed and stained as before (Appendix 20).

7.2.6 Plasmids of Cl.tetani

The 27 strains of Cl.tetani were grown for 48 hours anaerobically at 37°C on Columbia blood agar plates before being harvested with a loop into Tris-acetate buffer.(Appendix 40).

Plasmids were isolated by the method of Kado and Liu[229], and electrophoresed by the method of Meyers et al.[230] (Appendix 40).

7.2.7 Bacteriophages of Cl.tetani

The ability of each of the 27 strains of Cl.tetani to produce bacteriophages active against any other strain of Cl.tetani was tested by growing each of the strains on sterile membrane filters[233] (type 11357 pore size 0.2 um) placed on Columbia agar plates plus 2% Davis

agar for 48 hours. This permitted diffusion of bacteriocins into agar[234] without allowing the strains to swarm and in this way 14 strains could be tested on one plate (two per filter). The membranes were removed after growth and the plates were inoculated all over with the indicator strains of Cl.tetani using a sterile swab. Two plates were required to test all 27 strains using a single indicator strain. All 27 strains were used as indicator strain ensuring that each strain had been tested against all other strains both for production of, and susceptibility to, bacteriophages.

The inoculated plates were incubated anaerobically at 37°C overnight and subsequently examined for the production of areas of inhibition corresponding to the areas where the test strains had been grown.

7.2.8 Comparison of sporulating potential, toxin and haemolysin production, biochemical and metabolic activities, motility, antibiotic patterns, PAGE and IEF patterns and plasmid carriage in strains of Cl.tetani

If the 'competition' model for extracellular production[108] is true for Cl.tetani, and if the biosynthetic capability of the cell is subject to such a system, then distinct relationships ought to be seen between the above properties.

The findings from different parts of the study were therefore brought together for all 27 strains of Cl.tetani to see if this was true.

7.3 RESULTS

7.3.1. Spores of Cl.tetani

7.3.1.i The physiology of the sporulation process in Cl.tetani

When the sporulating potential was calculated for Cl.tetani NCTC 540 daily over a period of 7 days, sporulation occurred in an increasing percentage of cells after 3 days incubation in cooked meat medium at 37°C whereas at 30°C in the same medium 5 days incubation was required before sporulation occurred in an increased number of cells. In contrast, cells grown in FAB sporulated only poorly at either 37°C or at 30°C although vegetative growth was vigorous (Table 42.) When the VFAs produced by this strain at 37°C and 30°C were compared, no qualitative differences were observed although some quantitative differences could be seen (Table 43). Although the percentages of the total area produced by each peak were similar at each different time, the actual areas under the peak were much larger for the 37°C cultures than the 30°C cultures. In most cases the 37°C culture peak areas were almost three times as great as those produced at 30°C.

7.3.1.ii The sporulating potential of different strains of Cl.tetani

When the 27 strains of Cl.tetani were grown on 3 different media and the sporulating potentials estimated, two things became obvious. The first was that the figures obtained varied greatly from test to test even with the same strain grown in the same medium. Although most differences were in the ± 25% range, many were in the ± 50% range and some were greater than this. The second was that FAB supported sporulation poorly, rarely producing as many spores as either of the other two media (Table 44). Columbia blood agar plates supported sporulation quite well, in many cases almost as well as cooked meat broth and occasionally better,

but cooked meat broth appeared to be the best medium for sporulation overall.

There was great variety in the sporulation potentials observed with individual strains in the three media, some producing no spores in one or two of the media whilst producing large numbers in the third and vice versa. It was noted however, that strains L109, E88 and CN1342 produced scanty or no spores at all in all three media. CN1349, a subculture from strain E88 at some time in the past, had a sporing potential significantly greater than that of the parent strain.

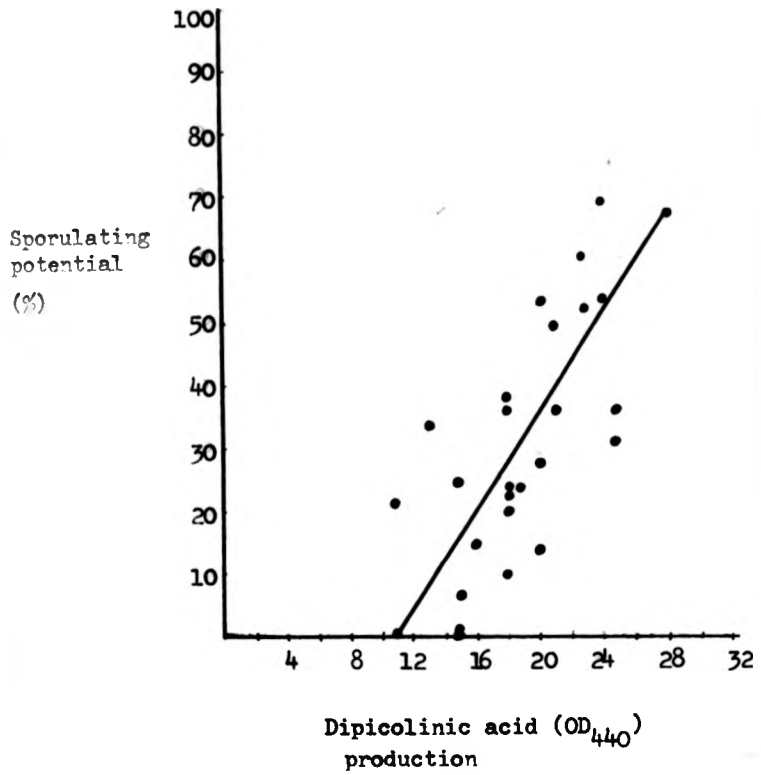
7.3.1.iii Dipicolinic acid production in sporulating cultures of Cl.tetani

When the 27 strains were investigated with regard to both their sporulating potential and their ability to produce dipicolinic acid, a definitive relationship was not observed (Table 45). Although in general, strains with high sporulating potentials tended to have high dipicolinic acid levels and strains with low sporulating potentials had lower levels, this was not always the case. When sporulating potential was plotted against dipicolinic acid levels (Fig.52) it could be seen that the relationship was non-linear and many strains with low sporulating potentials gave higher dipicolinic acid readings than strains with higher sporulating potentials. The variation observed between sporulating potentials for a single strain could be seen by comparing results in Tables 44 and 45.

7.3.2 Motility of Cl.tetani

7.3.2.i When the 27 strains were investigated by motility methods, there was complete correlation of results except in two cases (Table 46). Serotype VI strain (NCTC 9569) was non-motile by all four methods, and 24 of the other 26 strains were motile by all 4 methods, however strains E88 and CN1349 (the Harvard strain and a substrain of this strain) did not swarm and

FIG.52 Sporulating potential compared to dipicolinic acid production in cultures of Cl.tetani



appeared non-motile on columbia blood agar plates and motility test agar (Fig.53) whilst appearing sluggishly motile by the wet preparation method and exhibiting flagella by Leifsons staining method. This latter method was not very successful as it proved difficult to interpret due to large amounts of precipitated stain, and the majority of organisms appeared to have no flagella, only occasional bacteria showing clumps of flagella in each film.

7.3.2.ii Electron microscopy

On examining the electron micrographs of a motile Cl.tetani strain (NCTC 540) and a non-motile strain (NCTC 9569), little difference is seen between SEM preparations of the two strains, and no flagella were observed on either (Fig.54a). However, when the TEM preparations were examined, although no flagella were seen associated with strain NCTC 9569, strain 540 showed the presence of numerous fine hairlike strands, mainly in the surrounding medium and unconnected to the bacterial body, but occasionally joined directly to the cell wall (Fig.54b). It is probable that these are flagella that have become unattached during processing for some reason.

7.3.2.iii Dienes phenomenon in Cl.tetani

When the 27 strains of Cl.tetani were tested against each other, no inhibition of swarming was seen with any strain and no areas of demarcation (Dienes phenomenon) were observed between the spreading films of growth in any case. In all cases the test strains joined up to form a homogenous film over the agar plate surface (Fig.55).

7.3.2.iv Isolation of flagellin

When purified flagellin from Cl.tetani NCTC 540 was examined by 2 dimensional IEP, no peaks at all were seen.

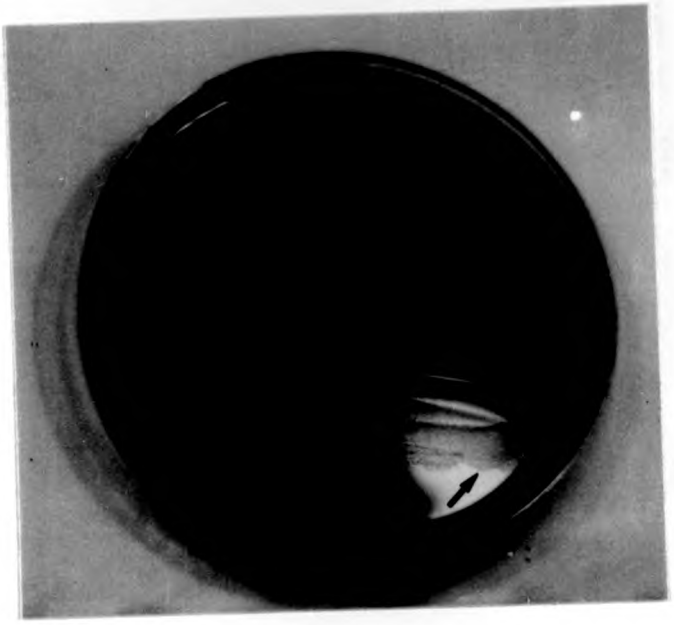
FIG.53a Non-swarming of Cl.tetani strains on Columbia blood agar plates

Note: distinct rhizoid colonies formed by strain NCTC 9569 (serotype VI) on Columbia blood agar (arrow)
Compare these with the smooth films of growth seen with motile strains (Fig.53c and Fig.55)

FIG.53b Cl.tetani strains in motility test agar

Note: diffuse growth produced by motile strain (left) compared to the well defined non-spreading growth exhibited by the non-motile strain (right)

FIG.53c **Swarming of Cl.tetani on Columbia blood agar plates**

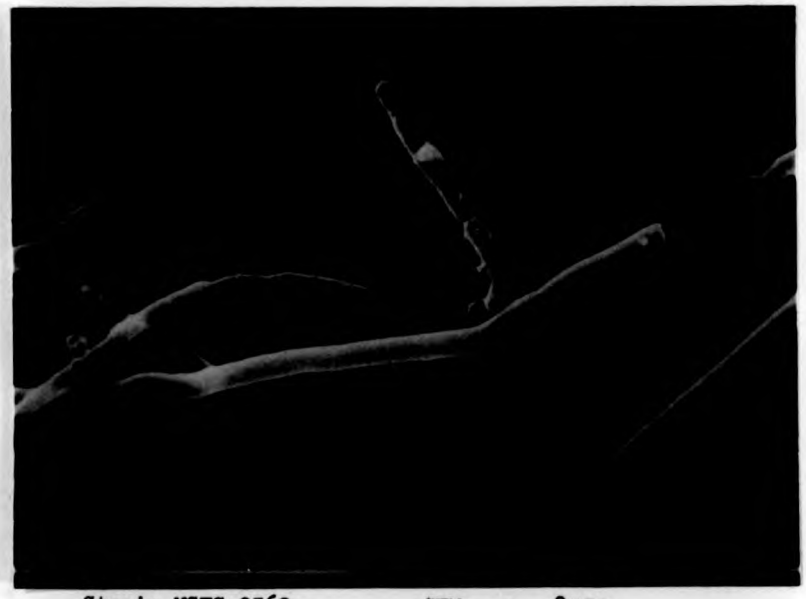


Note: The fine spreading film of growth and absence of distinct colonies (arrow)

(Strain NCTC 540)

FIG.54a Electron microscopy of Cl.tetani strains

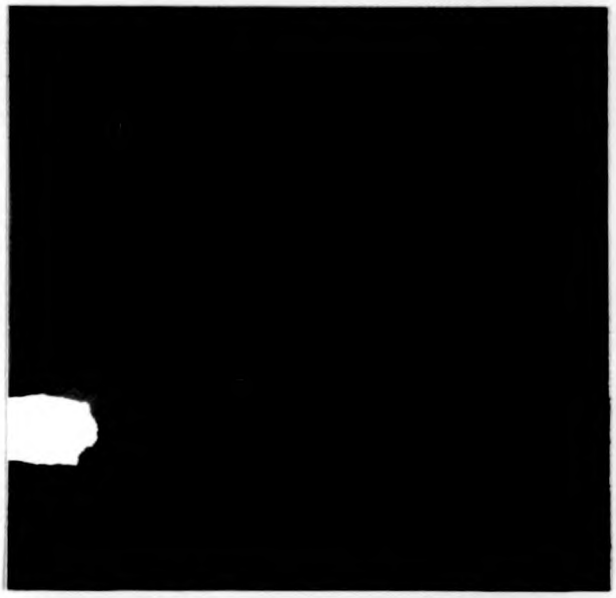
Strain NCTC 540 SEM x 8750



Strain NCTC 9569 SEM x 8750

Note: the similarity of appearance of both the motile (NCTC 540) and the non-motile (NCTC 9569) strains and the absence of any discernable flagella on both strains

FIG.54b Electron microscopy of Cl.tetani strains

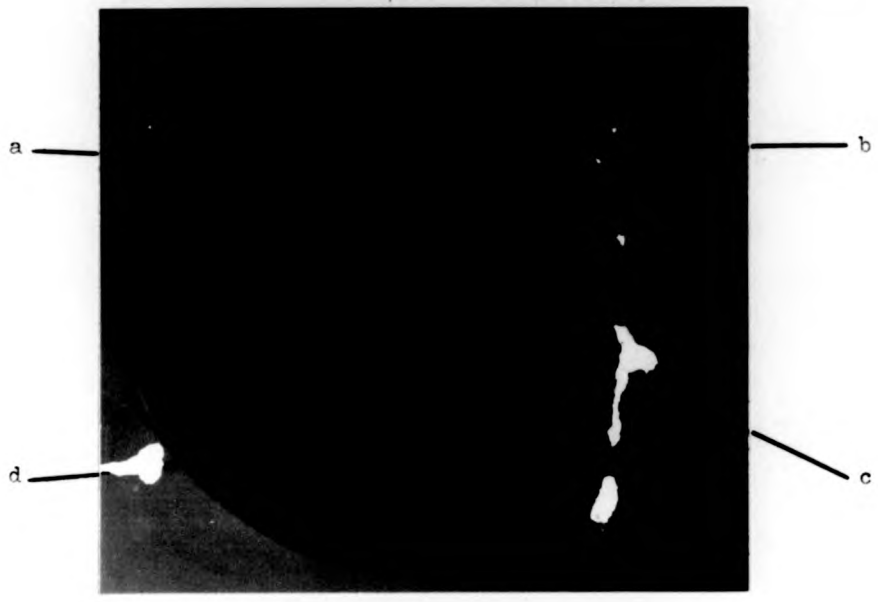


Strain NCTC 540 TEM x 33,000

Strain NCTC 9569 TEM x 43,000

Note: the presence of numerous fine hairlike structures (arrows) surrounding, and occasionally attached to, NCTC 540, and their absence in NCTC 9569. These structures are presumed to be flagella.

FIG.55 Swarming of Cl.tetani strains on the same Columbia blood agar plate



Strains a) serotype I b) serotype II c) serotype II
d) serotype IV - all spot-inoculated before incubation.
Note the smooth films of spreading growth which join completely, showing no areas of demarcation between strains

7.3.3 Antibiotic resistance patterns of Cl.tetani

When the 27 strains of Cl.tetani were tested against the 15 individual antibiotics contained in the two Sensititre plates a remarkably uniform MIC picture emerged (Table 47). All strains gave MICs to most antibiotics that were within two twofold dilutions of each other (e.g. Penicillin where the range was from 0.03 to 0.125 mg/l , and Erythromycin where the range was 0.125 to 0.5 mg/l). Even when the inter strain differences were greater than this (e.g. Metronidazole where the range was 0.125 to 2 mg/l - i.e. 5 twofold dilutions) the results were still such that the organisms were susceptible to the antibiotics and so the differences were not significant. (Fig.55)

7.3.4 Polyacrylamide gel electrophoresis (PAGE) of Cl.tetani proteins

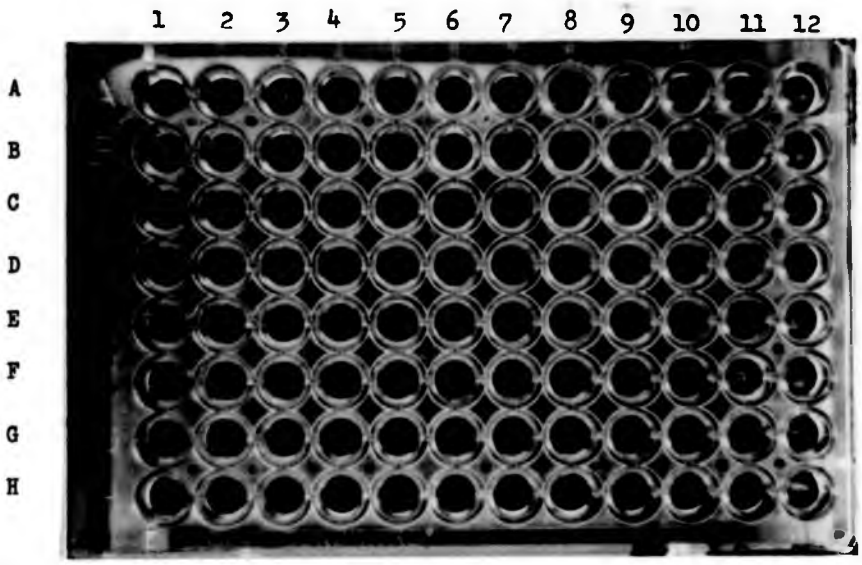
7.3.4.i Whole cell proteins

When whole cell proteins of the 27 strains of Cl.tetani were examined by PAGE, it soon became apparent that there were very few differences between the patterns produced by each strain (Fig.56). Slight differences in band pattern were not reproducible between gels and tended to disappear on retesting. Around 20 distinct bands were seen with most strains.

7.3.4.ii Culture supernatant proteins

When supernatants from the7 day FAB cultures of the 27 strains were examined by PAGE, relatively few bands were seen (between 1 and 7 per strain) and these formed distinctive patterns. (Fig.57) The 5 strongest bands were coded 1 to 5 (Fig.57) and were used to differentiate each strain into groups numerically by including the number of each band possessed by the strain into a code (i.e. serotype I possesses bands No.1 and 3 and is thereforecoded 1,3). These were then classed as pattern A (bands 1 and 3), pattern B (bands

FIG.55A **Antibiotic susceptibility testing of Cl.tetani using Sensititre AP 08 microwell plates**



Rows A) Penicillin (0.3-32 mg/l) B) Clindamycin (0.6-64 mg/l)
C) Metronidazole (0.6-64 mg/l) D) Chloramphenicol (0.6-64 mg/l)
E) Cefoxitin (0.125-128 mg/l) F) Erythromycin (0.6-64 mg/l)
G) Carbenicillin (0.25-256 mg/l) H) Cephalexin (0.125-128 mg/l)

vertical row 12 is the growth control.

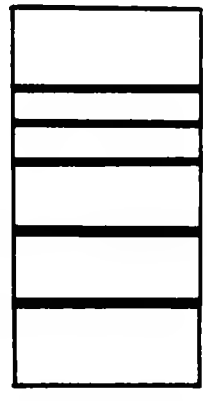
FIG.56 Polyacrylamide gel electrophoresis (PAGE) of Cl.tetani whole cell proteins

Lanes: 1) serotype I 2) L777A 3) LQ914 4) serotype II
5) serotype III 6) serotype IV 7) serotype V
8) serotype VI 9) serotype VII 10) serotype VIII
11) serotype IX 12) CN1349 13) molecular weight markers
14) L109 15) LQ1 16) L5500 17) CN1445 18) CN4878
19) CN1342 20) CN761 21) CN780 22) CN361 23) CN3973
24) Molecular weight markers

FIG.57a Polyacrylamide gel electrophoresis (PAGE of Cl.tetani culture supernatant proteins

Lanes: 1) Molecular weight markers 2) unused 3) serotype I
4) serotype II 5) serotype III 6) serotype IV
7) serotype V 8) serotype VI 9) serotype VII 10) serotype VIII
11) serotype IX 12) CN1349 13) 14) CN1342
15) CN4878 16) CN361 17) CN780 18) CN1445
19) CN761 20) L109 21) L91 22) L5500 23) L0914
24) Molecular weight markers

FIG.57b Diagrammatic representation of the 5 strongest bands and approximate molecular weights



2 and 4), pattern C (bands 3 and 4), pattern D (band 3) and pattern E (bands 2,4 and 5) (Table 48) It is probable that pattern E should really be classified as pattern B as the one extra band was not always present. Of the 27 strains 5 produced pattern A, 16 produced pattern B, 1 produced pattern C, 2 produced pattern D and 3 produced pattern E.

7.3.5 Isoelectric focussing (IEF) of Cl.tetani proteins

Neat culture supernatants produced occasionally only one or two weak bands by IEF therefore 10 x concentrated culture supernatants were used. When these were examined for all 27 strains, 3 - 4 bands were generally visible in most cases in the pH range 4.55 to 5.6 (Fig.58)

The patterns produced in this range were similar for most strains, however 9 of the 27 strains produced an additional band at around pH 4.25. This characteristic was reproducible although the bands were often quite faint. The strains possessing this extra band were NCTC 540 (serotype II), NCTC 5411 (serotype V), NCTC 9575 (serotype IX), CN4878, CN947, CN3973, L5500, LQ1 and LQ2. (Table 49)

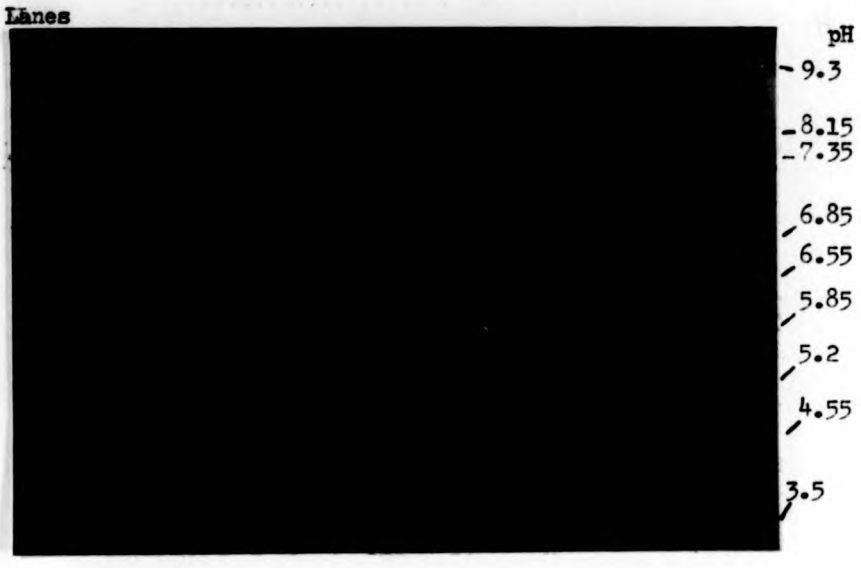
7.3.6 Plasmids of Cl.tetani

When the 27 strains of Cl.tetani were examined for plasmids, a single large plasmid was observed in 23 of the 27 strains (Fig.59). This plasmid was thought to be greater than 55 kd molecular weight by comparison to plasmids of known molecular weight contained in Esch.coli V517. No other plasmids were seen in strains of Cl.tetani. Strains which did not carry this large plasmid were CN1342, LQ931, LQ914 and L777A (Table 50).

7.3.7 Bacteriophages of Cl.tetani

When all 27 strains of Cl.tetani were tested against each other to demonstrate production of, or susceptibility to bacteriophages, no areas of inhibition were produced by any strainagainst any other strain.

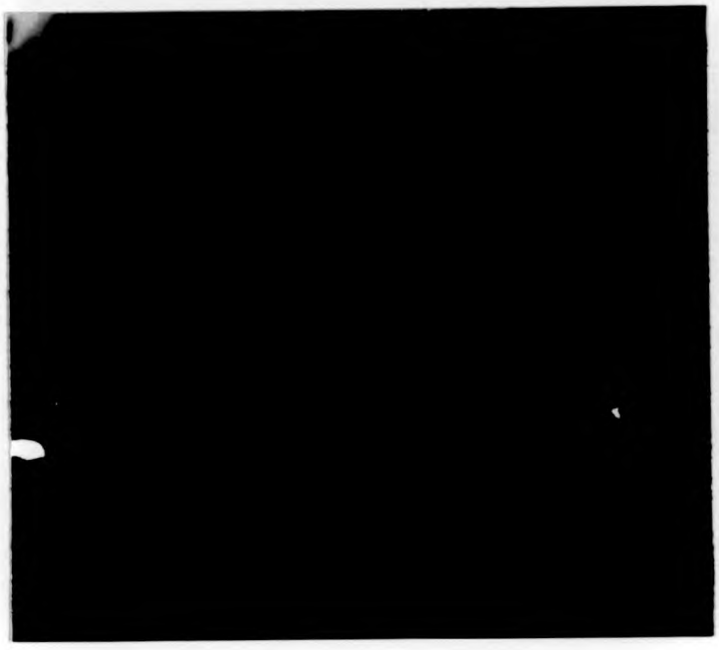
FIG.58 Isoelectric focussing of Cl.tetani proteins



Lanes: 1) serotype I 2) serotype II 3) serotype III
4) serotype IV 5) serotype V 6) serotype VI
7) serotype VII 8) serotype VIII 9) serotype IX
10) IEF markers 11) blank

Note: the presence of an extra band at around pH 4.25 with serotype II, V and IX strains. (arrow)

FIG.59 Plasmid carriage by Cl.tetani strains



Lanes 1) serotype I 2) E.coli V517 3) serotype II 4)serot ype III

5) serotype IV 6) serotype V 7) serotype VI 8) serotype VII

9) serotype VIII 10) serotype IX 11) L5500 12) CN1349

13) E88 14) E.Coli V517 15) LQ 730 16) CN 947 17) CN4878

Note: the presence of a single plasmid with each strain (arrow)

7.3.8 **Comparison of sporulating potential, toxin and haemolysin production, biochemical and metabolic activities, motility, antibiotic patterns, PAGE and IEF patterns and plasmid carriage in strains of Cl.tetani**

When the above characteristics were compared (Table 51), the only definite relationship to emerge was that between plasmid carriage and neurotoxin production. 24 strains produced neurotoxin and all possessed a single large plasmid with the exception of one, strain L777A, which did not appear to possess this plasmid but was toxigenic. The other 3 strains that lacked the plasmid were all nontoxigenic.

There were no other discernible relationships apparent. When sporulating potential was plotted against either neurotoxin production or haemolysin production (Fig.60 and 61) the distributions appeared almost random and the three properties appeared unconnected.

FIG.60 **Comparison of sporulating potential and neurotoxin production in 27 strains of Cl.tetani**

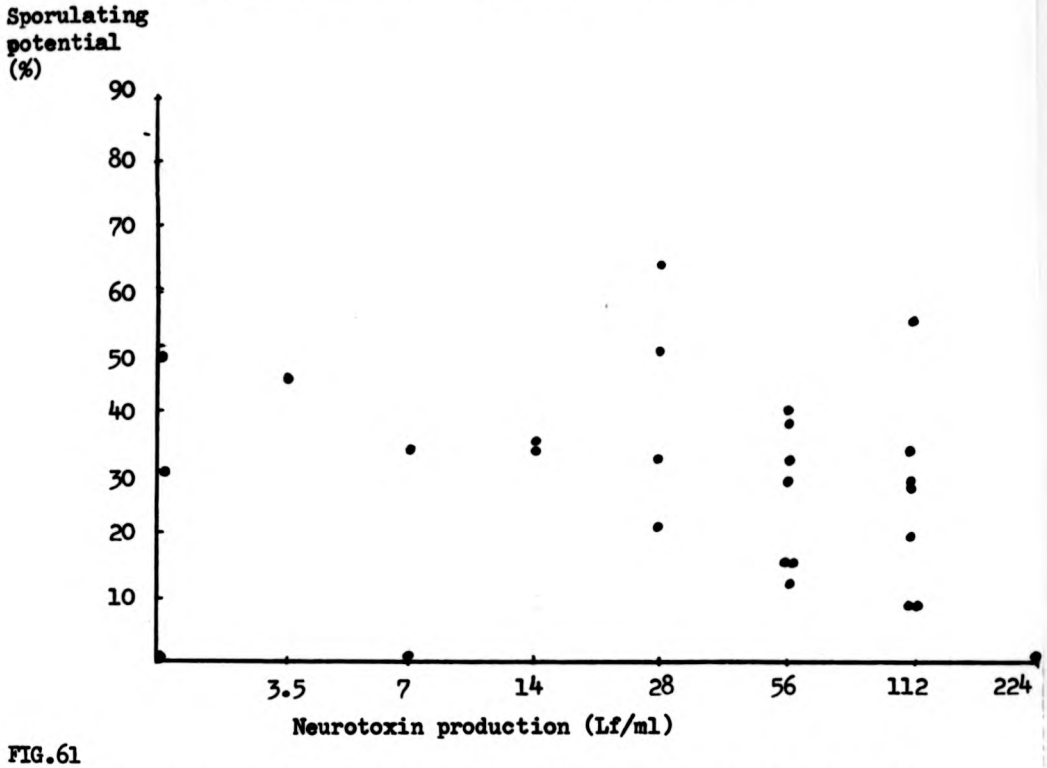
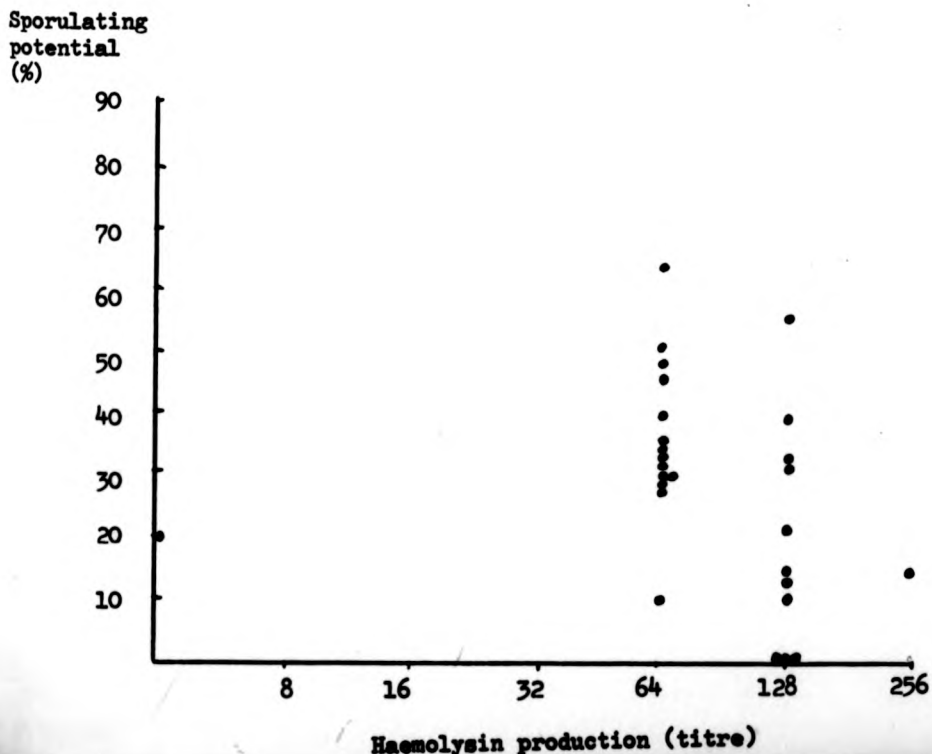


FIG.61 **Comparison of sporulating potential and haemolysin production in 27 strains of Cl.tetani**



7.4 DISCUSSION

To study the sporulation process of Cl.tetani, ideally perfect synchronicity is required. However, the methods used in this study have been found to give a degree of synchronicity in Cl.botulinum which, although not ideal, was sufficient to reflect functional and morphological changes in the cells accurately,[215] therefore perfect synchronicity was not sought.

Cl.tetani was observed to begin producing spores in an increasing number of cells after 3 days incubation at 37°C but not until the 5th day at 30°C when grown in cooked meat broth, and to produce spores very poorly at either temperature in FAB. It is known that media containing much unchanged protein may support sporulation less well than media containing digested protein[152] and it is possible that the different peptones contained in the two media may be responsible for the differences observed. Alternatively, as it is suggested that sporulation, coming at the end of exponential growth, is the result of the depletion of the medium of some essential nutrient[235], It is possible that in FAB, although environmental controls have brought about the end of exponential growth, the particular nutrient in question is not depleted and therefore sporulation does not proceed so readily as in cooked meat medium. The identity of this theoretical nutrient is, however, not known. The finding that cultures grown at 30°C (less than the optimum growth temperature) take longer to begin sporing is not surprising as obviously the growth rate is lower and therefore essential nutrients will not be depleted as quickly.

In common with the findings of Day and Costilow[215] working with Cl.botulinum, this study found that there was a significant decrease in the amount of acetic acid present in the culture supernatant as the number of spores increased, although the amount of butyric acid (measured as a % of the total volatile fatty acids present) remained relatively constant.

When determinations of the sporulating potential of all 27 strains were made repeatedly, up to ± 50% deviations were found between different observations from the same strains. This then throws a degree of doubt upon the significance of all conclusions drawn using these figures. Other workers have used the number of cells resistant to heating at 80°C or 100°C determined by viable counts and most probable number methods to determine the sporulating potential of strains[51,212], and this approach possibly gives greater accuracy in determining the potential, but it may also produce aberrant strains as a result of heating, and this was a factor which it was felt important to avoid in this study. Also, studies using Cl.novyí have used Gram-stained spore counts successfully and felt the technique to be useful,[214] but the results of this study suggest that the intrinsic inaccuracies of this technique may be a severe drawback.

Different strains of Cl.tetani appear to spore better on some media than on others but cooked meat medium tended to produce more spores from the greater majority of strains. Some strains did not sporulate on any of the media, or only sporulated very poorly, whilst others sporulated well on one or two of the media but not on the third, or vice versa. The reasons for these differences are unclear but it has been suggested that specific 'sporulation factors' may be required for sporulation[235] and it is possible that different strains of Cl.tetani are able to produce these in differing amounts, some better on one medium and some better on others, hence some strains sporulate better on one medium and some sporulate better on others.

Dipicolinic acid (pyridine 2,6-dicarboxylic acid) is a major component of clostridial spores[235] and is only found in spores, not in vegetative bacteria. There is a direct relationship between the synthesis of dipicolinic acid and the development of heat resistance[215] and the results of this study suggest that there is a general proportional

relationship between the sporulating potential and the amount of dipicolinic acid present in the cultures when different strains of Cl.tetani are investigated. The relationship is not perfectly linear however, although, as suggested earlier, the inaccuracies in determining the sporulating potential may have exacerbated this. On the other hand of course, and for the same reasons, any supposed relationship may be simply an artifact, but this is unlikely given the established relationships between the two properties[215,227].

As expected, the vast majority of strains investigated proved to be motile, and quite vigorously so. Only NCTC 9569, a known non-motile strain[167] was consistently non-motile and non-flagellate, but strains E88 and CN1349 both were non-swarming on agar plates and in semisolid motility test agar, although both showed some sluggishly motile organisms in wet preparations and had flagella demonstrable by flagella staining. Strain CN1349 is a subculture at some time in the past from strain E88 which is the specially adapted strain developed for toxin production (the Harvardstrain) and is known to be unusual. It is possible that these strains, because of their adaptation, have virtually lost the ability to swarm whilst retaining the ability to produce flagella (perhaps in reduced numbers). It was hoped that flagella staining would elucidate this but the results of these investigations were disappointing. Very few organisms in any preparation had visible flagella and even these seemed to occur only in patches, not completely surrounding the organism as in previous descriptions[151]. It was felt that during preparation for staining, the organisms must readily lose their flagella for some reason, leaving very few attached to the cells. It is possible that this loss of flagella has come about by repeated subcultures on solid media during the latter part of this study and is not a normal characteristic of the organism, as it does not appear to have been reported before. The suggestion that flagella loss occurs easily and is due to manipulation of the organism

is supported by the electron micrographs which show numerous fine hairlike structures, which could be flagella, around the cell bodies with motile strains, although relatively few appear still attached to the cells. The non-motile strain shows no similar structures. The fact that purified flagellin from Cl.tetani does not produce a precipitin peak with tetanus antitoxin when tested by two-dimensional IEP may be due to one of two reasons. Firstly, the flagellin isolation technique which was developed for Salmonella species, may have been inappropriate for Cl.tetani flagella, although this seems unlikely as bacterial flagella are all quite similar and it is logical to assume that methods developed for isolating one type should be effective with them all. Alternatively, and more likely, it is possible that the tetanus antitoxin, produced by inoculation of a mixture of 'intracellular' and 'extracellular' toxin, might not contain any precipitating antibodies to flagellar antigens, as there may have been no flagellar proteins in the original vaccinating dose.

Cl.tetani strainsshow no inhibition of swarming against each other and no areas of demarcation between swarming growths (Dienes' phenomenon). This phenomenon has been observed with Proteus species but is felt to occur only in intermittently swarming strains and to be due to mutually inhibitory substances produced into the medium. In strains that swarm continously Dienes' phenomenon is not seen, therefore. as Cl.tetani appear to swarm continuously (certainly there is no evidence of the rippled discontinuous swarming growth seen with Proteus species) it is not surprising that the phenomenon was not seen in this study.

Different strains of Cl.tetani appear to be markedly uniform in their antibiotic susceptibility patterns, even to the extent of producing MICs that were virtually identical. This was unexpected as the strains were collected from a wide geographic area and it has

been shown that with some clostridia, for example Cl.difficile notable differences were observed in susceptibilities to chloramphenicol, clindamycin, erythromycin, rifamycin and tetracycline. All of these with the exception of rifamycin were tested in this study.

Other workers have found that Cl.tetani strains can be differentiated by the different patterns their cellular proteins produced on PAGE,[223] and these techniques have been extended to include Cl.difficile[222,224], Cl.botulinum[236] and many other clostridia[223]. In fact, electrophoretic methods have now been accepted as having suitable resolution and reproducibility to form the basis for systems of bacterial taxonomy based on whole-cell protein patterns.[237] It has been suggested that the use of a standard technique to produce the patterns would be advantageous and allow comparability, and the suggested method of preparation is by boiling the cells in SDS.[237] However, when this technique was used in this study, there were no real differences in the patterns produced suggesting that either the strains investigated were extremely homogenous, a situation which is manifestly not so when the serotype, sporulation potential, toxigenicity and biochemical properties of the different strains are considered, or that this method of sample preparation is not applicable to Cl.tetani. Other workers have used mechanically disrupted cells to produce patterns that were useful in differentiating strains of Cl.tetani[223] but the equipment to disrupt cells in this manner was not available in this study. The synthesis of cellular proteins is the major expression of the microbial genome which is why the use of protein patterns show such reproducibility and good correlations with DNA-DNA hybridisation studies,[237] and it is only logical to extend this expression to extracellular proteins as well, as these are also thought to be under control of the genome.[108] The finding that culture supernatants from different strains of Cl.tetani produced distinctive

PAGE patterns would tend to support this view. It had been thought that IEF of whole cell or culture supernatant proteins might also produce patterns that could be used to differentiate the strains, but IEF of whole cell proteins was not successful, producing only dark smears and was not further investigated. IEF of culture supernatants produced only faint bands making the use of concentrated culture supernatants necessary. These produced distinct patterns but again these were generally very similar from strain to strain with the single exception that a distinct acidic band, around pH 4.25, was reproducibly produced by several strains. The identities of the proteins producing the PAGE and IEF patterns is not known. At first it was felt that at least one of the PAGE proteins might be a subfragment of the neurotoxin, but when PAGE patterns and toxicity of each of the strains was compared there was no correlation as some nontoxic strains produced identical band patterns to toxic strains. It was also felt that, as it has been shown that cellular PAGE protein patterns change during sporulation,[236] the sporulating potential of each strain may affect the culture supernatant PAGE patterns obtained here, but by comparison of the patterns observed and the sporulating potential for each strain no such relationship was proven. This may be due to thefact that it appears to be the low-molecular weight proteins that are mainly produced and altered during sporulation,[236] and it was high-molecular weight proteins that formed the basis of the culture supernatant PAGE patterns observed in this study.

Although plasmids with molecular weights of 76kb[226] and between 25 and 70 kb[40] have been observed in strains of Cl.tetani, in this study only a single large plasmid was found. This corresponded with neurotoxin production in all cases but one and was almost certainly the 75 kb plasmid which has been found to be the carrier for the structural gene for tetanus toxin production by others.[225,226]

The method of plasmid preparation used here was not, however ,

identical with the methods used by others but was quicker and less tedious than the 3 day method used by one study[225] and appeared more reproducible than that used by the other[226]. It has been proven to be suitable for isolating plasmids of both low and high molecular weight,[231] and plasmids with molecular weight of 55 kd as well as those with molecular weights of 2 kd were easily isolated during this study. It seems then, that the strains of Cl.tetani studied here do not possess any other plasmids and hence it must be concluded that neither haemolysin production nor other physical or biochemical characteristics are plasmid mediated.

Although a high percentage of Cl.perfringens strains have been noted to produce bacteriocins (or some inhibiting substance) detectable with suitable indicator strains,[234] none of the strains in this study was capable of producing any substance which could inhibit the growth of any other strain. This is perhaps not surprising as the production of inhibitory substances ought to have been observable to some extent in the swarming experiments (7.2.7).

7.5 CONCLUSION

Sporulation in Cl.tetani varies from strain to strain, in different media and at different temperatures. Sporulation began after 3 days incubation at 37°C in cooked meat medium but not until the 5th day at 30°C.

The amount of acetic acid in the culture supernatant decreases as sporulation proceeds, but the amount of butyric acid remains relatively constant, and the amount of dipicolinic acid present in the cultures appears to show a general proportional relationship to the number of spores present. However, quite major variations were observed between repeated determinations of sporulating potential (± 50%), and this means that any comparisons between other physical or biochemical properties and the sporulating potential of different strains of Cl.tetani must be viewed with caution.

Most strains of Cl.tetani are demonstrably motile by any method, but occasional strains appear non-motile by some methods and sluggishly motile by others. Flagella are difficult to demonstrate consistently and the organisms appear to lose them very easily.

None of the strains investigated produced bacteriophages or other inhibitor substances active against any other strain, and no Dienes' phenomenon was seen.

The Cl.tetani strains investigated here showed very similar antibiotic susceptibility patterns, and no strain was significantly different in this respect from the others.

PAGE patterns of cellular proteins were extremely homogenous between strains but patterns produced by culture supernatants allowed the strains to be differentiated into 5 groups on the basis of 4 high molecular weight proteins. Also, examination of concentrated culture supernatants by IEF allowed the strains to be differentiated into two groups based on the absence or presence of an acidic band around pH 4.25.

A high molecular weight plasmid was found in all toxigenic strains with one exception. This plasmid was not present in nontoxigenic strains. This direct relationship between plasmid carriage and neurotoxin production was the only distinct relationship observed between the different characteristics of Cl.tetani strains investigated in this study. These characteristics included sporulating potential, motility, PAGE and IEF patterns, Gelatin liquefaction, casein hydrolysis, RNase/DNase, fluorescence on MacConkey agar, amino acids deaminated, haemolysin titre, plasmid carriage and neurotoxin production.

CHAPTER 8

THE ANTIGENIC STRUCTURE OF CL.TETANI

8.1 SURVEY OF THE LITERATURE

In addition to the antigenic neurotoxin and haemolysin, Cl.tetani strains also possess flagellar antigens[2] and somatic or cell wall antigens[11,217].

It is claimed that nine serologic types of Cl.tetani (I - IX) can be found by agglutination reactions based on flagellar or 'H' antigens which were thermolabile.[239] Using agglutination techniques a heat stable somatic or 'O' antigen was also found which appeared to be common to all strains, and, after absorption of this antigen, a further heat stable antigen was found which was possessed by type II, IV, V and IX strains.[239]

Both flagellar and somatic antigens were involved in complement fixation tests and cross reactions were seen between all types at low titre due to the common somatic antigen and at high titre with type II, IV,V and IX strains due to the extra somatic antigens.[217]

Mandia[240] has incorporated Cl.tetani into a serological grouping system of Clostridia based primarily on two heat-stable somatic antigens, and has distinguished the nine types using 3 heat-labile antigens, but found that one of the somatic antigens was also shared by strains of Cl.sporogenes, Cl.parabotulinum and Cl.histolyticum.

The observation of more than one antigen by agglutination techniques requires tedious agglutination-absorption experiments and titrations, and typing by agglutination requires a separate antiserum to be produced for each serotype, therefore some workers have endeavoured to investigate the relationship of different strains of clostridia by double immunodiffusion (DID) using a single antiserum,[216] where each individual antigen-antibody reaction can be visualised separately. Using concentrated culture filtrate soluble antigens (which could be either somatic or flagellar) no fewer than 12 antigens were found from

6 different strains, none of which were common to all strains. The majority of strains possessed 4 to 6 antigens each and showed extreme heterogeneity.[216]

It was felt that an investigation along similar lines, using a single tetanus antiserum and both culture supernatant and cellular protein antigens investigated by the much more discriminatory technique of two-dimensional IEP might prove extremely valuable in that it might provide a method of serotyping Cl.tetani strains without having to resort to separate antisera and absorption experiments, and if so, the place of heat labile and heat-stable antigens in such a scheme might be more easily visualised.

Also, it has been suggested that bacterial spores contain specific precipitating antigens distinct from vegetative cells[241] and that these antigens are heat-labile therefore sporulating strains of Cl.tetani might show precipitin peaks specific for these spore antigens thus allowing their place in the scheme to be established.

Differentiation of Cl.septicum and Cl.chauvoei by using fluorescent labelled antibodies has been accomplished[242] and Cl.tetani has been identified using a similar technique,[243,244] therefore the use of such a technique was investigated in this study.

The following investigations were therefore undertaken.

1) **A study of the soluble antigens of Cl.tetani by agglutination, double immunodiffusion and immunoelectrophoresis techniques.**

2) **A study of the antigenicity of Cl.tetani strains by immuno-fluorescent antibody techniques.**

8.2 EXPERIMENTAL TECHNIQUES

8.2.1 Agglutination testing of Cl.tetani strains

Due to the relatively large amounts of antiserum required for these tests and the limited amounts available, it was decided to only investigate a single representative of each serological type in the first instance.

The nine NCTC strains of Cl.tetani representing serotypes I - IX were grown for 48 hours in FAB at 37°C. Following incubation the cells were deposited by centrifuging and washed once in sterile saline. The cells were then resuspended in saline to a turbidity equivalent to a MacFarland No.4 opacity tube (equivalent to approximately 12×10^8 orgs/ml) and formalin was added to a final concentration of 0.3%.

These suspensions were then tested by the tube agglutination technique (Appendix 42) and the resulting agglutination titres compared.

'O' agglutination tests were not performed as the common 'O' antigen would be expected to give positive reactions in all cases.

8.2.2 Examination of Cl.tetani culture supernatants by Double Immunodiffusion (DID)

As this technique has been used before to investigate Cl.tetani soluble proteins,[216] it was included in the study to investigate possible differences between different agglutination types, as this has not been investigated previously.

The nine Cl.tetani strains (serotypes I - IX) were grown in FAB medium for 4 days at 37°C before being centrifuged at 1,500 x g to deposit the cells. The culture supernatants were removed and tested against Wellcome tetanus antitoxin[71] by DID (Appendix 14).

8.2.3 Examination of Cl.tetani culture supernatants by two-dimensional IEP

Different workers have used strains grown for differing periods to produce their antigens for serological tests. These periods

vary from 24 or 48 hours[217,239,245] to 4 days[240,246] or even 6 days[216] therefore it was felt that it would be advisable to investigate the soluble antigenic pattern of strains grown for different periods by 2-dimensional IEP. Also, as heat-labile and heat stable antigens are known to be involved in serological reactions with Cl.tetani, the differences between heated and non-heated culture supernatants were also examined by 2-dimensional IEP.

Cl.tetani NCTC 279 (serotype I) was grown in FAB medium at 37°C for 5 days. Aliquots were removed on days 1, 2, 3 and 5, the cells removed by centrifugation and the culture supernatants stored at -40°C until required. These four aliquots were examined by two-dimensional IEP (Appendix 14) using Wellcome tetanus antitoxin.

In addition, an aliquot of the 3 day culture supernatant was heated by boiling for 10 minutes, and both this and the unheated 3 day culture supernatant were tested simultaneously by two-dimensional IEP.

8.2.4 Examination of Cl.tetani somatic antigens by two-dimensional IEP

8.2.4.i Mechanically disrupted antigens

The patterns produced by using Cl.tetani culture supernatants proved to be weak and difficult to visualise. Therefore, it was felt that the use of soluble protein antigens derived from deposited cells that had been mechanically disrupted[223] might produce stronger and more reproducible patterns.

The nine strains of Cl.tetani were grown for 3 days at 37°C in FAB medium before being centrifuged to deposit the cells. The supernatants were discarded and the deposits resuspended in 0.2ml of PBS. Approximately 0.5g of 1.5μm diameter glass beads[69] (approx.40) were added and the suspensions shaken at 150 rpm for 3 hours at room temperature on a rotary shaker.[247] Following this the mixtures were centrifuged to remove cell debris and beads and the supernatants examined by two-dimensional IEP. Again an aliquot of the serotype I extract was heated by boiling for 10 minutes and tested simultaneously with the unheated extract to investigate whether any heat-labile

proteins were present.

In addition to the 9 Cl.tetani strains already tested, a heat-treated variant of the serotype I strain which was highly sporing (Appendix 10) was grown and disrupted in an identical manner and investigated by IEP at the same time. At the time of investigation the sporulating potential of this strain was 44%.

8.2.4.ii Triton X-100 extracted antigens

To exclude the possibility that mechanical disruption of Cl.tetani cells might destroy or denature some antigens, a specific protein extraction technique which was somewhat gentler in action was sought. The non-ionic detergent Triton X-100 has been successfully used to solubilise membrane proteins prior to immunoelectrophoresis[248] and this technique was used here. The 9 strains of Cl.tetani were grown for 3 days at 37°C in FAB medium before being centrifuged to deposit the cells. These were resuspended in 0.2ml of 1% (v/v) Triton X-100[69] and left at 4°C overnight. The cells and debris were removed by centrifugation and the supernatants were examined by two-dimensional IEP.

8.2.5 Examination of Cl.tetani strains by Immunofluorescent antibody Techniques (IFAT)

8.2.5.i Use of Cl.tetani fluorescent conjugate absorbed with Cl.perfringens

A fluorescent antibody to Cl.tetani was produced using Wellcome tetanus antitoxin[71] (Appendix 25).

This was found to exhibit non-specific fluorescence with Cl.perfringens cultures, and was therefore absorbed with Cl.perfringens cells to remove this (Appendix 25). The optimal dilution of this Cl.perfringens absorbed fluorescent

conjugate (CPFC) was found to be neat.

The nine strains (serotypes (I - IX) were grown anaerobically at 37°C for 3 days on Columbia blood agar plates before each was emulsified in PBS to a turbidity approximately equivalent to a MacFarland No.1 opacity tube. 10 ul of each suspension was pipetted onto a 6mm diameter well on a multispot PTFE coated slide[250] and allowed to air dry. The slides were then fixed for 10 seconds in acetone and dried. 2 ul of CPFC was pipetted onto each well and the slides were incubated at 37°C for 30 minutes in a humidity chamber. Following incubation the slides were rinsed in PBS before being given two 10 minute washes in PBS, a final rinse in distilled water and then being air dried.

The stained slides were examined by epi-fluorescence on an SM-Lux microscope fitted with an ultraviolet Ploem-pak FITC system and a mercury vapour lamp.[251] The degree of fluorescence was assessed as +++ (very strong), ++ (strong), + (moderate), ± (weak), trace (barely visible) or - (negative).

The same experiment was repeated with 3 day FAB deposits of each of the strains resuspended in PBS to a MacFarland No.1 opacity tube and dried onto multiwell slides in the same manner as before.

8.2.5.ii <u>Use of Cl.tetani fluorescent conjugate absorbed with the nine serotype strains of Cl.tetani</u>

To investigate whether the fluorescent conjugate could be used as a simple technique to serotype unknown strains of <u>Cl.tetani</u> 100 ul aliquots of CPFC were absorbed using each of the nine serotype strains (I - IX) using the technique described in Appendix 25. These were then designated CPFC Abs I, CPFC Abs II etc. respectively.

Three day FAB deposits were prepared for each of the 9 serotype

strains as described earlier (8.2.5.i).

10 ul of each of these preparations was pipetted onto 10 wells of a multispot slide, was allowed to air dry and was then acetone fixed. 2 ul of CPFC was pipetted onto the first well of each slide and 2 ul of each of the serotype absorbed CPFC (CPFC Abs I to CPFC Abs IX) was pipetted onto the next nine wells respectively. The slides were stained, washed, dried and examined as before.

If this system was to be useful for serotyping then a strain stained with homologous absorbed conjugate should show minimal or no fluorescence while the same strain stained with any of the 8 heterologous absorbed conjugates should show staining as strong as the original CPFC reagent.

8.3 RESULTS

8.3.1 Agglutination testing of Cl.tetani strains

When the 9 serotypes of Cl.tetani were tested against Wellcome tetanus antitoxin, no agglutination was seen in 8 cases. One strain, serotype II, gave an agglutination titre of 1:80. (Fig.62a Table 52

8.3.2 Examination of Cl.tetani culture supernatants by Double immunodiffusion (DID)

When the 9 serotypes of Cl.tetani were tested, between one and three precipitin lines was seen with each strain (Fig.62). Serotypes I, II, III and IX produced 2 lines, serotypes IV, V, VII and VIII produced 3 lines and serotype VI produced only one line (Table 53). Reactions of identity were observed between the lines produced by different strains. One line appeared to be common to all strains.

8.3.3 Examination of Cl.tetani culture supernatants by two-dimensional IEP

When 1, 2, 3 and 5 day FAB culture supernatants were examined by two-dimensional IEP, a progressive pattern of peaks could be seen (Fig.63) increasing from one small strong peak and 3 very small weak peaks in the 1 day supernatant to 3 strong and 3 weak, 3 strong and 4 weak and 3 strong and 4 weak peaks in the 2 day, 3 day and 5 day supernatants respectively.

There were no differences between the 3 day and 5 day supernatants except that the peaks in the latter were slightly higher.

When the heated and non-heated 3 day supernatants were tested simultaneously, no qualitative differences were seen between the patters (Fig. 64) and only one peak (no.2) had its height slightly reduced by heating.

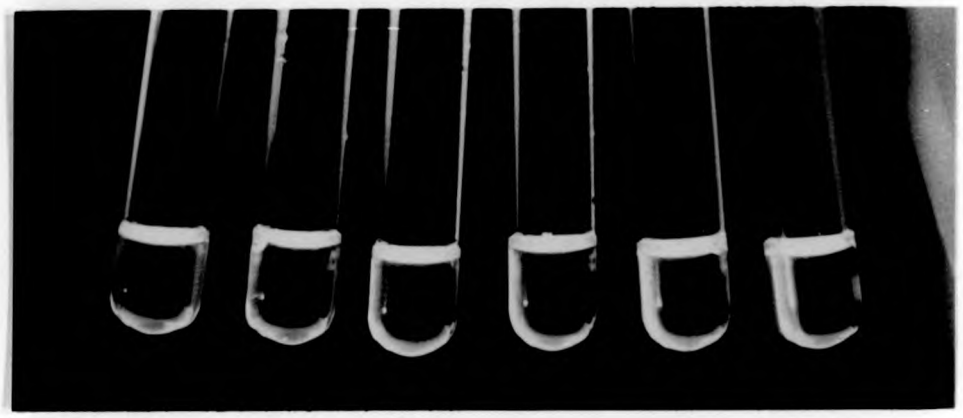
8.3.4 Examination of Cl.tetani somatic antigens by two dimensional IEP

8.3.4.i Mechanically disrupted antigens

When unheated and heated disrupted extracts from serotype I cells were examined simultaneously, no significant quantitative

FIG.62a

Agglutination testing of Cl.tetani



Wells 1 2 3 4 5 6

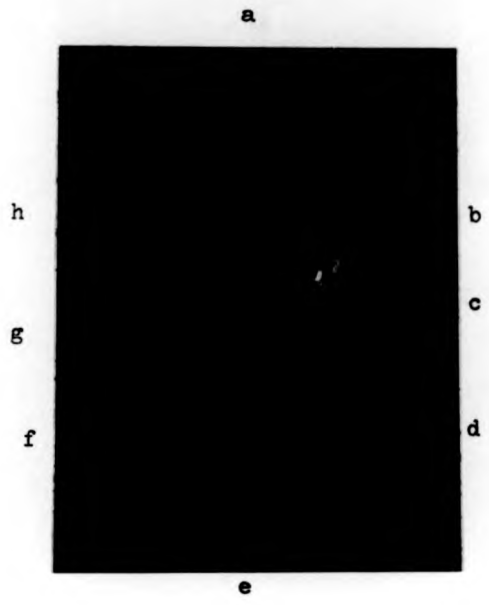
Dilution of antisera in wells:

1) 1:10 2) 1:20 3) 1:40 4) 1:80 5) 1:160 6) 1:320

Strain tested = NCTC 540 (serotype II)

Note: very faint agglutination in first 4 wells.

FIG. 62 Double immunodiffusion of Cl.tetani FAB culture supernatants



Wells:

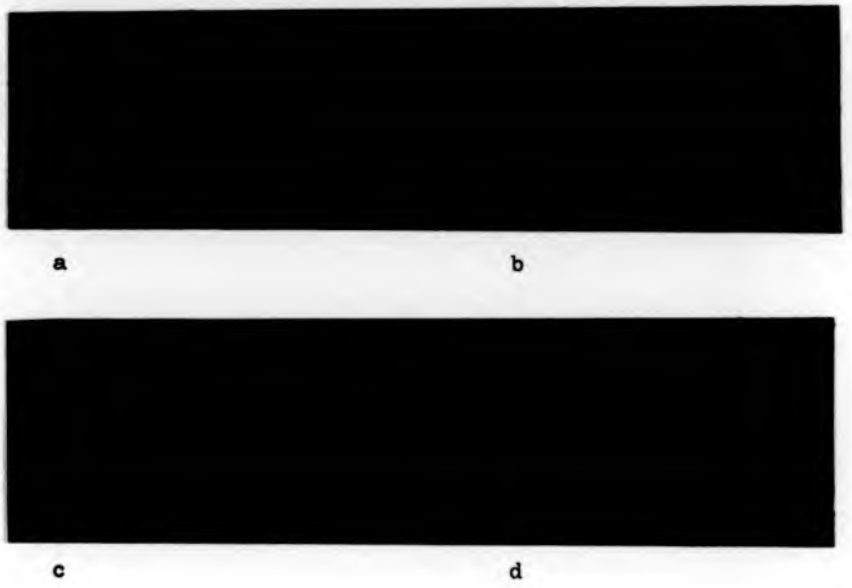
A = antitoxin

a, serotype II; b, serotype III; c, serotype IV; d, serotype V;

e, serotype VI; f, serotype VII; g, serotype VIII; h, serotype IX

Note: precipitin line common to all strains (arrow)

FIG. 63 Two-dimensional IEP investigation of 1, 2, 3 and 5 day FAB Cl.tetani culture supernatants



Wells: a- 1 day FAB culture supernatant (serotype I) 30 ul

b--2 day FAB culture supernatant (serotype I) 30 ul

c- 3 day FAB culture supernatant (serotype I) 30 ul

d- 5 day FAB culture supernatant (serotype I) 30 ul

diagrammatic representation:

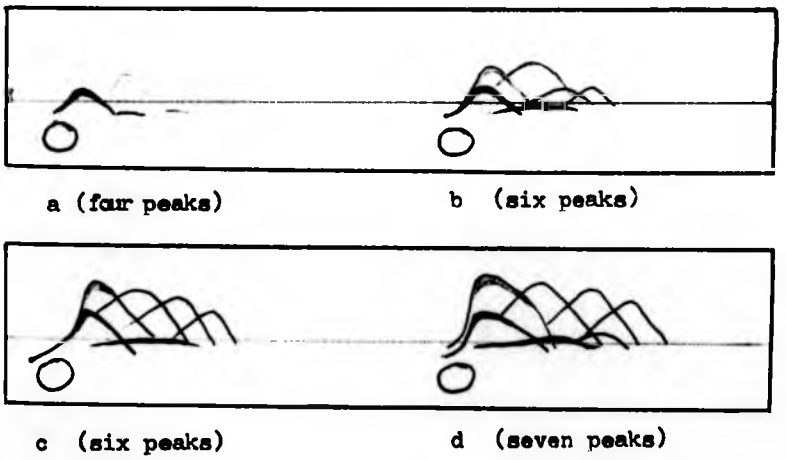
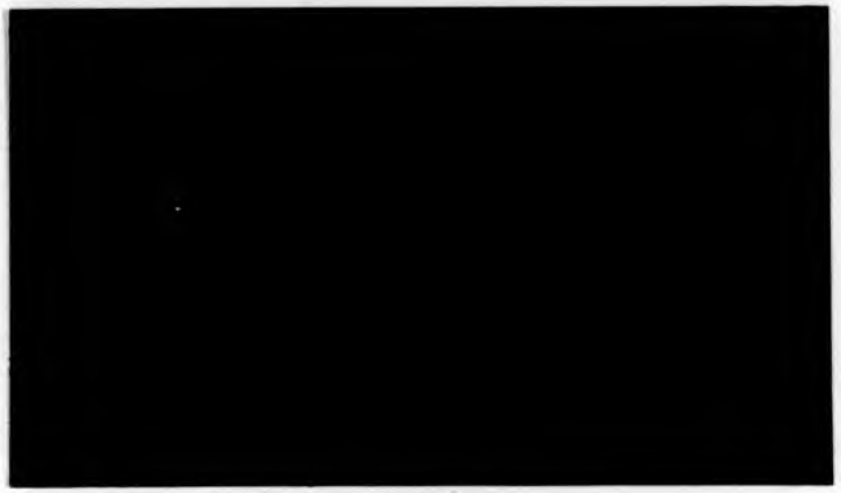


FIG. 64 Two-dimensional IEP investigation of unheated and heated 3 day FAB culture supernatant of Cl.tetani NCTC (serotype 1)

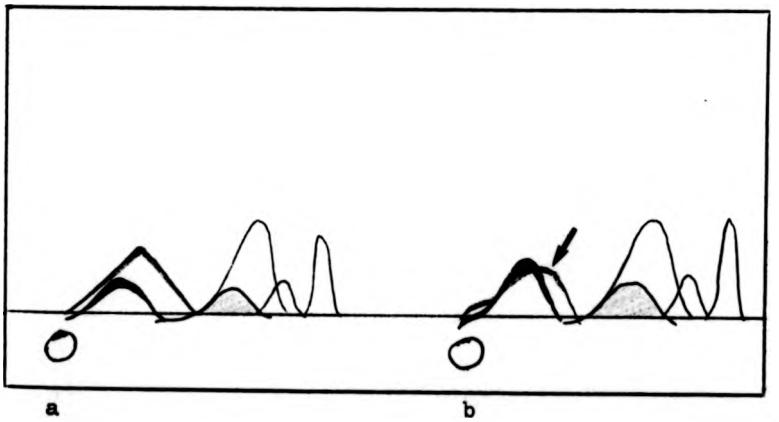


a b

Wells: a) unheated supernatant 30 ul

b) heated supernatant 30 ul

diagrammatic representation:



a b

Note: Peak 2 has been reduced slightly in height by heating.(arrow)

No peaks have been removed by heating.

Six peaks are present.

or qualitative differences were observed.

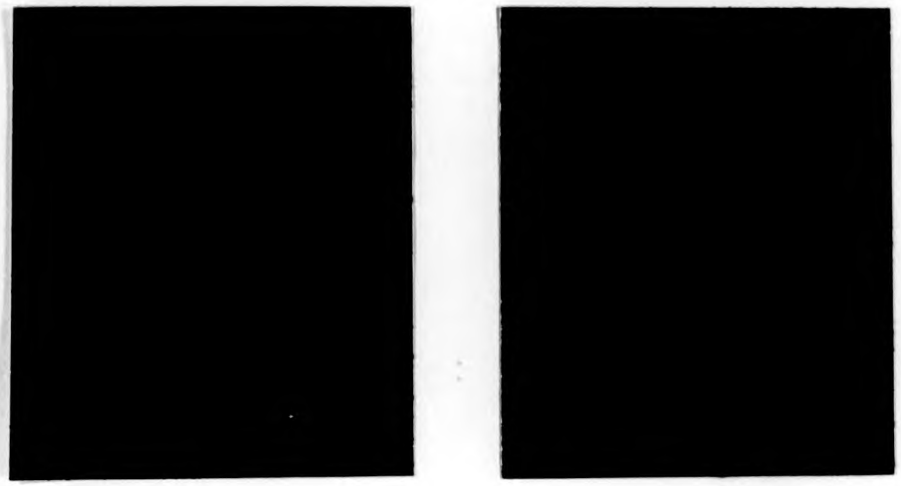
When the extracts produced by the serotype I strain and the highly sporulating, heat-treated variant of the serotype I strain were tested, virtually identical patterns were produced (Fig.65). Six peaks were produced by both extracts.

When extracts from all 9 strains of Cl.tetani were examined, between 5 and 8 precipitin peaks were produced by each strain (Fig.66)(Table 53). Serotypes I and IX produced 8 peaks, serotypes II, III, IV, V and VII produced 7 peaks, serotype VIII produced 6 peaks and serotype VI produced 5 peaks. Using the 8 peaks produced by the serotype I strain as a model, each peak was given a designated number from 1 to 8. The patterns produced by the other strains were compared to this standard pattern (Fig.66b) and found to be virtually identical save for occasional missing peaks. The missing peaks were almost exclusively those designated 1 or 2 (Fig.66)

8.3.4.ii Triton X-100 extracted antigens

When the 9 strains were examined using Triton X-100 extracted antigens, very similar precipitin patterns to those seen with disrupted antigens were found. In general though, the heights of the peaks produced by the Triton antigens were greater (Fig.67). Between 5 and 7 peaks were produced by each strain (Table 53). Serotypes I, III, IV, V, VI and IX all produced 7 peaks, serotype VII produced 6 peaks and serotype VIII produced 5 peaks. The peak designated No.7 was missing from all strains (Fig.67).

FIG.65 Two-dimensional IEP of disrupted cellular antigen extracts of Cl.tetani serotype I strain and a heat treated highly sporing variant of this strain

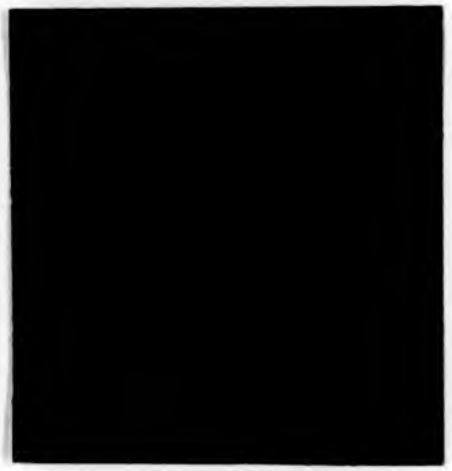


Wells: a) disrupted antigen of Cl.tetani serotype I cells (15 ul)

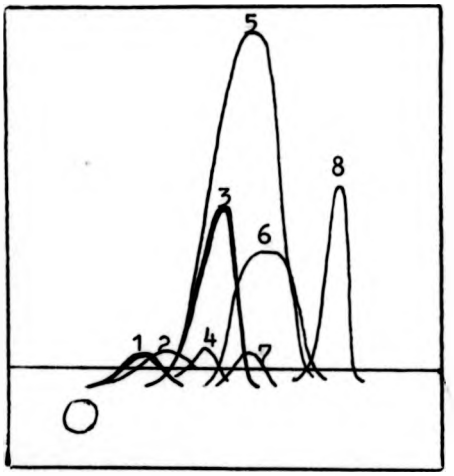
b) disrupted antigen of Cl.tetani serotype I heat-treated variant cells (15 ul)

Note: The presence of six peaks in both extracts

FIG.66 Two-dimensional IEP of mechanically disrupted cellular antigens of Cl.tetani strains



a) serotype I strain

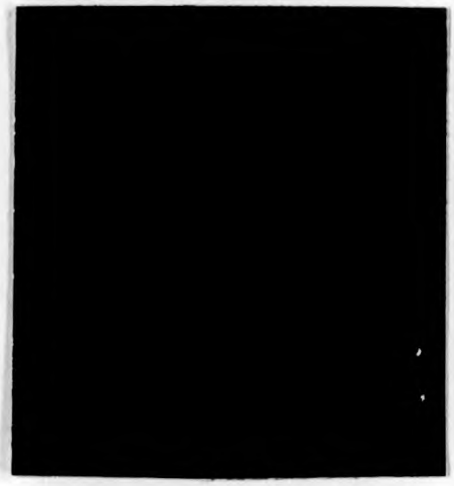


b) number and designation of the 8 peaks visualised with Serotype I.

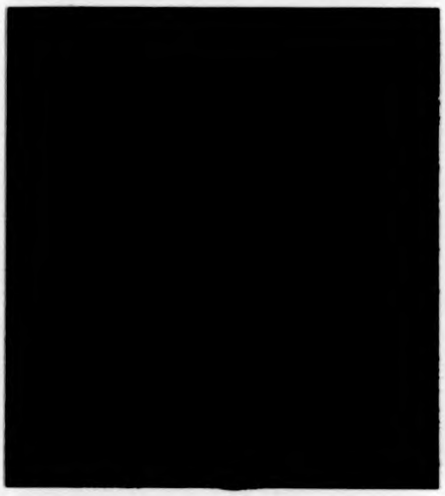
c) serotype II

d) serotype III

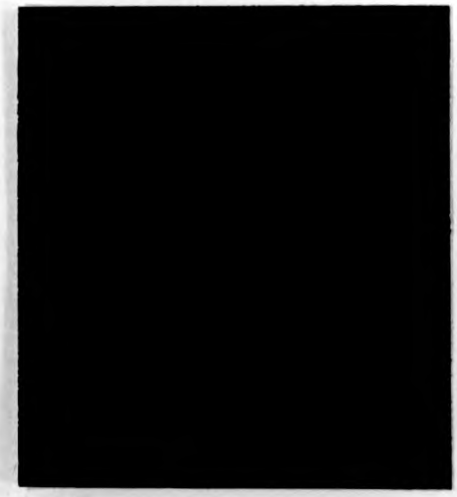
FIG. 66(continued)



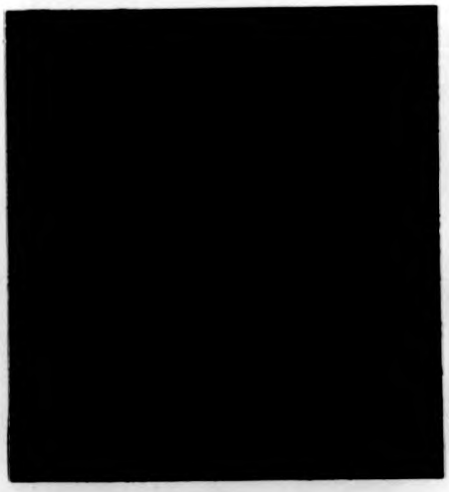
e) serotype IV



f) serotype V

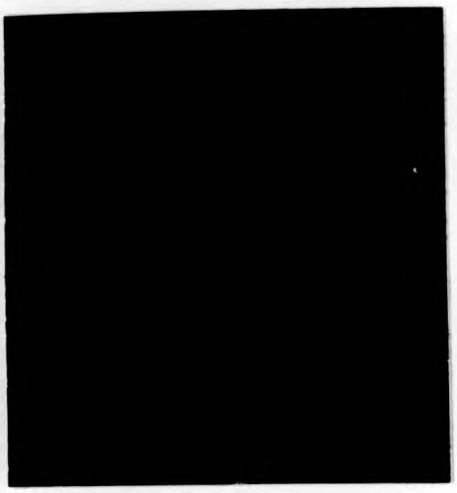


g) serotype VI

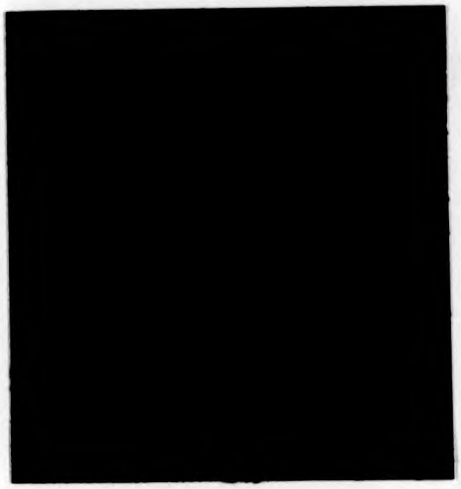


h) serotype VII

FIG. 66 (continued)

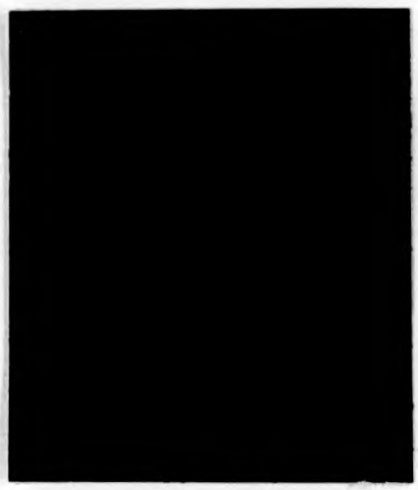


i) serotype VIII

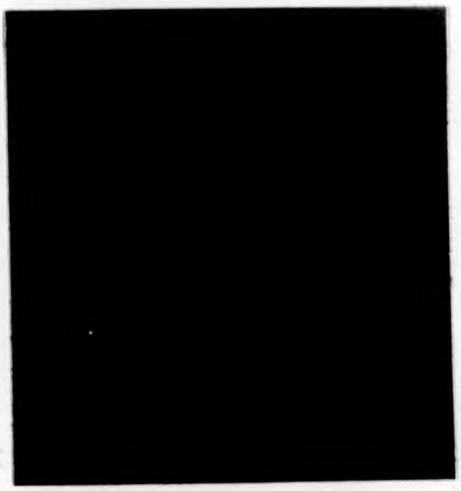


j) serotype IX

FIG. 67 Two-dimensional IEP of Triton X-100 extracted cellular antigens of Cl.tetani strains

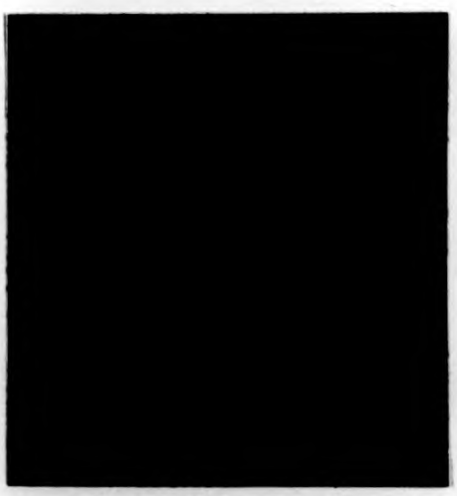


a) serotype I strain



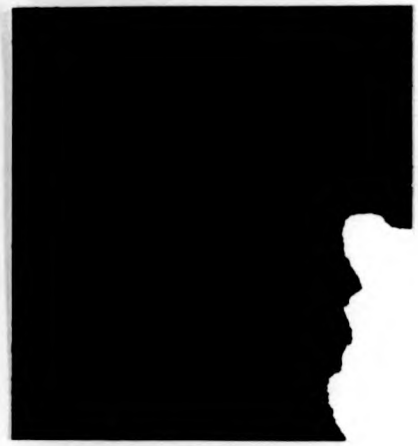
b) serotype II

c) serotype III

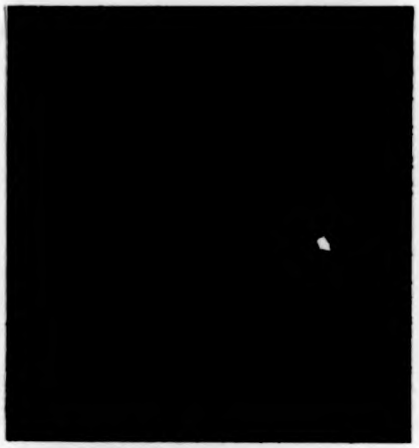


d) serotype IV

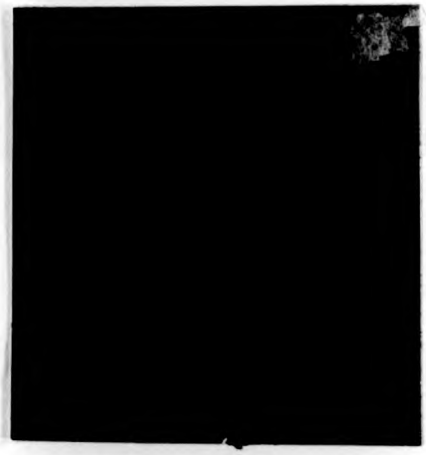
FIG. 67 (continued)



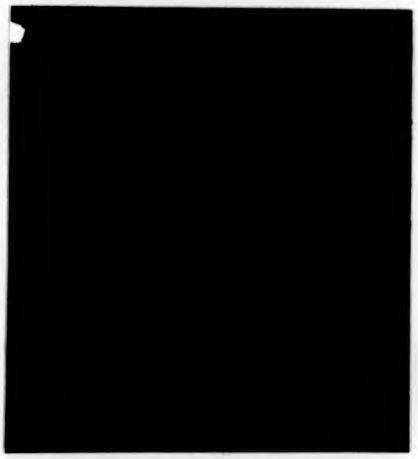
e) serotype V



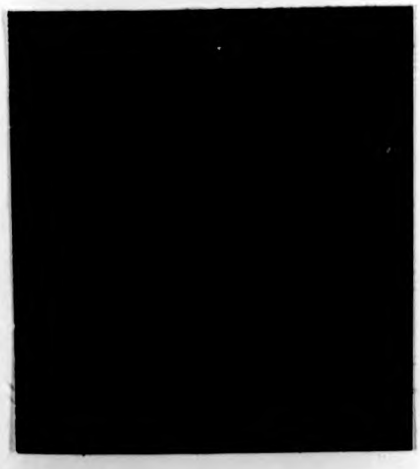
f) serotype VI



g) serotype VII



h) serotype VIII



i) serotype IX

8.3.5 Examination of Cl.tetani strains by Immuno-fluorescent antibody techniques (IFAT)

8.3.5.i Use of Cl.tetani fluorescent conjugate absorbed with Cl.perfringens

When the nine serotypes (I - IX) were examined using CPFC, it could readily be seen that the different strains showed differing degrees of fluorescence both from agar plates and broth deposits (Table 54).

Serotype III gave the strongest reactions regardless of cultural conditions followed by serotype II when grown in broth. The rest of the strains gave only moderate or weak reactions.

8.3.5.ii Use of Cl.tetani fluorescent conjugate absorbed with the nine serotype strains of Cl.tetani

When the results of this investigation were examined (Table 55) it could clearly be seen that absorbing CPFC with any strain of Cl.tetani removed all or most of its reactivity to other strains, regardless of serotype. Only the serotype III strain, which fluoresced brightly anyway, retained any noticable fluorescence and then only when stained with CPFC absorbed by serotypes V, VI, VII, VIII and IX which were amongst the weakest reactors.

8.4 DISCUSSION

The finding in this study that agglutination tests using the heat-labile flagellar 'H' antigens were unsuccessful was perhaps to be expected. There are several possible reasons for this. Firstly, given that the commercial antiserum used here is primarily directed against the neurotoxin,[101] it is possible that it contains no antibodies to flagellar proteins, however, as the toxoid used for the immunisation protocol (a mixture of culture filtrate and autolysate of Cl.tetani cells) was not available for study, this must remain a moot point. Other workers with 'H' agglutinations of Cl.tetani have recorded far higher titres than the maximum of 1:80 reached in this study (up to 1:640 and 1:1280[239]) therefore the results obtained here must all be regarded as negative. The single titre of 1:80 was possibly due to somatic antigens interfering. Secondly, the strains used here have already been demonstrated to readily lose their flagella, so the failure of 'H' agglutinations may simply be due to loss of flagella during the preparation of the cells.

Only between one and three precipitin lines were observed with different strains of Cl.tetani by DID although others have reported as many as 12 lines.[216] This discrepancy may be due to the fact that this study used simple 4 day old broth supernatants as antigens, whilst the other study used 6 day broth supernatants concentrated by ammonium sulphate fractionation and centrifugation.[216] The antiserum in that study was produced by immunisation of rabbits with this partially purified antigen and subsequent ammonium sulphate purification of the resulting antiserum, therefore it is hardly surprising that more antigens were detected. It is interesting to note however, that none of the antigens detected in six strains of Cl.tetani by Ellner and Green[216] were common to all strains, whereas at least one antigen detected in this study appeared to be common. As it is now established

that all strains of Cl.tetani share a common somatic antigen[216,217,239] it would be expected that this antigen would be demonstrated by most serological techniques, and the failure of the DID technique described by Ellner and Green[216] to do so is surprising.

When culture supernatants were examined by two-dimensional IEP over 5 days, a progressive pattern of soluble antigen production was seen starting with one major and three minor antigens and developing to three major and four minor antigens. It is suggested that the three strongest antigens are most probably the ones detected by DID. The finding that heating does not destroy any of the antigens detected confirms that only heat-stable somatic 'O' antigens are involved in these reactions, and that heat-labile antigens, if present at all, do not produce precipitin peaks in the system used in this study.

As culture filtrates produced only rather small peaks, and only somatic antigens were involved, it was felt that direct antigenic extracts from the bacterial cells might produce clearer and more reproducible patterns. This proved to be the case.

It was also felt that, if a nonsporing strain and a highly sporing variant of the same strain were examined simultaneously then perhaps a precipitin peak that was specific for spore antigens might be identified. This however, was not the case, as both strains produced identical precipitin patters. Perhaps the reason for this again lies with the antiserum used in this study. Generally, toxidity and sporulation potential of Cl.tetani strains are felt to be inversely related, and as the antiserum was produced primarily for its antitoxic properties it seems likely that the strains used to produce the immunising toxoid would be minimally sporulating therefore it is possible that the antiserum contains no spore-specific antibodies. Alternatively, as other studies have used boiling in acid to produce spore antigens[241], it may simply be the case that mechanical disruption of the cells may be too gentle a technique to free spore antigens from the spores. Two dimensional IEP of both mechanically disrupted

antigens and Triton X-100 extracted antigens of the nine serotype strains of Cl.tetani produced very similar patterns. The greatest number of precipitin peaks (8) were obtained with the serotype I and serotype IX strains and all 8 peaks were common to both strains. The other strains all exhibited the same pattern but with one or more peaks missing. It is interesting to note that all the Triton X-100 extracted antigens had peak 7 missing, and it is suggested that while the other 7 antigens are cell surface antigens and so are easily solubilised, antigen 7 is probably situated deeper in the cell wall, or in the actual body of the cell and is hence only released by disruption of the cell. Although it has been shown that, in addition to the common 'O' antigen possessed by all Cl.tetani strains, serotypes II, IV, V and IX possessed another antigen that was lacking in serotypes I,III, VI, VII and VIII,[217,239] no such antigen was seen in this study. It is, of course, possible that the antigen in question, which was detected by agglutination, was a non-soluble one and would therefore not be expected to appear in immunoprecipitin tests. The antigen common to all strains may have been antigen 3, 5, 6 or 8, but number 3 is by far the strongest and is surely the most likely candidate.

Given the marked homogeneity seen in these tests it is not surprising that IFAT results using absorbed sera also gave very uniform results. Absorption with any strain removed virtually all reactivity to all other strains even though the nine strains exhibited marked differences in intensity of fluorescence using CPFC serum. It is suggested that the major antigen involved in the IFAT reaction is probably the common 'O' antigen possessed by all strains, which explains why it is removed so readily by absorption with any strain. Absorption of the fluorescent conjugate with Cl.perfringens was required to remove non-specific fluorescence to that organism but the occurrence of non-specific fluorescence with other members of the clostridia was not investigated at this time.

It had been hoped that the different serotypes of Cl.tetani would produce different patterns of precipitation by DID or two-dimensional IEP, or that specific absorptions of the fluorescent conjugate by different serotypes of Cl.tetani would produce different staining patterns with different serotypes thus allowing serotyping of unknown strains to be easily accomplished using a single commercially available antiserum but this was not the case. The nine serotype strains could not be reliably differentiated by any of these methods.

Perhaps it was expecting too much of the commercial antiserum to expect sufficient different antibodies to be present to allow this differentiation, particularly since the actual number and serotypes of the strains used to produce the serum is unknown. However, the superior resolving power of techniques like two-dimensional IEP when compared to techniques like agglutination made the attempt worthwhile. Other workers have prepared antisera by immunisation with serotype-specific antigens or purified soluble antigen preparations, whereas the commercial antitoxin was produced using a toxoid. The presence of 8 antibodies to somatic antigens is almost certainly due to the presence of the antigens in the original toxoid as contaminants. It should be noted however, that these 'contaminants' must be fairly reproducible in different batches of toxoid, as experiments have shown that four different batches of antitoxin contain exactly the same antibodies.

8.5 CONCLUSION

The antiserum used in this study appeared to contain neither antibodies to flagellar 'H' antigens (or any other heat-labile antigens) nor antibodies to spore proteins.

The common 'O' antigen possessed by all strains of Cl.tetani appeared to be visualised by DID, two dimensional IEP and IFAT, but the further common antigen possessed by serotypes II, IV, V and IX was not detected.

The precipitation patterns produced by extracts of the different serotypes of Cl.tetani were extremely homogenous and therefore none of the techniques studies here including agglutination, DID, two-dimensional IEP or IFAT using absorbed antisera could differentiate any of the strains into serotypes.

The use of a single commercially available antiserum which was primarily produced as an antitoxin was probably the reason for the lack of differentiation between strains and its choice was probably inappropriate.

CHAPTER 9

DISCUSSION AND CONCLUSION

Clostridium tetani has fascinated medical researchers since its isolation in the late 19th century. It produces one of the most potent toxins found in nature and causes a dramatic disease - Tetanus - which explains its singular importance in the eyes of many early bacteriologists.

Much of the work on the physical, biochemical, antigenic and metabolic characteristics of Cl.tetani was therefore carried out some time ago,[5,7] and there are differences in results reported by different workers in some areas. Also, the advent of modern biochemical and immunological techniques for the study of bacteria which appear not to have been applied to Cl.tetani has left our understanding of the organism incomplete.

The purpose of this present study was to review existing knowledge of Cl.tetani and reinvestigate as many aspects as possible using both conventional and new techniques in an attempt to rectify this situation. It was also hoped that a comprehensive investigation of many different properties of the organism might reveal hitherto unsuspected relationships.

Tetanolysin, the haemolysin produced by Cl.tetani was studied first and previous work indicating that the rate of haemolysis was directly proportional to the concentration of haemolysin present and that reaction rates were analogous to those obtained in reactions catalysed by enzymes[55] was confirmed. It was also found that, regardless of haemolysin concentration, the majority of haemolysis occurred within one hour, that the optimum temperature for the haemolysin reaction appears to be 37°C, and that the optimum temperature for haemolysin production appears to be 37°C also.

The haemolysin was produced during the period of active growth of the organism and the majority was liberated outside the cell, although some remained intracellularly. After around 3 days when growth had ceased both intracellular and extracellular levels fell rapidly

confirming the previous suggestion that haemolysin is rapidly destroyed once formed.[2]

It has already been shown that this destruction is due to inactivation by oxidation or heat[46] and the findings of this study are in agreement, with the rate of heat inactivation increasing with temperature.

Although it has been noted that oxygen-inactivated haemolysin can be reactivated by the addition of reducing substances,[46] this study has shown that heat-inactivated haemolysin can be reactivated to a degree by incubation at 4°C in the absence of oxygen. Total reactivation was not seen but the degree of reactivation appeared to be dependent on the original temperature used for inactivation, a linear relationship being observed between the two below 70°C. It appears that the greater the degree of inactivation, the greater the potential for reactivation. The mechanism of heat inactivation is felt to be by protein denaturation [46,50] and although the hypothesis that this can be reversible has been made[50], it has not been supported until the results of the present study were analysed.

The use of a number of additives and storage temperatures proved incapable of halting the rapid destruction of the haemolysin with the exception of storing for short periods under a liquid paraffin seal or at -40°C for up to one month. Although the effect of pH on heat-inactivation of the haemolysin has been studied,[50] the effect of direct pH inactivation appears not to have been studied previously with the exception of a suggestion that acid inactivation has been observed at pH 1 - 6.[50] This study has shown that the haemolysin was inactivated below pH 5 and that alkaline inactivation appeared at pH 10 and above. Between pH 5 and 9 little pH inactivation takes place.

The addition of glucose or sucrose to the haemolysin produced only a slight inhibition of its action, in contrast to previous findings with Cl.septicum where the haemolysin reaction could be completely interrupted.[53] The reason for this was not clear but it is possible that the modes of action of the two lysins are significantly different.

This study confirmed the finding that the haemolysin was specifically inhibited by antitoxin raised against tetanus toxin[2,5] but found that it was also non-specifically inhibted by the cholesterol content of the antiserum used and so could be inhibited by antisera to other organisms and by normal sera. The effect of cholesterol has also been noted before[45]. Antistreptolysin-O is antigenically related to tetanolysin[47], however in this study Antistreptolysin-O produced no inhibition of the haemolysin reaction. The reason for this is not known, but one possibility is that purification of the Antistreptolysin-O may have altered it to the extent that it would no longer combine with tetanolysin.

The finding that the majority of Cl.tetani strains produce haemolysin (the only exception being NCTC 9569 - a serotype VI strain) is in agreement with the findings of Kerrin.[45]

When the haemolysin was purified it was found to be a protein with a molecular weight between 66 and 68.75 kilodaltons, had an isoelectric point of 5.0 and contained isoleucine, alanine, lysine, threonine, glutamine, glutamic acid and serine.

Although the haemolysin was specifically inhibited by the antitoxin, no precipitating or agglutination reactions could be found between the two.

In studies of the haemolysin reaction it was found that whilst the tube haemolysin technique used by most other workers[45,46,47,49,50] was most suitable for investigation of the kinetics of the reaction, simple measurement of the quantity of haemolysin present was most easily accomplished using the microtitre plate haemolysin assay.

In studies of the neurotoxin of Cl.tetani it was found that the Haemagglutination Inhibition (HAI) technique was the best in-vivo method of assay. This is in agreement with the findings of other workers.[119,121] Newer immunological techniques proved disappointing in that some, such as latex agglutination did not work and others such as Rocket IEP and Single radial immunodiffusion suffered from non-specific antigen-

antibody reactions. When the antitoxin was absorbed to remove these the tests became very insensitive. Only partial antigenic identity was observed between different toxin preparations with Rocket-line IEP. The flocculation test[116,117] was found to be wasteful of reagents, very difficult to read and gave inaccurate values of toxins in this study.

Although Massachussetts medium, which has been specially formulated for toxin production[95] produced the highest toxin levels on the whole with the strains tested, both Cooked meat broth and FAB medium also proved capable of supporting toxigenesis. The same strains were found to be toxigenic in both Massachussetts medium and FAB medium.

Although native tetanus toxin and its enzymatic breakdown products have been investigated immunologically previously using Double Immuno-diffusion[18,122,126,128,140] and has been shown to comprise a single precipitin line in the native state and three distinct immunologically related components designated α , β and β_1 (a subfragment of fragment β), this study appears to be the first time these components have been individually visualised by the powerful tool of two-dimensional IEP, and the findings here confirm those previous results.

Using two-dimensional IEP and direct visualisation of the toxin and its breakdown products, support for the theory that gangliosides are the major toxin receptor in brain tissue[40,66] and that a subfragment of fragment β (fragment β_1) was responsible for tetanus toxin binding <u>in-vivo</u> [40,42] was obtained when it was found that ganglioside containing brain extracts absorbed whole tetanus toxin, and prefer ential absorption of fragment β by these extracts occurred.

The findings that native tetanus toxin had a molecular weight of approximately 150 kd and that fragments α , β and β_1 have molecular weights of approximately 50, 100 and 40 kd respectively are in agreement with established views[43,44,126,127] as is the finding that native toxin has an isoelectric point of 5.2[15]. However, the breakdown fragments also appear to all have isoelectric points of 5.2, a fact not

established before.

It had been hoped that the binding of tetanus toxin to histological brain sections could be visualised directly by immunofluorescent assay techniques, but this was not possible as no distinct fluorescent areas were seen.

Although other workers have apparently demonstrated a third tetanus toxin, the nonspasmogenic peripherally acting toxin,[16,148,149] this study was unable to isolate or detect any component which may have been this toxin. It is possible that the techniques used by the original investigators, which were extremely sensitive, detected the toxin in quantities too small to be detected by the techniques used in this study.

When the biochemical reactions of Cl.tetani were investigated, the findings agreed with established views that Cl.tetani is relatively biochemically inactive. Of 120 different tests studied only 25 proved positive and many of these could be clustered together into related groups, for instance the four different phosphatase tests and the three different esterase tests, and some, such as gram reaction, shape of cell, spore production and Polymyxin B resistance were not true biochemical reactions. In the final analysis there appeared to be 13 positive biochemical reactions or groups of related reactions and all or most strains of Cl.tetani gave positive results in all of these. Many of these results are in agreemant with established beliefs, however the findings that all strains tested in this study were H_2S positive, phosphatase positive and grew on bile medium were unexpected as all three of these tests have been previously reported as negative.[12,151,154] The reasons for these discrepancies and discrepancies between the differing reported results for other tests such as indole, DNase, gelatin hydrolysis, milk hydrolysis and fibrinolysin production and the almost universally positive results obtained in this study is almost certainly due to differences in the test media and protocols.

There was no evidence of any saccharolytic activity in this study even though numerous sugars were tested by a number of different methods. This is in agreement with the views of most workers although there are reports that Cl.tetani may occasionally be saccharolytic.[153] This may again be due to differences in test procedures, or alternatively may be due to the chance emergence of occasional aberrant strains.

The finding that milk and casein hydrolysis and precipitation was neutralised by tetanus antitoxin confirms previous findings[154] but of the other positive reactions only fibrinolysis and indole production were likewise inhibited.

As Cl.tetani has been shown to possess several proteolytic enzymes it is clear that the organism probably obtains much of its energy from the breakdown of proteins during growth and this study found that all strains tested were capable of deamination of 5 amino acids with a further 6 amino acids being utilised by different strains. This is in agreement with the earlier suggestion that the utilisation of many aminoacids varies from strain to strain[197] although it was felt that the assay technique used here was probably too insensitive to detect all amino acid utilisations present. This view is supported by the observations that, in the absence of casein hydrolysate or other amino acid source, 7 individual amino acids could support growth with L-cystine apparently the most important, even though the majority of strains did not show deamination of L-cystine. Again it was felt that discrepancies in previous reports regarding which amino acids are essential or stimulatory to growth were due to differences in experimental techniques and the use of single strains. Unfortunately, the present study is also liable to the same criticisms and the true picture of amino acid requirements of Cl.tetani is still unclear.

All the strains of Cl.tetani tested here produced acetic, butyric, propionic, lactic and succinic acids in similar proportions as metabolic products during growth. The removal of iron from the growth

medium resulted in significant decreases in growth levels.

Casein hydrolysate and yeast extract were found to be necessary for maximum growth of the organism and the amount of each present in the culture medium was proportional to the growth obtained. Casein hydrolysate could be replaced by 21 individual L-amino acids but a reduction in growth level was seen suggesting that it contains some other growth stimulator in addition to amino acids. Yeast extract could be replaced by four individual vitamins although none appeared to be essential for growth. This disagrees with previous findings, where some vitamins were felt to be essential for growth,[111,209] and it was felt that the most likly explanation for these discrepancies was the possibility that the casein digest might contain sufficient of these essential substances to allow growth to continue, although this was not further investigated in this study. Unfortunately, this meant that vitamin requirements of Cl.tetani could not be defined, however, it has been suggested that precise vitamin requirements may vary from strain to strain in any case.[209]

Sporulation in Cl.tetani varies from strain to strain and in different media and at different temperatures, however the method of estimating sporulating potential used in this study was found to be subject to major experimental errors ($\pm$ 50%) and therefore any conclusions drawn from these results must be treated with caution. The use of viable counts following heat-treatment has been used to estimate sporulating potential in Cl.tetani previously[51,212] and possibly gives greater accuracy but it is also likely that the heat-treatment may alter some of the strains other physical or metabolic properties and so this approach was avoided in this study. That heat-treatment can produce aberrant strains was shown by the apparent relationship between heating, spore production and haemolysin production, where heat treated, highly sporing substrains produced significantly less haemolysin that their parent strains. However, when the sporulating potentials of non heat-

treated strains was compared to haemolysin production, there did not appear to be any direct relationship, although the possible errors in estimating the sporulating potentials throws some doubt on this finding.

Of all the physical and cultural properties investigated, only neurotoxin production and plasmid carriage could be shown to have a direct relationship. All toxigenic strains possessed a single high molecular weight plasmid which was not present in non-toxigenic strains with one exception. This is in accord with the results of other workers [225,226]. None of the strains possessed bacteriophages active against any other strain.

The majority of strains were demonstrably motile but flagella were difficult to demonstrate effectively and appeared to be lost easily,. perhaps by manipulation of the organism. Whether this is a normal characteristic of Cl.tetani or an artifact brought about by repeated subcultures is not known. Purified flagellin did not produce a precipitin peak when examined by two-dimensional IEP, but this does not necessarily mean that it is non-antigenic. Indeed other workers have used flagella antibodies to differentiate Cl.tetani strains into a number of serotypes using agglutination reactions[239] but this approach was also unsuccessful in this study. These findings suggest that the tetanus antitoxin used here did not contain flagellar antibodies. This suggestion was further supported by the finding that agglutination tests to detect flagellar 'H' antigens did not work with this antitoxin. Indeed, the antitoxin appeared to contain no antibodies to any heat-labile antigens or to spore proteins when examined by two-dimensional IEP, and it is suggested that perhaps the original immunising antigen or toxoid was devoid of these antigens.

Antibiotic susceptibility patterns were very similar with all strains tested, there being no significant differences seen between any strains.

Although PAGE cellular protein profiles were extremely homogenous

between strains, culture supernatant protein profiles allowed strains to be differentiated into 5 groups on the basis of 4 high molecular weight proteins, and IEF culture supernatant protein patterns allowed differentiation into 2 groups on the basis of an acidic band around pH 4.25. However, none of these different groups showed any significant assocation with any other property, either physical, immunochemical or metabolic.

The detection of a common heat-stable 'O' antigen in the different serological types of Cl.tetani by DID in this study agrees with the work of others,[239] but a further suggestion that some serotypes have a further specific heat stable antigen was not confirmed. In fact, all 9 serotypes examined in this investigation appeared extremely homogenous by two-dimensional IEP whether culture filtrates, mechanically disrupted cells or cells extracted with Triton X-100 were used. A total of 8 precipitin peaks were visualised and most of these were found to be present in varying quantities in most cases. Some strains had one, two or even three peaks missing but this was insufficient to differentiate the serotypes.

Immunofluorescent staining of the cells using FITC conjugated tetanus antitoxin stained all serotypes to varying degrees, but absorption of the conjugate by any serotype of Cl.tetani removed all activity to any other serotype. This is most probably due to the common 'O' antigen which appeared to be the single most reactive antigen possessed by Cl.tetani and is likely to be the one involved in the fluorescent staining. It follows therefore that absorption with any stain, regardless of serotype, will remove most of the fluorescent reaction.

It seems therefore that strains of Cl.tetani show marked homogeneity in virtually all their metabolic and immunochemical characters with only occasional strains showing occasional differences. Thus, the great majority of strains are motile, are haemolytic, produce neurotoxin, produce spores, are positive in the same few biochemical tests, have the same metabolic end products, the same cellular protein profiles, the

same antibiotic susceptibility patterns, the same somatic antigen profiles, the same plasmid profiles and possess the same general physical and cultural properties. There are quantitative differences between strains in most of these areas but these are rarely enough to differentiate the species into different groups. The only times this is possible is using PAGE and IEF of culture supernatants and amino acid deamination patterns but these distinctions are probably meaningless as none of the other properties show any positive relationships except neurotoxin production and plasmid carriage. It would appear that there is little support in this study for the 'competition' model of bacterial extracellular enzyme and toxin biosynthesis[108] at least as far as Cl.tetani is concerned.

Much of the information in this study is not new. However, many of the areas investigated contained gaps or contradictions in established knowledge and it is hoped that these have now been clarified, and where ever possible explanations for the results have been made.

TABLE 1

Comparison of methods for measurements of haemolytic activity of Cl.tetani culture supernatants

Method	Volume required	Age of Culture (hrs)	Haemolysin* titre	H.U./ml	Comments on procedure
Tube haemolysin method	1ml	12	256	256	Laborious, relatively large volumes required and endpoints vague
		18	1024	1024	
		24	2048	2048	
Microtitre plate haemolysin method	25ul	12	64	2560	Simple and rapid endpoints very clear
		18	128	5120	
		24	256	10240	
Radial haemolysis plate method	100ul in wells 25ul on discs	12	2	20 in wells	Simple, titres low and haemolysis zones very small
		18	2		
		24	2	80 on discs	

* titres expressed as reciprocal of dilution showing 50% haemolysis

TABLE 2

Comparison of haemolysin titres obtained using the Microtitre plate assay with different concentrations of red blood cells

Suspension	OD_{545} of RBCs	Haemolysin titre
1	100	64
2	80	64
3	60	256
4	40	512*
5	20	1024*

* endpoints difficult to read

TABLE 3

The effect of different incubation temperatures, atmospheres and buffers on haemolysin titres using the microtitre plate assay

Incubation temperature (°C)	Atmospheric conditions	Haemolysin titre with:			
		Saline	ASO buffer	Saline + gelatin	FAB
37	Anaerobic	256	256	256	256
20	Anaerobic	128/256	128/256	128/256	128/256
37	Aerobic	64	256	128	256
20	Aerobic	32	128	64	128

TABLE 4

Comparison of different growth temperatures on production of haemolysin by Cl.tetani

Growth temperature (°C)	Haemolysin titre	OD_{600}
56	0	2.5
44	0	2.5
37	256	34
30	64	20
20	0	1.5
4	0	2

TABLE 5

Comparison of the effects of different growth media on haemolysin production by Cl.tetani

Growth medium	Haemolysin titre	OD_{600}
FAB	256	17
Wilkins-Chalgren broth	256	11
Cooked meat medium	2	12
Serum broth	0	0.5
Massachussets	256	20
Iso-sensitest broth	0	1

TABLE 6

The effects of storage of haemolysin at different temperatures

Storage temperature °C	Haemolysin titre after storage for:					
	0 days	1 day	2 days	3 days	5 days	7 days
- 40	NT	256	256	256	256	256
- 18	NT	256	256	256	128	128
4	NT	256	256	64	neat	0
20	NT	128	64	32	0	0
30	NT	64	32	8	0	0
37	256	64	32	8	0	0
42	NT	16	0	0	0	0
56	NT	0	0	0	0	0

NT = Not tested

TABLE 7

Comparison of titres obtained with haemolysin incubated with different additives at 37°C

Additive	Final Concentration	haemolysin titre at* 2 hours	6 hours	24 hours
Chloroform	0.5%	64	32	32
Sodium azide	0.1%	0	0	0
Gelatin	1%	64	64	64
BSA	1%	64	32	32
Horse serum	2%	0	0	0
Peptone	1%	64	64	64
Phenol	0.8%	64	16	16
Cholesterol	1%	neat	0	0
Lecethin	1%	64	64	64
Formalin	0.1%	0	0	0
PBS (Control)	10%	128	128	64
Under liquid paraffin	-	256	256	256
Cooked meat granules	5%	64	8	8

* Initial titre 256

TABLE 8

Comparison of titres obtained with haemolysin incubated in different volumes at 37°C for 24 hours

Volume of medium/ml	Haemolysin titre	Oxidation ofmedium
1ml	2	+
2	4	+
3	16	+
5	64	+
10	64	+
20	64	±
50	128	-
100	256	-
Original culture supernatant	256	-

TABLE 9

The effects of different antitoxins and sera on the haemolysin titre of Cl.tetani NCTC 279 culture supernatant

Antiserum	Haemolysin inhibition titre *	Cholesterol level (m.mol l^{-1})
Normal horse serum	64	2.37
Tetanus antitoxin 4	2	0
Tetanus antitoxin 3	2	0
Tetanus antitoxin 2	2	0
Cl.perfringens type A antitoxin	128	0.54
Cl.novyi type A antitoxin	16	0.86
Cl.novyi type B antitoxin	16	0.80
Cl.septicum antitoxin	8	1.01
Cl.chauvoei antitoxin	8	0.70
Normal human serum	0	3.75
Human tetanus antitoxin	8	2.76
Pneumococcalomniserum	0	0
Antistreptolysin O	0	0
Rabbit plasma	0	0
Tetanus antitoxin 4 absorbed with NCTC 279 (haemolytic)	0	NT
Tetanus antitoxin 4 absorbed with NCTC 9569 (non-haemolytic)	2	NT
Original titre of supernatant was	256	

0 = no inhibition of haemolysis

NT = Not tested

* = For calculation of haemolysin inhibition titre see p34

TABLE 10

Comparison of haemolysin titres obtained from Cl.tetani haemolysins incubated at different pHs for 24 hours at 4°C

pH of storage	Haemolysin titre
1	0
2	0
3	0
4	0
5	64
6	64
7	64
8	64
9	64
10	32
11	2
12	0
Original sample	128

TABLE 11

The production of tetanolysin by different strains of Cl.tetani after 24 hours incubation at 37°C in FAB medium

* Average of 4 different assays ..... + Not available .....

Strain No.	Serotype	haemolysin titre*	OD_{600}*
NCTC 279	I	128	19
" 540	II	64	20.5
" 539	III	64	20
" 5410	IV	64	19
" 5411	V	128	19
" 9569	VI	0	0.5
" 9568	VII	128	18.5
" 9574	VIII	128	18.5
" 9575	IX	64	20
CN 1349 (Harvard)	NA+	64	18.5
" 1445	NA	256	21
" 4878	NA	128	24.5
" 361	NA	128	19
" 1342	NA	128	21.5
" 761	NA	64	19.5
" 947	NA	64	18.5
" 780	NA	128	20
" 3973	NA	64	21.5
L5500	NA	64	20
L109	NA	128	20
LQ1	NA	64	20
LQ2	NA	64	19.5
LQ931	NA	64	20
LQ914	NA	64	19
LQ730	NA	64	18.5
L777A	NA	128	21.5
E88	NA	128	20.5

TABLE 12

Comparison of haemolysin production, spore production and amount of growth in heated and unheated strains of Cl.tetani

Strain designation	Original Suspension % Spores	Unheated			Heated			
		Haemolysin titre	OD_{600}	Spores (%)	Haemolysin titre	OD_{600}	Spores (%)	Subsequent growth on Blood Agar
*1-H-80	40	8	11.5	66	0	6	50	+
I	0	64	11	1	0	6	1	-
II	8	64	14	1	16	18.5	1	+
III	8	64	16	1	8	22.5	1	+
IV	5	64	12.5	1	16	27	1	+
V	2	64	13.5	1	0	4.5	1	+
VI	1	0	2.5	11	0	3.5	1	+
VII	16	64	13.5	1	32	18	1	+
VIII	1	64	12	1	0	7.5	1	+
IX	1	64	13	1	0	5	1	+
CN1349(Harvard)	24	128	16	1	0	6	12	+
" 1445	1	128	6.5	1	0	6	1	+
" 4878	1	64	16	1	0	4	1	+
" 361	10	128	7.5	1	32	11	1	+
" 1342	1	64	16.5	1	0	9	1	+
" 761	8	64	16	1	32	12	16	+
" 947	4	64	16	1	0	35	2	+
" 780	1	64	16	1	0	5	1	+
" 3973	1	64	14	1	0	5	1	+
L5500	1	64	9.5	4	0	6.5	1	+
L109	1	64	15	1	0	3.5	1	+
LQ1	1	64	13	10	0	6.5	1	+
LQ2	1	64	14	8	0	7.5	1	+
LQ931	1	64	13	10	0	6	1	+
LQ914	1	64	16	8	0	6	1	+
LQ730	1	64	13	6	0	6.5	1	+
L777A	1	128	16.5	1	0	4.5	1	+
E88	1	128	16	1	0	6.5	1	+

TABLE 13

Comparison of Haemolytic activity, antigenic activity and PAGE bands of Sephadex G-200 fractions of Cl.tetani haemolysin (method 1)

Fraction Number	Haemolysin titre	Antigenic activity (DD and Rocket EIEP)	PAGE bands
1	0	-	-
2	0	-	-
3	0	-	-
4	2	+	+
5	8	++	++
6	64	+	+
7	512	trace	trace
8	512	-	trace
9	512	-	trace
10	128	-	trace
11	32	-	-
12	2	-	-
13	0	-	-
14	0	-	-

TABLE 14

Agglutination reactions of Sephadex G-200 fractions of Cl.tetani haemolysin and concentrated haemolysins

SAMPLE	Reactions with latex reagent		Reactions with Staphylococcal reagent	
	Tetanus antitoxin	absorbed antitoxin	tetanus antitoxin	absorbed antitoxin
Sephadex fraction 1	-	trace	AA	AA
2	-	trace	"	"
3	-	trace	"	"
4	-	trace	"	"
5	-	trace	"	"
6	-	±	"	"
7	-	±	"	"
8	-	±	"	"
9	-	±	"	"
10	-	±	"	"
11	-	trace	"	"
12	-	trace	"	"
13	-	trace	"	"
14	-	trace	"	"
Original culture supernatant	-	trace	"	"
Concentrated supernatant	+++	+++	"	"
Concentrated fraction 8	-	±	"	"
Final product of Method 2	-	trace	"	"
Final product Method 3	-	trace	"	"

KEY

-	negative
trace	trace agglutination
±	weak "
+	moderate "
++	strong "
+++	very strong "
AA	Autoagglutination

TABLE 15

Comparison of methods of assay of Cl.tetani neurotoxin

Method	Volume of reagents required	Amount of toxin used (Lf/ml)	Amount of toxin calculated (Lf/ml)	Comments on procedure
Tube flocculation	0.5 - 1ml	14	5 - 7.4	Slow, cumbersome endpoints vague
HAI	25 ul	74	70	Quick, easy
		28	28	Sensitised cells difficult to standardise.
Latex Agglutination	20-40 ul	74	0	No observable strong
		28	0	agglutinations
Single Radial Immunodiffusion	10 ul	74	50	Very insensitive
		28	25	Precipitin zones small and hard to measure. Absorbed antisera required.
Double Immunodiffusion	25 ul	74	NOT QUANTITATIVE	Interference from other antigen-antibody reactions
		28		
Rocket IEP	10-15 ul	74	60	Absorbed antisera required. Rocket
		28	30	peaks vague
Rocket-line IEP	10-15 ul	74	85	Technically difficult. On ly
		28	30	partial antigenic identity.

TABLE 16

Assay of Cl.tetani strains for toxin production by the HAI test

Strain	MASSACHUSSETTS MEDIUM* OD_{600}	MASSACHUSSETTS MEDIUM* Toxin(Lf/ml)	FAB MEDIUM* OD_{600}	FAB MEDIUM* Toxin(Lf/ml)
NCTC I	15	28	18	28
" II	10	14	18	28
" III	14	28	30	14
" IV	4.5	112	17.5	56
" V	10	56	18	28
" VI	4	112	6.5	56
" VIII	10	112	12	28
" VIII	12	112	11	28
" IX	12	28	15	28
CN4878	14	28	21	28
" 1349(Harvard)	14	56	11	28
" 761	17	112	13.5	56
" 361	15	56	9	56
" 1445	18	56	18	56
" 3973	14	56	11	56
" 947	21	56	13	28
" 1342	5	0	13	0
" 780	10	14	11	14
L5500	12	112	11	28
LQ1	14	3.75	15	28
LQ2	17	7.5	13.5	28
LQ914	12	0	13	0
LQ931	15	0	18	0
LQ730	14	112	11	28
L777A	17	56	17	56
L109	17	7.5	9	56
E88	28	224	28	28
NCTC I (3 day FAB)				0

* Grown at 34°C for 7 days

TABLE 17

Assay of Cl.tetani strains for toxin production by SRID and Rocket IEP

Strain	Amount of toxin detected (Lf/ml)			
	SRID		Rocket IEP	
	MASS *	FAB *	MASS *	FAB *
NCTC I	-	-	-	-
" II	6	-	-	-
" III	-	-	-	-
" IV	-	-	-	-
" V	-	-	-	-
" VI	-	-	-	-
" VII	-	-	-	-
" VIII	-	-	-	-
" IX	-	-	-	-
CN 4878	-	-	-	-
" 1349	-	-	6	-
" 761	8	-	6	-
" 361	-	-	-	-
" 1445	-	-	7.5	-
" 3973	7	-	-	-
" 947	-	-	-	-
" 1342	-	-	-	-
" 780	-	-	-	-
L 5500	-	-	-	-
LQ 1	-	-	-	-
" 2	-	-	-	-
" 914	-	-	-	-
" 931	-	-	-	-
" 730	-	-	-	-
L 777A	-	-	-	-
L 109	-	-	-	-
E 88	-	-	9	-
3 day FAB (NCTC I)		-		-

* Grown at 34°C for 7 days

- = toxin not detected

TABLE 18

Production of toxin in other media and by extraction of cells

	Amounts of toxin detected (Lf/ml)		
	HAI	SRID	Rocket IEP
Massachussetts medium (strain E88)	112	0	9
Wilkins Chalgen broth (strain E88)	0	0	0
Cooked Meat medium (strain E88)	112	0	0
Isosensitest broth (strain E88)	0	0	0
Serum broth (strain E88)	0	0	0
Thiol broth (strain E88)	0	0	0
Raynaud extract (strain 279)	448	8	12

TABLE 19

Binding of tetanus toxin to brain tissue extracts

Brain extract	Absorption of whole toxin	Absorption of fragments	
		α	β
A	++	++	++
C	+++	++	+++
F	+++	++	+++
H	+++	++	+++
J	+++	++++	++++
P	-	-	-
Y	-	-	-
Z	+++	+	++++

KEY:

- \- no lowering of precipitin peak
- \+ slight lowering of precipitin peak
- ++ moderate lowering of precipitin peak
- +++ marked lowering of precipitin peak
- ++++ removal of precipitin peak

TABLE 20

Results of testing 10 strains of Cl.tetani by the API ZYM system

Test reaction	Cl.tetani serotype									CN
	I	II	III	IV	V	VI	VII	VIII	IX	1342
1. Control	-	-	-	-	-	-	-	-	-	-
2. Alkaline phosphatase	+	+	+	+	+	+	+	+	+	+
3. Esterase (C4)	-	-	-	-	-	-	-	-	-	-
4. Esterase lipase (C8)	+	+	+	+	+	+	+	+	+	+
5. Lipase (C14)	-	-	-	-	-	-	-	-	-	-
6. Leucine arylamidase	-	-	-	-	-	-	-	-	-	-
7. Valine	-	-	-	-	-	-	-	-	-	-
8. Cystine	-	-	-	-	-	-	-	-	-	-
9. Trypsin	-	-	-	-	-	-	-	-	-	-
10. Chymotrypsin	-	-	-	-	-	-	-	-	-	-
11. Acid phosphatase	+	+	+	+	+	+	+	+	+	+
12. Naphthol-AS-BI-phospholydrolase	+	+	+	+	+	+	+	+	+	+
13. α - galactosidase	-	-	-	-	-	-	-	-	-	-
14. β - galactosidase	-	-	-	-	-	-	-	-	-	-
15. β - glucuronidase	-	-	-	-	-	-	-	-	-	-
16. α - glucosidase	-	-	-	-	-	-	-	-	-	-
17. β - glucosidase	-	-	-	-	-	-	-	-	-	-
18. N-acetyl- β glucosaminidase	-	-	-	-	-	-	-	-	-	-
19. α mannosidase	-	-	-	-	-	-	-	-	-	-
20. α fucosidase	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction

\- : Negative reaction

TABLE 21

Results of testing 10 strains of Cl.tetani by the API STREP system

Test reaction	Serotype of strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Acetoin production	-	-	-	-	-	-	-	-	-	-
2. Hippurate hydrolysis	-	-	-	-	-	-	-	-	-	-
3. β- Glucosidase	-	-	-	-	-	-	-	-	-	-
4.Pyrrolidonylarylamidase	-	-	-	-	-	-	-	-	-	-
5. α- Galactosidase	-	-	-	-	-	-	-	-	-	-
6. β- Glucuronidase	-	-	-	-	-	-	-	-	-	-
7. β Galactosidase	-	-	-	-	-	-	-	-	-	-
8. Alkaline phosphatase	tr	+	+	+	+	tr	+	+	+	+
9. Leucine arylamidase	-	-	-	-	-	-	-	-	-	-
10.Arginine dehydrolase	-	-	-	-	-	-	-	-	-	-
11.Ribose fermentation	-	-	-	-	-	-	-	-	-	-
12.L-Arabinose "	-	-	-	-	-	-	-	-	-	-
13.Mannitol "	-	-	-	-	-	-	-	-	-	-
14.Sorbitol "	-	-	-	-	-	-	-	-	-	-
15.Lactose "	-	-	-	-	-	-	-	-	-	-
16.Trehalose "	-	-	-	-	-	-	-	-	-	-
17.Inulin "	-	-	-	-	-	-	-	-	-	-
18.Raffinose "	-	-	-	-	-	-	-	-	-	-
19.Starch "	-	-	-	-	-	-	-	-	-	-
20.Glycogen "	-	-	-	-	-	-	-	-	-	-

KEY:

- \- Negative reaction
- \+ Positive reaction
- tr Trace positive

TABLE 22

Results of testing 10 strains of Cl.tetani by the API Anaerobic enzyme system. Strip ANI - 1 - 20

Test reaction	Serotype of test strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Alkaline phosphatase	+	+	+	+	+	+	+	+	+	+
2. Acid phosphatase	+	+	+	+	+	+	+	+	+	+
3. Lipase C10	-	-	-	-	-	-	-	-	-	-
4. α-Galactosidase	-	-	-	-	-	-	-	-	-	-
5. β-Galactosidase	-	-	-	-	-	-	-	-	-	-
6. α-Glucosidase	-	-	-	-	-	-	-	-	-	-
7. α-Fucosidase	-	-	-	-	-	-	-	-	-	-
8. L.Arabinose	-	-	-	-	-	-	-	-	-	-
9. Phospho-β-Galactosidase	-	-	-	-	-	-	-	-	-	-
10.N-acetyl-β-Glucosaminidase	-	-	-	-	-	-	-	-	-	-
11.Argnine arylamidase	-	-	-	-	-	-	-	-	-	-
12.Proline "	-	-	-	-	-	-	-	-	-	-
13.Ornithine "	-	-	-	-	-	-	-	-	-	-
14.Glutamine "	-	-	-	-	-	-	-	-	-	-
15.Histidine "	-	-	-	-	-	-	-	-	-	-
16. glutamyl transpeptidase	-	-	-	-	-	-	-	-	-	-
17.Leucyl-glycine arylamidase	-	-	-	-	-	-	-	-	-	-
18.Histidyl-pheynylalamine-arylamidase	-	-	-	-	-	-	-	-	-	-
19.Glutamyl-histidine arylamidase	-	-	-	-	-	-	-	-	-	-
20.Alanine-phenylalamine proline arylamidase	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction

\- : Negative reaction

TABLE 23

Results of testing 10 strains of Cl.tetani by the API Anaerobic enzyme system. Strip AN2. tests 21-40

Test reaction	Serotype of test strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Esterase C4	+	+	+	+	+	+	+	+	+	+
2. Naphthol-AS-Bl-phosphate	+	+	+	+	+	+	+	+	+	+
3. β-glucuronidase	-	-	-	-	-	-	-	-	-	-
4. α-mannosidase	-	-	-	-	-	-	-	-	-	-
5. β-mannosidase	-	-	-	-	-	-	-	-	-	-
6. β-glucosidase	-	-	-	-	-	-	-	-	-	-
7. β-D-fucosidase	-	-	-	-	-	-	-	-	-	-
8. Trypsin	-	-	-	-	-	-	-	-	-	-
9. Leucine arylamidase	-	-	-	-	-	-	-	-	-	-
10. Valine "	-	-	-	-	-	-	-	-	-	-
11. Serine "	-	-	-	-	-	-	-	-	-	-
12. Lysine "	-	-	-	-	-	-	-	-	-	-
13. Hydroxyproline "	-	-	-	-	-	-	-	-	-	-
14. Aspartic "	-	-	-	-	-	-	-	-	-	-
15. Pyrrolidonic "	-	-	-	-	-	-	-	-	-	-
16. Phenylalanine"	-	-	-	-	-	-	-	-	-	-
17. Glutamylglutamic arylamidase	-	-	-	-	-	-	-	-	-	-
18. Lysylalanine "	-	-	-	-	-	-	-	-	-	-
19. Phenylalanine-arginine arylamidase	-	-	-	-	-	-	-	-	-	-
20. Proline-arginine arylamidase	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction
\- : Negative reaction

TABLE 24

Results of testing 10 strains of Cl.tetani by the API Anaerobic enzyme system. Strip AN3. Tests 41-60

Test reaction	Serotype of test strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Nitrate	-	-	-	-	-	-	-	-	-	-
2. Indole	-	-	-	-	-	-	-	-	-	-
3. Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
4. Urea	-	-	-	-	-	-	-	-	-	-
5. Glucose fermentation	-	-	-	-	-	-	-	-	-	-
6. Glycerol "	-	-	-	-	-	-	-	-	-	-
7. L-Arabinose "	-	-	-	-	-	-	-	-	-	-
8. D-ribose "	-	-	-	-	-	-	-	-	-	-
9. D-xylose "	-	-	-	-	-	-	-	-	-	-
10. D-galactose "	-	-	-	-	-	-	-	-	-	-
11. D-fructose "	-	-	-	-	-	-	-	-	-	-
12. Mannitol "	-	-	-	-	-	-	-	-	-	-
13. ∝- methyl-D-mannoside fermentation	-	-	-	-	-	-	-	-	-	-
14. ∝- methyl-D-glucoside fermentation	-	-	-	-	-	-	-	-	-	-
15. Arbutin fermentation	-	-	-	-	-	-	-	-	-	-
16. Cellobiose "	-	-	-	-	-	-	-	-	-	-
17. Lactose "	-	-	-	-	-	-	-	-	-	-
18. Melibiose "	-	-	-	-	-	-	-	-	-	-
19. Trehalose "	-	-	-	-	-	-	-	-	-	-
20. Tetrathionate reductase	+	+	+	+	+	+	+	+	+	+

\+ : Positive reaction

\- : Negative reaction

TABLE 25

Results of testing 10 strains of Cl.tetani by the Sensititre AP60 system

Test reaction	Serotype of test strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Nitrate reduction	-	-	-	-	-	-	-	-	-	-
2. Glucose fermentation	-	-	-	-	-	-	-	-	-	-
3. Decarboxylase blank	-	-	-	-	-	-	-	-	-	-
4. Oxidase	-	-	-	-	-	-	-	-	-	-
5. β-galactosidase	-	-	-	-	-	-	-	-	-	-
6. Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-
7. Indole production	-	-	-	-	-	-	-	-	-	-
8. Urease production	-	-	-	-	-	-	-	-	-	-
9. Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-
10.Voges-Proskaur	-	-	-	-	-	-	-	-	-	-
11.Citrate utilisation	-	-	-	-	-	-	-	-	-	-
12.Malonate utilisation	-	-	-	-	-	-	-	-	-	-
13.Tryptophan deaminase	-	-	-	-	-	-	-	-	-	-
14.Hydrogen sulphide	+	+	+	+	+	+	+	+	+	+
15.Aesculin hydrolysis	-	-	-	-	-	-	-	-	-	-
16.Gelatin liquefaction	-	+	+	+	+	-	+	-	+	+
17.Inositol fermentation	-	-	-	-	-	-	-	-	-	-
18.Mannitol "	-	-	-	-	-	-	-	-	-	-
19.Adonitol "	-	-	-	-	-	-	-	-	-	-
20.Arabinose "	-	-	-	-	-	-	-	-	-	-
21.Maltose "	-	-	-	-	-	-	-	-	-	-
22.Rhamnose "	-	-	-	-	-	-	-	-	-	-
23.Sorbitol "	-	-	-	-	-	-	-	-	-	-
24.Sucrose "	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction

\- : Negative reaction

TABLE 26

Results of testing 10 strains of Cl.tetani by the Abbott BID system

Test reaction	Serotype of Test strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Glucose fermentation	-	-	-	-	-	-	-	-	-	-
2. Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-
3. Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-
4. Citrate utilisation	-	-	-	-	-	-	-	-	-	-
5. Malonate utilisation	-	-	-	-	-	-	-	-	-	-
6. Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-
7. Urease production	-	-	-	-	-	-	-	-	-	-
8. Adonitol fermentation	-	-	-	-	-	-	-	-	-	-
9. Arabinose "	-	-	-	-	-	-	-	-	-	-
10. Inositol "	-	-	-	-	-	-	-	-	-	-
11. Lactose "	-	-	-	-	-	-	-	-	-	-
12. Mannitol "	-	-	-	-	-	-	-	-	-	-
13. Rhamnose "	-	-	-	-	-	-	-	-	-	-
14. Sorbitol "	-	-	-	-	-	-	-	-	-	-
15. Sucrose "	-	-	-	-	-	-	-	-	-	-
16. Xylose "	-	-	-	-	-	-	-	-	-	-
17. Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
18. Indole production	-	-	-	-	-	-	-	-	-	-
19. Acetoin	-	-	-	-	-	-	-	-	-	-
20. Polymyxin B resistance	+	+	+	+	+	+	+	+	+	+
21. Oxidase	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction

\- : Negative reaction

TABLE 27

Results of testing 10 strains of Cl.tetani by the Anaerobe-Tek system

Test reaction	Serotype of test strain I	II	III	IV	V	VI	VII	VIII	IX	CN 1342
1. Gram reaction	+	+	+	+	+	+	+	+	+	+
2. Bacillus	+	+	+	+	+	+	+	+	+	+
3. Spore production	-	+	+	+	+	-	+	+	+	+
4. H_2S production	+	+	+	+	+	+	+	+	+	+
5. Indole production	-	-	-	-	-	-	-	-	-	-
6. Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-
7. Catalase production	-	-	-	-	-	-	-	-	-	-
8. Phospholipase C production	-	-	-	-	-	-	-	-	-	-
9. Lipase production	-	-	-	-	-	-	-	-	-	-
10. Bile tolerance	+	+	+	+	+	+	+	+	+	+
11. Milk digestion or precipitation	-	-	-	-	-	-	-	-	-	-
12. Gelatinase production	-	+	+	+	+	-	+	-	+	+
13. Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
14. DNase production	+	+	+	+	+	+	+	+	+	+
15. Glucose fermentation	-	-	-	-	-	-	-	-	-	-
16. Mannitol "	-	-	-	-	-	-	-	-	-	-
17. Lactose "	-	-	-	-	-	-	-	-	-	-
18. Trehalose "	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction

\- : Negative reaction

TABLE 28

Results of testing 10 strains of Cl.tetani on commercial nutrient test agar plates

Test reaction	Serotype of test strain									CN
	I	II	III	IV	V	VI	VII	VIII	IX	1342
1. Hydrogen sulphide production	±	±	±	±	±	±	±	±	±	±
2. Gelatinase production	-	-	-	-	-	-	-	-	-	-
3. Indole production	-	-	-	-	-	-	-	-	-	-
4. Glucose fermentation	-	-	-	-	-	-	-	-	-	-

\+ : Positive reaction

± : Weak positive reaction

\- : Negative reaction

TABLE 29

Results of testing 27 Cl.tetani strains by laboratory prepared biochemical test agar plates

Test reaction		No.of strains giving Positive reaction	No.of strains giving Negative reaction
I	Collagenase	0	27
II	Albumin hydrolysis	0	27
III	Chitinase	0	27
IV	Lipolytic activity	27	0
V	Chondroitinase	0	27
VI	Esterase	0	27
VII	Arbutin hydrolysis	0	27
VIII	Elastase	0	27
IX	Mucinase	0	27
X	DNase	26	1
XI	RNase	26	1
XII	Gelatinase	23	4
XIII	Casein hydrolysis/ precipitation	26	1
XIV	Casein hydrolysate digestion	0	27
XV	Whole milk digestion/ precipitation	0	27
XVI	Fibrin hydrolysis	26	1
XVII	Desulfoviridin production	0	27
XVIII	Glutamic acid decarboxylase	27	0
XIX	NAD glycohydrolase	0	27
XX	Hyaluronidase	0	27
XXI	H_2S production	27	0
XXII	Indole production	0	27
XXIII	Phosphatase activity	27	0
XXIV	Fluorescence on MacConkey	25	2
XXV	Tetrazolium reduction	0	27

TABLE 30

Results of testing 27 *Cl.tetani* strains by laboratory prepared bottled media

Test		No.of strains giving positive result	No.of strains giving negative result
i	Meat digestion	0	27
	meat reddening*	27	0
ii	Milk digestion+	26	1
	Acid production	0	27
iii	Ammonia production from peptone	27	0
iv	Gas production in deep agar stabs ‡	27	0

* slight reddening after 7 days incubation

\+ becoming visible after 48 hours incubation

‡ becoming visible after 4-5 days incubation

TABLE 31

Results of testing 27 strains of Cl.tetani for milk digestion and precipitation using different milk products as substrate

Milk Product	No.of strains giving Positive result	No.of strains giving Negative result
Whole milk	0	27
Powdered milk 1	26	1
Powdered milk 2	26	1
Skim milk 1	26	1
Skim milk 2	26	1
Skim milk 3	26	1
Skim milk 4	26	1

TABLE 32

Results of testing 27 strains of Cl.tetani for indole production using 3 different reagents and 3 different test protocols

Reagent	Test Protocol	No.of strains Positive	No.of strains Negative
Kovacs'	Spot test	0	27
Reagent	Direct test*	3	24
	Extracted test*	20	7
Aqueous	Spot test	27	0
Kovacs'	Direct test	0	27
reagent	Extracted test	0	27
DMACA	Spot test	27	0
	Direct test	0	27
	Extracted test	0	27

* Direct and extracted tests of FAB and RCM gave comparable results

TABLE 33

Effect of tetanus antitoxin on the beiochemical reactions of Cl.tetani

Reaction	Inhibition of reaction by antitoxin
1. phosphatase	-
2. lipase	-
3. gelatinase	-
4. DNase	-
5. RNase	-
6. Casein hydrolysis/precipitation	+
7. Fluorescence on MAC	-
8. fibrinolysis	+
9. H_2S	-
10.indole	+
11.Glutamic acid decarboxylase	-
12.skim milk hydrolysis/precipitation	+

\+ : inhibition by antitoxin

\- : no inhibition by antitoxin

TABLE 34

Positive biochemical reactions exhibited by Cl.tetani strains

Biochemical test	Group
Alkaline phosphatase	phosphatase group
acid phosphatase	
Naphthol-AS-Bl-phosphohydrolase	
phosphatase test	
Esterase C4	esterase/lipase group
Esterase lipase C8	
Glycerol tributyrate hydrolysis	
Skim milk hydrolysis	milk hydrolysis group
casein hydrolysis	
milk digestion	
DNase	DNA/RNA hydrolysis group
RNase	
Ammonia production from peptone	ammonia production group
gas production	
glutamic acid decarboxylase	
reddening of meat particles	reducing group
tetrathionate reductase	
H_2S production	
gelatinase production	
indole production	
bile tolerance	
fluorescence on MacConkey agar	
fibrin hydrolysis	
casein precipitation	

TABLE 35

Effect of Casein hydrolysate and yeast extract on growth of Cl.tetani NCTC 539

Medium	OD_{600}	% of complete medium growth
Complete (3% casein hydrolysate and 1% yeast extract)	44	100
Complete with only 1% casein hydrolysate	33	74.9
Complete with only 0.1% casein hydrolysate	28	63.6
Complete minus casein hydrolysate	0	0
Complete minus 8 vitamins (with 1% yeast extract)	48	109
Complete minus 8 vitamins with 0.1% yeast extract	18	40.9
Complete minus 8 vitamins minus yeast extract	1.5	3.4
Complete minus 8 vitamins, yeast extract and casein hydrolysate	0	0

TABLE 36

Effect of removing other chemicals from the complete medium on the growth of Cl.tetani NCTC 539

Formula The complete medium minus	OD_{600}	% of complete medium growth
1. Nothing	40	100
2. $MgSO_4$	37.5	93.7
3. $FeCl_3$	5	12.5
4. Cysteine hydrochloride	22.5	56.2
5. NaCl	36.5	91.2
6. The 8 vitamins	37.5	93.7
7. Nothing	36	100
8. $MgSO_4$ and $FeCl_3$	13	36.1
9. NaCl and Cysteine HCl	20	55.5
10. NaCl, cysteine CHl and yeast extract	16	44.4
11. NaCl, cysteine HCl, $MgSO_4$ and $FeCl_3$	3.5	9.7
12. NaCl, cysteine HCl, yeast extract $MgSO_4$ and $FeCl_3$	2.5	6.9
13. NaCl, yeast extract, $MgSO_4$ and $FeCl_3$	1	2.8
14. NaCl, $MgSO_4$ and $FeCl_3$	14	38.9
15. Vitamins, NaCl, yeast extract cysteine HCl, $MgSO_4$ and $FeCl_3$	1	2.8

TABLE 37

Effect of removing or adding vitamins to the complete medium on the growth of Cl.tetani NCTC 539

Formula The complete medium minus Yeast extract	OD_{600}	% of complete medium growth
1. complete	60	100
2. minus Uracil	42	70
3. " Thiamine	70	116.7
4. " Riboflavin	60	100
5. " Pyridoxine	44	73.3
6. " Nicotinic acid	48	80
7. " Calcium pantothenate	42	70
8. " Biotin	53	88.3
9. " Vitamin B_{12}	49	81.7
10. Complete	41	100
11. Minus all Vitamins except Uracil	1	2.4
12. " " " " Thiamine	1.5	3.6
13. " " " " Riboflavin	18	43.7
14. " " " " Pyridoxine	35	85
15. " " " " Nicotinic acid	1.5	3.6
16. " " " " Calcium pantothenate	38	92.3
17. " " " " Biotin	39	94.8
18. " " " " Vitamin B_{12}	2	4.9

TABLE 38

Amino acids necessary for growth of Cl.tetani NCTC 539

Complete medium minus casein hydrolysate	OD_{600}	% of complete medium growth	% of growth with all 21 amino acids
1. Complete medium plus casein hydrolysate	44	100	-
2. " " plus all 21 amino acids	11	25	100
3. " " plus L-phenylalanine	1	2.2	9
4. " " plus L-lysine	0	0	0
5. " " plus Hydroxy-L-proline	0.5	1.1	4.5
6. " " plus L-alanine	1	2.2	9
7. " " plus L-aspartic acid	2.5	5.7	22.7
8. " " plus L-valine	0	0	0
9. " " plus L-glutamine	0	0	0
10. " " plus L-tryptophan	5	11.4	45.4
11. " " plus L-methionine	3.5	8	31.8
12. " " plus Glycine	0	0	0
13. " " plua L-isoleucine	0	0	0
14. " " plua L-leucine	0	0	0
15. " " plus L-cysteine	0	0	0
16. " " plus L-histidine	0	0	0
17. " " plus L-arginine	0.5	1.1	4.5
18. " " plus L-asparagine	2.5	5.7	22.7
19. " " plus L-glutamic acid	5	11.4	45.4
20. " " plus L-proline	0.5	1.1	4.5
21. " " plus L-serine	0	0	0
22. " " plus L-threonine	4	9	36.3
23. " " plus L-cystine	10	22.7	90.9

TABLE 39

Deamination of amino acids by Cl.tetani NCTC 539 in tubes

Amino acid	Absorbance *
1. L-phenylalanine	0.076
2. L-lysine	0.069
3. Hydroxy-L-proline	0.038
4. L-alanine	0.032
5. L-aspartic acid	0.049
6. L-valine	0.039
7. L-glutamine	1.026
8. L-tryptophan	0.064
9. L-methionine	0.078
10. Glycine	0.031
11. L-isoleucine	0.031
12. L-leucine	0.043
13. L-cysteine	0.036
14. L-histidine	0.026
15. L-arginine	0.048
16. L-asparagine	0.053
17. L-glutamic acid	0.032
18. L-proline	0.045
19. L-serine	0.032
20. L-threonine	0.109
21. L-cystine	0.030

* = Absorbance read on dual beam spectrophotometer[163] at wavelengths 492 and 600 nm

TABLE 40

Deamination of amino acids by 27 strains of Cl.tetani in plastic trays

Strain	Deamination of amino acid										
	5	6	7	9	12	13	16	17	19	20	21
Serotype I	tr	-	+	+	-	-	tr	+	-	+	tr
II	tr	-	+	+	-	-	-	+	-	+	-
III	tr	-	+	+	-	-	-	+	-	+	-
IV	tr	-	+	+	-	-	-	+	-	+	tr
V	tr	-	+	+	-	-	-	+	-	+	-
VI	tr	-	+	+	-	tr	-	+	-	+	-
VII	tr	-	+	+	-	-	-	+	-	+	-
VIII	tr	-	+	+	-	tr	-	+	-	+	-
IX	tr	-	+	+	-	-	-	+	-	+	-
CN 1445	tr	-	+	+	-	-	-	+	-	+	-
" 4878	tr	-	+	+	-	tr	-	+	-	+	-
" 361	tr	-	+	+	-	tr	-	+	-	+	-
" 1342	tr	-	+	+	-	-	-	+	-	+	-
" 761	tr	-	+	+	-	-	-	+	-	+	-
" 947	tr	-	+	+	-	-	-	+	-	+	-
" 780	tr	-	+	+	-	-	-	+	-	+	-
" 3973	tr	-	+	+	-	-	tr	+	-	+	tr
" 1349	tr	-	+	+	-	-	-	+	-	+	-
L5500	tr	-	+	+	-	+	-	+	-	+	-
L109	tr	-	+	+	-	+	-	+	tr	+	-
LQ730	tr	-	+	+	-	-	-	+	-	+	-
LQ1	tr	-	+	+	-	-	-	+	-	+	tr
LQ2	tr	+	+	+	-	-	tr	+	-	+	-
LQ931	tr	-	+	+	tr	-	-	+	-	+	-
LQ914	tr	-	+	+	-	-	-	+	-	+	-
777A	tr	-	+	+	tr	-	-	+	-	+	tr
E88	tr	-	+	+	-	-	tr	+	-	+	tr

KEY: + = Positive reaction. tr = trace reaction. - = negative reaction

5 = L.aspartic acid 6 = L.valine 7 = L.glutamine 9 = L.methionine

12 = L.Leucine 13 = L.cysteine 16 = L.asparagine

17 = L.glutamic acid 19 = L.serine 20 = L.threonine 21 = L.cystine

The 16 other L.-amino acids and the 16 D-amino acids (Appendix 37) all gave consistantly negative reactions with all strains of Cl.tetani tested.

TABLE 41

Volatile and non-volatile fatty acid production in Cl.tetani strains

Strain	VFA's(% of total)+ Acetic	Propionic	butyric	NonVFAs (% of total)+ lactic	succinnic
serotype I	15 *	11.51 *	73.48 *	61.79	38.21
II	18.57	6.25	75.18	71.89	28.11
III	20.43	15.06	64.51	72.13	27.87
IV	16.38	10.94	72.68	70.40	29.60
V	21.87	15.42	62.72	73.29	26.71
VI	13.17	14.67	72.15	68.60	31.40
VII	14.11	11.79	74.20	66.39	33.40
VIII	15.81	23.12	61.08	70.25	29.75
IX	21.85	13.26	64.89	72.41	27.59
CN 1445	15.21	13.40	71.39	71.38	28.62
4878	13.98	10.37	75.65	63.28	36.72
361	14.55	11.82	73.63	69.97	30.03
1342	18.37	14.97	66.66	71.88	28.12
761	17.41	9.125	73.465	66.23	33.77
947	15.32	21.53	63.15	68.85	31.15
780	23.43	24.80	51.77	71.78	28.22
3973	16.91	9.628	73.462	69.43	30.57
1349	17.30	9.53	73.17	61.98	30.02
L5500	15.73	11.98	72.29	66.21	33.79
L109	13.38	12.15	74.47	69.91	30.09
LQ1	17.64	10.51	71.85	64.30	35.70
LQ2	17.10	9.11	73.79	69.12	30.88
LQ931	17.02	15.79	67.19	61.49	38.51
LQ914	18.01	15.67	66.31	70.58	29.42
L777A	29.20	26.0	44.79	74.40	25.60
E 88	21.33	12.10	66.57	70.53	29.47
LQ730	17.79	16.41	65.80	68.66	31.34

* values ± 20% approximately

\+ The area of each individual peak is expressed as a percentage of the total area of all the peaks, as a measure of the relative amounts of each acid in the extract

TABLE 42

Sporulating potential of Cl.tetani NCTC 540 grown for different times at 30°C and 37°C in different media

Days of incubation	Spores (%) in* Cooked meat broth 37°C	Cooked meat broth 30°C	FAB 37°C	FAB 30°C
1	1	2	0	0
2	4	2	<1	0
3	17	4	3	0
4	26	4	3	2
5	28	10	4	2
6	34	20	4	3
7	36	24	3	3

* Sporulating potential is taken as the percentage of the total number of cells counted that possess spores or forespores. The figures given here are the mean of 3 determinations. These showed a maximum difference of roughly ± 10%.

TABLE 43

Volatile fatty acids (VFAs) produced by sporing Cl.tetani cells at 30°C and at 37°C in Cooked meat medium

Days of incubation	VFA*	Temperature of incubation 37°C % of total area	Individual Peak Area	30°C % of total area	Individual Peak Area
1	A	21.32	158939	30.47	53471
	P	-		-	
	B	78.69	586719	69.53	121999
3	A	15.2	915279	17.28	99184
	P	10.24	613225	13.22	75889
	B	72.97	4418034	69.51	399070
5	A	16.41	909724	15.35	334309
	P	11.60	643413	8.53	186283
	B	70.21	3893565	76.10	1657389
7	A	12.29	1946807	14.26	777672
	P	12.90	2043390	9.105	496726
	B	72.94	11553453	74.87	40845584

*

A = Acetic acid

P = Propionic acid

B = Butyric acid

TABLE 44

The sporulating potential of different strains of Cl.tetani on different media

Strain	Sporulating potential (% of total)*		
	Columbia blood agar plates 37°C	FAB 37°C	Cooked meat 30°C
Serotype I	2	0	22
II	11	14	36
III	66	4	64
IV	12	20	28
V	60	7	40
VI	8	0	20
VII	30	2	55
VIII	15	3	9
IX	29	4.5	50
CN 1445	17	4	16
" 4878	< 1	0	32
" 361	1	14	12
" 1342	0	2,5	< 1
" 761	63	12.9	35
" 947	0	8	29
" 780	20	11	33
" 3973	25	8	37
" 1349	14	0	32
L5500	37	2	9
L109	< 1	< 1	< 1
LQ730	32	0	30
LQ1	27	9	45
LQ2	14	4	34
LQ931	39	19	48
LQ914	28	7	30
L777A	3	4	16
E 88	2	0	< 1

* Figures are each the mean of 3 observations to thenearest whole number. A ± 50% deviation was observed in many cases between different observations for the same strain.

TABLE 45

Sporulating potential and dipicolinic acid production in cultures of Cl.tetani

Strain	Sporing potential(%)	Dipicolinic acid (OD_{440})
Serotype I	32	25
II	36	25
III	66	28
IV	28	20
V	54	24
VI	26	18.5
VII	60	23.5
VIII	12	20
IX	68	24
CN1445	14	16
" 4878	38	17
" 361	10	18
" 1342	1	15
" 761	52	23
" 947	24	17
" 780	36	21
" 3973	50	21
" 1349	26	18
L5500	6	15
L109	< 1	11
LQ730	36	18
LQ1	54	20
LQ2	20	18
LQ931	34	13
LQ914	26	15
L777A	22	11
E88	< 1	15

TABLE 46

Motility of Cl.tetani strains

Strain	Motility method			
	swarming	Motility test agar	Wet film	Leifson stain
Serotype I	+	+	+	tr
II	+	+	+	tr
III	+	+	+	tr
IV	+	+	+	tr
V	+	+	+	tr
VI	-	-	-	-
VII	+	+	+	tr
VIII	+	+	+	tr
IX	+	+	+	tr
CN1445	+	+	+	tr
" 4878	+	+	+	tr
" 361	+	+	+	tr
" 1342	+	+	+	tr
" 761	+	+	+	tr
" 947	+	+	+	tr
" 780	+	+	+	tr
" 3973	+	+	+	tr
" 1349	-	-	+	tr
L5500	+	+	+	tr
L109	+	+	+	tr
LQ730	+	+	+	tr
LQ1	+	+	+	tr
LQ2	+	+	+	tr
LQ931	+	+	+	tr
LQ914	+	+	+	tr
L777A	+	+	+	tr
E88	-	-	+	tr

+ = Positive

- = negative

tr = very weakly positive

TABLE 47 a

Minimum Inhibitory Concentrations (MICs) of 15 antibiotics to Cl.tetani strains

Strain	Antibiotic P	M	A	Ct	G	K	E
Serotype I	.06	.5	<.12	<.5	>16	>64	.5
II	.125	.5	<.12	<.5	>16	>64	.25
III	.06	1	<.12	<.5	>16	>64	.25
IV	.03	.5	<.12	<.5	>16	>64	.5
V	.125	.5	<.12	<.5	>16	>64	.125
VI	.06	.5	<.12	<.5	>16	>64	.5
VII	.03	.5	<.12	<.5	>16	>64	.5
VIII	.125	1	<.12	<.5	>16	>64	.125
IX	.03	.5	<.12	<.5	>16	>64	.125
CN 1445	.06	.5	<.12	<.5	>16	>64	.5
" 4878	.03	.5	<.12	<.5	>16	>64	.25
" 361	.03	.5	<.12	<.5	>16	>64	.25
" 1342	.125	.5	<.12	<.5	>16	>64	.25
" 761	.03	.5	<.12	<.5	>16	>64	.5
" 947	.125	1	<.12	<.5	>16	>64	.125
" 780	.06	1	<.12	<.5	>16	>64	.5
" 3973	.06	.5	<.12	<.5	>16	>64	.25
" 1349	.06	.5	<.12	<.5	>16	>64	.5
L5500	.06	.5	<.12	<.5	>16	>64	.125
L109	.06	.5	<.12	<.5	>16	>64	.25
LQ730	.03	.5	<.12	<.5	>16	>64	.25
LQ1	.06	.5	<.12	<.5	>16	>64	.125
LQ2	.125	.5	<.12	<.5	>16	>64	.25
LQ931	.125	1	<.12	<.5	>16	>64	.25
LQ914	.03	.5	<.12	<.5	>16	>64	.5
L777A	.06	.5	<.12	<.5	>16	>64	.125
E88	.06	.5	<.12	<.5	>16	>64	.125

(MIC expressed in mg/l.)

P = Penicillin G M = Methicillin A = Ampicillin

Ct = Cephalothin K = Kanamycin E = Erythromycin

TABLE 47 b

Minimum Inhibitory Concentrations (MICs) of 15 antibiotics to Cl.tetani strains

Strain	Antibiotic							
	Ch	T	V	Cl	Mz	Cf	Cb	Cx
Serotype I	2	<.12	.25	<.06	.5	.25	.5	.5
II	2	<.12	.5	<.06	2	.25	.5	1
III	1	<.12	.25	<.06	.5	.25	.5	1
IV	.5	<.12	.25	<.06	.25	.5	.25	.5
V	1	<.12	.25	<.06	1	.5	1	1
VI	.5	<.12	.5	<.06	.5	.5	.5	.5
VII	.5	<.12	.25	<.06	.25	.5	.5	1
VIII	.5	<.12	.5	<.06	.5	.5	.5	.5
IX	.5	<.12	.5	<.06	.25	.5	.25	.5
CN 1445	.25	<.12	.25	.125	.5	1	.5	1
" 4878	1	<.12	.25	<.06	.5	.5	.5	1
" 361	.25	<.12	.25	<.06	.25	.25	.5	1
" 1342	1	<.12	.5	<.06	.25	.25	.25	.5
" 761	2	<.12	.25	<.06	.25	.5	.5	1
" 947	1	<.12	.25	.125	.5	.5	.25	1
" 780	.25	<.12	.5	<.06	.25	.25	.25	.5
" 3973	1	<.12	.25	<.06	.5	.25	.5	1
" 1349	2	<.12	<.25	.125	1	.25	.25	.5
L5500	2.5	<.12	.25	<.06	.125	1	.5	.5
L109	.5	<.12	.25	<.06	.25	.25	.25	1
LQ730	.5	<.12	.5	<.06	.5	.25	.25	1
LQ1	.5	<.12	.25	<.06	.125	.25	.25	1
LQ2	.5	<.12	.25	<.06	.5	.5	.5	.5
LQ931	.25	<.12	.5	<.06	.5	.5	.5	.5
LQ914	1	<.12	.5	.125	.5	.5	.5	.5
L777A	1	<.12	.25	.125	.5	1	.5	.5
E88	2	<.12	.25	.125	.5	.25	.5	.25

(MIC expressed in ug/ml)

Ch = Chloramphenicol T = Tetracyclines V = Vancomycin

Cl = Clindamycin Mz= Metronidazole Cf = Cefoxitin

Cb = Carbenicillin Cx = Cephalexin

TABLE 48

Grouping of Cl.tetani strains by Polyacrylamide gel electrophoresis (PAGE) of culture supernatants

Strain	PAGE Pattern Code	Pattern
Serotype I	1,3	A
II	2,4	B
III	1,2,4	B
IV	2,4	B
V	2,4	B
VI	2,4	B
VII	2,4	B
VIII	3,4	C
IX	2,4	B
CN1445	1,3	A
"4878	2,4	B
" 361	1,3	A
" 1342	1,3	A
" 761	1,3	A
" 947	2,4	B
" 780	2,4	B
" 3973	2,4	B
" 1349	3	D
L5500	2,4,(5)*	E
L109	2,4	B
LQ730	2,4	B
LQ1	2,4,(5)*	E
LQ2	2,4	B
LQ931	2,4	B
LQ914	2,4,(5)*	E
L777A	2,4	B
E88	3	D

* band 5 was not always present

TABLE 49

Isoelectric focussing (IEF) of Cl.tetani proteins

Strain	No.of bands present	pH 4.25 band
Serotype I	3	-
II	4	+
III	4	-
IV	4	-
V	4	+
VI	2	-
VII	4	-
VIII	4	-
IX	5	+
CN 1445	3	-
" 4878	4	+
" 361	3	-
" 1342	4	-
" 761	3	-
" 947	4	+
" 780	4	-
" 3973	4	+
" 1349	2	-
L5500	5	+
L109	3	-
LQ730	3	-
LQ1	4	+
LQ2	4	+
LQ931	4	-
LQ914	3	-
L777A	3	-
E88	2	-

+ = positive

- = negative

TABLE 50

Plasmid carriage in Cl.tetani strains

Strain	Plasmid carriage
Serotype I	+
II	+
III	+
IV	+
V	+
VI	+
VII	+
VIII	+
IX	+
CN 1445	+
" 4878	+
" 361	+
" 1342	-
" 761	+
" 947	+
" 780	+
" 3973	+
" 1349	+
L5500	+
L109	+
LQ730	+
LQ1	+
LQ2	+
LQ931	-
LQ914	-
L777A	-
E88	+

+ = positive

- = negative

TABLE 51 a

Comparison of sporulating potentials and other cultural and metabolic characteristics in Cl.tetani strains

Strain	Sporulating* Potential	Neurotoxin+ Production (Lf/ml)	Haemolysin* titre	motility¶	PAGE¶ Pattern
Serotype I	22	28	128	+	A
II	36	14	64	+	B
III	64	28	64	+	B
IV	28	112	64	+	B
V	40	56	128	+	B
VI	20	112	0	-	B
VII	55	112	128	+	B
VIII	9	112	128	+	C
IX	50	28	643	+	B
CN 1445	16	56	256	+	A
" 4878	32	28	128	+	B
" 361	12	56	128	+	A
" 1342	< 1	0	128	+	A
" 761	35	112	64	+	A
" 947	29	56	64	+	B
" 780	33	14	128	+	B
" 3973	37	56	64	+	B
" 1349	32	56	64	trace	D
L5500	9	112	64	+	E
L109	< 1	7	128	+	B
LQ730	30	112	64	+	B
LQ1	45	3.5	64	+	E
LQ2	34	7	64	+	B
LQ931	48	0	64	+	B
LQ914	30	0	64	+	E
L777A	16	56	128	+	B
E88	< 1	224	128	trace	D

* in cooked meat medium at 30°C for 7 days (table 44)

\+ in Massachussetts medium by HAI (table 16)

* in FAB (table 11)

§ Chapter 5

‖ Chapter 6

¶ Chapter 7

TABLE 51 b

Comparison of sporulating potentials and other cultural and metabolic characteristics in Cl.tetani strains

Strain	IEF ¶ pH 4.25 band	large § plasmid carriage	Gelatin § liquefaction	Casein § hydrolysis	DNA/ § RNA -se	Amino ¶ acid deamination	Fluorescence ¶ on MacConkey
Serotype I	-	+	-	+	+	Cy	+
II	+	+	+	+	+		+
III	-	+	+	+	+		+
IV	-	+	+	+	+	Cy	+
V	+	+	+	+	+		+
VI	-	+	-	+	+	C	+
VII	-	+	+	+	+		+
VIII	-	+	-	+	+		+
IX	+	+	+	+	+	C	+
CN1445	-	+	+	+	+		-
" 4878	+	+	+	+	+	C	+
" 361	-	+	+	+	+	C	+
" 1342	-	-	+	+	+		+
" 761	-	+	+	+	+	C	+
" 947	+	+	-	-	-		+
" 780	-	+	+	+	+		+
" 3973	+	+	+	+	+	As.,Cy.	+
" 1349	-	+	+	+	+		+
L5500	+	+	+	+	+	C	+
L109	-	+	+	+	+	L.S.	-
LQ730	-	+	+	+	+		+
LQ1	+	+	+	+	+	Cy.	+
LQ2	+	+	+	+	+	As.V	+
LQ931	-	-	+	+	+	L	+
LQ914	-	-	+	+	+		+
L777A	-	-	+	+	+	L.Cy.	+
E88	-	+	+	+	+	As.Cy.	+

C = L.Cysteine
Cy= L.Cystine
As= L.Asparagine
S = L.Serine
L = L.Leucine
V = L.Valine

TABLE 52

Agglutination testing of Cl.tetani strains

Strain	'H' Agglutination titre
Serotype I	10
II	80
III	10
IV	10
V	10
VI	10
VII	10
VIII	10
IX	10

TABLE 53

Soluble antigens of Cl.tetani strains

Strain serotype	Number of precipitin lines or peaks produced by Culture supernatants (examined by DID)	Mechanically disrupted cells (examined by 2DIEP)	Triton X-100 extracts (examinedby 2DIEP)
I	2	8 -	7 (7)*
II	2	7 (1)*	6 (7)
III	2	7 (7)	7 (7)
IV	3	7 (1)	7 (7)
V	3	7 (1)	7 (7)
VI	1	5 (1,2,4)	7 (7)
VII	3	7 (2)	6 (2,7)
VIII	3	6 (1,2)	5 (1,2,7)
IX	2	8 -	7 (7)

* Figures in parenthesis indicate the number of the missing precipitin peak when compared to the pattern produced by Serotype I.

TABLE 54

Examination of strains of Cl.tetani strains using Cl.tetani fluorescent conjugate absorbed with Cl.perfringens

Strain and serotype	Degree of fluorescence with 48 hour cultures from Columbia blood agar	FAB
I	±	+
II	+	++
III	+++	+++
IV	+	±
V	+	+
VI	+	+
VII	±	±
VIII	±	+
IX	±	+

+++ = very strong fluorescence

++ = strong

+ = moderate

± = weak

TABLE 55

Examination of strains of Cl.tetani using Cl.tetani fluorescent conjugate absorbed with serotype strains of Cl.tetani

Serotype of strain examined	Degree of fluorescence with fluorescent conjugate absorbed with strains of serotypes:								
	I	II	III	IV	V	VI	VII	VIII	IX
I	tr	tr	tr	tr	tr	tr	tr	tr	tr
II	tr	tr	-	-	tr	tr	tr	tr	tr
III	+	tr	-	-	++	++	+	+	++
IV	-	-	-	-	-	tr	tr	tr	-
V	tr	-	-	tr	-	tr	tr	-	-
VI	-	-	-	-	-	-	-	-	-
VII	-	-	-	-	-	-	-	-	-
VIII	-	-	-	-	-	-	-	-	-
IX	tr	-	-	-	-	-	-	-	-

++ = strong fluorescence

+ = moderate

± = weak

tr = trace (barely visible)

- = no fluorescence

APPENDIX 1

NCTC Strains of Cl.tetani

NCTC*	279	Serotype	I
"	540		II
"	539		III
"	5410		IV
"	5411		V
"	9569		VI
"	9568		VII
"	9574		VIII
"	9575		IX

* NCTC - National Collection of Type Cultures

APPENDIX 2

Wellcome Collection strains of Cl.tetani

and other strains used in this study

CN	1445	(Wellcome Collection)§
"	4878	"
"	361	"
"	1342	"
"	761	"
"	947	"
"	780	"
"	3973	"
"	1349	(Harvard strain) subculture "
L	5500	(Laboratory isolate) - soil ‡
L	109	(Laboratory isolate) - soil ‡
LQ	730	(DMRQC)*
LQ	1	"
LQ	2	"
LQ	931	"
LQ	914	"
L	777A	(Laboratory isolate) - infected wound ‡
E	88	+ (Harvard strain)

* Division of Microbiological Reagents and Quality Control, Central PHL, 61 Colindale Avenue, London.

\+ Courtesy of Dr. Ulrich Eisel, Institut für Medizinische Virologie der Justus-Leibig-Universität, Geissen, West Germany.

‡ Microbiology Department, St. George's Hospital, Stafford.

§ Wellcome Research Laboratories, Beckenham, Kent.

APPENDIX 3

Standard RBC suspension for haemolysin assays[55]

Defibrinated horse red blood cells[29] were washed twice in Antistreptolysin O buffer, pH 7.0[29] and resuspended to their original volume. The concentration of this suspension was adjusted so that a sample, after haemolysis with zapoglobin[90] and addition of an equal volume of buffer, gave an absorbance of 60 at 545 mu on an EEL Spectrophotometer[91].

APPENDIX 4

Tube haemolysis technique

Serial dilutions of haemolysin were made in ASO buffer from neat to 1; 2048 in 3 x $\frac{1}{2}$" glass test tubes in 1ml quantities. 1ml of the standard RBC suspension was added to each dilution. Positive controls were distilled water plus RBCs and negative controls were ASO buffer plus RBCs.

The suspensions were mixed and incubated in a 37°C waterbath for 1 hour, before being centrifuged briefly.

The colour of the haemoglobin in the supernatant fluids was compared visually with that of the positive and negative controls and the dilution haemolysing 50% of the RBCs was interpolated. This was confirmed by reading the absorbance of each of the supernatants at 545 u on an EEL Spectrophotometer blanked on distilled water and plotting the percentage haemolysis, taking the positive control as 100% haemolysis and the negative control as 0% haemolysis.

The dilution which showed the 50% endpoint was taken as the haemolysin titre.

APPENDIX 5

Microtitre plate haemolysis test

Serial dilutions of haemolysin were made in ASO buffer from neat to 1:2048 in 'U' bottomed plastic microtitre trays[92] in 25 ul volumes. 25 ul of the standard RBC suspension was added to each dilution and to positive and negative controls and the plates were shaken for 5 seconds on a microtitre plate shaker[93] before being covered with plastic film and incubated at 37°C for 1 hour.

The wells were examined for the presence of a button of unlysed RBCs at the bottom, or for the presence of free haemoglobin and the well showing 50% lysis was determined. The dilution showing the 50% endpoint was taken as the haemolysin titre.

APPENDIX 6

Radial haemolysis plate test

Serial dilutions of haemolysin were made in ASO buffer from neat to 1:2048. 100 ul of each were pipetted into 5mm wells cut into agarose plates (2% in ASO buffer) containing 50% (v/v) standard RBC suspension and the plates were incubated for 4 hours at 37°C.

Following incubation, the plates were examined for radial zones of haemolysis around the wells. The last dilution showing evidence of visible haemolysis was taken as the haemolysin titre.

In some experiments 25 ul of each dilution was pipetted onto sterile 5mm filter paper discs which were subsequently placed on the surface of plates and incubated and examined as before.

APPENDIX 7

Physiological saline

NaCl - 0.85 grams

Distilled water - 100 ml

This was autoclaved at 121°C for 15 minutes before use

Physiological saline + 1% gelatin

As above but with 1 gram gelatin[29] added

ASO buffer[29]

25ml of sterile distilled water was added to 1 vial of dried ASO buffer and mixed until dissolved. It was used within 24 hours of reconstitution

FAB broth[61]

FAB powder - 29 grams

Distilled water - 100 ml

This was distributed into bottles in the required amounts and autoclaved at 121°C for 15 minutes.

It was used as soon as possible.

If the medium began to turn pink it was not used.

Motility test agar[30,183]

FAB powder - 2.9 g

Triphenyltetrazolium chloride (TTC) - 0.005g

Agar - 0.2g

Distilled water - 100 ml

This was autoclaved at 121°C for 15 minutes and distributed into sterile bijoux bottles in 3ml amounts.

Gram's stain[183]

1) a) Crystal violet 10g

Ethanol (95%) 100ml. Mix and dissolve

b) Ammonium oxalate 1% aqueous solution

20ml of a) and 80ml of solution b) were mixed for use.

2) Lugol's Iodine

Iodine 5g

Potassium iodide 10g

Distilled water 100ml

This was diluted 1:5 with distilled water for use

3) Iodine-acetone decolouriser

Iodine 10g

Potassium iodine 6g

Distilled water 10ml

Ethanol (90%) to 100ml

For use 3.5ml of the above was added to 96.5ml acetone.

4) Carbol fuchsin

A) Basic fuchsin 10g

Ethanol (95%) 100ml

This was mixed and allowed to dissolve and was stored at 37°C overnight.

B) Phenol 5g

Distilled water 100ml

This was mixed and allowed to dissolve.

10ml A was added to 100ml B.

This was diluted 1:10 in distilled water for use.

Slides were flooded with crystal violet solution for 30 seconds, washed with water and flooded with Lugol's iodine for 30 seconds. This was washed off thoroughly with iodine-acetone solution which was left on for 30 seconds before washing in water and counterstaining with carbol fuchsin for 30 seconds. This was washed off with water, the slides were air dried and examined.

<u>Leifson's stain</u>

1) Basic fuchsin 3g

95% ethyl alcohol 250ml

This was shaken and allowed to stand until dissolved.

2) NaCl 3.75g

tannic acid[69] 7.5g

distilled water 500ml

This was added to the basic fuchsin-alcohol mixture and shaken thoroughly.

When made up, the stain was stored in the freezer and allowed to stand for 24 hours before using. Before each subsequent use the stain was equilibrated at room temperature for 2 to 4 hours.

Slides were flooded with 2ml of stain for between 6 and 15 minutes. (When the dark red colour was replaced by lighter pink precipitated stain and a gold-green sheen had formed around the edges.) The slides were then rinsed with distilled water, air dried and examined.

2) NaCl 3.75g

tannic acid[69] 7.5g

distilled water 500ml

This was added to the basic fuchsin-alcohol mixture and shaken thoroughly.

When made up, the stain was stored in the freezer and allowed to stand for 24 hours before using. Before each subsequent use the stain was equilibrated at room temperature for 2 to 4 hours.

Slides were flooded with 2ml of stain for between 6 and 15 minutes. (When the dark red colour was replaced by lighter pink precipitated stain and a gold-green sheen had formed around the edges.) The slides were then rinsed with distilled water, air dried and examined.

APPENDIX 8

Measurements of bacterial density as a measure of the amount of growth in cultures of Cl.tetani

Fluid cultures of Cl.tetani were centrifuged to deposit the cells. The supernatants were carefully removed and the cells were resuspended in PBS to a volume equal to the original. These suspensions were washed by centrifuging and removing the supernatant once more.

Following this, the cells were resuspended in PBS to the original volume and the bacterial density was measured at 600 u on an EEL spectrophotometer. The blank was distilled water.

It has been stated by Mellanby[94] that there is a linear relationship between dry weight of organisms and extinction (absorption) at 600 u. The cells were washed in this method to remove any supernatant broth which might interfere with the readings.

APPENDIX 9

Massachussetts Medium[95]

*Casein digest	25g
Glucose	8g
NaCl	2.5g
$MgSO_4$	0.1g
Cystine	0.125g
Calcium pantothenate	1mg
Uracil	1.25mg
Nicotinic acid	0.25mg
Thiamine	0.25mg
Riboflavin	00.25mg
Pyridoxine	0.25mg
Biotin	2.5ug
Vitamin B_{12}	0.05ug
$FeCl_3.6H_2O$	32mg
Distilled water	1000ml

Distribute in 50ml amounts in 250ml glass bottles and autoclave at 121°C for 20 minutes.

Use as soon as possible.

* The Casein digest used was Sheffield Products N-Z-Case[12]TT.Lot No. 4NB14. Kindly supplied by Morham Limited.[109]

Before use Calcium phosphate precipitation[110] was performed to remove inhibitory substances as follows:

A20% solution of the material was heated to boiling and 3.75g Anhydrous $CaCl_2$ added followed by 7.5g K_2HPO_4. The pH was adjusted to 9.3 with 6N NaOH and the solution filtered through a Whatman No.1 filter[103]. The pH of the filtrate was then adjusted to 7.2 - 7.6 with concentrated HCl before use.

All other chemicals were from BDH.[69]

APPENDIX 10

Production of heat-treated variant of Cl.tetani NCTC 279

Cl.tetani NCTC 279 is a very weakly sporing strain and in many cultures is virtually asporogenous. The following procedure had to be attempted many times before a variant strain could be isolated.

A heavy suspension of Cl.tetani NCTC 279 from 7 day old Columbia blood agar plates incubated anaerobically at 30°C was made in 0.5ml sterile saline. This suspension was heated at 80°C for 10 minutes in a waterbath before being subcultured (0.1ml) onto a fresh Columbia Blood Agar plate which was subsequently incubated anaerobically at 37°C until growth appeared, and also into fresh FAB medium which was incubated at 37°C and subcultured onto Columbia blood agar plates daily. These were incubated anaerobically at 37°C until visible growth appeared. Any growth following heat-treatment was examined for sporulation by gram-stained film.

Once a sporing variant was isolated, it was stored as a heavy suspension in distilled water at -40°C until needed when it was grown overnight on Columbia blood agar at 37°C anaerobically before use.

The sporing variant rapidly reverted to the weakly sporing parent strain on subculture. Usually only 3 or 4 subcultures were necessary for full reversion.

APPENDIX 11

Estimation of spore production in Cl.tetani strains[50]

Films were made from cultures under examination and heat-fixed in a bunsen burner. The films were subsequently Gram-stained and examined microscopically.

Sporulating potential and spore production was calculated by examining 20 microscopic fields containing approximately 50 to 150 cells per field for the number of spores present versus the total number of cells. Gram-positive forespores were also included in the number of spores. From these figures the % of cells bearing spores was calculated, using the following formula:

$$\frac{B \times 100}{T}$$

where T = the total number of cells and B = the number of cells bearing spores.

APPENDIX 12

Protein Determination[79]

Stock reagents were made up as follows:

1) A solution of about 20% sodium carbonate was added slowly, with stirring, to a solution of copper sulphate-potassium tartrate to give final concentrations of 0.1% copper sulphate (pentahydrate), 0.2% potassium tartrate and 10% sodium carbonate. This solution is stable for at least 2 months at room temperature.

2) 10% sodium dodecylsulphate (SDS)

3) 0.8 M sodium Hydroxide.

4) Folin-Ciocalteu phenol reagent.

Reagent A was made by mixing equal volumes of stock solutions 1, 2 and 3 and distilled water.

Reagent B was made by mixing one volume of stock solution 4 with 5 volumes of distilled water.

Method

Samples (containing between 5 and 100 ug protein) were brought to a total volume of 1ml with distilled water. 1ml of Reagent A was added, mixed and allowed to stand for 10 minutes at room temperature. Then 0.5ml of Reagent B was added, mixed and incubated at room temperature for 30 minutes.

The absorbance was read within 2 hours at 700 nm on an EEL spectrophotometer.

The standard curve was constructed using solutions containing 25, 50, 75 and 100 ug/ml Bovine serum albumin, fraction V (Sigma), prepared from a stock solution containing 0.5mg/ml.

Sodium carbonate, copper sulphate, potassium tartrate, sodium hydroxide, SDS and Folin-Ciocalteu reagent were from BDH[69], bovine serum albumin was from Sigma.[27]

APPENDIX 13

Double Immunodiffusion technique

Double immunodiffusion (DID) in gel was performed according to the principles laid out by Nilsson.[80]

Agarose (1%) in PBS containing 0.02% sodium azide was poured onto glass microscope slides (3ml) or glass electrophoresis plates (6ml) to a depth of 1.5mm. Wells were cut with a gel cutter[99] in a circular pattern with one central well 5mm in diameter and eight peripheral 5mm diameter wells at a distance of 5mm edge-to-edge. 25ul of antibody was pipetted into the central well and 25ul of the test solutions were pipetted into the peripheral wells. The slides were incubated at room temperature for 48 hours before being pressed between filter paper sheets and dried in a hot-air oven. The dried slides were stained in 0.5% Naphthalene black in a solution of Methanol:Acetic Acid:Water (5:1:4) for 10 minutes and destained in the dye-free solution.

Precipitin lines were easily seen as sharp, blue-black stained bands between the central and peripheral wells.

Agarose was from BDH[69] and Naphthalene black was from Gurrs.[96]

Single Radial Immunodiffusion[134]

Slides were prepared as above except they contained either 0.3ml of unabsorbed Wellcome horse antitoxin, or 0.3ml of the same antitoxin absorbed with a young culture of Cl.tetani cells (Appendix 18) to remove all antibodies except that to the toxin.

3mm wells were cut as before and 10 ul of antigen pipetted into them. The slides were placed in a humidity chamber and incubated at room temperature for 24 hours before being dried and stained as before.

Precipitin rings were seen around wells containing specific antigens and their diameters are proportional to the amount of antigen present. More than one ring indicates more than one antigen-antibody reaction.

A standard curve was produced by diluting either TOX or VAC in PBS

to contain 25, 10 and 5 Lf/ml or 28, 14 and 7 Lf/ml respectively. The diameter of the precipitin rings produced by these standards was measured and plotted against Lf/ml and the diameter of any unknown could be read off this plot directly in Lf/ml.

APPENDIX 14

Immunoelectrophoresis techniques

a) Two-dimensional Immunoelectrophoresis[84]

Stock buffer Tris/barbital buffer, pH 8.6, ionic strength (u) = 0.1

Tris (hydroxymethyl methylamine)	44.3g
5 S-diethylbarbituric acid (barbital)	22.4g
Calcium lactate	2g
Sodium azide	1g
Distilled water	1 litre

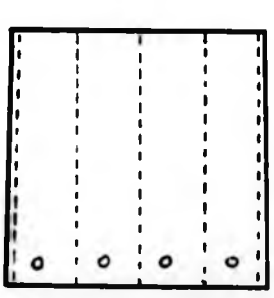
All chemicals from BDH[69]

Agarose = Agarose 15 ($-M_r$= 0.15)[69]

Preparation

4.5ml of melted 1% (w/v) Agarose in Tris barbital buffer pH8.6 u=0.02 (stock diluted 1:5) was poured onto a 5 x 5cm glass plate and allowed to gel, on a horizontal table.

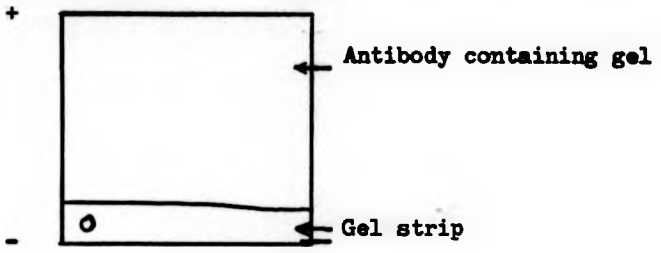
Four wells (3mm or 5mm diameter) were made in the gel, using gel punches, along the cathodic (-ve) edge of the plate and about 5mm from the edge, as in the template.



The antigen samples (10 ul to 25 ul) were placed in the wells and the gel placed in the electrophoresis chamber. Connection to the buffer in the electrode chambers (Tris/barbital pH 8.6u = 0.1) was made by Cambrelle wicks[97] and electrophoresis was performed for 45 minutes with a constant current of 12MA (2.5MA/cm).

The gel was then sliced with a razor blade along the dotted lines

shown in the template and each strip was removed and transferred to the cathodal end of a clean glass plate (5 x 5cm) overlaid with Gelbond[98] cut to the same size, as in the diagram.



3.5ml of antibody containing gel (usually 0.5ml antibody to 3ml agarose unless otherwise stated) was poured onto the rest of the plate and allowed to set. Electrophoresis in the second dimension was performed at a constant current of 5mA (1mA/cm) for 18 - 20 hours (overnight).

After electrophoresis the gel is pressed, dried and stained as described for double diffusion (Appendix 13).

Measurement of the relative mobility of an antigen was accomplished using the above system and adding 1 ul of Bovine serum albumin (BSA) (0.005% in PBS)[27] to the antigen and adding 33 ul of Antibovine serum albumin[98] to the agarose containing the antitoxin. This produced an internal BSA/antiBSA reference peak. The distance this had migrated from the sample well could be measured and the mobility of other peaks relative to this figure could be calculated as follows:

$$\frac{R}{T} = Rm \qquad \text{(Relative mobility)}$$

where R is the distance of migration of the reference peak and T is the distance of migration of the test peak.

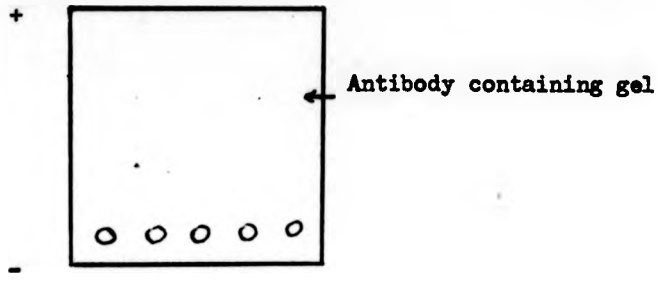
b) Rocket Immunoelectrophoresis[81]

The same buffers and agarose as Two dimensional IEP were also used here.

1% Agarose (w/v) in Tris barbital buffer pH 8.6 u = 0.02 was melted and cooled to 56°C, before antibody was added. For 5 x 5cm glass plates, 0.5ml antiserum was added to 3ml of agarose and for 5 x 10cm glass plates, 1ml antiserum was added to 6ml of agarose before pouring

and allowing to gel as before.

Wells were cut using a gel cutter[99] (3mm or 5mm diameter) along the cathodic edge of the gel, as shown in the template.



10 ul samples were placed in the wells and electrophoresis was performed at a constant current of 5mA for 5 x 5cm plates and 10mA for 5 x 10cm plates for 18 - 20 hours.

After electrophoresis gels were pressed, dried and stained as before.

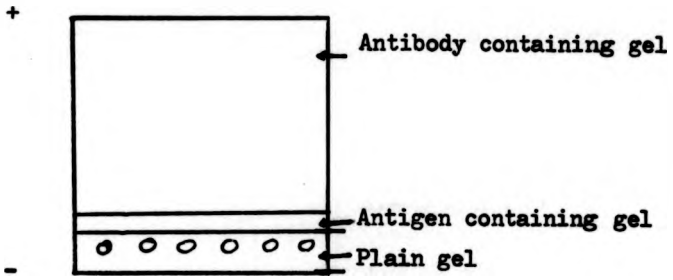
Standard curves were prepared using TOX diluted in PBS to give 75, 50, 25, 10 and 5 Lf/ml and VAC diluted in PBS to give 28, 14, 6.6 and 2.8 Lf/ml. In addition, 20 ul and 40 ul samples of VAC (giving equivalent to 56 and 112 Lf/ml) were also used to complete this curve. Peak heights were measured and plotted against Lf/ml. The height of unknown peaks could then be measured and read directly off the plot in Lf/ml.

c) Rocket-Line Immunoelectrophoresis[123]

This technique is similar to rocket immunoelectrophoresis but a trough containing another antigen is cut across the plate slightly above the sample wells as in the diagram. 6ml of 1% Agarose (w/v) in Tris barbital buffer pH 8.6 u=0.02 was melted and cooled to 56°C before 1ml of antiserum was added. This was mixed and poured onto a 5 x 10cm glass plate and allowed to gel. A 1.5cm wide strip was cut from the cathodic end with arazor blade and removed. This was replaced by pouring 2ml of molten agarose without antiserum onto the vacant area of the plate and allowing to gel.

A trough was cut 3mm wide and 10cm long across this gel at the end closest to the antiserum containing gel, as in the template. Into this trough was poured 0.7ml of molten agarose containing the antigen under study. 3mm sample wells were cut along the cathodic side of this trough and electrophoresis was performed at a constant current of 10mA/plate for 18 - 20 hours.

Pressing, drying and staining were as before as was preparation of the standard curve.



APPENDIX 15

Polyacrylamide Gel Electrophoresis (PAGE)

This technique is taken from the method of Laemmli[82] as modified by Byrne[4], and using a technical manual from Pharmacia.[83]

Slab gels (180 x 160mm) composed of 10% (w/v) acrylamide separating gel with a 20mm 4% (w/v) stacking gel were used to run as many as 24 samples simultaneously, in a Shandon Southern slab gel apparatus.[97]

Acrylamide (22.2g) and methylbisacrylamide (0.6g) were dissolved in water to give 100ml of stock solution. Separating gel, 10% (w/v) was cast by mixing 16.875ml of 0.75 mol/L Tris HCL buffer pH 8.8 containing 0.2% (w/v) Sodium dodecyl sulphate (SDS), with 15.18ml of acrylamide stock. This mixture was degassed at 2000 millitorr for 10 minutes on a Virtis freeze dryer[100] before adding 50ul of NNN'N' tetramethylethylene diamine (TEMED) and 1.65ml ammonium persulphate (15mg/ml) solution. The gel was poured between glass plates and allowed to polymerise.

A 4% stacking gel composed of 5ml of 0.25 mol/L Tris HCL buffer pH 6.8 containing 0.2% (w/v) SDS, 3.3ml Acrylamide solution (1g acrylamide and 0.25g methylbisacrylamide in 10ml distilled water) and 1.7ml ammonium persulphate (15mg/ml) was poured on top of the separating gel and allowed to polymerise with a 24 well forming comb in place.

The electrode buffers were made up as follows:

Tris (3.02g), glycine (14.4g) and SDS (1g) were dissolved in 1 litre of distilled water. The pH was 8.3 and rarely needed adjustment.

Samples were solubilised by mixing with an equal volume of sample buffer made up as follows; 0.0625mol/L Tris HCL buffer pH 6.8 containing 20% glycerol, 10% 2-mercaptoethanol and 0.001% (w/v) Bromophenol blue; and heated at 100°C for 5 minutes. 50 ul of each sample was applied per well and electrophoresis was performed for 5 - 6 hours at 45 mA constant current. Staining was carried out overnight in 0.25% Coomassie Brilliant Blue R in Methanol: Acetic Acid: Water (40:7:53) and destaining

was in the dye free buffer.

Acrylamide, methylbisacrylamide, TEMED and Bromophenol blue were obtained from Shandon Southern[97] as was all the electrophoresis equipment. Ammonium persulphate was from Sigma[27]; Methanol, Acetic Acid, Tris SDS and mercaptoethanol were from BDH[69] and glycerol was from Difco.[29] Molecular weight marker proteins were obtained from Sigma[27],(Myosin, MW205. B.Galactosidase, MW16. Phosphoylase B, MW 97.4. Bovine serum albumin, MW 66. Egg albumin, MW45 and Carbonic anhydrase MW 29. Kilo daltons were used).

APPENDIX 16

Latex agglutination test[87]

Reagents

1) Glycine buffered saline pH 8.2 (GBS)

Glycine 7.3g and Sodium chloride 10g were dissolved in 900ml distilled water and the pH adjusted to 8.2 using 1.0N Sodium hydroxide before the final volume was brought to 1 litre.

2) Glycine buffered saline plus Bovine serum albumin (GBS-BSA)

Bovine serum albumin was added to GBS to a final concentration of 0.1%.

3) Latex.

The suspension of polystyrene latex particles (0.81mu, Difco)[29] were used unwashed and undiluted and stored at 4°C.

Latex sensitization

To one part of antibody diluted 1:2 in GBS an equal amount of latex suspension was added. The mixture was shaken well and allowed to stand in a water bath at 37°C for 2 hours. Subsequently, two parts of GBS-BSA were added. This sensitised latex suspension is ready for use and can be stored for at least one year at 4°C.

Latex agglutination test

To 40 ul of test suspension, 20 ul sensitised latex was added on a ring slide. After 3 minutes shaking the agglutination was read with the naked eye. Results were expressed as 0, +, ++, +++ or ++++.

The titre is the reciprocal of the highest dilution of the sample that gives a ++ or greater agglutination.

APPENDIX 17

Staphylococcal Coagglutination test[88]

Staph aureus Cowan 1 strain was incubated for 18 hours at 37°C on Columbia blood agar plates and harvested by washing off the plates in PBS. The organisms were washed twice in PBS and treated for 3 hours in 0.5% buffered Formalin, pH 7.4.

The suspension was incubated for 1 hour at 80°C and adjusted to 10% (v/v) suspension in PBS with 0.01% sodium azide.

An equal amount of Staph aureus suspension and tetanus antitoxin (both neat and absorbed with serotype VI - see Appendix 18) was mixed and incubated at room temperature for 1 hour with occasional shaking. The suspensions were washed six times in PBS and diluted to make a 1% (v/v) suspension in PBS with 0.01% sodium azide. The reagents were stable at 4°C for at least 4 months.

40 ul of sample was mixed with 40 ul of sensitised Staph aureus suspension on a glass slide and rocked for 10 minutes. Reactions were determined in indirect light against a black background and graded 0 to 4+ as before (Appendix 16).

APPENDIX 18

Absorption of Cl.tetani antitoxin

The strain of Cl.tetani to be used in the absorption was grown on Columbia Blood Agar plates for 24 hours at 37°C anaerobically. The growth was scraped off 10 plates using a plastic inoculating loop and emulsified in 10ml of Tetanus antitoxin[101]. After mixing for 10 seconds on a Miximatic vortex mixer[102] the suspension was incubated for 48 hours at 37°C and subsequently at 4°C for 7 days. The cellular debris was removed by centrifugation and the supernatant used as the absorbed antitoxin.

APPENDIX 19

Two dimensional Paper Chromatography for Amino Acids[89]

Standard Solutions

Standard amino acid 'marker' solutions were made up to a cencentration of 5mg/ml in distilled water with the addition of a drop of HCl to aid dissolution as follows:

Standard Solution I	Standard Solution 2
Phenylalanine	**Asparagine**
L-Lysine	**Glutamic acid**
Hydroxy-L proline	**Proline**
Alanine	**Serine**
Glycine	**Threonine**
Isoleucine	**L-Cystine**
Leucine	**Aspartic acid**
L-Cysteine	**Valine**
Histidine	**L-Glutamine**
Arginine	**Tryptophan**
	Methionine

Solvents

All obtained from Sigma[27]

1) Butanol-Acetic Acid

N-Butanol	120ml
Glacial Acetic Acid	30ml
Water	50ml

2) Phenol-Ammonia

Phenol	500g
Water	125ml

Just before use add 0.65ml of Ammonia to the above. All from BDH.[69]

Chromatography Paper

Whatman No.1 Chromatography Paper (100 x 100mm) was used throughout.[103]

Chromatography

Samples were hydrolysed in 0.2N HCl at 100°C for 10 minutes prior to testing.

The samples and standards were spotted onto the corners of separate chromatography papers and allowed to dry. The papers were fitted onto a frame to hold them taut and the bottom edges placed in the buffer. First dimension chromatography was performed in Butanol-Acetic acid buffer overnight (16 hours) in the ascending manner.

The frame and papers were removed from the buffer and air dried (30 minutes) before being rotated through 90° laterally and the bottom edges placed in the next buffer. Second dimension chromatography was performed in Phenol-Ammonia buffer for 8 hours, again in the ascending manner.

Following chromatography, the frame and papers were removed from the buffer and air dried in the cold overnight (18 hours).

When the papers were dry, the amino acids were revealed by developing the chromatograms with a Ninhydrin spray. (Freshly made up solution of 0.2% Ninhydrin in acetone (w/v) containing a small quantity of 2% pyridine).

The chromatograms were developed in the cold overnight or at 105°C for 3 minutes.

Individual amino acids were identified by comparison of developed colour, general shape and position and Rf values (calculated as relative mobility of a spot compared to the solvent front) in both directions, with the colour, shape, position and Rf values of the standard amino-acid 'marker' solutions.

Quantitation of relative amounts of each amino acid was determined by visual comparison of spots with comparison spots in the standard solutions.

All α -amino acids react with the ninhydrin reagent in the cold, usually within 3 hours, or certainly overnight and give, in the main,

purple colours, although significant variations are obtained (i.e. asparagine - brown).

Chromatography tank, frame and spray bottle were all from Shandon Southern.[97]

All chemicals were from BDH.[69]

APPENDIX 20

Isoelectric focusing (IEF)[104,105]

Gel Preparation

0.22g Agarose IEF

2.66g sorbitol

20ml distilled water boil and cool to 70°C

Add 1.9ml Pharmalyte, mix and pour onto 120 x 100mm glass plate. (A sheet of Gelbond cut to the same size as the glass plate and held in place by a drop of water between the two was incorporated in some experiments). Once set, the gel was allowed to harden at room temperature overnight in a moist box.

Use of the gel

A cooling platten was positioned in the electrophoresis chamber and connected to the tap. Tap water was circulated through the platten to cool it (approx 10°C). The gel plate was positioned on the cooling platten with a drop of water between the two to ensure good contact.

The electrode strips were soaked in the appropriate electrode solutions (Anode - 0.05 M H_2SO_4. Cathode - 1 M NaOH) before being thoroughly blotted on filter paper to remove excess liquid. These were then placed on the anodic and cathodic edges of the gel ensuring that there was no overlap at the sides.

The samples were applied either in wells formed atthe time of pouring the gel, or on blotting paper strips.

The IEF electrode assembly was put into place, with the electrode wires making good contact along the length of the electrode strips and the lid was closed.

Running conditions

The power pack was set to constant power and adjusted to give a reading of 5 WATTS. The voltage and current were also noted and monitored every 15 minutes.

When the current had ceased to fall by 10% of its value in 15 minutes, it was assumed focusing had taken place and the power was turned off. This usually required 1 - 1½ hours.

Fixing and staining

After completion of focusing, the gel was fixed in 5% sulphosalicylic acid plus 10% trichloroacetic acid for 30 minutes and then washed in two lots of destaining solution (Methanol: acetic acid: water (3:1:6)) for 25 minutes each. The gel was then dried between filter paper layers followed by 15 minutes in a drying cabinet, before staining in 0.2% Coomassie Brilliant Blue R-250 in destaining solution for 10 minutes.

Destaining was carried out until the background was clear and the gel was again dried before being viewed against a white background.

Interpretation

10 ul of the IEF marker calibration standard was run on every IEF plate. This allowed the pH gradient profile to be determined by plotting the stated pH for each of the 11 marker proteins in the standard against the distance of migration from the cathode.

The pI of unknown proteins could then be determined by reading directly from the graph once their migration distance had been measured.

Three different Pharmalyte carrier ampholytes were used. pH range 3 - 10, pH range 4 - 6.5 and pH range 6.5 - 9.[78]

Electrode solutions for pH range 4 - 6.5, 6.5 - 9 and 3 - 10 were 0.01 M H_2SO_4 (Anode) and 1 M NaOH (cathode).

Agarose IEF, IEF electrode strips, Pharmalytes and IEF broad PI calibration kit were all from Pharmacia.[78]

Power packs, electrophoresis chambers, cooling plattens and IEF electrodes were all from Shandon Southern.[97]

APPENDIX 21

The Tube Flocculation Test[119]

2.5ml VAC was added to 2.5ml PBS to produce a solution containing 14 Lf/ml toxoid.

0.5ml of this was added to each of 10 3 x ½" round bottomed glass test tubes (i.e. 7.5 Lf/tube)

The working standard antitoxin (Wellcome Standardised tetanus antitoxin 32 units/ml)[71] was added in decreasing volumes as in the table, to produce a range of concentrations.

Tube No.	Volume of TOX(ml)	Volume of antitoxin (ml) neat	1:10	Antitoxin Units/tube
1	0.5	1	-	32
2	0.5	0.5	-	16
3	0.5	0.25	-	8
4	0.5	0.1	-	3.2
5	0.5	-	0.75	2.4
6	0.5	-	0.5	1.6
7	0.5	-	0.25	0.8
8	0.5	-	0.1	0.32
9	0.5	-	0.05	0.16
10	0.5	-	-	0

The tubes were incubated at 50°C and inspected at intervals for visible flocculation. The first tube to show flocculation is taken as the tube containing an equal number of Lf doses of the toxin or toxoid and of *in vitro* units of antitoxin. Therefore the Lf value of the toxin under test is equal to the number of units of antitoxin in the most rapidly flocculating tube.

APPENDIX 22

The Haemagglutination Inhibition test[120,121,124]

Sensitised RBCs

3ml of sheep RBCs in Alsevers solution[28] were washed 3 times in PBS. The washed, packed cells were then mixed with 24ml of cold 3% formaldehyde in PBS and stirred at 4°C for 24 hours.

6ml of cold 40% formaldehyde was added, mixed and stirred at 4°C for 24 hours. Following this the cells were washed 8 times with 25ml volumes of PBS before being resuspended to approximately 5% by volume.

5ml of this suspension was mixed with 5ml of 1:40,000 tannic acid[69] and incubated at 37°C for 15 minutes before being centrifuged and the deposited cells washed in 10ml of PBS. The cells were resuspended in 5ml of PBS and 5ml of Wellcome Tetanus vaccine (VAC) 28 Lf/ml was added. The mixture was incubated at 56°C for 1 hour following which the cells were washed twice and resuspended in 5ml of PBS containing 0.2% Bovine serum albumin[27] as a stock reagent.

The working dilution of sensitised Red cells had to be determined by experiment for each batch, but was normally a 1:5 or 1:7 dilution of the stock reagent.

Haemagglutination Inhibition test procedure

Serial dilutions of toxin preparations in PBS were made in 25 ul volumes in round bottomed microtitre trays.[92] 25 ul of the appropriate dilution of Wellcome Standard tetanus antitoxin[71] was added to each well, the plates were shaken gently and incubated at room temperature for 1 hour.

A volume of 50 ul of sensitised sheep RBCs was added to each well, the plates shaken gently again and incubated at room temperature overnight covered with a cellophane seal.[26] Controls included Sensitised RBCs in 50 ul of PBS (negative control - no agglutination) and Sensitised RBCs plus antitoxin only (positive control - agglutination).

The plates were examined for haemagglutination and for inhibition of haemagglutination. The highest dilution of toxin preparation to give 50% haemagglutination inhibition was taken as the endpoint.

A sample of reference tetanus toxin (VAC) was included in every run and the Lf/ml of each of the test samples could be determined against the values recorded for this reference. For example, if the reference sample shows marked haemagglutination inhibition in the 1:64 dilution and an unknown sample in the same run shows the same degree of haemagglutination inhibition in the 1:128 dilution then it must contain twice as much toxin as the reference. As the reference is known to contain 28 Lf/ml then the sample must contain 56 Lf/ml.

Several different levels of sensitivity were used in this test. They are referred to as the LA/10, LA/100, LA/200 and LA/400 doses of tetanus toxoid and are defined respectively as the minimum amounts of tetanus toxoid which, when mixed with 0.1, 0.01, 0.005 or 0.0025 IU/ml of standard tetanus antitoxin, prevented the agglutination of sensitised sheep RBCs added subsequently.

In practice these levels of sensitivity are achieved by adding 25 ul of dilutions containing 0.1, 0.01, 0.05 or 0.025 1U/ml Standard tetanus antitoxin respectively to the wells.

It has been shownthat the amount of toxin in a sample that can be detected by the LA/10, LA/100, LA/200 and LA/400 levels of sensitivity is 1.107, 0.095, 0.050 and 0.0175 Lf/ml respectively.

In this study the LA/10 and LA/100 levels were used mainly.

APPENDIX 23

Extraction of neurotoxin from cells of Cl.tetani[11,15]

Cultures of Cl.tetani were grown in Massachussetts medium (Appendix 9) at 34°C for 2 days. The bacterial cells were deposited by centrifugation and extracted at 4°C for 2 days in isotonic solutions of 1 M NaCl - 0.1 M Na Citrate, using 1/10 of the original culture volume. Usually 25ml of culture was deposited and 2.5ml salts solution used.

After extraction the cells were removed and the supernatant containing the toxin stored at -40°C until required.

APPENDIX 24

Extraction of Brain tissue[130]

The brain was freed of membranes and washed in sterile distilled water. 100g was ground slowly in a mortar and pestle until a thick paste was produced. This was transferred to a 1 litre beaker and 300ml acetone[69] added slowly. This was blended using the mortar for 2 minutes then another 100ml of acetone was added, mixed for 2 minutes and the mixture filtered through Whatman No.1 filter paper.[103] The acetone was designated extract A.

The precipitate was extracted in an identical manner with a further 400ml acetone which was then discarded. The precipitate was then extracted once with 400ml absolute alcohol[103] (extract C) followed by two extractions with 400ml petroleum ether (b.p.30-60°)[103]. The two petroleum ether extracts were combined (extract F) and concentrated to dryness.

The residue was dissolved in 20ml ethyl ether[103] which was stored in the refrigerator for 2 days, when it began to settle out. It was then centrifuged and the precipitate removed.

The ether supernatant (extract H) was concentrated to dryness and the residue was dissolved in 5ml of ethyl ether. This was stored in the refrigerator overnight before being centrifuged to remove any precipitate.

To the ether supernatant 5ml of ethyl ether and 25ml of absolute alcohol were added slowly, with stirring, to precipitate the cephalin. The mixture was incubated at room temperature for 1 hour before being centrifuged to collect the precipitated cephalin.

This was dissolved in 5ml chloroform[103](extract J).

In addition, 10g of fresh brain was homogenised in 40ml sterile distilled water for 5 minutes using a mortar and pestle before being filtered as before to remove the tissue. The aqueous extract was designated extract Y.

Also, Protagon was extracted by the method of Van Heyningen[66]. Briefly, a 5 g portion of brain tissue was exhaustively extracted with acetone then ether. The supernatants were discarded and the precipitate was extracted with 85% ethanol at 56° for 1 hour, before being filtered. The ethanolic filtrate was Protagon and was designated extract Z.

A protein containing extract was made by suspending 5 g of brain tissue in 10ml of 1% aqueous Triton X-100[27] and grinding slowly with a pestle and mortar for 2 minutes. This was incubated at room temperature for 30 minutes before being filtered to remove particulate matter. The filtrate was designated extract P.

Heated acid extracts were made by suspending 5 g of ground tissue in 10ml of 0.4 M HCl and boiling for 5 minutes. The cooled mixture was filtered to remove particulate matter and the filtrate was designated extract S.

Extracts:
- A - Acetone extract
- C - Alcohol extract
- F - Petroleum ether extract
- H - Ethyl ether extract
- J - Cephalin extract
- Y - Aqueous extract
- Z - Protagon extract
- P - Triton X-100 extract
- S - Acid-heat extract

APPENDIX 25

Fluorescent labelling techniques

a) Fluorescent labelling of tetanus toxin[131,132]

0.5mg fluorescein isothiocyanate (FITC)[69] was dissolved in 0.2ml of 0.5 M sodium carbonate - sodium bicarbonate buffer (pH 8.3) in a screw capped bottle. 0.2ml of 0.005 M phosphate buffer containing 0.1mg TOX protein was added. The bottle was shaken for 3 minutes, centrifuged at 500 x g for 3 minutes and the supernatant removed. This was applied to a column (18cm x 1cm) of Sephadex G.25[78] equilibrated with 0.02 M phosphate buffer (pH 6.5), and the first coloured fraction to elute was collected. This was the labelled toxin free from unconjugated FITC, and was designated F-TOX.

b) Fluorescent labelling of Wellcome Tetanus antitoxin[132,249]

1 mg FITC was dissolved in 2ml of 0.1M Na_2HPO_4, pH 9,0. 0.5ml of 0.2 M Na_2HPO_4 was added dropwise over 2 to 3 minutes to 2ml of Wellcome tetanus antitoxin[71] with constant vigorous stirring (without frothing). 1ml of the fresh FITC solution was added to this in the same way. The pH was adjusted to 9.5 with 0.1M Na_3PO_4 and the total volume adjusted to 4ml with 0.145 M NaCl. The container was swirled to mix and left at 25°C for 30 minutes then placed in an ice bath. The solution was then centrifuged at 1,500 x g for 30 minutes to remove any precipitate, and the solution was dialysed overnight against distilled water at 4°C.

Following another centrifugation the supernatent was passed through a Sephadex G-25[78] column equilibrated with PBS (pH 7.2). The first coloured band to elute was collected and utilised as the fluorescent conjugate. (FC).

c) Absorption of the conjugate

The conjugate was absorbed with Cl.perfringens cells and various strains of Cl.tetani as follows:

Cl.perfringens NCTC 8237 was grown anaerobically for 1 week on two

Columbia blood agar plates at 37°C. The growth from both plates was harvested with a plastic loop and emulsified in 1ml of the fluorescent conjugate. This was then left at room temperature for 3 days in the dark with occasional gentle shaking. The cells were deposited by centrifugation and the supernatant used as the Cl.perfringens absorbed fluorescent conjugate (CPFC).

The same procedure was used to absorb 100 ul aliquots with the growth from half a 48 hour Columbia blood agar culture of each of the 9 strains of Cl.tetani (serotypes I - IX - Appendix 1).

APPENDIX 26

The API ZYM System[22]

Nineteen constitutive enzymes and one control are contained in cupules in a plastic strip.

A suspension of Cl.tetani cells was made in 2ml of distilled water to a turbidity between a McFarland No.5 and No.6 opacity standard[29] from a pure Columbia blood agar culture.

The enzyme strip was placed inside an incubation tray with water in the bottom to provide a humid atmosphere. Two drops of the bacterial suspension were inoculated into each cupule of the strip using a pasteur pipette and the lid was replaced. The tray was incubated for 4 hours at 37°C. After incubation, one drop of ZYM A reagent[22](Tris hydroxmethyl-amino-methane 250g; distilled water to 1 litre) and one drop of ZYM B reagent[22](Fast Blue B 3.5g in 1 litre of 2 methoxyethanol) was added to each cupule and left for 5 minutes.

Colour development was recorded from 0 - 5 with 0 corresponding to a negative reaction and 5 corresponding to a maximum positive reaction. Values 1 to 4 are intermediate between the two. Value 1 corresponds roughly to the liberation of 5 nanomoles, 2 to 10 nanomoles, 3 to 20 nanomoles, 4 to 30 nanomoles and 5 to 40 or more nanomoles of enzyme.[22] Enzymes, substrates, pH and the colour of both positive and negative reactions are shown in the following table.

Summary of results of API ZYM Reactions

No.of Cupule	Enzyme assayed for	pH	Result	
			Positive	Negative
1	Control		Colourless or colour of sample	
2	Alkaline phosphatase	8.5	Violet	Very pale yellow or colour of control
3	Esterase (C4)	6.5	Violet	
4	Esterase Lipase (C8)	7.5	Violet	
5	Lipase (C14)	7.5	Violet	
6	Leucine arylamidase	7.5	Orange	
7	Valine arylamidase	7.5	Orange	
8	Cystine arylamidase	7.5	Orange	
9	Trypsin	8.5	Orange	
10	Chymotrypsin	7.5	Orange	
11	Acid phosphatase	5.4	Violet	
12	Naphthol-AS-Bl-phosphohydrolase	5.4	Blue	
13	α galactosidase	5.4	Violet	
14	β galactosidase	5.4	Violet	
15	β glucuronidase	5.4	Blue	
16	α glucosidase	5.4	Violet	
17	β glucosidase	5.4	Violet	
18	N-acetyl- β glucosaminidase	5.4	Brown	
19	α mannosidase	5.4	Violet	
20	α frucosidase	5.4	Violet	

APPENDIX 27

The API 20 Strep system[22]

Nine constitutive enzymes and the fermentation of 11 substrates are tested in a plastic strip.

A suspension of Cl.tetani was made in 2ml of sterile distilled water to a turbidity approximately equal to a No.4 McFarland opacity standard.[29]

The strip and incubation tray was prepared as previously and 3 drops of the suspension placed in each of the first 9 cupules with a pasteur pipette. The tenth well was filled half full and the remainder of the suspension added to an ampoule of API strep medium[22] and mixed. This was then used to fill each of the remaining wells half full. Wells 10 to 20 were then overlaid with mineral oil and the tray was incubated at 37°C for 4 hours.

After incubation, 1 drop of VP 1 reagent[22] (Potassium Hydroxide 40% in water) and 1 drop of VP 2 reagent[22] (6% Alpha Naphthol in ethyl alcohol) was added to well 1 and left 10 minutes before reading.

2 drops of Ninhydrin reagent[22] (7% in 2-methoxyethanol) were added to well 2 and left for 10 minutes before reading.

1 drop of Zym A[22] and 1 drop of ZYM B[22] was added to wells 4 to 9 and read after 10 minutes.

Following the reading of the first 9 wells, the tray was re-incubated for a further 18 hours and the last 11 wells read.

Enzymes, and the colour of both and negative reactions are shown in the following table.

Summary of Results of API 20 STREP Reactions

No. of Cupule	Test	Enzyme assayed or reaction tested	Result: Positive	Result: Negative
1	VP	Acetoin production	Pink-red	Colourless
2	HIP	Hippurate hydrolysate	Violet	Colourless or pale blue
3	ESC	β-glucosidase	Black-grey	Colourless or pale yellow
4	PYRA	Pyrrolidonylarylamidase	Orange	Colourless
5	GAL	α-galactosidase	Violet	Colourless
6	GUR	β-glucuronidase	Blue	Colourless
7	GAL	β-galactosidase	Violet	Colourless
8	PAL	Alkaline phosphatase	Violet	Colourless
9	LAP	Leucine arylamidase	Orange	Colourless
10	ADH	Arginine dehydrolase	Red	Yellow
11	RIB	Ribose fermentation	Yellow	Orange-red
12	ARA	Arabinose fermentation	Yellow	Orange-red
13	MAN	Mannitol fermentation	Yellow	Orange-red
14	SOR	Sorbitol fermentation	Yellow	Orange-red
15	LAC	Lactose fermentation	Yellow	Orange-red
16	TRE	Trehalose fermentation	Yellow	Orange-red
17	INU	Inulin fermentation	Yellow	Orange-red
18	RAF	Raffinose fermentation	Yellow	Orange-red
19	AMD	Starch fermentation	Yellow	Orange-red
20	GLYG	Glycogen fermentation	Yellow	Orange-red

APPENDIX 28

The API Anaerobic enzyme system[162]

Forty constitutive enzymes and 20 conventional substrates (carbohydrates, nitrate, indole, urea etc.) are contained in the cupules in three plastic strips labelled AN1, AN2 and AN3.

Two suspensions of Cl.tetani are required from a pure culture on Columbia blood agar.

Suspension A - in 3ml of phosphate buffer[22] ≥ MacFarland No.5 opacity standard.

Suspension B - in 2 ml of distilled water ≥ MacFarland No.3 opacity standard.

Strips and incubation trays were prepared as previously and the AN1 and AN2 were inoculated with 2 drops of suspension A in each well. The AN3 strip was inoculated with 2 drops of Suspension B in each well and wells 3 to 19 on the AN3 strip were overlaid with mineral oil.

All trays were incubated at 37°C for 4 hours.

After incubation 1 drop of ZYM A[22] and 1 drop of ZYM B[22] reagents were placed in wells 1 - 3 and 11 - 20 (inclusive) of the AN1 strip and wells 1 - 2 and 8 - 20 (inclusive) of the AN2 strip. These tests were examined after 10 minutes.

1 drop of NIT 1 reagent[22] and 1 drop of NIT 2 reagent[22] were added to well 1 of strip AN3 and 1 drop of IND reagent[22] was added to well 2 on this strip. Results were recorded within 10 minutes.

Enzymes and the colour of both positive and negative reactions are shown in the following tables.

Summary of results of the API ANI strip reactions

No.of Cupule	Test	Enzyme assayed for	Result Positive	Result Negative
1	PAL	Alkaline phosphatase	Red-violet	Colourless
2	PAC	Acid phosphatase	Red-violet	Colourless
3	LIP	Lipase C10	Red-violet	Colourless
4	AGAL	α-Galactosidase	Yellow	Colourless
5	BGAL	β-Galactosidase	Yellow	Colourless
6	AGLU	α-Glucosidase	Yellow	Colourless
7	AFUC	Fucosidase	Yellow	Colourless
8	LARA	L.Arabinosidase	Yellow	Colourless
9	PBGA	Phospho-β-Galactosidase	Yellow	Colourless
10	BAGL	N-acetyl-β-Glucosaminidase	Yellow	Colourless
11	ArgA	Arginine arylamidase	Orange	Colourless
12	ProA	Proline arylamidase	Orange	Colourless
13	OrnA	Ornithine arylamidase	Orange	Colourless
14	GluA	Glutamine arylamidase	Orange	Colourless
15	HisA	Histidine arylamidase	Orange	Colourless
16	GluT	Glutamyl transpeptidase	Orange	Colourless
17	LGA	Leucyl-glycine arylamidase	Orange	Colourless
18	HPA	Histidyl-phenylalamine-arylamidase	Orange	Colourless
19	GHA	Glutamyl histidine arylamidase	Orange	Colourless
20	APPA	Alanine-phenylalanine-proline arylamidase	Orange	Colourless

Summary of results of the API AN2 strip reactions

No.of Cupule	Test	Enzyme assayed for	Result Positive	Result Negative
1	EST	Esterase C4	Red-violet	Colourless
2	NPO_4	Naphthol-ASBi-phosphate	Blue	Colourless
3	BGUR	β-Glucuronidase	Yellow	Colourless
4	AMAN	α-Mannosidase	Yellow	Colourless
5	BMAN	β-Mannosidase	Yellow	Colourless
6	BGLU	β-Glucosidase	Yellow	Colourless
7	BFUC	β-D-fucosidase	Yellow	Colourless
8	TRY	Trypsin	Orange	Colourless
9	LeuA	Leucine arylamidase	Ora nge	Colourless
10	ValA	Valine arylamidase	Orange	Colourless
11	SerA	Serine arylamidase	Orange	Colourless
12	LysA	Lysine arylamidase	Orange	Colourless
13	HPrA	Hydroxyproline arylamidase	Orange	Colourless
14	AspA	Aspartic arylamidase	Orange	Colourless
15	PYRA	Pyrrolidonic arylamidase	Orange	Colourless
16	PheA	Phenylalanine arylamidase	Orange	Colourless
17	GGA	Glutamylglutamic arylamidase	Orange	Colourless
18	LAA	Lysylalanine arylamidase	Orange	Colourless
19	PhAA	Phenylalanine-arginine arylamidase	Orange	Colourless
20	PrAA	Proline-arginine arylamidase	Orange	Colourless

Summary of results of API AN3 strip reactions

No.of Cupule	Test	Enzyme assayed for or reaction tested	Result	
			Positive	Negative
1	NO_3	Nitrate	Red	Colourless
2	IND	Indole	Pink	Colourless
3	ADH	Arginine dehydrolase	Pink	Yellow
4	URE	Urea	Pink-red	Yellow
5	GLU	Glucose fermentation	Yellow	Red
6	GLY	Glycerol fermentation	Yellow	Red
7	ARA	L-arabinase fermentation	Yellow	Red
8	RIB	D-ribose fermentation	Yellow	Red
9	XYL	D-xylose fermentation	Yellow	Red
10	GAL	D-galactose fermentation	Yellow	Red
11	FRU	D-fructose fermentation	Yellow	Red
12	MAN	Mannitol fermentation	Yellow	Red
13	MDM	α-Methyl-D-mannoside fermentation	Yellow	Red
14	MDG	α-Methyl-D-glucoside fermentation	Yellow	Red
15	ARB	Arbutin fermentation	Yellow	Red
16	CEL	Cellobiose fermentation	Yellow	Red
17	LAC	Lactose fermentation	Yellow	Red
18	MEL	Melibiose fermentation	Yellow	Red
19	TRE	Trehalose fermentation	Yellow	Red
20	TTR	Tetrathionate reductase	Yellow	Green

APPENDIX 29

The Seward AP60 System[26]

Media for performing 23 standard biochemical tests and 1 control are dried into wells of a microtitre plate in 3 vertical rows in this system.

A suspension of Cl.tetani was made in 4ml distilled water to a turbidity equivalent to a MacFarland No.2 opacity standard.[29] 100 ul of this suspension was inoculated into each of the 24 wells and wells 3, 6, 8, 9 and 14 were overlaid with mineral oil.

A transparent adhesive seal was placed over the plate and a small hole punctured in this seal over wells 11 and 12.

Plates were incubated at 37°C in an anaerobic atmosphere for 48 hours before being examined.

Following incubation the seal was removed and the following reagents added:

1 drop of Nitrate reagent 1[26] (Sulphanilic acid (0.8% w/v) in 5N Acetic acid) and 1 drop of Nitrate reagent 2[26] (Dimethyl-1-naphthylamine (5.7% v/v) in 5 N Acetic acid) to well 1. Read after 1 minute. If Negative add a small quantity of zinc dust and leave 4 minutes.

1 drop of Oxidase reagent[26] (Tetramethyl-p-phenylene diamine dihydrochloride (1% w/v) in distilled water) to well4 and read within 20 seconds.

4 drops of Kovac's reagent[26] (p-Dimethylaminobenzaldehyde (5% w/v) in isoamyl alcohol: Concentrated Hydrochloric acid (3:1) to well 7, read within 10 minutes.

1 drop of VP 1 reagent[26] (Alpha naphthol (6% w/v) in absolute ethanol) and 1 drop of VP 2 reagent[26] (37% w/v) Aqueous Potassium hydroxide) to well 10 and read after 20 minutes.

1 drop of TDA reagent[26] (10% w/v) Aqueous Ferric Chloride) to well 13 and read within 10 minutes.

Tests, substrates and colour of both Positive and Negative reactions are shown in the table..

Summary of results of Sensititre AP60 test system reactions

No.of well	Code	Reaction tested	Result	
			Positive	Negative
1	NIT	Nitrate reduction	Pink-red	Colourless*
2	GLU	Glucose fermentation	Yellow	Blue-green
3	DBB	Decarboxylase broth base	NA	Pale yellow-grey-brown
4	OXI	Oxidase	Purple	Colourless
5	ONPG	β-galactosidase	Yellow	Colourless
6	LDC	Lysine decarboxylase	Purple	Yellow
7	IND	Indole production	Pink-red	Colourless
8	URE	Urease production	Red	Yellow
9	ODC	Ornithine decarboxylase	Purple	Yellow
10	VP	Voges-Proskauer	Red-pink	Colourless
11	CIT	Citrate utilisation	Blue	Green
12	MAL	Malonate utilisation	Blue	Yellow-green
13	TDA	Tryptophan deaminase	Red-brown	Yellow
14	H_2S	Hydrogen Sulphide production	Black precipitate	No precipitate
15	AES	Aesculin Hydrolysis	Black precipitate	No precipitate
16	GEL	Gelatin liquefaction	Diffusion	No diffusion
17	INOS	Inositol fermentation	Yellow	Blue-green
18	MAN	Mannitol fermentation	Yellow	Blue-green
19	ADO	Adonitol fermentation	Yellow	Blue-green
20	ARA	Arabinose fermentation	Yellow	Blue-green
21	MLT	Maltose fermentation	Yellow	Blue-green
22	RHA	Rhamnose fermentation	Yellow	Blue-green
23	SOR	Sorbitol fermentation	Yellow	Blue-green
24	SUC	Sucrose fermentation	Yellow	Blue-green

* If addition of zinc dust produces a red colour, the result is a true negative. If the reaction is still colourless, it should be regarded as positive.

APPENDIX 30

The Abbott BID System[163]

20 biochemical tests are contained in a plastic cartridge. A suspension of Cl.tetani was prepared from a pure 24 hour old Columbia blood agar plate in 10ml of sterile distilled water equivalent to between a MacFarland No. 0.5 and a No.1 opacity standard.

The protective top cover was perforated using the supplied perforator and 200 ul of the suspension was inoculated into each of the wells. A cartridge cover is placed over the cartridge and the reagents mixed thoroughly with a vigorous side-to-side motion. Incubate at 37°C for 5 hours.

Following incubation, 50 ul of Indole reagent[163] (5% w/v) p-dimethylaminobenzaldehyde in 10% (v/v) concentrated hydrochloric acid) was pipetted into chamber 18 and observed for colour reaction. The outer surface of the cartridge was carefully wiped with a tissue and inserted into the operational part of an Abbott 'Quantum II' spectrophotometer equipped with a 'BID' module.[163] The reading of the reactions was automatic but could be done manually by visual examination as shown in the following table.

Summary of results of Abbott 'BID' system reactions

No.of Chamber	CODE	Reaction tested	Result Positive	Result Negative
1	GLU	Glucose fermentation	Yellow	Blue-green
2	LYS	L-Lysine monohydrochloride decarboxylase	Blue-dark green	Yellow-light green
3	ORN	L-ornithinine monohydrochloride decarboxylase	Blue-dark green	Yellow-light green
4	CIT	Citrate utilisation	Blue-dark green	Yellow-light green
5	MAL	Malonate utilisation	Blue-dark green	Yellow-light green
6	ESC	Esculin hydrolysis	Black	Colourless
7	URE	Urease production	Blue	Green-yellow
8	ADO	Adonitol fermentation	Yellow	Blue-green
9	ARA	Arabinose fermentation	Yellow	Blue-green
10	INO	Inositol fermentation	Yellow	Blue-green
11	LAC	Lactose fermentation	Yellow	Blue-green
12	MAN	Mannitol fermentation	Yellow	Blue-green
13	RHA	Rhamnose fermentation	Yellow	Blue-green
14	SOR	Sorbitol fermentation	Yellow	Blue-green
15	SUC	Sucrose fermentation	Yellow	Blue-green
16	XYL	Xylose fermentation	Yellow	Blue-green
17	ARG	L-arginine hydrochloride fermentation	Yellow	Blue-green
18	IND	Indole production	Pink	Clear
19	ACE	Acetamide	Blue	Green
20	PLB	Polymyxin B resistance	Clear	Turbid

APPENDIX 31

The Anaerobe-Tek system[164]

Fifteen biochemical tests are contained in a round multi-compartment plastic plate. Gram reaction, spore production and cell shape are also considered. Suspensions of Cl.tetani were made in Anaerobe-Tek broth from pure cultures on Columbia blood agar plates to a turbidity approximately equal to a MacFarland No.3 opacity standard.

The lid was removed from an Anaerobe-Tek plate and, using a pasteur pipette, one drop of the suspension was streaked across the central well. The pipette was then stabbed into the medium at the point of inoculation and discarded.

Using an absorbent cotton wool swab the suspension was inoculated onto the surface of the 11 peripheral wells starting with the Esculin compartment and inoculating clockwise. The swab was recharged between each compartment.

The plates were incubated anaerobically at 37°C for 48 hours inside a loosely sealed plastic bag containing a piece of moist cotton wool to provide humidity.

Following incubation the plates were exposed to air for 30 minutes and dilute aqueous bromothymol blue added to the carbohydrate containing chambers (9, 10, 11 and 12).

A cotton swab soaked in Indole reagent[31](1% w/v) p-dimethylamino-cinnamaldehyde in 3% v/v) hydrochloric acid) was rotated on the growth in the centre well and observed for 30 seconds for colour reaction.

One drop of 3% (v/v) Hydrogen peroxide[69] was pipetted onto the growth in the Esculin chamber (No.2) and observed for the production of bubbles.

An aliquot of Gelatinase reagent[31](mercuric chloride (8% w/v) in 5% (V/V) hydrochloric acid) was pipetted into the Gelatin chamber (No.6) and observed for 30 seconds for reaction.

An aliquot of Gram's Iodine[155] was pipetted into the starch chamber (No.7) and the reaction read immediately.

The reactions and both positive and negative results are shown in the following table.

Summary of Results of Anaerobe-Tek reactions

No.of Chamber	Location	Test reaction	Result Positive	Negative
1	Centre well	Hydrogen sulphide production	Black	Beige
	Centre well	Indole production	Blue/green	Pink/Violet
2	Esculin well	Esculin hydrolysis	Diffusable Brown	Beige
		Catalase production	Bubbles produced	Nobubbles
3	Egg Yolk well	Lecithinase	White precipitate	Yellow
	Egg Yolk well	Lipase	Pearly irridescent layer	Yellow
4	Bile well	Bile tolerance	Growth	No growth
5	Milk well	Milk proteolysis	Clear zone	Cloudy
6	Gelatin well	Gelatin hydrolysis	Clear zone	Opaque
7	Starch well	Starch hydrolysis	Clear zone	Brown/Blue
8	DNA well	DNAse	Purple/Pink	Blue/Clear
9	Glucose well	Glucose fermentation	Yellow	Green
10	Mannitol well	Mannitol fermentation	Yellow	Green
11	Lactose well	Lactose fermentation	Yellow	Green
12	Trehalose well	Trehalose fermentation	Yellow	Green

APPENDIX 32

Commercial Biochemical Test Plates [30]

Glucose agar plates, Gelatin agar plates, H_2S agar plates and Indole agar plates were made up following the manufacturers' instructions (1 sachet dissolved in 200ml of distilled water) plus 2% Davis agar. This was autoclaved at 121°C for 20 minutes prior to pouring the plates. (25ml per 9mm petri dish).

Inoculation of plates

Suspensions of the Cl.tetani strains were made from pure Columbia blood agar cultures in 2ml of sterile distilled water to a turbidity equal to a MacFarland No.10 opacity standard.[29] 200 ul of each suspension was pipetted into a separate sterile plastic autoanalyzer cup which was then placed in a metal rack accommodating 20 cups in all. The loaded rack was placed on a multipoint inoculator[93] which was then used to inoculate the plates one after another. The plates were incubated for 48 hours anaerobically at 37°C in a humid atmosphere produced by putting them in a slackly sealed plastic bag containing moist cotton wool before examination.

Interpretation of results

H_2S agar plates. A positive result was indicated by a blackening of the inoculum spot and the area around it.

Gelatin agar plates. A positive result was indicated by a clear area around the inoculum spot following flooding of the plate with acidic mercuric chloride.[69] (Mercuric chloride 12g. water 80ml, Conc.HCl 16ml).

Indole agar plates. A positive result was indicated by a blue colour on the inoculum.spot when the plate was flooded with DMACA reagent[31] (4-Dimethylaminocinnamaldehyde 1g in 100ml of 10% v/v concentrated hydrochloric acid). Colourless or a pink colour is negative.

Glucose agar plates. A positive result was indicated by a yellow colour in or around the inoculum spot.

APPENDIX 33

Biochemical tests on plate media

All inocula for plate media tests were made as follows:

Suspensions of Cl.tetani strains were made from pure Columbia blood agar cultures in 2ml of distilled water to a turbidity equal to a MacFarland No.10 opacity standard. 200 ul of each suspension was pipetted into a separate sterile plastic autoanalyser cup which was subsequently placed in a metal rack accommodating 20 cups in all. The loaded rack was placed on a multipoint inoculator,[93] which was used to inoculate the plates unless otherwise specified.

To reduce spreading of spot inoculated organisms 2%(w/v) Davis agar was added to all plates unless otherwise specified. All media were autoclaved at 121°C for 20 minutes prior to pouring the plates. Unless otherwise specified all plates were incubated for 48 hours anaerobically in a humid atmosphere produced by putting the plates in slackly sealed plastic bags containing moist cotton wool.

i) Collagenolytic activity[158]

Collagenolytic activity was tested on Columbia agar plates supplemented with 2% Davis agar containing type 1 Collagen (0.02% w/v)[27] and Sodium alginate (0.02% w/v)[69].

Collagenolytic activity was indicated by clearing around the inoculum.

ii) Albumin hydrolysis[158]

Albumin hydrolysis was tested on Columbia agar plates supplemented with 2% Davis agar containing 1% (w/v) Bovine serum albumin (fraction V)[27].

Positive reactions were indicated by clear zones around inocula.

iii) Chitin hydrolysis[158]

Chitin hydrolysis was indicated by clear zones around the inoculum on Columbia agar plus 2% Davis agar supplemented with 0.25% purified Chitin[69]. This was produced by treating crude chitin alternatively

with 1N N_aOH and 1N HCl and then with ethanol to remove foreign material. The remaining material was dissolved in cold conc. HCl, filtered through glass wool, precipitated in distilled water and washed until neutral before adding to the medium.

iv) Lipolytic activity[158]

Lipolytic activity was tested on Columbia agar plates supplemented with 2% Glycerol tributyrate[69] and 2% Davis agar. Lipolysis was indicated by clear zones around the inoculum spots.

v) Chondroitinase activity[158]

Chondroitinase activity was tested by incorporating filter-sterilized chondroitin sulphate[27] into Columbia agar plates supplemented with 2% Davis agar to a final concentration of 400 ug/ml.

Bovine serum albumen[27] was also added to a final concentration of 1%.

Reactions were determined by flooding the plates with 2N acetic acid after incubation and observing. The appearance of clear zones around the inoculum indicated chondroitinase activity.

vi) Esterase activity[158]

Esterase activity was tested on Columbia agar plates supplemented with 2% Davis agar and with 1% Tween 20, 40, 60 and 80.[69] A positive reaction was indicated by a turbid halo around the inoculum spot.

vii) Arbutin hydrolysis[158]

Arbutin hydrolysis was tested on Columbia agar plus 2% Davis agar supplemented by 0.1% Arbutin[27] and 0.05% Ferric citrate[69]. Positive reactions gave a black halo around the inoculum spot.

viii) Elastase activity[158]

Elastase activity was tested on Columbia agar plus 2% Davis agar supplemented with 0.25. Elastin powder.[27] A clear zone around the inoculum spot indicates a positive reaction.

ix) Mucinase activity[158]

Mucinase activity was tested on Columbia agar plates plus 2%

Davis agar supplemented with 0.28% Mucin (type II Crude)[27]. Cultures were flooded with 1% Calcium chloride[69] solution and positive reactions were indicated by clear zones around the inoculum.

x) DNase activity[168]

DNase was tested on DNase test agar[29] (3g/100ml distilled water) plates plus 1.5% Davis agar. A positive result was indicated by a clear zone around the inoculum spots after floodingwith 1N Hydrochloric acid.

xi) RNase activity[168]

RNase activity was tested on Columbia agar plates plus 2% Davis agar supplemented with 0.4% RNA (Sodium salt)[69]. Results were obtained by flooding cultures with 1N Hydrochloric acid. Clear halos around inoculum spots indicated positive results.

xii) Gelatinase activity[168]

Gelatinase activity was tested on Columbia agar plates plus 2% Davis agar supplemented with 1% Gelatin[29]. Results were obtained by flooding the plates with 15% (w/v) Mercuric chloride in 1N Hydrochloric acid.

A clear zone around the inoculum indicated a positive result.

xiii) Casein agar plates[151]

Casein digestion or precipitation was tested on Columbia agar plates supplemented with 2% Davis agar and 2% Casein.[27] Digestion was indicated by clear zones around the growth and precipitation was indicated by a diffuse opacity around the growth following flooding of the plates with 10% (v/v) HCl.

xiv) Casein hydrolysate agar plates[151]

These were prepared and examined as above, but substituting Casein hydrolysate[27] for Casein.

xv) Milk digestion

Milk digestion was examined on Columbia agar plates plus 2% Davis agar containing 10% (v/v) whole milk. The milk was added to the plates after autoclaving and just prior to pouring. Digestion was

indicated by clear zones around the growth and precipitation by zones of diffuse opacity around the growth. If the clear zones were difficult to see, they could be intensified by flooding the plates with 10% (v/v) HCl.

xvi) Fibrin agar plates[160]

Fibrinolysin activity was tested on Columbia agar plates plus 2% Davis agar plus 250 units of sterile bovine thrombin[170] (dissolved in 2ml distilled water) per 400ml of agar base and 400mg of sterile bovine fibrinogen[170] dissolved in 20ml of distilled water. Thrombin and fibrinogen solutions were added after autoclaving.

Fibrinolysis is indicated by clearing around inocula. Where this was not immediately evident the plates were overstained with 0.2% Naphthalene black in Methanol: Acetic acid: water (5:1:4) for 20 minutes. This was decanted and the plate was rinsed with tap water. Zones of clearing could be seen around the colonies as clear areas against a blue background.

xvii) Desulfoviridin production[151]

Strains were grown in FAB for 24 hours at 37°C. 3ml of culture showing good growth was removed and centrifuged. A few drops of each deposit was placed in waxed circles in apetri dish and 2 drops of 2N NaOH was added to each. The dish was examined under longwave ultraviolet light for the presence of a red fluorescence which indicated desulfoviridin.

xviii) Glutamic acid decarboxylase[165]

The glutamic acid test substrate was prepared with 0.05g of bromocresol green sodium salt[171], 0.1g L-glutamic acid,[27] and 0.3ml of sodium dodecyl sulphate[27] in 1 litre of distilled water.

The test was performed by scraping 48 hour anaerobic Columbia blood agar cultures with a sterile cotton swab and suspending the growth in 2ml of the test substrate to a turbidity approximately equal to a MacFarland No.1 opacity tube.[29] This was then incubated aerobically

at 37°C for 24 hours.

A definite blue colour was recorded as positive whereas any other colour was recorded as negative.

xix) Nicotinamide adenine dinucleotide glycohydrolase[166](NADG)

A strain of Haemophilus parainfluenzae (clinical isolate) was used as the indicator organism. Five colonies from a 24 hour chocolate blood agar plate were emulsified in 1ml sterile distilled water. This was flooded over the surface of a diagnostic sensitivity test (DST) agar plate[29] and allowed to dry in.(10 minutes at room temperature). V factor (NAD) discs[30] were placed on the surface of the plate and the test organisms were stab inoculated into the seeded plate approximately 8 - 10mm from a disc. Plates were incubated at 37°C in an anaerobic atmosphere (containing 10% CO_2) and examined after 24 hours for zones of inhibition in the indicator organisms areas of growth.

xx) Hyaluronidase activity [158]

A mucoid, hyaluronic acid producing strain of Streptococcus equi[162] was used as indicator organism.[172] This was inoculated as a streak across a Columbia blood agar plate and test organisms were cross-streaked at 90° to this so that they almost, but not quite, touched the original streak. The plates were incubated at 37°C anaerobically for 48 hours before examining. Hyaluronidase activity was indicated by destruction of mucoid streptococcal growth adjacent to the test organism streak.

xxi) H_2S production[151]

SIM medium[28] was made by adding 3g of powder and 2g of Davis agar to 100ml of distilled water, autoclaving at 121°C for 15 minutes and pouring into plates. These were inoculated using the multipoint inoculator as before, incubated for 48 hours anaerobically at 37°C before examination. A black colour around the inoculum spots indicates H_2S production.

xxii) Indole production

For indole demonstration, a filter paper strip impregnated

with oxalic acid[69] (dipped in hot saturated oxalic acid and dried) was touched to each inoculum spot on the above SIM plate. The development of a pink to red colour indicated indole formation.

xxiii) Phosphatase activity[169.,173]

Phosphatase activity was tested on Columbia blood agar plates plus 2% Davis agar supplemented with 2ml of 0.6% Sodium phenolphthalein phosphate[27] solution per 100ml. This was added after autoclaving and just prior to pouring the plates. One ml of ammonia[69] was poured into the lid after incubation and the presence of phosphatase was indicated by a pink colouration of the growth at the inoculum and the surrounding medium.

xxiv) Fluorescence on MacConkey agar[6]

MacConkey agar plates were made by adding 52g of powder and 20g of Davis agar to one litre of distilled water, autoclaving at 121°C for 15 minutes and pouring into plates. After anaerobic incubation for 4 days at 37°C the plates were examined for green fluorescence of the growth using a Blak-Ray long wave ultraviolet lamp[174].

xxv) Tetrazolium reduction[175]

This was tested on Columbia agar plates plus 2% Davis agar containing 1% glucose[69] and 0.0025% 2,3,5-triphenyl tetrazolium chloride.[69] The latter two components were added as sterile solutions following autoclaving and just prior to pouring the plates. Tetrazolium reduction was indicated by the formation of a deep red colour by the growth and in the medium surrounding it.

APPENDIX 34

Biochemical tests on bottled media

All inocula for bottled media tests were made as follows:

Loopfuls of pure Cl.tetani strains from Columbia blood agar plates were inoculated into 20ml FAB medium in a glass universal. This was incubated at 37°C for 48 hours. One loopful (10 ul) of each strain was inoculated into each fluid test medium, and a loopful of each strain was stabbed to the bottom of each agar medium.

i) Meat digestion and reddening[150]

This was tested using 15ml cooked meat medium[61] in sterile glass universals. After inoculation the cultures were incubated at 37°C for 7 days with daily examination. Digestion is shown by blackening of the meat. Reddening indicates reduction of the meat.

ii) Milk digestion and acid production[173]

Skim milk[176] was dispensed in 10ml aliquots in glass universals and 0.1ml of a 0.1% solution of bromothymol blue[69] was added. The inoculated bottles were incubated at 37°C for 14 days with daily examination. An equivalent number of uninoculated bottles were also incubated as sterility controls. Acid production was indicated by a yellow reaction of the medium whilst digestion may be seen by the production of a transparent solution.

iii) Ammonia production from peptone[151]

This was tested using FAB broths which were incubated for 48 hours after inoculation. 100 ul of culture fluid was then removed from each and placed in test tubes. 100 ul of Nessler's solution[27] was then added. Ammonia production was indicated by the production of an orange colour.

iv) Gas production in deep agar cultures[151]

This was tested using 20ml Columbia agar plus 2% Davis agar in glass universal bottles. Each was inoculated by stabbing to the bottom of the agar with a loop. Incubation at 37°C was continued for 7 days with daily examination. Gas production was seen by the presence

of gas bubbles in the agar. The presence of large amounts of gas may cause the agar to split or raise up the bottle.

APPENDIX 35

a) Indole reagents

i) Kovacs' reagent[183,184]

P-dimethyl aminobenzaldehyde	5g
Amyl alcohol	75ml
Conc. HCl	25ml

Dissolve the aldehyde in the alcohol by gently warming. Cool and add the acid.

ii) Aqueous Kovacs' reagent[163,184]

P-dimethylaminobenzaldehyde	5g
Conc. HCl	10ml
Distilled water	90ml

Dissolve the ddehyde in the water by gently warming. Cool and add the acid.

iii) P-dimethylaminocinnamaldehyde reagent (DMACA)[26]

P-dimethylaminocinnamaldehyde	1g
Con.HCl	3ml
Distilled water	97ml

Dissolve the aldehyde in the water and add the acid.

b) Indole test protocols

i) The spot indole test[184]

Whatman No.1 filter paper[103] was moistened with each of the 3 indole reagents and test organisms were scraped off Columbia blood agar plates with clean applicator sticks and gently rubbed onto the saturated filter paper. The appearance of a red colour with the Kovacs' reagents or a blue colour with the DMACA was regarded as indole positive. Any other colour, or colourless, was negative.

ii) The direct indole test[183,185]

3ml of each of the 48 hour FAB and RCM cultures were placed

in test tubes and 0.5ml of each reagent was added. The tubes were well shaken and examined after 1 minute. A red colour in the upper layer with the Kovacs' reagents or a blue colour with DMACA was regarded as positive.

iii) The extracted indole test[151,155]

To 2ml of the 48 hour FAB and RCM cultures of each strain 1 ml xylene[69] was added, shaken, and left to stand for 2 minutes. 0.5ml of each reagent was then slowly added down the side of the tubes. A red ring with the Kovacs' reagents or a blue one with DMACA appearing in the upper layer within 15 minutes was regarded as positive.

APPENDIX 36

Complete growth medium for Cl.tetani

This was made up as follows:

Casein hydrolysate	30g
NaCl	2.5g
Cystine	0.125g
Calcium pantothenate	1mg
Uracil	1.25mg
Nicotinic acid	0.25mg
Thiamine	0.25mg
Riboflavin	0.25mg
Pyridoxine	0.25mg
Biotin	2.5 ug
Vitamin B_{12}	0.05 ug
$FeCl_3.6H_2O$	32mg
$MgSO_4$	0.1g
Cysteine hydrochloride	0.5g
Agar	7mg
Yeast extract	10g
Distilled water	1 litre

The reagents were added to the water, put into glass 250ml bottles in 50ml aliquots and sterilised byautoclaving at 121°C for 15 minutes.

Cystine, calcium pantothenate, uracil, nicotinic acid, thiamine, riboflavin, pyridoxine, biotin, vitamin B_{12} and cysteine hydrochloride were all prepared in 100 ml amounts in distilled water at ten times the required strength. The solutions were filter sterilised and kept in the refrigerator until required. For use, 10ml of each solution was added to the medium aseptically just prior to autoclaving.

Casein hydrolysate, cystine, calcium pantothenate, uracil, nicotinic acid, thiamine, riboflavin, pyridoxine, biotin, vitamin B_{12}

and cysteine hydrochloride were from Sigma[27]. NaCl, $FeCl_3.6H_2O$ and $MgSO_4$ were from BDH[69]. Agar and Yeast extract were from Oxoid.[28]

APPENDIX 37

Amino acids

1 L-phenylalanine

2 L-lysine

3 Hydroxy-L-proline

4 L-alanine

5 L-aspartic acid

6 L-valine

7 L-glutamine

8 L-tryptophan

9 L-methionine

10 Glycine

11 L-isoleucine

12 L-leucine

13 L-cysteine

14 L-histidine

15 L-arginine

16 L-asparagine

17 L-glutamic acid

18 L-proline

19 L-serine

20 L-threonine

21 L-cystine

22 D-methionine

23 D-alanine

24 D-B-3,4 Dihydroxy phenylalanine

25 D-glutamic acid

26 D-cystine

27 D-phenylalanine

28 D-serine

29 D(-)isoleucine

30 D-asparagine

31 D-leucine

32 D-aspartic acid

33 D-histidine

34 D-norleucine

35 D-valine

36 D-threonine

37 D-tryptophan

PAGINATION
ERROR

APPENDIX 38

Gas Liquid Chromatography[151,200,204]

Sample preparation

VOLATILE FATTY ACIDS

1ml of 18 hour old FAB culture supernatant was placed in a glass bottle and 3 drops of 50% H_2SO_4 added.

A small amount of NaCl and 1ml of diethyl ether[69] was subsequently added and the mixture agitated for 30 seconds before centrifuging for 5 minutes.

1 ul of the ether supernatant was injected onto the column using a microsyringe.[205]

NON VOLATILE FATTY ACIDS (methyl derivatives)

1ml of 18 hour old FAB culture supernatant was placed in a glass bottle and 2ml of methanol[69] and 0.4ml 50% H_2SO_4 were added. The mixture was incubated at 56°C for 30 minutes and then cooled. 1ml of water and 0.5ml chloroform[69] were added and the mixture was agitated for 30 seconds before centrifuging for 5 minutes. 2 ul of the chloroform layer (under the aqueous layer) was aspirated and injected onto the column using a microsyringe.[205]

Operating conditions

Chromatography was carried out using a Packard model 430 chromatograph fitted with dual flame ionisation detectors, temperature programmer, pressure and flow controls and built in integrator. The recorder was a Packard model 641. The column was 1.5 metres of 4mm inner diameter glass with a packing of 10% SP1000 on 100/120 Chromosorb W.AW.[205a]

To detect volatile fatty acids the operating conditions were as follows:

Preprogrammed run with initial oven temperature 100°C with a temperature rise of 4°C/min to 160°C.

Injectors and Detectors at 150°C.

Nitrogen flow rate 15 ml/min

Air flow rate 250ml/min

Hydrogen flow rate 25ml/min

Integrator settings: Slope 2

APKW 0.1

PKW 0

Minimum Area 200

Integrate delay 0.5

LSTC 125

Attenuation 8.

Standards: VFA - Capco Anabac, containing Acetic, propionic, isobutyric butyric, isovaleric, valeric, isocaproic and caproic acids.

NON VFA - Capco Anabac containing Pyruvic, lactic, oxalacetic and succinic acids.

Identification of VFAs and Non-VFAs was by comparing the retention times of each peak in the test extracts as measured by the chromatograph with the retention times of the known acids in the standard mixtures.

The integrator was set to measure the total area produced by all the peaks, and then to calculate the percentage of the total produced by each peak, as a measure of the relative amounts of each acid in the test extracts.

No internal standard was included in the test extracts therefore it was not possible to calculate the actual amounts of each acid present.

APPENDIX 39

Assay for dipicolinic acid in bacterial spores[227]

5ml of cell suspension was autoclaved for 15 minutes at 121°C before being cooled, acidified with 0.1ml of 1N acetic acid and left at room temperature for 1 hour. The suspension was then centrifuged at 1,500 x g for 10 minutes and 4ml of the clear supernatant carefully aspirated into a clean test tube. 1ml of freshly prepared reagent, consisting of 1% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1% ascorbic acid in 0.5M acetate buffer at pH 5.5 was added. The colour developed immediately and the optical density was read at 440m u on an EEL spectrophotometer. The blank was 1ml of water plus 4ml of treated culture supernatant.

No standard dipicolinic acid curve was made because of the unavailability of purified dipicolinic acid therefore the amount of dipicolinic acid in each culture was expressed as the absorbance value rather than in ug/ml.

APPENDIX 40

Isolation and detection of Plasmids[229,230]

Plasmid extraction

Bacteria were harvested from 48 hour Columbia blood agar plates into 0.1ml of 'E' buffer (40 mM Tris-acetate and 2mM EDTA adjusted to pH 7.9 with glacial acetic acid) to form a turbid suspension (MacFarland No.10 opacity standard).

The cells were lysed by adding 0.2ml of lysing solution (50mM Tris and 3% Sodium dodecyl sulphate, pH 12.6) and heating at 56°C for 20 minutes.

The solution was cooled, 0.3ml of phenol/chloroform (1:1 vol/vol) solution was added, mixed and centrifuged at 1,500 x g for 15 minutes to break the emulsion.

Avoiding the precipitate at the interface, samples could be withdrawn directly from the aqueous phase.

Plasmid electrophoresis

The plasmid preparations (15 ul) were electrophoresed in 0.7% agarose[69] dissolved in Tris-borate buffer (89mM Tris, 2.5mM disodium EDTA and 8.9mM boric acid).

A dye solution consisting of bromophenol blue (0.07%), SDS(7%) and glycerol (33%) in water was added to each sample (5 ul/sample) prior to electrophoresis. Gels were 150 x 150mm and wells were made using a plastic well former.[97]

Electrophoresis was at 30mA until the dye reached the bottom of the plate. (Approx. 4 hours)

Chamber buffer was Tris-borate as above.

The gel was then placed in a solution of ethidium bromide[27] in water (0.4 ug/ml) and stained for 15 minutes before being examined under ultraviolet illumination.

The sizes of plasmids were estimated by comparison with plasmids extracted by an identical procedure from Egch.coli V517 which contains

8 plasmids (A - H) of differing size[231] and was a gift from Dr. P. Rochelle.[232]

The sizes of the plasmids are as follows:

pVA517 A - 55.1 kb (kilobases)

pVA517 B - 7.4 kb

pVA517 C - 5.6 kb

pVA517 D - 5.2 kb

pVA517 E - 4.1 kb

pVA517 F - 3.1 kb

pVA517 G - 2.7 kb

pVA517 H - 2.1 kb

Tris, EDTA, SDS, boric acid, bromophenol blue, glycerol and acetic acid were from BDH.[69]

APPENDIX 41

Isolation of Cl.tetani flagellin[218]

Cl.tetani NCTC 540 was grown in 100ml FAB medium[61] for 18 hours at 37°C. After incubation the cells were harvested by centrifugation at 1,500 x g for 30 minutes and then mixed with sterile saline to produce a thick suspension. This was then adjusted to pH 2.0 with 1M HCl and stirred at room temperature for 30 minutes.

The bacterial cells now devoid of flagella were removed by centrifugation at 1,500 x g for 30 minutes and the supernatant, containing detached flagellin in monomeric form was adjusted to pH 7.2 with 1M NaOH. Ammonium sulphate was added slowly with vigorous stirring to two-thirds saturation (2.67M) and the mixture was held overnight at 4°C and then centrifuged at 1,500 x g for 1 hour. The precipitate was dissolved in 5ml of distilled water and then dialysed in visking tubing[238] at 4°C overnight against distilled water. The dialysed flagellin preparation was then freeze-dried and reconstituted in 0.5ml of distilled water.

APPENDIX 42

Tube agglutination technique for detection of Cl.tetani heat-labile 'H' antigens [217,239]

Wellcome tetanus antitoxin[71] was serially diluted in glass test tubes from 1:10 to 1:5120 in PBS using 0.5ml amounts.

0.5ml of the formolised cell suspensions was added to each and the tubes were incubated at 56°C for 4 hours and overnight at 4°C before examining. 'H' agglutination appears as fine flocculation which resuspends on gentle shaking.

The titre of each strain was taken as the highest dilution showing visible flocculation.

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