A STUDY OF THE CONDITIONS GOVERNING THE FORMATION AND GERMINATION OF SPORES OF CLOSTRIDIUM PERFRINGENS TYPE A

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INTRODUCTION

The true bacterial spores with which this thesis is concerned are produced mainly by members of the family Bacillaceae. These spores, of which only one is produced per vegetative cell, are formed intracellularly and differ markedly from:-

(1) The large numbers of spores produced by distinct fruiting cells of the Actinomycetales and fungi.

(2) The spores produced by the Myxobacteriales and the chlamydospores of fungi, where the entire vegetative cell becomes encysted.

The bacterial spore differs from the vegetative cell in its method of formation, structure and chemical composition. The most interesting of these differences in chemical composition is the presence of dipicolinic acid in spores. This compound is not found in vegetative cells and its presence in spores was thought to be unique in living cells until its recent discovery in certain fungi. For practical purposes the bacterial spore is easily recognised by its marked light refractility, impermeability to stains and high degree of resistance to many physical and chemical agents. Accordingly spores possess higher ability to survive in the presence of adverse surroundings. This exceptional potential for survival make spores a considerable nuisance in the preservation of foods and the sterilisation of hospital supplies.

Since the first observation of spore formation by Cohn (1872) and spore germination by Prazmowski (1880, cited by Thimann 1963), many workers have tried to elucidate the factors controlling sporulation. The limited knowledge of these processes has been summarised by Ordal (1957) who stated that sporulation is a normal metabolic process which will occur within the bacterial cell only when:-

(1) The cell is of a sporogenous type.

(2) The cell acquires the proper physiological condition.

(3) The cell is surrounded by the proper environment.

The recent advances in these studies have been predominantly concerned with the formulation of synthetic and semi-synthetic media and have led to the elucidation of specific organic and inorganic requirements for sporulation of several species of bacteria. Using these media certain biosynthetic pathways operative during spore formation have been identified. The most interesting development in this field has been the use of the replacement culture technique, whereby vegetative cells produced in a medium favourable for growth are transferred to a second medium favourable for sporulation. In this way vegetative growth and sporulation can be separated and studied independently.

Similarly, the conditions promoting and inhibiting the transformation of the dormant, metabolically-inactive spore, to the metabolically-active vegetative cell have been elucidated for a

number of bacteria.

Recently the immunological characterisation of antigens which are specific for the spore and for the vegetative cell has proved of great value for the study of cell differentiation and for taxonomic purposes.

Most of the recent large numbers of papers dealing with the biochemistry of sporulation and germination have dealt with members of the genus Bacillus. This is undoubtedly due to the relative ease of cultural procedures employed when working with these organisms. Nevertheless the spores of Cl.perfringens type A have received considerable attention for many years because of their undoubted importance in putrefactive processes and disease production. However, due to the difficulties involved in promoting spore formation by this organism, these publications have been restricted to the development of sporulation media.

The spores of Cl.perfringens type A are particularly interesting for a number of reasons, not least of these is the existence of two distinct varieties of Cl.perfringens type A. On the one hand there are isolates which are beta-haemolytic on horse blood agar and produce spores which will survive boiling for only a few minutes, while on the other hand there are isolates which are not beta-haemolytic on horse blood agar, but produce spores which are viable even after boiling for several hours. Organisms of the latter group

have been designated heat-resistant Cl.perfringens type A and have been isolated on numerous occasions from the faeces of individuals suffering from transient attacks of food-poisoning. The spores of these two closely related groups of organisms, differing widely in heat resistance, provide excellent material for research into the fundamental mechanisms of spore heat resistance. The immunological characterisation of the spore antigens of these two groups could also be of considerable help in taxonomy.

A further substantial gap in our knowledge is the absence of any studies of the organic and inorganic requirements for the germination of the spores of this organism. Research on this topic could lead to advances in the treatment of wounds contaminated with spores of Cl.perfringens type A, as well as leading to the development of improved media for the enumeration of spores of this organism.

The present work aimed to extend knowledge of the formation and germination of the spores of Cl.perfringens type A. A necessary prerequisite for this work was the development of a medium in which large numbers of spores of Cl.perfringens could be reproducibly produced. Recent claims by Ellner (1956) may have gone some way towards providing such a medium. In his hands spores were produced by each of the 138 isolates examined and spore yields amounting to 80 per cent of the inoculum were frequently obtained. However, several text-books of bacteriology (Topley and Wilson 1955, Bigger 1959 and Henrici and Ordal 1948) maintain that Cl.perfringens type A

does not form abundant spores *in vitro*. An extensive search of the early literature confirmed that numerous authors have been unable to demonstrate spores of this organism microscopically. In order to demonstrate spores at all, these authors have resorted to subjecting cultures to periods of heat treatment sufficient to kill the vegetative cells, followed by subculture. The appearance of turbidity in these cultures was then taken as presumptive evidence for the existence of spores in the original cultures.

The first part of this thesis was therefore devoted to an assessment of the usefulness of Ellner's medium for adequate preparation of *Cl.perfringens* spores and a comparison with other media for the purpose of spore formation. Included in this part of the thesis is a study of a number of factors influencing sporulation in Ellner's medium and several attempts were made to improve sporulation of weakly sporing isolates by modifying this medium. A number of experiments are also described in which attempts have been made to develope a defined medium based on Ellner's medium. Further sections have been devoted to :-

1) Variation in sporulation and its association with other factors.
2) Microscopical observations of sporulation in slide culture.
3) The morphology of *Cl.perfringens* spores as revealed by the electron microscope.

The second part of the thesis deals with factors promoting and inhibiting spore germination.

NOMENCLATURE AND CLASSIFICATION

The specific epithet "perfringens" (L.part.adj. breaking through) was first used by Veillon and Zuber (1898) and is the designation used in the seventh edition of Bergey's Manual of Determinative Bacteriology (1957) by virtue of its selection by the Permanent Standards Commission of the Health Organisation of the League of Nations (1931).

Between 1890 and 1920, this organism was isolated from a wide variety of habitats by a number of workers. Accordingly a number of synonyms are found in the literature for this period. These are:-

1. The Bacillus of Acute Articular Rheumatism (Achalme 1891).
2. Bacillus aerogenes capsulatus (Welch and Nuttall 1892).
3. Bacillus phlegmonis emphysematosa (Fraenkel 1893).
4. Clostridium Welchii (Migula 1894).
5. Bacillus enteritidis sporogenes (Klein 1895).
6. Bacillus emphysematosus (Kruse 1896).
7. Bacillus emphysematis vaginae (Lindenthal 1897).
8. Bacillus cadaveris butyricus (Buday 1898).
9. Bacillus emphysematosum (Migula 1900).
10. Granulo-bacillus saccharobutyricus liquefaciens immobilis (Schattenfroh and Grassberger 1900).
11. Bacillus egens (Stoddard 1919).

The first attempt to classify the Cl.perfringens group of

organisms was made by Simmonds (1915a) who divided the group into four types on the fermentation of glycerol and inulin. This classification was shown to be unsound by Humphreys (1924) who found that during glycerol fermentation a bactericidal aldehyde (acrolein) was formed. This compound was also metabolised by the bacterium, so the final pH of the medium was dependent on the relative rates of production and breakdown of acrolein, rather than the ability of the organism to ferment glycerol.

Attempts to classify Cl.perfringens using serological methods were also initially unsuccessful. Simmonds (1915a) and Howard (1928) were unable to establish a classification using the agglutination test.

The discovery by Bull and Pritchett (1917) of an antigenic, heat-labile exotoxin, eventually led to the serological classification of Cl.perfringens as we know it today. Bull and Pritchett found that toxic filtrates from Cl.perfringens cultures were neutralised by antisera prepared against the toxins of other strains. The specificity of this reaction was confirmed by the Medical Research Committee Report of 1919. Dalling (1928) and McEwan (1930) however, each isolated a distinct anaerobic bacillus from sheep which bore close morphological and cultural relationships to Cl.perfringens but differed in their toxin-antitoxin reactions. They designated these organisms the "Lamb Dysentery Bacillus" and

Bacillus paludis respectively.

Wilsdon (1931) did not consider these organisms worthy of species status and carried out a series of experiments in which he compared the production, heat stability, haemolytic power and minimum lethal dose of the toxins from fifty two isolates, as well as the ease of preparing toxoid and the clinical course of disease following injection of each toxin. From these experiments he found that the toxins of the 52 isolates fell into four distinct groups, these he designated types A B C and D. Type A corresponded to the original group described by Bull and Pritchett, type B to the "Lamb Dysentery Bacillus" and type C to B.paludis, while type D was another closely related group. Using representative isolates from each of these four types Wilsdon carried out a detailed series of toxin-antitoxin neutralisation tests from which he postulated the existence of three lethal toxic factors, W X and Z. All four types produced factor W, which was the only factor produced by type A isolates. Types B and C produced factors W and Z, while types B and D produced factors W and X. Using this system of classification Wilsdon (1932-33) showed that the organism isolated from the intestine of sheep by Bennetts (1932) and called by him B.ovitoxicus, was identical to Cl.perfringens type D.

In a further more detailed series of toxin neutralisation tests, carried out using Wilsdon's four toxic types, Glenny, Barr, Llewellyn-

Jones, Dalling and Ross (1933) were able to distinguish five distinct toxins which they designated by the first five letters of the Greek alphabet. This was followed by the isolation of another type of Cl.perfringens from calves by Bosworth (1940-43). This organism produced alpha-toxin and a hitherto unrecognised lethal toxin which was designated iota. As this organism represented a type not covered by Wilsdon's classification it has been included as an additional type, namely type E (Oakley, Warrack and Warren 1948).

A further series of closely related organisms were isolated by Zeissler and Rassfeld-Sternberg (1949) from portions of the intestine of individuals with enteritis necroticans. These organisms differed from previously described types of Cl.perfringens in that they produced exceptionally heat-resistant spores. The spores of these organisms resisted boiling for periods ranging from one to four hours, in contrast to the spores of types A,B,C,D and E which did not survive boiling for more than fifteen minutes. By including these organisms in an additional group, type F, on the basis of heat resistance plus a slight variation in the minor antigens produced (Oakley 1949), a new criterion for type-differentiation was introduced.

This criterion of high spore heat resistance was also used as a differentiating factor by Hobbs, Smith, Oakley, Warrack and Cruikshank (1953) to classify a number of strains of Cl.perfringens which they isolated from the faeces of individuals suffering from food-poisoning.

These organisms produced the same major lethal antigens as Cl. perfringens type A, i.e. they produced alpha-toxin but not beta-epsilon-or iota-toxins. Unlike the typical type A isolates these were non-or only weakly-haemolytic on horse blood agar, i.e. as they did not produce the haemolytic antigen, theta-toxin. These organisms were therefore included in the classification as heat-resistant or food-poisoning Cl.perfringens type A.

The identification of Cl.perfringens types A to F has been considerably simplified by the discovery of Macfarlane, Oakley and Anderson (1941) that the alpha-toxin of this group of organisms is a lecithinase and is responsible for the opalescent zone produced around colonies on nutrient agar plates containing serum or egg yolk. Several other members of the genus Clostridium also produced lecithinases, although only those of Cl.perfringens, Cl.bifermentans and Cl. sordellii are inhibited by Cl.perfringens type A antitoxin. Of these only Cl.perfringens isolates ferment lactose (Willis and Hobbs 1958). As outlined previously, differentiation within the Cl.perfringens group can only be effected by examining the toxins produced. Suitable procedures for demonstrating these toxins have been developed by Oakley and Warrack (1953). These workers prepared a table showing the distribution of toxins produced by types A to F. A modified version of this table, including the results of their own work, has been compounded by Brooks, Sterne and Warrack (1957). This table is

reproduced in Table 1 of this thesis.

TABLE 1

Characters of the antigens and their distribution in the Cl.perfringens types.

Type		Occurrence	Major lethal antigens				Minor lethal and non-lethal antigens								Non-α98 hæmolysis	
			α	β	ε	ι	γ	δ	η	θ	κ	λ	μ	ν		
			Lethal, necrotising Ca^{++} dependent lecithinase	Lethal, necrotising	Lethal, necrotising	Lethal, necrotising	Lethal	Lethal, hæmolytic	Lethal (validity doubtful)	Hæmolytic oxygen-labile (? lethal)	Collagenase	Proteinase	Hyaluronidase	Deoxyribonuclease	"Ox"	"Horse"
A	Classical	Gas-gangrene (man) Intestinal commensal (man and animals) Putrefactive processes Soil, etc.	+++	−	−	−	−	−	(+)	++−	++−	−	+−	++−	(+)	(+)
	Food-poisoning	Patients and food in outbreaks of food-poisoning	+++	−	−	−	−	−	−	(+)	+±−	−	+−	++	−	−
B	Classical	Lamb-dysentery Foals	+++	+++	+±−	−	++	(+)*	−	++	−	+++	+++	++	−	+−†
	Iranian	Hæmorrhagic enteritis of goats and sheep, Iran	+++	+++	+++	−	?	−	−	+++	+++	−	−	+−	−	−
C	Classical	"Struck" in sheep	+++	+++	−	−	++	+++	−	+++	+++	−	−	+−	−	−
	Colorado	Neonatal hæmorrhagic enteritis in calves and lambs	+++	+++	−	−	?	−	−	+++	+++	−	−	+−	−	−
	Piglet	Enteritis in piglets	+++	+++	−	−	?	−	−	+++	+++	−	(+)	++	+++	+++
D		Enterotoxæmia in adult sheep, lambs, goats and bovines Intestine of man	+++	−	+++	−	−	−	−	++	++	++	++−	+±−	(+)†	+−†
E	. .	Sheep, cattle (? pathogenic)	+++	−	−	+++	−	−	−	++	++	++	(+)	++−	+−†	+−†
F	. .	Enteritis necroticans (man)	+++	++−	−	−	++	−	−	−	−	−	−	+++	++†	++†

+++ = Produced by all strains; ++ = produced by most strains; ++−, +− = produced by some strains;
(+) = produced by very few strains. Heavy type denotes production of large amounts of antigen.

* = Antibody present in antisera of hyperimmune horses.

† = Hæmolysis produced only in the presence of antisera.

PART 1

SPORULATION OF CLOSTRIDIUM PERFRINGENS TYPE A

CONTENTS

REVIEW OF THE LITERATURE

CONTENTS

Introduction

Many authors who have dealt extensively with the sporulation of Cl.perfringens, including Simmonds (1915a,b), Adamson (1918-19) and Smith (1954) have emphasised that spores are not formed in routinely used bacteriological culture media. Similarly, spores of this organism have not been seen in films prepared from infected tissues (Henry 1916-17, Jablons 1919-20, Kendal, Day and Walker 1922, Hall 1922 and Weinberg, Nativelle and Prévot 1937). In view of these findings it is not surprising that Welch and Nuttall (1892), Fraenkel (1893) and Veillon and Zuber (1898) failed to observe spores in cultures used for the first description of this organism. Even when using the so called sporogenic media, Jacqué (1904), Simmonds (1915a), Henry (1916-17), Jablons (1919-20), Becker (1920), Hall (1922), Weinberg et al. (1937) and Bethge (1947-8) have emphasised the necessity for using cultural rather than microscopical techniques for the demonstration of spores. Using this technique Simmonds (1915a), unlike many of his predecessors, was able to demonstrate spores in all the cultures he examined. To do this he used seven media which he had found to be sporogenic and although, like Jacqué (1904) and Noguchi (1907-8), he was aware that there was a marked variation in the ability of isolates to form spores, he maintained that large numbers of spores were not found in these cultures. This is apparently not the case as a number of workers ranging from Jacqué in

1904 to Hall, Angelotti, Lewis and Foter in 1963, have observed abundant sporulation of certain isolates in specific sporulation media.

Some strains will even produce abundant spores in media which are generally considered to be asporogenic and Eastoe and Long (1959) worked with a single isolate which spored profusely in Robertson's meat broth. More comprehensive surveys of sporulation of a number of isolates in this medium by: Robertson(1915-16), Henry (1916-17), McKillop (1958) and Yamamoto, Sadler, Adler and Stewart (1961) have shown that sporulation is invariably weak, while Gibbs and Hirsch (1956) estimated that only 0.0056 per cent of the viable cells were present as spores.

In the following literature review the factors influencing sporulation have been gathered together under ten headings. Six of these are directly concerned with the composition of sporulation media. Under these headings are reviewed the effect of pH, carbohydrates, proteins, peptones, inorganic ions and a number of comparatively simple peptone media. The media included in the last of these sections have been formulated since 1955 and appear to be a significant advance over the complex media used prior to this date. Three sections have been devoted to: the influence of the oxidation-reduction potential, temperature, and the period of incubation required for sporulation. The remaining section deals with the

association of sporulation with the variability in colonial morphology within an isolate, and the association of sporulation with the colonial morphology and virulence of separate isolates.

1. The effect of pH and carbohydrate on sporulation in artificial media.

The advantage of alkaline media for the production of spores by Cl.perfringens was first reported by Fraenkel (1893), who observed spores of this organism on Cholera agar.

By far the most widely studied aspect of the sporulation of Cl.perfringens is the inhibitory effect of fermentable carbohydrate. This was first observed by Jacqué in 1904 and confirmed by Noguchi (1907-8), Fitzgerald (1911), Simmonds (1915a), Henry (1916-17), Hall (1922) and Torrey, Kahn and Salinger (1930). Similarly Adamson (1918-19) did not observe spores of Cl.perfringens on glucose agar, while Gibbs and Hirsch (1956) reported that the spore to total viable count ratio, fell from 0.0056 per cent in a reinforced cooked meat broth medium, to 0.0012 per cent in the same medium containing 2 per cent glucose. The inhibitory effect of fermentable carbohydrate was shown by both Fitzgerald and Henry to be directly attributable to the low pH produced in these media as a result of carbohydrate fermentation. To overcome the inhibition of sporulation by fermentable carbohydrate Savini (1911, cited by Simmonds 1915a), Fitzgerald (1911) and Zeissler (1930) used sugar-free media, while Torrey et al. rendered their sporulation media sugar free by growing

Escherichia coli in them before inoculating with Cl.perfringens. The last mentioned of these workers adjusted the pH of their media before inoculating with Cl.perfringens.

With non-fermentable carbohydrate Noguchi and Fitzgerald found that sporulation was enhanced. Fitzgerald however, claimed that that there were two exceptions to this rule, namely, that the sugars mannitol and raffinose, which were fermented, increased sporulation. The effect of these two sugars on sporulation in buffered media has been studied by a number of workers and is dealt with in detail towards the end of this section.

The study of the effect of pH on sporulation was then taken a stage further by Simmonds (1915a), Robertson (1915-16), Henry (1916-17) and Kendal, Day and Walker (1922), who reported that sporulation only occurs on neutral or alkaline media. This pH effect is also apparent in the observation made by Hall (1922) who found that one of the best methods of obtaining spores of Cl.perfringens is by growing on blood agar under alkaline pyrogallol.

The optimum degree of alkalinity for sporulation of Cl.perfringens has been determined by Schattenfroh and Grassberger (1900), Noguchi (1907-8), Fitzgerald (1911), Becker (1920) and Zeissler (1930). Unfortunately these workers expressed the reaction of their media in terms of either normality or equivalent to a concentration of sodium carbonate. Using the conventional terminology, namely pH, it

has been shown that spores of Cl.perfringens are only formed in media with a pH between 6.5 and 7.6 (Brosteanu 1937, cited by Weinberg et al. 1937), the optimum pH for sporulation being 7.2 (Bethge 1947-8). Similar results were obtained by Torrey et al. (1930) using a buffered sugar-free broth. These workers observed abundant sporulation (between 40 and 80 per cent of the cells contained spores) of each of their isolates at its pH optimum, this was isolate specific in the pH range 7.4 to 8.0. Spores were not observed below pH 6.6.

Most of the papers so far cited dealing with the effect of carbohydrate on sporulation have emphasised that sporulation is prevented by the presence of fermentable carbohydrate. However in 1900, Schattenfroh and Grassberger recommended an alkaline starch medium for inducing Cl.perfringens to form spores. The utility of this medium was confirmed by Stolz (1902, cited by Simmonds 1915a), Jacqué (1904) and Runeberg (1908, cited by Simmonds 1915a). Simmonds (1915a) however, was unable to produce spores in an alkaline medium containing starch as all his isolates fermented starch vigorously. A possible explanation of this discrepancy was put forward as early as 1908 by Hibler (cited by Bethge 1947-8), who maintained that fermentable carbohydrate was not inhibitory to sporulation provided there was sufficient alkali (in the form of sodium carbonate) present in the medium to neutralise the acid formed. Bethge (1947-8) confirmed this report by obtaining a large increase in the number of

spores in a protein-rich medium, when 0.5 per cent dextrose and 1.5 per cent sodium carbonate were added. In spite of this large increase in sporulation, Bethge still found it necessary to estimate sporulation using the viable spore count method which has been described earlier.

Undoubtedly the medium most widely and apparently most successfully used to produce spores of Cl.perfringens is that devised by Ellner (1956). This medium is buffered at pH 7.8 with potassium dihydrogen phosphate and disodium hydrogen phosphate and contains fermentable carbohydrate (0.3 per cent starch). Albrycht and Trembowler (1959) studied the effect of pH on sporulation in this medium and in common with previous papers on the effect of pH in unbuffered media, found that the optimum pH for sporulation lay between pH 7 and 8, with inhibition of sporulation below pH 6.5 and above pH 8.5. Thus in both buffered and unbuffered media, the observation made by Bethge (1947-8),that the upper pH limit for sporulation is the same as for vegetative growth, whereas the lower pH level for growth (pH 4.82, Medical Research Council 1919) is well below that for sporulation, has not been discredited.

The influence of carbohydrate on sporulation in Ellner's medium was investigated by Albrycht and Trembowler (1959). Removal of the starch resulted in decreased vegetative growth and with certain isolates the percentage sporulation was also decreased. Increasing the starch content from 0.3 to 0.9 per cent resulted in increased

vegetative growth, but did not increase the percentage sporulation. Ellner (1956) found that replacing the starch content of his medium by mannitol or raffinose was without affect on sporulation, although glucose and lactose inhibited sporulation. Albrycht and Trembowler (1959) however, found that whereas vegetative growth was not affected when starch was replaced by mannitol, raffinose or maltose, sporulation was delayed. Of the three, raffinose alone inhibited sporulation.

There are then three conflicting views regarding the influence of raffinose and mannitol on sporulation. Fitzgerald (1911) and Ellner (1956) claim that Cl.perfringens isolates form spores in media containing these compounds. Torrey and colleagues (1930) maintain that they will not, while Albrycht and Trembowler believe that sporulation is supported by mannitol and inhibited by raffinose. The view of Hibler (1908, cited by Bethge 1947-8) and Bethge (1947-8) that fermentable carbohydrate enhances sporulation provided there is sufficient alkali in the medium to neutralise the acid formed, is not entirely substantiated by these experiments using Ellner's medium.

The inhibitory effect of fermentable carbohydrate on sporulation appears to be specific for the species Cl.perfringens, as Gibbs and Hirsch (1956) found that the addition of small amounts of glucose to a reinforced meat broth medium was advantageous for spore formation of all the other species of the genus Clostridium they studied. In

the genus Bacillus, fermentable carbohydrate is to a certain extent a limiting factor in sporulation. With Bacillus cereus var. terminalis, glucose is oxidised to acetic acid until all the glucose has been removed from the medium. At this stage the Kreb's cycle enzymes become detectable and the acetic acid is oxidised to carbon dioxide, with the concomitant production of energy, sporulation and rise in pH (Hanson, Srinivasan and Halvorson 1963).

From the preceding work dealing with the effect of pH on sporulation by Cl.perfringens, along with the absence of an oxidative pathway of glucose catabolism (Kreb's cycle), it appears that the biochemical pathways operative during spore formation by Cl.perfringens differ markedly from those so far elucidated in the genus Bacillus.

2. The effect of pH on sporulation illustrated by the incidence of spores in the intestine and faeces

Despite the difficulties encountered in inducing Cl.perfringens to form spores in artificial culture media, spores are readily found in faeces. This led Simmonds (1915a,b) to investigate sporulation in sterilised and unsterilised 10 per cent suspensions of human faeces.

The effect of pH in the sterilised faecal suspensions (with or without dextrose added) was the same as in the media reviewed in the previous section, namely, that spores were formed only if the

suspensions remained neutral or alkaline. The experiments carried out with unsterilised faecal suspensions utilised the observation that there was a large increase in the Cl.perfringens spore count of faeces upon anaerobic incubation for 24 to 48 hours. Here again dextrose did not necessarily inhibit sporulation. In this instance however, only when the pH of the faeces fell below 5.0 was there no increase in the spore count (Nye (1927) converted Simmonds alkalinity value to pH units). Simmonds attributed this to symbiosis, but was unable to reproduce the effect by culturing Cl.perfringens in sterile suspensions of 10 per cent faeces in symbiosis with Bacterium coli, Bacterium subtilis and Bacterium prodigiosus. Although Passini (1905, cited by Simmonds 1915a) and Sittler (1908, cited by Simmonds 1915b) had previously claimed to have induced Cl.perfringens to spore by growing in mixed culture with Escherichia coli.

The effect of pH on the incidence of Cl.perfringens spores in human faeces is illustrated by the finding of Herter (1906-7) that spores of Cl.perfringens are unusually abundant in the faeces of individuals with pernicious anaemia. This was confirmed by Simmonds (1915a), Kahn (1924) and Moench, Kahn and Torrey (1925), although it was not until 1927 that Nye found that this increase in spore count was secondary to gastric achylia. Nye therefore attributed this increase to the fact that the pH of the intestinal contents of these

individuals is nearer the pH optimum for sporulation of Cl.perfringens than the pH prevailing in healthy adults. These observations were confirmed by Davidson (1928) and Torrey et al. (1930). The studies of the pH of the contents of the upper-and mid-portions of the small intestine cited by both Nye and Torrey et al. in support of their conclusions, are included in Table 2.

Diet has also been shown to have an important effect on the incidence of Cl.perfringens spores in faeces. Thus Cannon (1921), Cannon and McNease (1923), Hudson and Parr (1924), Braafladt (1923), Hines (1923) and Moench, Kahn and Torrey (1925), all demonstrated that a carbohydrate-rich diet produced an aciduric flora with a decrease or absence of Cl.perfringens spores, while a protein-rich diet had the reverse effect.

3. The protein content of media and sporulation.

Running parallel with the development of simple broth media, either sugar-free or supplemented with non-fermentable carbohydrate, several workers have recommended a number of proteinaceous media. The advantages of proteinaceous media were realised as early as 1908 by Hibler (cited by Bethge 1947-8) and more recently by Robertson (1915-16), Zeissler (1930), Headlee (1931), Brosteanu (reported by Weinberg et al. 1937) and Bethge (1947-8). The media which have been recommended, along with numerous references to their

TABLE 2

The pH of the contents of the small intestine of healthy individuals and of individuals with gastric anacidity.

	Healthy individuals	Individuals with gastric anacidity
pH of the upper-portion of the small intestine	(1) 5.9-6.6 (2) 6.6-7.0 (av.7.0)	(2) 7.0-7.8 (av. 7.4)
pH of the mid-portion of the small intestine	(1) 6.2-6.7 (3) 4.1-6.5 (av.5.2) (4) 5.83	(5) 6.8-7.9 (av.7.35)

(1) Van der Reis (1925)

(2) Knott (1927)

(3) McClendon, Bissell, Lowe and Meyer (1920)

(4) Long and Fenger (1917)

(5) Torrey, Kahn and Salinger (1930)

efficacy are summarised in Tables 3, 4 and 5.

There are unfortunately few comparisons of these media. Adamson (1918-19) claimed that inspissated serum was better than brain medium, which in turn was better than egg broth or Dorset's egg medium. Headlee (1931) also found that a serum medium (Loeffler's serum medium) was efficient, coming second only to Aronson's alkaline egg medium. Few spores were produced in nutrient broth supplemented with alkaline egg or serum, beef extract broth, and a sugar-free brain-heart infusion broth. Unfortunately both of these good sporulation media had their disadvantages. Loeffler's medium did not support good vegetative growth prior to sporulation and was therefore unsuitable for the mass production of spores. While in alkaline egg medium, the acid produced during vegetative growth resulted in coagulation of the egg, from which it was impossible to separate the spores.

The best of these sporulation media appear to be the media containing serum and Aronson's alkaline egg broth. It seems probable that the serum media would have an alkaline reaction, especially if the serum had been kept for some time before use. Both of these media would therefore provide the high pH requirement of a good sporulation medium.

There are then two distinct concepts of a good sporulation medium. On the one hand there are the comparatively simple peptone

TABLE 3

Blood and serum media recommended to induce sporulation of Cl.perfringens type A

Medium	Recommended by:	Abundant sporulation of some isolates observed by:
Coagulated serum	Welch (reported by Dunham 1897) Dunham (1897) Klein (1901-2) Herter (1906-7) Medical Research Council (1919)	Henry (1916-17) Adamson (1918-19) Jablons (1919-20) Weinberg et al. (1937)
Serum broth	Dunham (1897) Muscatello and Gangitano (1900)[1].	
Loefflor's serum medium	Hewlett (1899) Gwyn (1899) Klein (1901-2) Herter (1906-7) D'Agata (1910)[1]. Weinberg et al. (1937)	Headlee (1931) Fraenkel (1902)
Serous fluid media	Dunham (1897) Howard (1899) Rist (1901)[1]. Herter (1906-7) Bethge (1947-8)	
Media containing blood	Hall (1922) Weinberg et al. (1937)	

[1]. Cited by Simmonds (1915 a.)

TABLE 4

Egg media recommended to induce sporulation of Cl.perfringens type A

Medium	Recommended by:	Abundant sporulation of some isolates observed by:
Saline plus a piece of coagulated egg albumin	Loris-Melikov (1909) De Gasperi and Savini (1911)*	
Nutrient broth plus pieces of coagulated egg albumin	Simmonds (1915 a)	Jablons (1919-20)
Aronson's alkaline egg broth	Robertson (1915-16) Henry (1916-17) Medical Research Council (1919) Hall (1922) Willis (1957 a)	

* Cited by Simmonds (1915 a)

TABLE 5

Other proteinaceous media recommended to induce sporulation of Cl.perfringens type A

Medium	Recommended by:	Abundant sporulation of some isolates observed by:
Gelatin medium	Hewlett (1901) Simmonds (1915a)	
Brain-infusion broth	Hibler (1908)* Adamson (1918-19) Becker (1920) Hall (1922) Headlee (1931)	
Liver media	Zeissler (1930) Bethge (1947-8) York and Vaughn (1954)	

*Cited by Bethge (1947-8)

media,which, particularly in recent years, have been claimed to promote abundant sporulation. While on the other hand there are the complex proteinaceous media,which, particularly in the first half of this century, were found to be more efficient than the simple media.

The role of complex media in promoting spore formation by members of the genus Bacillus has been attributed by Foster and Heiligman (1949) to the mineral content of these media. The efficiency of recently devised, comparatively simple media, in promoting spore formation by Cl.perfringens leads one to suppose that the utility of complex media could lie in their inorganic content. There are unfortunately no direct comparisons of complex and simple media and it would be foolhardy to cast aside the complex media before settling this point.

4. The influence of peptone and protein digest media on sporulation.

Although Cl.perfringens does not form spores in 1 per cent peptone water (Noguchi 1907-8, Fitzgerald 1911) or in 10 per cent peptone water (several authors cited by Bethge 1947-8),media containing peptone have been extensively used to produce spores of this organism.

When added to a complex medium (alkaline, sugar, liver broth) Bethge (1947-8) found that the optimum peptone concentration for sporulation was 9 to 10 per cent. With higher concentrations of

peptone there was a gradual decrease in the number of spores produced, until at a peptone concentration of 20 per cent, no spores were formed.

Although peptone has been used to supplement complex proteinaceous sporulation media, it has been most successfully used as the chief nitrogenous constituent of a number of relatively simple media. In these media much lower concentrations of peptone have been used. There is unfortunately only one comparison of peptone and proteinaceous media. In this instance Hibler (1908, cited by Bethge 1947-8) found that a simple alkaline agar medium containing carbohydrate and 2 per cent peptone, was a better sporulation medium than a proteinaceous medium containing an equivalent amount of alkali. Although no other comparison of simple peptone and proteinaceous media has been made, the buffered peptone media devised by Torrey et al. (1930) and Ellner (1956) appear to support much better sporulation than proteinaceous media. Torrey and colleagues used a sugar-free nutrient broth containing 1 per cent peptone buffered with calcium and sodium carbonates, hydrochloric acid and either potassium or sodium dihydrogen phosphate. Ellner's medium which has been extensively used, contains 1 per cent peptone, 0.3 per cent yeast extract, starch and magnesium sulphate. This medium is buffered at pH 7.8 with sodium and potassium phosphates. The influence of peptone on sporulation in this medium has been studied by Albrycht and

Trembowler (1959), who found that reducing the concentration of peptone to 0.2 per cent, or using a tryptic digest of peptone instead of peptone, had no effect on sporulation.

A number of media similar in composition to Ellner's medium (i.e. buffered peptone media containing carbohydrate) were also found to yield spores of a number of strains by Hall, Angelotti, Lewis and Foter (1963). These workers also compared Ellner's medium with an unbuffered medium containing 2 per cent trypticase, 2 per cent vitamin-free casamino acids, thiamine and sodium thioglycollate. In this experiment Ellner's medium was found to be superior to the unbuffered medium in terms of the number of spores produced, although only the unbuffered carbohydrate-free medium, consistently yielded spores of known heat-resistant types, which were capable of resisting boiling for prolonged periods.

5. The influence of the inorganic composition of the medium on sporulation.

In recent years a considerable amount of research has been devoted to the determination of the effect of inorganic ions on sporulation. In this work Cl.perfringens has been completely neglected.

Most of the media used to promote spore formation by Cl.perfringens have been of complex organic constitution or based on peptone. A number of the comparatively simple peptone media have been supplemented with yeast extracts. All these classes of media are rich in

inorganic ions, although their precise inorganic composition is not known. Difficulties are also encountered in assessing the effect on sporulation of calcium carbonate; sodium carbonate and sodium and potassium phosphates, as these compounds have been used to buffer media containing carbohydrate. It seems probable that the control of pH by these compounds was more important than the stimulatory effect of the ions themselves. Nevertheless, by critically surveying the sporulation media used, it is possible to evaluate the effect of large amounts of certain inorganic ions.

Media containing agar have frequently been observed to promote sporulation of Cl.perfringens, in some cases better sporulation has been obtained simply by solidifying the medium with agar (Schattenfroh and Grassberger 1900). Kahn (1922), Rita (1940, cited by Bethge 1947-8) and Bethge (1947-8) have also recommended the use of agar media to obtain spores of Cl.perfringens, while Hobbs et al. (1953), who encountered considerable difficulty in producing spores, obtained irregular sporulation in an agar medium in slide culture. Although these media have not been compared with similar media without agar, the importance of agar cannot be dismissed, especially as Curran (1957) has pointed out that agar contains relatively large amounts of Mg^{++}, K^{+} and Ca^{++} as well as biologically significant amounts of Mn^{++} and $Fe^{++(+)}$.

Addition of certain inorganic ions to peptone media has shown more clearly their effect on sporulation. Thus, sodium chloride was found to have no effect on sporulation in the concentrations used by Fitzgerald (1911) (0.5 to 5.0 per cent) and Brosteanu (1937) (0.1 to 0.7 per cent). Brosteanu however, found that sporulation was prevented when calcium chloride was added to the medium at concentration of 1 per cent.

The stimulatory effect of the potassium ion on sporulation can be clearly seen in the work of Torrey et al. (1930). These workers prepared a series of tubes of sugar-free nutrient broth containing 1 per cent peptone. To each tube a piece of calcium carbonate was added and the pH adjusted by adding an appropriate amount of a 10 per cent solution of sodium carbonate or hydrochloric acid. To these tubes was then added either KH_2PO_4 or NaH_2PO_4. Relatively weak sporulation (2 to 9 per cent) was invariably obtained in the tubes containing NaH_2PO_4, while in tubes containing the potassium salt sporulation in the order of 50 per cent was generally observed.

Similar results have been obtained with Ellner's medium. In this case Albrycht and Trembowler (1959) found that when the sodium and potassium phosphates were omitted from the medium there was a dramatic reduction in sporulation. While Ellner observed that magnesium sulphate (0.01 per cent) increased sporulation of several but not all isolates.

6. The influence of the oxidation-reduction potential on sporulation.

In the vast majority of experiments dealing with the sporulation of Cl.perfringens the media have been incubated anaerobically. In two instances only (Rita 1940, cited by Bethge 1947-8 and Bethge 1947-8), have the media been incubated aerobically. In these experiments sloppy agar media were used and although spores were formed under these conditions, vegetative growth was weak. For good vegetative growth it was essential to incubate the media anaerobically. This led Bethge to suggest that a low oxidation-reduction potential is probably more important for growth than sporulation. Bethge however, did not explain why greater numbers of spores were formed in an alkaline serum liver broth when incubated anaerobically. A possible explanation of this finding is that in the simple sloppy agar media, as in recently developed peptone media (e.g. Ellner's medium), there is very little vegetative growth before the cells spore, while in the complex media there is considerable vegetative growth prior to sporulation. By analogy with the genus Bacillus, where increased aeration has frequently been found essential for optimum sporulation, it seems probable that the optimum oxidation-reduction potential for sporulation of Cl.perfringens is higher than that for vegetative growth. Taking this to its extreme, Cl.perfringens might well sporulate in aerated cultures in Ellner's medium. This possibility has been examined

in the experimental section.

7. The influence of temperature on sporulation.

The influence of temperature on sporulation was first studied by Jacqué in 1904. Using an isolate which spored well on a starch agar medium at 37°, Jacqué was unable to produce any spores in this medium at 0° or room temperature (14-20°). Yamamoto, Sadler, Adler and Stewart (1961) however, found that spores of Cl.perfringens were produced at room temperature. These workers on feeding vegetative cells of a heat-resistant isolate to chickens were unable to isolate spores of this organism from the faeces. When the faeces were held at room temperature for two to three days spores were found. In this instance the relatively high body temperature of the chicken (42°) was excluded as the inhibitory factor by inoculating sterile 10 per cent suspensions of faeces with the organism and incubating at 37 and 42°. There was no significant difference between the numbers of spores formed at the two temperatures.

Apart from these two papers all the other studies of the sporulation of Cl.perfringens have been carried out at 37°, presumably because this is thought to be the optimum growth temperature. According to Topley and Wilson (1955) and Bergey (1957), 37° is the optimum growth temperature. However, Greer (1926) and Boyd, Logan and Tytella (1948), maintain that the optimum growth

temperature is usually higher than 37°, while Săsărman and Horodniceanu (1961)-working with thirty isolates of Cl.perfringens - found that the optimum growth temperature was isolate specific between 42 and 45°, although the optimum temperature for toxin production was 37°. In view of these findings it appears that an important environmental factor influencing sporulation has been overlooked, especially as it is known that in the genus Bacillus the optimum temperature for sporulation is close to that for growth, but the range is narrower (Ordal 1957).

8. The period of incubation required for sporulation.

The period of incubation required for sporulation is dependent upon the media and methods used. At the extreme end of the scale Hobbs et al. (1953) resorted to procedures lasting up to 7 weeks to produce spores. These workers grew the isolate in meat extract broth for 48 hours, washed in distilled water and left the organisms in distilled water for six weeks at room temperature. A few millilitres of this suspension were then inoculated into cooked meat broth and incubated for 24 hours. To this culture, which showed no evidence of growth, sterile starch solution was added to a concentration of 0.1 per cent. After a further five hours, growth, gas production and spores were observed.

Fortunately in all the other media used, sporulation has been

observed within one week. Proteinaceous media generally require one to four days incubation for sporulation, although Simmonds (1915a) using a gelatin medium found that an incubation period of one week was necessary. Within these limits different workers have recommended varying periods of incubation, even when using the same medium. Thus, Robertson (1915-16) found that spores were formed in 3 to 4 days on inspissated serum, while Henry (1916-17) using the same medium maintained that spores were formed within 24 hours. Bethge (1947-8) using a number of alkaline protein rich media, observed good sporulation after 24 hours incubation, although Becker (1920) using soda agar and alkaline brain broth and Willis (1957a) using alkaline egg medium, obtained maximum sporulation after incubation periods of 3 and 4 days respectively.

"Rapid" sporulation has been observed by Jablons (1919-20) and Weinberg et al. (1937) in broth containing pieces of coagulated egg white, while Eastoe and Long (1959) found that spores were formed in 18 to 24 hours in Robertson's meat broth. Unfortunately in these media not all isolates will form spores.

Cells also spore rapidly in the simpler media. Thus Kahn (1922) using a sugar-free agar medium, obtained spores of all the isolates he examined in forty eight hours, while in peptone media Ellner (1956), Angelotti, Hall, Foter and Lewis (1962), and Hall et al. (1963) have recommended incubating cultures for 16 to 24 hours. Torrey et al.

(1930) however, although they obtained good sporulation in 24 hours, found that the ratio of spores to vegetative cells was higher after 12 days. Whether this was due to sporulation or lysis of vegetative cells was not determined.

The only detailed observations of the cytology of sporulation have been carried out using Ellner's medium. Smith and Ellner (1957) found that forespores arose terminally, enlarged and became encysted from 4 to 5 hours after subculture into the sporulation broth. After 6 hours the forespores appeared to be retracting from their terminal positions; this was accompanied by contraction of the sporangia. After 7 hours an occasional morphologically mature spore was seen, and from 7 to 9 hours the proportion of forespore cores resistant to the mordanting effect of potassium permanganate increased By 12 hours the forespore cores were, in most cases, resistant to hydrolysis (i.e. mature spores). This maturation effect is explained by Smith and Ellner as being due to lack of synchrony in the culture. Cash and Collee (1962) confirmed these observations using other criteria of spore maturation. In these experiments heat-resistant forms were first observed after 4 to 5 hours incubation, increasing in number up to 12 hours. Acid fast spores were not observed until 6 hours had elapsed. This corresponds to the numerical increase in spores resistant to hydrolysis noted by Smith and Ellner.

The inhibitory effect of fermentable carbohydrate and acid pH on

sporulation have been reviewed previously. Both of these factors delay sporulation. This has been illustrated by Bethge (1948) who found that in media with a pH of 6.6 to 6.0 spores were not formed until after the first week of incubation. In media containing fermentable carbohydrate, such as brain infusion media, Hall (1922) found that spores were formed only when the sugar had been metabolised. Fermentable carbohydrate however, does not always inhibit sporulation. The most noteworthy of these exceptions is the incorporation of starch in Ellner's medium; however when the starch was replaced by mannite, maltose or raffinose, Albrycht and Trembowler (1959) found that sporulation was delayed by 28 to 58 hours.

9. <u>The association of sporulation with colonial morphology and virulence in separate isolates, and the variability of sporulation within an isolate associated with a change in colonial morphology</u>.

Many workers ranging from Jacqué in 1904 to Hall <u>et al</u>. in 1963 have noticed a marked variation in the ability of isolates of <u>Cl.perfringens</u> to form spores. Bredemann (1909, <u>cited by</u> Simmonds 1915a) however, attached no significance to this obeervation as he claimed that a non-sporing isolate could be made sporogenic by growing on the surface of a potato which had been rubbed with chalk. This observation has not been confirmed.

A few workers have correlated sporulation with colonial morphology

and virulence. Three colonial types, smooth, rough, and mucoid have been observed, of which the most common is smooth. Of these three types, Simmonds (1915a) and Henry (1916-17) reported that the mucoid isolates formed spores the most readily. Stevens (1935) however, apparently unaware of the earlier work, observed better sporulation of smooth than mucoid or rough types. Two variants (non-mucoid and rough) were isolated by McGaughey (1933) from the spreading granular edge of a three day old colony of a smooth isolate. These variants differed markedly in their ability to form spores. The smooth parent colonies produced few spores, the non-mucoid variant produced numerous spores, and like Stevens, McGaughey found that the rough variant did not produce spores.

A rather unusual way of producing spores, which is associated with an unstable variation in colonial morphology, was developed by Hobbs et al. (1953). These workers consistently obtained spores by growing an isolate in meat extract broth for 48 hours, followed by washing and suspending in distilled water for six weeks at room temperature. A few millilitres of this suspension were inoculated into cooked meat broth and incubated for 24 hours. To this culture, which showed no evidence of growth, sterile starch solution was added to a concentration of 0.1 per cent. After a further five hours, growth, gas production and spores were observed. A blood agar plate inoculated with this five hour culture produced three

colonial types. Forty per cent of the colonies were normal and made up of non-sporing bacilli. The remainder were either small "poached egg" or "pin point" colonies, these contained numerous spores. On subculture on blood agar plates or in meat broth, these atypical colonies reverted to the non-sporing parent type.

In addition to these papers associating sporulation and colonial morphology, a single paper by Thorsness (1934) drew attention to the association of sporulation and virulence. Thorsness injected rabbits intramuscularly with a culture of Cl.perfringens which was virulent for guinea-pigs but non-virulent for rabbits and isolated the organisms daily from the lesions. Organisms re-isolated within six days of injection were pathogenic for guinea-pigs and formed spores, while those isolated after this time were non-pathogenic for guinea-pigs and asporogenic. Attempts to decrease virulence in vitro and thereby reproduce this phonomenon, were unsuccessful.

As can be seen from the preceding paragraphs, a number of workers have associated colonial morphology and virulence with sporulation. Other workers have associated colonial morphology with toxin production and virulence. Unfortunately there is little agreement amongst this latter group of workers. Buchaly (1931, cited by Weinberg et al. 1937) and Roe (1934) claimed that rough variants were more toxic and pathogenic than smooth types, while Orr, Josephson, Baker and Breed (1933), Orr and Breed (1933) and Basilevsky and Melnik (1936,

cited by Weinberg et al. 1937) maintained the opposite to be true. To complete the spectrum, Kossovsky (1936, cited by Weinberg et al. 1937) found no difference in pathogenicity between rough and smooth types; while McGaughey (1933) isolated two rough types, one of which was more toxic and the other less toxic than the parent. Similar confusion occurs regarding the pathogenicity of mucoid variants. Roe claimed that they were avirulent, while Buchaly stated that they were virulent.

10. Observations of sporulation on recently developed simple sporulation media.

The sporulation media devised for Cl.perfringens fall into two distinct groups. On the one hand there are the complex proteinaceous media which yield high viable spore counts, but apparently low percentage sporulation; while on the other hand there are a number of recently devised media in which high percentages of spores have been produced. This latter group comprises the media devised by Torrey et al. (1930), Ellner (1956) and Angelotti et al. (1962). These media contain from one to two percent peptone and those devised by Torrey et al. and Ellner are buffered, although only Ellner's medium contains fermentable carbohydrate. By far the most widely used of these media has been Ellner's medium. In this medium Ellner found that "greater than 90 per cent sporulation was not unusual" and Collee, Knowlden and Hobbs (1961) found on

microscopical examination that "large numbers of spores" were produced by 16 of the 39 heat-resistant isolates examined. Further reports of the efficiency of this medium have been made by Yamamoto, Sadler, Adler and Stewart (1961), who found that spores were "readily observed" in 55 out of 63 isolates examined, while Meisel, Albrycht and Rymkiewicz (1959a,b; 1962) and Rymkiewicz (1960) have obtained sufficient spores for biochemical investigations.

In an attempt to determine the relative importance of the components of Ellner's medium, Albrycht and Trembowler (1959) found that:-

1. Omitting the yeast extract did not affect sporulation.
2. Dissolving the components of Ellner's medium in ox-heart broth resulted in a dramatic reduction in the number of spores formed.
3. Omitting the starch reduced the percentage sporulation of some isolates, although decreasing the concentration of starch from 0.3 to 0.1 per cent did not affect growth or sporulation. Increasing the starch content resulted in increased vegetative growth but not sporulation.
4. Omitting the phosphates did not affect growth but there was a dramatic reduction in sporulation. Increasing the concentration of phosphates was without effect.
5. Reducing the peptone concentration from 1.0 to 0.2 per cent did not affect sporulation.

In recent years the only sporulation medium which has challenged the supremacy of Ellner's medium is that formulated by Angelotti et al. (1962). This medium contains 2 per cent trypticase, 2 per cent

vitamin-free casamino acids, 0.1 per cent sodium thioglycollate and thiamine. When this medium was compared with Ellner's medium, Zoha and Sadoff's medium (1958)(1.5 per cent trypticase, 1 per cent peptone, 0.2 per cent glucose, 0.5 per cent NaCl and 0.25 per cent K_2HPO_4) and an unspecified medium devised by Sames; Angelotti et al. claimed that their medium generally yielded the highest sporulation rate. In a second paper by these workers (Hall et al. 1963), in which the previous four and an additional medium (Ellner's medium containing 0.1 per cent $MnSO_4$ in place of $MgSO_4$) were compared, they found that the number of spores produced in Ellner's medium was greater than in their own medium, although only the medium which they devised in 1962 consistently yielded spore crops of known heat-resistant strains which were capable of resisting boiling for prolonged periods. This deficiency in spores produced in Ellner's medium has also been observed by Horodniceanu and Săsărman (1961). Albrycht and Trembowler (1959) however, found no difference between the heat resistance of spores produced in Ellner's medium and brain infusion broth.

A further method for producing spores of Cl.perfringens has been recommended by Schneider, Grecz and Anellis (1963). These workers grew a single isolate in dialysis sacs suspended in a previously unpublished medium. This medium contained 5 per cent

trypticase, 0.5 per cent peptone and 0.5 per cent thioglycollate supplement (Baltimore Biological Laboratories). Good spore crops were obtained, which these workers attributed to the influence of the dialysis membrane. Unfortunately the results of control experiments using this medium are not included in this paper, although these workers state that "the strain of Cl.perfringens used is generally known for its poor sporulation in conventional cultures."

MATERIALS AND METHODS

In this section are included the materials and methods which have been repeatedly used throughout Part 1. of the thesis. Each set of experiments has its own Materials and Methods section in which are presented methods peculiar to those experiments. In certain instances where minor modifications peculiar to a particular experiment have been used, these have been included with the experiment. This I hope will simplify reading of the Experimental part of the thesis, particularly where reference to the methods used for a particular experiment may be necessary.

Organisms. Most of the experimental work recorded in this thesis has been carried out using isolates (strains) of Cl.perfringens type A from the National Collection of Type Cultures. In all, 48 isolates have been used. These are listed in Table 6, together with their origin. Sixteen of these are type A strains from the National Collection of Type Cultures, comprising 14 heat-resistant and 2 beta-haemolytic isolates. Seven isolates, assumed to be type A, including three beta-haemolytic and four heat-resistant types were obtained from Dr.Collee (Bacteriology Department, Edinburgh University). A single beta-haemolytic isolate, again assumed to be type A, was received from Miss Nicholson (Bacteriology Department, Newcastle

TABLE 6

Isolates of Cl.perfringens type A

Strain designation	Type	Hobb's Serotype	Source
NCTC 8359	Heat-resistant	1	National Collection of Type Cultures
NCTC 8238	" "	2	"
NCTC 8239	" "	3	"
NCTC 8247	" "	4	"
NCTC 8678	" "	5	"
NCTC 8679	" "	6	"
NCTC 8449	" "	7	"
NCTC 8235	" "	8	"
NCTC 8798	" "	9	"
NCTC 8799	" "	10	"
NCTC 9851	" "	11	"
NCTC 10239	" "	12	"
NCTC 10240	" "	13	"
NCTC 8797	" "		"
NCTC 2836	Beta-haemolytic		"
NCTC 2837	" "		"
032	" "		Dr J.G. Collee
F.4637/60	" "		"
F.6599/59	" "		"
F.1546/52	Heat-resistant	10	"
F.2985/50	" "		"
133	" "		"
134	" "		"
19	Beta-haemolytic		Miss J. Nicholson
J.M.S.	" "		Department of Bacteriology, Glasgow University

TABLE 6 (Continued)

Strain designation	Type	Hobb's Serotype	Source
21	Beta-haemolytic		Human faeces
22	" "		" "
23	" "		" "
25	" "		" "
26	" "		" "
S1	" "		" "
S2	" "		" "
S3	" "		" "
S5	" "		" "
S6	" "		" "
S9	" "		" "
S11	" "		" "
S12	" "		" "
S14	" "		" "
S16	" "		" "
S20	" "		" "
S21	" "		" "
S23	" "		" "
S24	" "		" "
R5	" "		" "
R6	" "		" "
R10	" "		" "
R11	" "		" "

University). The remainder were all beta-haemolytic and were isolated from the faeces of hospital patients, as described in Appendix One. The toxins produced by five of these isolates were examined by Dr. I. Batty (Wellcome Research Laboratories) who found that they were all Cl.perfringens type A, producing alpha and kappa toxins.

Lyophilised cultures were taken up in 0.5ml nutrient broth and the resulting suspensions "plated out" on horse blood agar. These were incubated anaerobically at 37° for forty eight hours, when typical colonies were selected and transferred to tubes of Robertson's meat broth. These were incubated at 37°. The identification and purity of each isolate was ascertained by the following tests:-

1. Failure to grow aerobically.
2. Typical saccharolytic growth in Robertson's meat broth.
3. Typical colonial morphology and haemolysis on horse blood agar plates.
4. The lecithovitellin reaction on serum agar plates and its inhibition by appropriate antisera.
5. Production of a "stormy clot" in litmus milk medium.
6. Fermentation with the production of acid and gas of the following peptone water sugars:-
   a. Glucose,
   b. Sucrose,
   c. Lactose.
7. Absence of motility.

Routine subcultures were made in Robertson's meat broth and a subculture of each isolate in Robertson's meat broth was stored for reference.

Media. Unless otherwise stated, media were prepared as described by Mackie and McCartney (1960). Ellner's medium and other sporulation media are described below. These were distributed in 10ml amounts in universal containers and unless otherwise stated were sterilised by autoclaving at 15lb/sq in for 15 minutes.

1. Ellner's medium

Peptone (Oxoid L.37)	10g
Yeast extract (Oxoid)	3g
Soluble starch (B.D.H.)	3g
$MgSO_4$	0.1g
KH_2PO_4	1.5g
$Na_2HPO_4.12\ H_2O$	67.0g
Distilled water	1 l.

These components were dissolved by steaming, the product cooled and the pH adjusted to 7.8 by addition of a few drops of a solution of sodium hydroxide.

2. A medium devised by Bethge (1947-8)

Sheep serum	60ml
Beef infusion broth (pH 7.2)	40ml
A mixture of chopped guinea-pig heart, liver and kidney	2g

As autoclaving this medium resulted in coagulation, sterilisation was effected by autoclaving the beef infusion broth and organ pieces, then aseptically adding the Seitz filtered serum. The sterile medium was aseptically distributed in 10ml amounts in universal containers. The pH of this medium was 7.8.

3. Mannitol peptone water

Peptone (Oxoid L.37)	1g
Mannitol	1g
NaCl	0.85g
Distilled water	100ml

This medium was sterilised by Seitz filtration and aseptically distributed in 10ml amounts in universal containers.

4. Alkaline egg medium

The yolk of one egg and the white of two eggs were beaten up in a beaker. 6ml N.sodium hydroxide were added and 500ml tap water. The mixture was slowly heated to 95° and held at this temperature for 60 minutes. It was then filtered through muslin and diluted one in five in infusion broth. The medium was distributed in 10ml amounts in universal containers and sterilised by autoclaving at 10lb/sq in for 20 minutes.

5. Brain-heart infusion broth

Difco dehydrated culture medium was reconstituted as directed.

6. Serum peptone water

Sheep serum	10ml
Peptone (Oxoid L.37)	1.5g
NaCl	1.275g
Distilled water	150ml

This medium was sterilised by Seitz filtration and aseptically distributed in 10ml amounts in universal containers. Preliminary experiments showed that this was a better sporulation medium than undiluted serum.

7. Liver broth

Dehydrated Ox liver (Oxoid L.25)	6.4g
Peptone (B.D.H.)	50.0g
NaCl	2.5g

Made up to 500ml with distilled water.

8. A medium used by Halvorson (1957) for the sporulation of anaerobes.

Bacto-casitone (Difco)	1.5 per cent (w/v)
NaCl	0.5 per cent (w/v)
Glucose	0.2 per cent (w/v)
K_2HPO_4	M/30
Tris buffer	M/80
$FeSO_4.7H_2O$	0.00005 per cent (w/v)

$CuSO_4 . 5H_2O$	0.0005 per cent (w/v)
$ZnSO_4 . 7H_2O$	0.0005 per cent (w/v)
$MnSO_4 . 4H_2O$	0.005 per cent (w/v)
$MgSO_4$ (anhydrous)	0.02 per cent (w/v)

9. Yeast extract medium (used for the preparation of inocula by Halvorson)

Bacto-casitone (Difco)	1.5 per cent (w/v)
Yeast extract (Oxoid)	0.5 per cent (w/v)

10. A medium similar to that recommended by Angelotti et al. (1962)

Bacto-casitone (Difco)	2.0 per cent (w/v)
Casein hydrolysate (Oxoid)	2.0 per cent (w/v)
Thioglycollic acid	0.1 per cent (v/v)

The pH of this medium was adjusted to 7.0 with concentrated sodium hydroxide and sterilised by autoclaving at 15lb/sq in for 15 minutes. A Seitz filtered solution of aneurin (Roche) was added to a concentration of 1µg/ml. The medium was aseptically distributed in 10ml amounts in universal containers.

Period and temperature of incubation of media. Unless otherwise stated all media were incubated anaerobically (see over) at 37°. Cultures in Ellner's medium and media based on Ellner's medium were incubated for 18 hours.

Preparation of inocula for sporulation media. Halvorson (1957) has shown that in order to produce good spore crops in comparatively simple peptone media, it is advantageous to inoculate these media with a young, actively growing culture. The cells in these cultures are to a certain extent synchronised and when transferred to the sporulation broth, spore rapidly and in unison. Ellner (1956) also recommended that an actively growing 4 to 12 hour broth culture should be used. In the following experiments, in which Ellner's medium and modifications to this medium have been used, inocula were prepared by growing each isolate for 18 hours in a universal container of Robertson's meat broth (incubated aerobically) followed by two four hour growth periods in fresh Robertson's meat broth. For these last two growth periods an inoculum of approximately 10 per cent of the total volume was used. 2ml of this actively growing culture was used to inoculate 10ml of Ellner's medium.

For the more complex media used in experiment two, in which there is considerable vegetative growth prior to sporulation, smaller inocula have been used. In this experiment overnight cultures in Robertson's meat broth (incubated aerobically) were used to inoculate fresh tubes of Robertson's meat broth. After nine hours incubation 0.2ml amounts of each of these cultures were used to inoculate the sporulation media.

Anaerobiosis. Cultures were incubated in anaerobic jars (Baird and

Tatlock (London) Limited.) with room temperature catalyst and indicator. Anaerobic conditions were established by evacuating the jars using a water pump and filling with hydrogen to atmospheric pressure. By this method the anaerobic indicator remained colourless in the atmosphere established in the jar. The cap liners were removed from the universal containers of inoculated media to allow passage of hydrogen into them.

Estimation of sporulation. Each experiment was carried out in duplicate and the spore content of each sample was estimated by direct microscopy and/or viable spore counts. As growth in Ellner's medium is poor, it was essential to centrifuge each culture (10ml) before attempting to count spores microscopically. Each culture was therefore centrifuged at 5,000 r.p.m. for 15 minutes, the supernatant carefully decanted and the sediment resuspended by adding 1ml sterile distilled water and shaking. The percentage of spores in wet films prepared from this material was determined by counting the number of spores as a percentage of the total number of cells, using a phase contrast microscope (a Baker Series 4, Modern Research Microscope, with Trilux condenser and Projectolux illumination was used). A minimum of 200 cells were counted for each estimation. Total viable and spore counts (see over) were not used to determine the percentage sporulation.

Viable spore counts were obtained using the following technique: 0.5ml of the resuspended culture was pipetted into a 4 x ½" test tube. The test tube was then placed in a water bath at 75° for 20 minutes to kill vegetative cells. (This low temperature long duration technique, recommended by Gibbs and Hirsch (1956), was used as it minimises errors in the heating and cooling of the sample). After heating, a series of tenfold dilutions in sterile distilled water were prepared. Four standard loopfuls[1] of each dilution were then spread over the surfaces of horse blood agar plates, so that one loopful occupied one-half of each plate. Before use, the horse blood agar plates were placed in an incubator at 37° for two hours to remove excess moisture. Inoculated plates were incubated anaerobically at 37° for two days before counting. Half-plates containing between 15 and 150 colonies were selected for counting, except in instances where the undiluted culture gave less than this number. From the results the number of spores present in one loopful of undiluted culture was calculated.

A preliminary analysis on the counts obtained using this technique, showed that there was a tendency for the variances between

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[1] Standard Loopful. A wire loop was made which was used throughout these experiments. The loop was loaded by dipping horizontally into the culture and carefully withdrawing. During this procedure the tube was held as near the horizontal as possible.

counts for different loopfuls to be greater than would be expected if the numbers of colonies developing from different loopfuls had identical Poisson distributions. Therefore, confidence limits were worked out from the variances between counts for different loopfuls on the basis of Students t- distribution. On the average, at least, this leads to wider confidence intervals than would be obtained by assuming identical Poisson distributions.

This procedure for counting spores has a number of advantages over more widely used methods. Firstly, plate count methods are statistically more accurate than methods using tubes of liquid media. Secondly, this method uses only half the number of plates that would be required for the pour plate method, while yielding the same number of counts - so saving on valuable anaerobic jar space. In addition, the use of horse blood agar provides a useful check on the purity of the cultures.

EXPERIMENTAL

SECTION A

The Influence of Environment on the Sporulation of Cl.perfringens.

CONTENTS

1. Screening of Forty Eight Isolates for Spore Formation in Ellner's Medium

Introduction

It has been pointed out in the review that there is a wide discrepancy as to the ability of isolates of Cl.perfringens type A to form spores. The most widely used of the media purported to promote abundant sporulation of Cl.perfringens type A is Ellner's medium. This medium appears from the literature to induce almost 100 per cent of the isolates tested to produce abundant spores. However, initial attempts to promote sporulation of two isolates using Ellner's medium resulted in the production of less than one spore per hundred cells examined. In order to assess more fully the utility of this medium, the sporulation of forty eight isolates was examined.

Results

The results of this experiment are shown in Tables 7, 8 and 9. As the beta-haemolytic isolates were found to produce far greater numbers of spores than the heat-resistant types, the results are included separately in Tables 7 and 8. A comparison of the results in these two tables is shown in Table 9. From these tables it can be seen that although 13/30 (43.3 per cent) of the beta-haemolytic

TABLE 7

Sporulation of beta-haemolytic isolates of Cl.perfringens type A in Ellner's medium

Isolates producing 1-100% intracellular spores*		Isolates producing up to 1% intracellular spores*	Isolates in which no intracellular spores were seen	
Isolate	Percentage spores	Isolate	Isolate	Mean spore count per standard loopful
NCTC 2836	76	NCTC 2837	F.6599/59	10
032	30	F.4637/60	S9	400
19	75	22	S11	200
21	90	S1	S21	0
23	70	S12	R5	15
25	90	S14	R6	120
26	90	S16	R10	2
S2	10	S20	R11	62
S3	80	S24		
S5	5			
S6	5			
S23	2			
J.M.S.	27			

*These isolates yielded spore counts of more than 400

TABLE 8

Sporulation of heat-resistant isolates of Cl.perfringens type A in Ellner's medium

Isolates producing up to 1% intracellular spores*	Isolates in which no intracellular spores were seen	
Isolate	Isolate	Mean spore count per standard loopful
NCTC 8359	NCTC 8247	0
NCTC 8238	NCTC 8235	0
NCTC 8239	NCTC 8797	0
NCTC 8678	NCTC 8798	36
NCTC 8679	NCTC 8799	10
NCTC 8449	F.1546/52	0
NCTC 9851	134	60
NCTC 10239	133	400
NCTC 10240		
F.2985/50		

*These isolates yielded spore counts of more than 400

TABLE 9

Sporulation of beta-haemolytic and heat-resistant isolates in Ellner's medium (analysis of results)

Isolate	Percentage of isolates displaying various abilities to sporulate			
	1 to 100% spores	Less than 1% spores	Spores not detected microscopic-ally but detected after heating and sub-culture	Spores not detected after heating and sub-culture
Heat-resistant	0 (0/18)	55.6(10/18)	22.2(4/18)	22.2(4/18)
Beta-haemolytic	43.3(13/30)	30.0(9/30)	23.3(7/30)	3.3(1/30)

isolates produced between 1 and 100 per cent spores, none of the heat-resistant isolates produced more than 1 per cent spores. Although accurate spore counts were not carried out on cultures in which spores were seen, in all these cultures the spore count exceeded four hundred. This was higher than the maximum spore count from cultures in which spores were not seen. The spore counts on cultures in which spores were not seen further illustrates the tendency of the heat-resistant strains to produce extremely low numbers of spores. Thus, spores were detected in all but a single culture (3.3 per cent) of the beta-haemolytic strains, compared with 22.2 per cent of the heat-resistant isolates.

2. A Comparison of Spore Formation on Several Recommended Media

Introduction

The survey carried out in the previous section has shown that good spore crops of a number of beta-haemolytic Cl.perfringens type A isolates can be obtained using Ellner's medium. A number of isolates, predominantly heat-resistant types, produced only small numbers of spores in this medium. It was therefore decided to compare the ability of a number of media to promote sporulation of two heat-resistant isolates which produced less than 1 per cent spores in Ellner's medium. Two beta-haemolytic isolates which produced abundant spores in Ellner's medium were included to determine whether these other recommended media were more, less, or equally effective in promoting sporulation than Ellner's medium.

Materials and Methods

Media. Besides Ellner's medium seven media were used which have been recommended for the sporulation of Cl.perfringens. These were: mannitol peptone water, alkaline egg medium, brain-heart infusion broth, serum peptone water, liver broth and media devised by Bethge (1948) and Angelotti et al. (1962). These have been described in the literature review and on pages 47 to 50 of the Materials and Methods

section. Two media used by Halvorson (1957a) to promote sporulation of other anaerobes have also been used, these have been designated Halvorson and Yeast extract. In addition two routinely used bacteriological culture media, infusion broth and Robertson's meat broth, have been used.

Organisms. Two heat-resistant isolates, NCTC 8798 and NCTC 8799, which produced less than 1 per cent spores in Ellner's medium, and two beta-haemolytic isolates, 032 and NCTC 2836, which produced more than 30 per cent spores, were used.

Period of incubation. Inoculated media were incubated for one week. This was done because several of the media supported considerable vegetative growth prior to sporulation.

Estimation of sporulation. Estimation of sporulation using phase contrast microscopy was impossible with several of these media, viz:-

1. Robertson's meat broth often contains globules of fat or oil which look similar to free spores.
2. Liver broth contains highly refractile components which can be confused with spores.
3. In alkaline egg and Bethge's medium a precipitate was formed on incubation with Cl.perfringens. This made microscopical estimation of sporulation difficult.

TABLE 10

Sporulation of two heat-resistant and two beta-haemolytic isolates on several recommended media

Medium	Initial pH	Mean number of spores per standard loopful[2].				Estimation of vegetative growth[1].	
		NCTC 8798	NCTC 8799	032	NCTC 2836	Heat-resistant types	Beta-haemolytic types
Bethge	7.8	>3,000	1,125	2,160	>3,000	+++	+++
Mannitol peptone water	7.4	0	0	595	300	+	+
Alkaline egg	7.4	60	2	505	>3,000	++++	++++
Brain-heart	7.4	82	0	1,020	>3,000	+	++
Infusion broth	7.2	320	1	77	140	±	±
Serum peptone water	NT	23 >300*	0 52*	1 >300*	>3,000 >300*	±	±
Liver broth	NT	0	0	3	0	+	+++
Halvorson	7.8	30	0	>3,000	>3,000	+	+
Yeast extract	7.3	8	0	>3,000	45	±	±
Angelotti	7.0	76	7	>3,000	>3,000	NT	NT
Robertson's meat broth	7.2	890	7	1,125	1,300	++	++
Ellner	7.8	36	10	>3,000	>3,000	±	+

1. Vegetative growth was estimated visually and assigned to one of the values, ±, +, ++, +++, or ++++.

*In a subsequent experiment an inoculum of 1.0ml was used. This experiment yielded the results marked *.

2. This is the mean spore count per loopful after centrifugation of the culture and resuspension in 1ml of sterile distilled water.

NT No test.

These factors, together with the expected production of only small numbers of spores, led to estimation of sporulation by the viable spore count method. As this method was used, the amount of vegetative growth was also estimated,-to give a crude indication of the degree of sporulation. Growth in these media was estimated visually and assigned to one of the values ±, +, ++, +++ or ++++.

Results

The results of this experiment are shown in Table 10. Although precise figures of the percentage sporulation were not obtained, the beta-haemolytic isolates - both of which produced more than 30 per cent spores in Ellner's medium - did not spore so well in any of the media examined as they did in Ellner's medium. In seven of the eleven media examined, the beta-haemolytic isolates still produced larger numbers of spores than the heat-resistant types. In the remaining four media mixed results were obtained, i.e. one of the heat-resistant types produced more spores than one of the beta-haemolytic types and vice-versa.

The four peptone media, i.e. Angelotti, Halvorson, mannitol peptone water and Ellner's medium, all yielded similar results. In these media the heat-resistant types spored weakly if at all, while the beta-haemolytic types produced much larger numbers of spores. Vegetative growth of all four isolates was comparatively weak in

these media, although as can be seen from the results, this is no index of sporulation.

Two of the media examined are particularly noteworthy, these are liver broth and a medium recommended by Bethge. In liver broth it was only possible to show the existence of spores with isolate 032, and then only three spores were found per loopful. In Bethge's medium however, all the strains spored equally well. This medium was also unique in that the heat-resistant isolates produced more spores than in any of the other media examined, including Ellner's medium. Although the percentage sporulation in this medium was difficult to assess due to the formation of a precipitate during growth and sporulation, none of the isolates appeared to produce more than 1 per cent spores. This is borne out by the viable spore count data. Thus, although the heat-resistant isolates produced more spores in this medium than in Ellner's medium, the opposite is true of the beta-haemolytic strains.

3. Examination of Some of the Factors Influencing Sporulation in Ellner's Medium

CONTENTS

Introduction

In this section experiments have been carried out to investigate the effect of a number of factors which either from preliminary experiments, or by analogy with the genus Bacillus, could result in improved sporulation of Cl.perfringens. Ellner's medium was used as previous experiments have shown this medium to be superior to the other media examined. In addition, even though this medium has been widely used, no attempts have been made to define the optimum conditions for sporulation.

Preliminary experiments indicated that the medium used to prepare the inoculum for Ellner's medium had a pronounced effect on sporulation. It was therefore decided to compare the ability of eight media to produce cells capable of forming spores when transferred to Ellner's medium. Other factors also affect sporulation in Ellner's medium and it was apparent that one of the most important of these was the volume of inoculum used to seed Ellner's medium. As outlined in the literature review, Cash and Collee (1962) have shown that in Ellner's medium each cell of the inoculum undergoes no more than four divisions prior to sporulation. Therefore in order to obtain the maximum number of spores per millilitre of medium, it is essential to use the largest possible inoculum. Albrycht and Trembowler (1959) however, pointed out that if the water content of

Ellner's medium is replaced by infusion broth, sporulation is reduced. Thus, if too large an inoculum is used it would appear that the percentage sporulation will fall off. Experiments were therefore carried out to determine the maximum volume of inoculum which could be transferred to Ellner's medium without decrease in the percentage sporulation. The effect of inoculating with washed cells was also examined.

Also included in this section are experiments to determine the influence of temperature and aeration on sporulation. Both of these factors are important for sporulation in the genus Bacillus, but their effect on sporulation of Cl.perfringens has not been studied. In the genus Bacillus the optimum temperature for sporulation has been found to be the same as for vegetative growth, but the range is narrower (Ordal 1957). Contrary to popular belief, Boyd et al. (1948) have shown that the optimum growth temperature for Cl.perfringens is usually higher than 37°, while Săsărman and Horodniceanu (1961) found that for the 30 isolates they examined, the optimum growth temperature lay between 42 and 45°, although the optimum temperature for toxin production was 34 to 36°. Experiments were therefore carried out to determine which of these temperature optima was the best for sporulation of an isolate of Cl.perfringens.

In the genus Bacillus oxygen has frequently been found to be a factor limiting sporulation and many workers have found it essential

to use shake cultures, or to sparge their sporulation media with oxygen to obtain good spore yields (Knaysi 1945, Roth, Lively and Hodge 1955, Tinelli 1955, Grelet 1957 and Halvorson 1957a). Although the inhibitory effect of oxygen on vegetative growth of Cl.perfringens is well known, its effect on sporulation has not been studied. Using Ellner's medium a sharp division is created between vegetative growth and sporulation, enabling sporulation to be studied independently. It was therefore decided to study the effect of aeration on sporulation in Ellner's medium, to determine what effect if any, this would have.

Materials and Methods

Organisms. Except where otherwise stated, isolate NCTC 2836 was used,

Media. Ellner's medium was prepared as described on page 47 with the following modification. After adjusting the pH to 7.8 the medium was steamed and cooled, followed by centrifugation to remove the precipitate formed. This medium was distributed in 10ml amounts in universal containers and sterilised by autoclaving. This modification was found to have no effect on sporulation, although the cleaner medium so produced simplified the determination of percentage sporulation.

Experimental

3.1 The effect of the pre-sporulation medium on sporulation in Ellner's medium

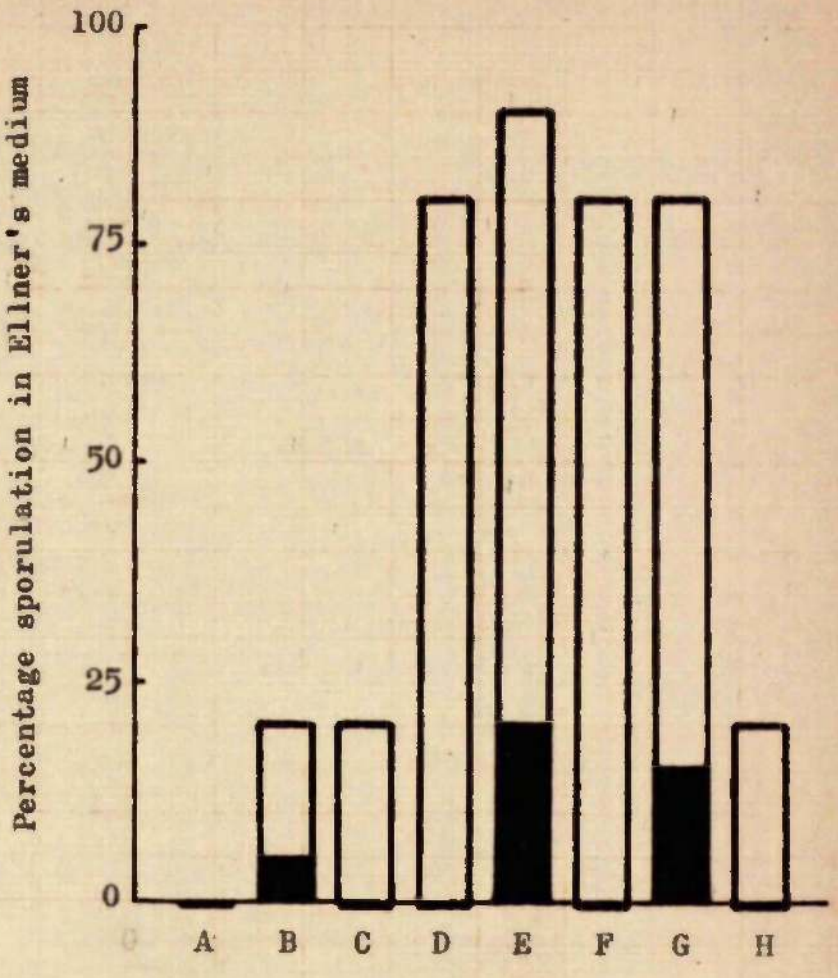
Method. In this experiment four strains were used. These were: 032, NCTC 2836, NCTC 8798 and NCTC 8799. Each strain was grown overnight in Robertson's meat broth followed by two four hour growth periods in each of the media examined. The media examined were: Brewer's medium, liver broth, digest broth, brain-heart infusion broth, Robertson's meat broth, yeast extract broth, serum peptone water and infusion broth. 1ml of the last of these subcultures was used to inoculate 10ml Ellner's medium.

Results. Isolates NCTC 8798 and NCTC 8799 produced less than 1 per cent intracellular spores regardless of the pre-sporulation medium used. The other two isolates, 032 and NCTC 2836, produced widely varying percentages of spores depending upon the pre-sporulation medium used. The results obtained using these isolates are illustrated in Figure 1.

It can be seen from these results that the best of the pre-sporulation media used was Robertson's meat broth, with serum peptone water coming a close second. With brain-heart infusion broth and yeast extract broth, isolate NCTC 2836 spored well, while isolate 032 produced only small numbers of spores. Sporulation of both strains

Fig. 1

The effect of the pre-sporulation medium on sporulation in Ellner's medium

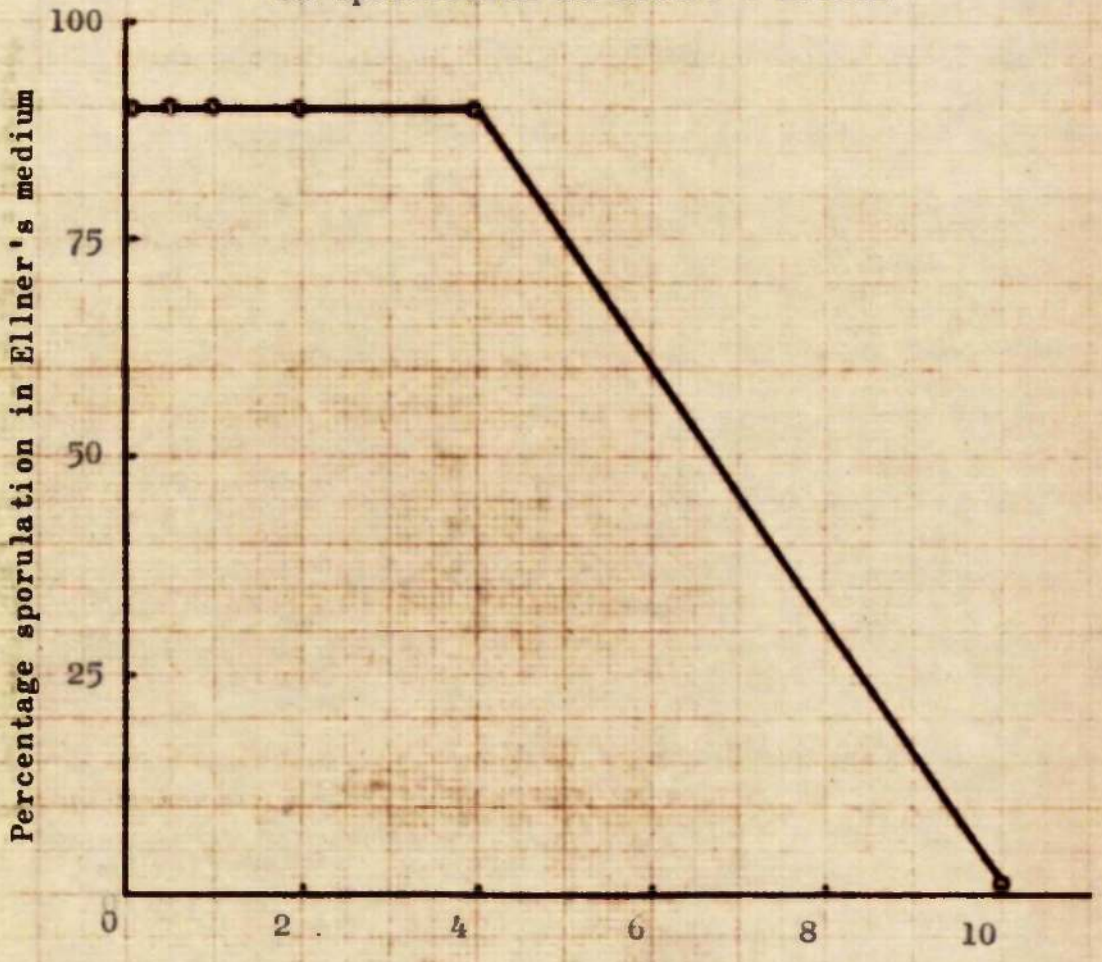


A. Brewer's medium
B. Liver broth
C. Digest broth
D. Brain-heart infusion broth
E. Robertson's meat broth
F. Yeast extract broth
G. Serum peptone water
H. Infusion broth

White areas, strain NCTC 2836
Black areas, strain 032

Fig. 2

The influence of inoculum volume on sporulation in Ellner's medium



was comparatively weak when liver broth, digest broth and infusion broth were used as pre-sporulation media; while with the remaining medium, Brewer's medium, spores were not seen on microscopical examination. Neither were spores shown to be present after heating and subculture. The possibility that some factor inhibiting sporulation was being carried over into Ellner's medium with the inoculum, was eliminated by washing the vegetative cells from a four hour culture in Brewer's medium in freshly boiled and cooled physiological saline. Even when this washed culture was used to inoculate the sporulation broth no spores were formed.

3.2 Determination of the maximum volume of culture which can be used to inoculate Ellner's medium without decrease in sporulation

Method. A series of volumes of a four hour culture of isolate NCTC 2836 in Robertson's meat broth, ranging from 0.1 to 10ml were used to inoculate 20ml amounts of Ellner's medium.

Results. In this experiment (see Figure 2) up to 4ml of inoculum was added to 20ml of Ellner's medium before any decrease in the percentage sporulation was observed. When an inoculum of 10ml was used the percentage sporulation was reduced from 90 per cent to 2 per cent.

3.3 The effect of washing the inoculum and reconstituting washed cells with Robertson's meat broth, on sporulation in Ellner's medium

To overcome the inhibition of sporulation when large volumes of inoculum were used, an attempt was made to increase the spore yield of Ellner's medium by inoculating the sporulation broth with washed cells. In this experiment Ellner's medium was added to a centrifuged pellet of cells after first washing the cells in freshly heated and cooled physiological saline. The low percentage sporulation obtained using this technique lead to further investigation of the role of Robertson's meat broth on sporulation in Ellner's medium. This was done by reconstituting washed cells with the original or fresh Robertson's meat broth.

Method. Four 1ml amounts were withdrawn from a four hour culture in Robertson's meat broth and subjected to one of the following procedures:-

1. Used to inoculate 10ml Ellner's medium (i.e. control).

2. Added to 20ml freshly boiled and cooled physiological saline. The cells were then spun down, the supernatant discarded and 10ml Ellner's medium added to the washed cells.

3. The cells were washed as described above. To these cells were added 1ml fresh sterile Robertson's meat broth and 10ml Ellner's medium.

4. The cells were washed as described above. To these cells were added 1ml of the original culture after Seitz filtration and 10ml Ellner's medium.

Results. The results of this experiment (see Figure 3) show that although washed cells formed spores in Ellner's medium, the percentage sporulation was markedly reduced. When washed cells were reconstituted with Seitz filtered Robertson's meat broth from the original four hour culture, sporulation attained its original level, indicating that washing the cells did not affect their viability. This effect was reproduced only to a limited extent when fresh broth was used. It appears from this experiment that in order to obtain high percentage sporulation in Ellner's medium it is essential to add, along with the vegetative cells, a certain amount of the pre-sporulation medium.

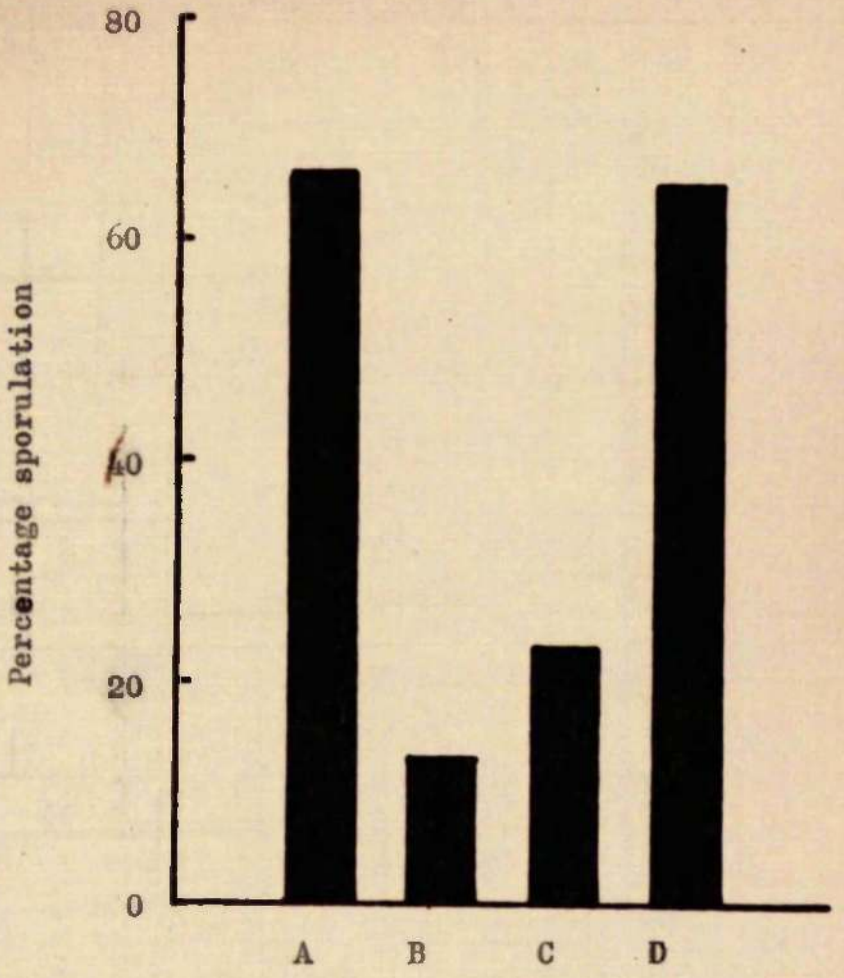
3.4 Comparison of vegetative growth and sporulation at 37 and 45°

Methods

1. Vegetative growth. Vegetative growth in 1 per cent (w/v) glucose broth at 37 and 45° was compared using a nephelometer (Evans Electroselenium Limited, Harlow, Essex). Preliminary experiments indicated that the nephelometer reading was not directly proportional to the density of the culture. A control curve was therefore

Fig. 3

The effect of washing the inoculum and reconstituting washed cells with Robertson's meat broth on sporulation of strain NCTC 2836 in Ellner's medium



A. Ellner's medium inoculated with a four hour culture in Robertson's meat broth

B. Ellner's medium inoculated with washed cells

C. Ellner's medium inoculated with washed cells plus fresh Robertson's meat broth

D. Ellner's medium inoculated with washed cells plus Seitz filtered Robertson's meat broth from the original four hour culture

prepared. This was done by taking 1,2,3,4,5,6,7,8,9 and 10ml amounts of an 18 hour culture in glucose broth and making up to 10ml with sterile glucose broth. The sensitivity control of the nephelometer was adjusted so that the undiluted broth gave a reading of one hundred and ten. With this arbitrary value fixed, the reading given by a density control tube was taken. Immediately before each reading the instrument was checked with this tube and adjustment made to the sensitivity control when necessary. The density of each dilution of the glucose broth culture was then measured and a control curve prepared (Figure 4).

Vegetative growth was then compared at 37 and 45° in 1 per cent glucose broth. Anaerobic conditions were established in the broths (400ml) by steaming for 30 minutes. Each broth was then allowed to cool to the test temperature in a water bath and inoculated with 2ml of an 18 hour culture of the organism in Robertson's meat broth. 10ml samples were withdrawn at half-hourly intervals for density determination. The nephelometer readings obtained were then corrected using the control curve and a graph of density against time plotted (Figure 5).

2. Sporulation. Four hour cultures in Robertson's meat broth incubated at 37 and 45° were used to inoculate Ellner's medium. In addition, cultures grown at 45° were used which had been subcultured

Fig. 4

Nephelometer control curve

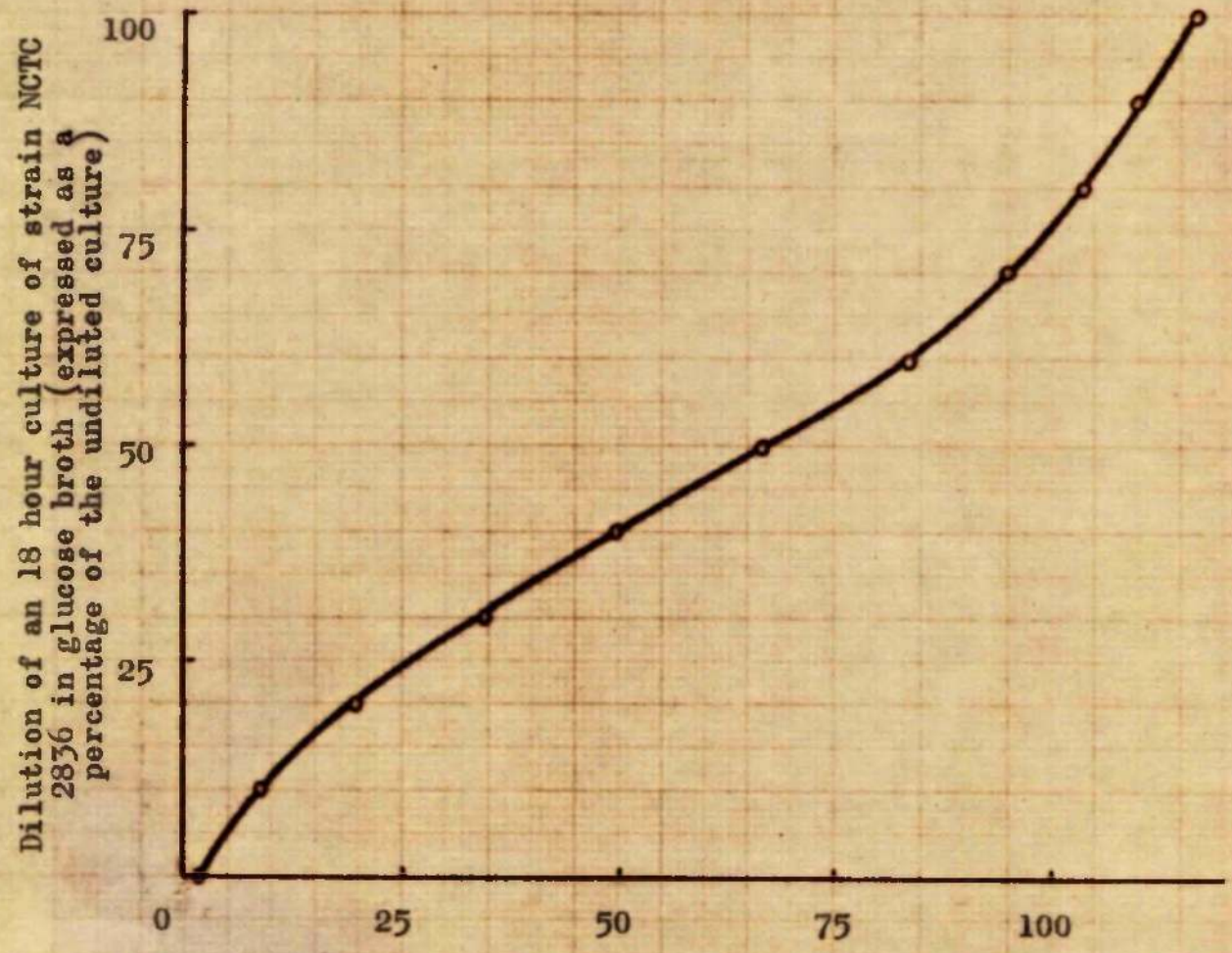
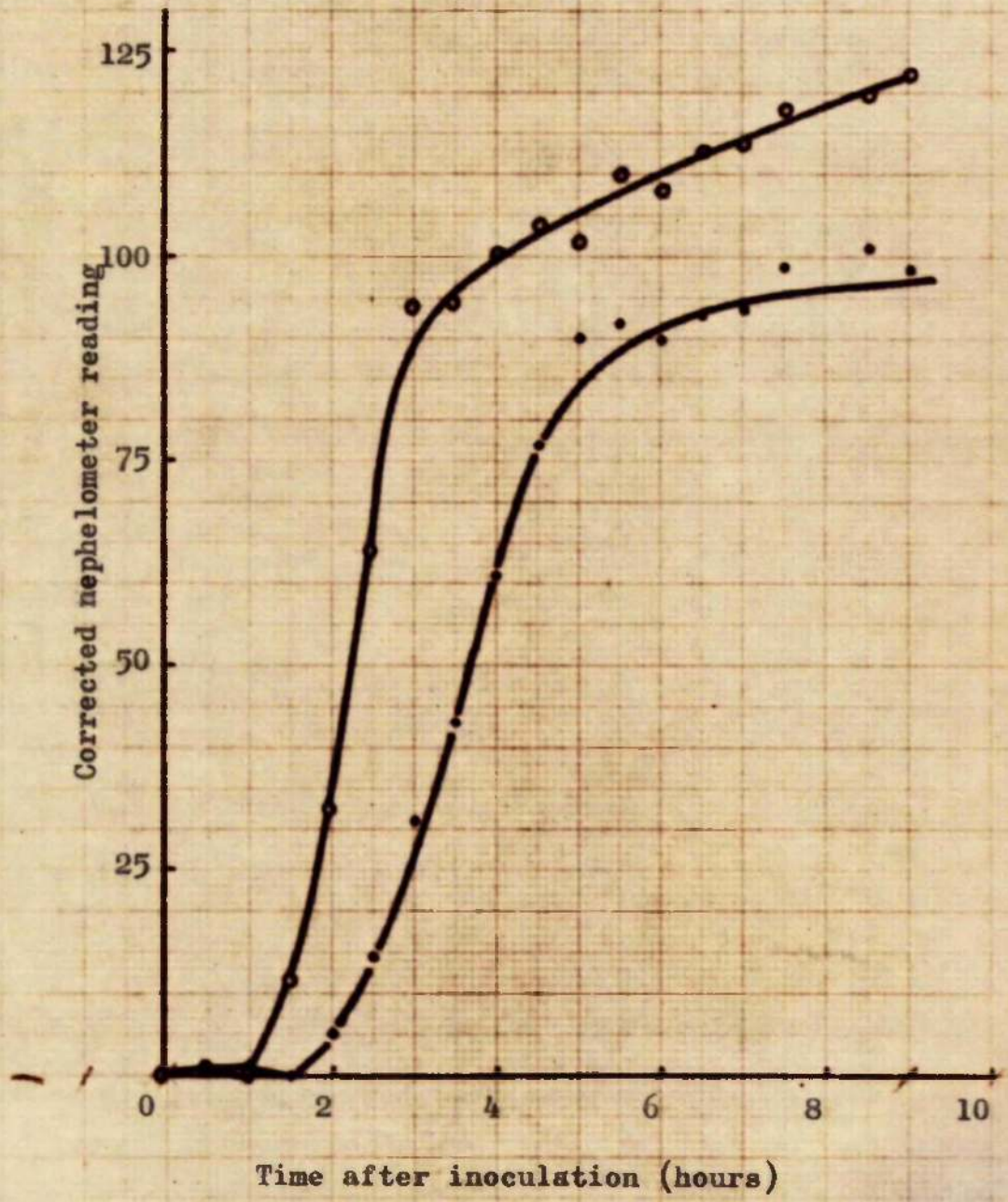


Fig. 5

Comparison of vegetative growth of Cl.perfringens (strain NCTC 2836) in glucose broth at 37 and 45°



Closed circles, growth at 37°
Open circles, growth at 45°

every two hours. This was done to ensure that log. phase cultures were used, as at the higher temperature growth was more rapid (see Figure 5).

Results

From Figure 5 it can be seen that vegetative growth was both more rapid at 45 than at 37° and that the final density produced was greater. Sporulation, however (see Figure 6), was reduced forty-fold at 45° and there was little difference in sporulation at 45° whether the inoculum was grown at 37 or 45°. Similarly, when the inoculum was grown at 45° there was no significant difference in sporulation whether the medium was inoculated with two or four hour cultures.

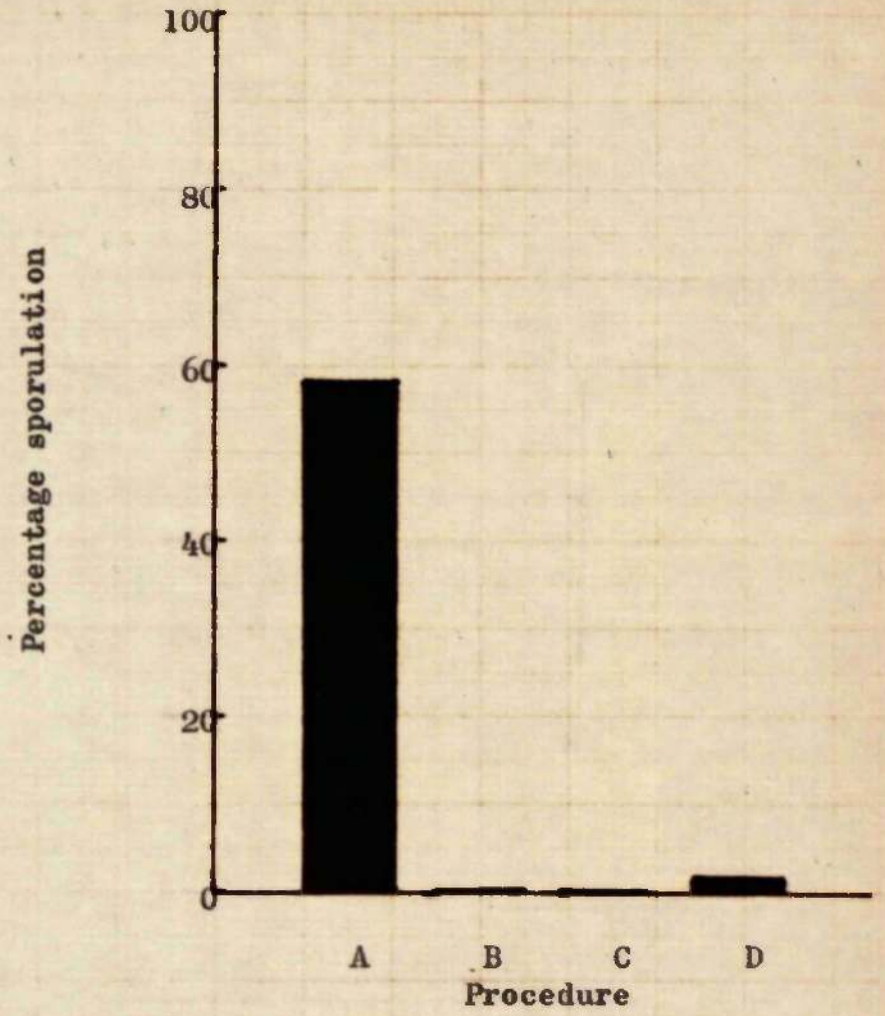
3.5 Determination of the effect of aeration on sporulation

Method. Four 100ml flasks each containing 20ml Ellner's medium and closed with cotton wool plugs were inoculated with 2ml of a four hour culture in Robertson's meat broth. Two flasks were incubated anaerobically and two on a shaker (Microid Flask Shaker, Griffin and Tatlock Limited).

Results. In the cultures incubated anaerobically sporulation in the order of 80 per cent was obtained. Spores were not observed in the shake cultures.

Fig. 6

Comparison of sporulation of Cl.perfringens (strain NCTC 2836) in Ellner's medium at 37 and 45°



A. The inoculum was grown for 4 hours at 37° and the sporulation medium incubated at 37°

B. The inoculum was grown for four hours at 37° and the sporulation medium incubated at 45°

C. The inoculum was grown for two hours at 45° and the sporulation medium incubated at 45°

D. The inoculum was grown for four hours at 45° and the sporulation medium incubated at 45°

4. Attempts to increase sporulation of weakly sporing isolates by modifying Ellner's medium

CONTENTS

Introduction

In the previous section an isolate which spores abundantly in Ellner's medium has been used to study the effect of a number of cultural conditions on sporulation. In this section attempts have been made to improve sporulation of two isolates which produced only small numbers of spores in Ellner's medium, by modifying this medium. As a control, two isolates which produced high percentages of spores were cultivated in parallel. The modifications made and the reasons for selecting these particular changes are enumerated below.

1. Preparation of Ellner's medium with different commercial preparations of peptone and yeast extract

Different commercial preparations of peptone are known to vary widely in amino acid composition. Ellner's medium was therefore prepared with a number of these preparations and sporulation in the resulting media compared. In addition, a number of amino acids are necessary for vegetative growth of Cl.perfringens and for the sporulation of other anaerobes. In order to determine the effect of a mixture of amino acids and breakdown products of peptone, without drastically altering the composition of Ellner's medium, one half of the peptone content was replaced with pancreatic digest of peptone.

2. Enrichment with inorganic salts

The importance of inorganic salts in promoting sporulation was not realised until 1931, when comparatively simple media such as peptone water and infusion media became widely used. The inorganic requirement for sporulation in both the genus Bacillus and the genus Clostridium appears to be species specific, although Mg^{++} and Mn^{++} are necessary for sporulation of several members of the genus Bacillus. The only report of inorganic ions enhancing sporulation of Cl.perfringens was made by Ellner (1956) who found that sporulation of a number of strains was improved by adding Mg^{++}. This is the only inorganic salt in Ellner's medium besides the buffer. Sporulation was therefore studied in Ellner's medium with and without Mg^{++}, and in Ellner's medium without Mg^{++} supplemented with a mixture of inorganic salts including Mg^{++}. A salts mixture was used initially, in preference to the individual salts, as a preliminary screening experiment. Should a positive result be obtained, this would be followed up with single salts to determine which salt is responsible for the change.

3. Enrichment with thiamine

Lund (1955-6, 1957) in a series of experiments to improve sporulation of a weakly sporing, putrefactive anaerobe, commonly designated P.A. 3679, found that sporulation was enhanced when the

organism was grown in a broth in which Cl.sporogenes or Cl.parasporogenes had been grown, although spent broths of other anaerobes were ineffective in this respect. Concluding that this was due to supplementation of the medium, Lund went on to show that addition of thiamine overcame the asporogenic property of this medium. The effect of addition of thiamine to Ellner's medium on sporulation of Cl.perfringens was therefore studied.

4. Enrichment with unknown factors in spent media and sporing cells

It has been pointed out in the previous paragraph that Lund found that sporulation of P.A. 3679 was greatly improved by growing in spent media. Furthermore, Srinivasan and Halvorson (1964) have demonstrated the existence of a low molecular weight factor which can be extracted from sporing cells and used to initiate sporulation of cells in the exponential phase of growth. An attempt was therefore made to improve sporulation of a weakly sporing isolate by culturing in the supernatant Ellner's medium in which a good sporing isolate had been grown for varying periods of time. To these spent media were also added the cells (after disintegration) which had been removed from the media by centrifugation. This was done in order to demonstrate the presence of a "sporing factor".

5. The effect of pH

The effect of pH on sporulation of Cl.perfringens has been dealt with in detail in the literature review. In summary, a number of workers found the optimum pH for sporulation to be strain specific. The effect of small changes in pH on sporulation was studied to see whether improved sporulation could be effected by this means. In addition, the effect of pH on vegetative growth was studied to determine whether the pH optimum for vegetative growth of sporing and non-sporing strains was the same.

Materials and Methods

Organisms. Poorly sporing isolates NCTC 8798 and NCTC 8799 and good sporing isolates NCTC 2836 and 032 were used.

Experimental

4.1 The effect of different commercial preparations of peptone

Method. The following peptones were used in the preparation of Ellner's medium:-

1. Allen and Hanbury's (Eupepton)
2. Evans (Bacteriological Peptone)
3. Oxoid (L.37)
4. Difco (Proteose Peptone)
5. B.D.H. (Bacteriological Peptone)

Results. There was no appreciable difference in sporulation of the two weakly sporing strains regardless of the peptone used (see Table 11). In all these instances sporulation was less than 1 per cent. The two good sporing strains produced varying percentages of spores. With these strains the best sporulation was effected by Oxoid (L.37) peptone, followed by Difco (Proteose Peptone). Allen and Hanbury's (Eupepton) and Evans (Bacteriological Peptone) produced 50 per cent and 35 per cent sporulation of strain NCTC 2836 respectively, while sporulation of strain 032 was weak. B.D.H. (Bacteriological Peptone) was the worst examined, inducing sporulation in the order of seven per cent with both of the good sporing strains. In this medium the two weakly sporing strains autolysed, making estimation of the percentage sporulation impossible, although judging from the number of spores present in these cultures, sporulation must have been less than 1 per cent.

4.2 The effect of different commercial preparations of yeast extract

Method. In this experiment Oxoid (L.37) peptone was used in the preparation of Ellner's medium, as the previous experiment has shown that this peptone induces better sporulation than the other peptones examined. The yeast extracts compared were Oxoid Yeastrel and Marmite Yeast Extract.

TABLE 11

The effect on sporulation of different commercial preparations of peptone in Eliner's medium

Peptone preparation	Percentage spores produced by isolate			
	NCTC 2836	032	NCTC 8798	NCTC 8799
Allen & Hanbury's (Eupepton)	50	10	< 1	< 1
Evans (Bacteriological Peptone)	35 plenty of forespores	5	< 1	< 1
Oxoid (L.37)	60	95	< 1	< 1
Difco (Proteose peptone)	40	40	< 1	< 1
B.D.H. (Bacteriological Peptone)	5	10	vegetative cells autolysed, sporulation estimated to be less than 1 per cent	

Results. Both of the yeast extracts induced sporulation to the same extent. The two weakly sporing strains produced less than one per cent spores and the good sporing strains produced in the order of 60 per cent spores (see Table 12).

4.3.1. Enrichment of Ellner's medium with peptone digest

Method. In place of 1 per cent peptone (Oxoid L.37), a mixture of 0.5 per cent peptone (Oxoid L.37) and 0.5 per cent pancreatic digest of peptone (Difco) was used in the preparation of Ellner's medium.

Results. Sporulation of the weakly sporing strains was again less than 1 per cent in both media. However, in the modified medium sporulation of the good sporing strains was considerably reduced (see Table 13).

4.3.2. Enrichment of Ellner's medium with inorganic salts

Method. Sporulation was compared in Ellner's medium, Ellner's medium with the magnesium sulphate omitted and Ellner's medium in which the magnesium sulphate was replaced by a mixture of salts including magnesium sulphate. The salts mixture used was that formulated by Halvorson (1957)(previously described on page 49).

Results. Here again abundant sporulation of the weakly sporing strains was not effected, although there was a marked effect on sporulation of the other strains (see Table 14). Omission of magnesium sulphate resulted in diminished sporulation of strain NCTC 2836. When a mixture

TABLE 12

The effect on sporulation of different commercial preparations of yeast extract in Ellner's medium

Yeast extract preparation	Percentage spores produced by isolate			
	NCTC 2836	032	NCTC 8798	NCTC 8799
Oxoid yeastrel	50	70	<1	<1
Marmite yeast extract	60	60	<1	<1

TABLE 13

The effect on sporulation of replacing the peptone content of Ellner's medium with a mixture of equal parts peptone and pancreatic digest of peptone

Medium	Percentage spores produced by isolate			
	NCTC 2836	032	NCTC 8798	NCTC 8799
Ellner	70	30	<1	<1
Modified Ellner	8	1	<1	<1

TABLE 14

The effect of inorganic salts on sporulation in Ellner's medium

Medium	Percentage spores produced by isolate			
	NCTC 2836	032	NCTC 8798	NCTC 8799
Ellner's medium $MgSO_4$ omitted	8	NT	Less than 1 per cent spores, no appreciable change in the percentage sporulation was observed in any instance	
Complete Ellner's medium	93	10		
Ellner's medium $MgSO_4$ omitted Halvorson's salt mixture added	90	10		

NT Not tested

of salts was added to the medium, sporulation was the same as in the unmodified medium. This indicates that magnesium sulphate improves sporulation of at least one strain of Cl.perfringens, while the other inorganic salts ($FeSO_4$, $CuSO_4$, $ZnSO_4$ and $MnSO_4$) did not inhibit or enhance sporulation in the presence of magnesium.

4.3.3. Enrichment of Ellner's medium with thiamine

Method. Thiamine (Aneurin, Roche Products Limited) was added to Ellner's medium at a concentration of 1µg/ml and the medium sterilised by autoclaving.

Results. (See Table 15). The addition of thiamine to Ellner's medium did not improve sporulation of any of the four strains. In this particular experiment strains NCTC 2836 and 032 produced lower percentages of spores than in previous experiments. This decreased sporulation has been observed occasionally while working with these strains, even when the same batch of Ellner's medium has been used. This is probably brought about by slight variations in the state of the culture used to inoculate the sporulation medium, as in subsequent experiments initiated from the same parent culture, high percentage sporulation has been observed.

TABLE 15

The effect on sporulation of addition of thiamine to Ellner's medium

Medium	Percentage spores produced by isolate			
	NCTC 2836	032	NCTC 8798	NCTC 8799
Ellner	10 (>400)	5 (>400)	0 (12)	0 (0)
Ellner supplemented with thiamine (1 µg/ml)	10 (>400)	5 (>400)	0 (10)	0 (0)

Figures in brackets are the mean spore counts per standard loopful of centrifuged culture.

4.4 Sporulation in spent Ellner's medium supplemented with disintegrated sporing cells

Method. 1ml of a four hour culture in Robertson's meat broth of strain 032 was used to inoculate each of nine universal containers holding 10ml Ellner's medium. The inoculated cultures of Ellner's medium were incubated anaerobically at 37° and one container was withdrawn after 1,2,4,6,8,10,14,18 and 24 hours. The cellular material was removed from these cultures by centrifugation and disintegrated using Balotini number 12 beads in a Mickle Disintegrator (H. Mickle, Gomshall, Surrey). Fifty minutes in this apparatus resulted in almost complete disintegration of the cellular material, which was reconstituted with its supernatant and Seitz filtered. Each of these nine 10ml preparations was inoculated with 1ml of a four hour culture of the weakly sporing strain NCTC 8798 in Robertson's meat broth and incubated anaerobically for 18 hours, when sporulation was estimated. The microscopic appearance of strain 032 prior to disintegration is shown in Table 16.

Results. Sporulation of strain NCTC 8798 was not improved by culturing in the medium, reconstituted with disintegrated cells, in which strain 032 had been cultured. In all instances sporulation was less than 1 per cent (see Table 16). The spore count was greater when strain NCTC 8798 was grown in fresh Ellner's medium than in any of the spent reconstituted media.

TABLE 16

The effect on sporulation of growing a weakly sporing isolate (NCTC 8798) in spent Ellner's medium supplemented with disintegrated vegetative cells or spores of a good sporing isolate (032)

Isolate 032 prior to disintegration		Sporulation of NCTC 8798 in the spent medium reconstituted with disintegrated cells	
Time in Ellner's medium (hours)	Morphology	Percentage spores	Mean spore count*
		less than 1 per cent[1].	74[1].
1	vegetative	" " " " "	5
2	"	" " " " "	6
4	"	" " " " "	9
6	"	" " " " "	8
8	"	" " " " "	8
10	forespores a few spores	" " " " "	9
14	forespores 20% spores	" " " " "	9
18	60% spores	" " " " "	12
24	60% spores	" " " " "	14

*The figures in this column are the mean spore count per standard loopful of centrifuged culture (see page 52).

1. Control. Strain NCTC 8798 added to fresh Ellner's medium.

4.5 The effect of pH

Methods

Vegetative growth. Vegetative growth of the four isolates was compared in nutrient broth. Ellner's medium could not be used for this experiment as in this medium seldom more than four divisions take place before the cessation of vegetative growth and the onset of sporulation. The nutrient broth was divided into five batches and the pH adjusted by adding solutions of hydrochloric acid or sodium hydroxide. These media were sterilised by autoclaving, the pH recorded and distributed in 10ml amounts in 6 x 5/8" test tubes. Each tube was inoculated with 0.04ml of a four hour culture in Robertson's meat broth of either isolate NCTC 8798, NCTC 8799, 032 or NCTC 2836. The tubes were incubated anaerobically at 37^{0} for 18 hours, when the density of the resultant growths were compared using a nephelometer.

Sporulation. The pH of four batches of Ellner's medium was adjusted by adding concentrated solutions of hydrochloric acid or sodium hydroxide. These media were sterilised by autoclaving and the pH recorded. After inoculation and incubation with each of the four strains, the pH was again measured.

Results

The results of this experiment are shown in Table 17 and Figure 7.

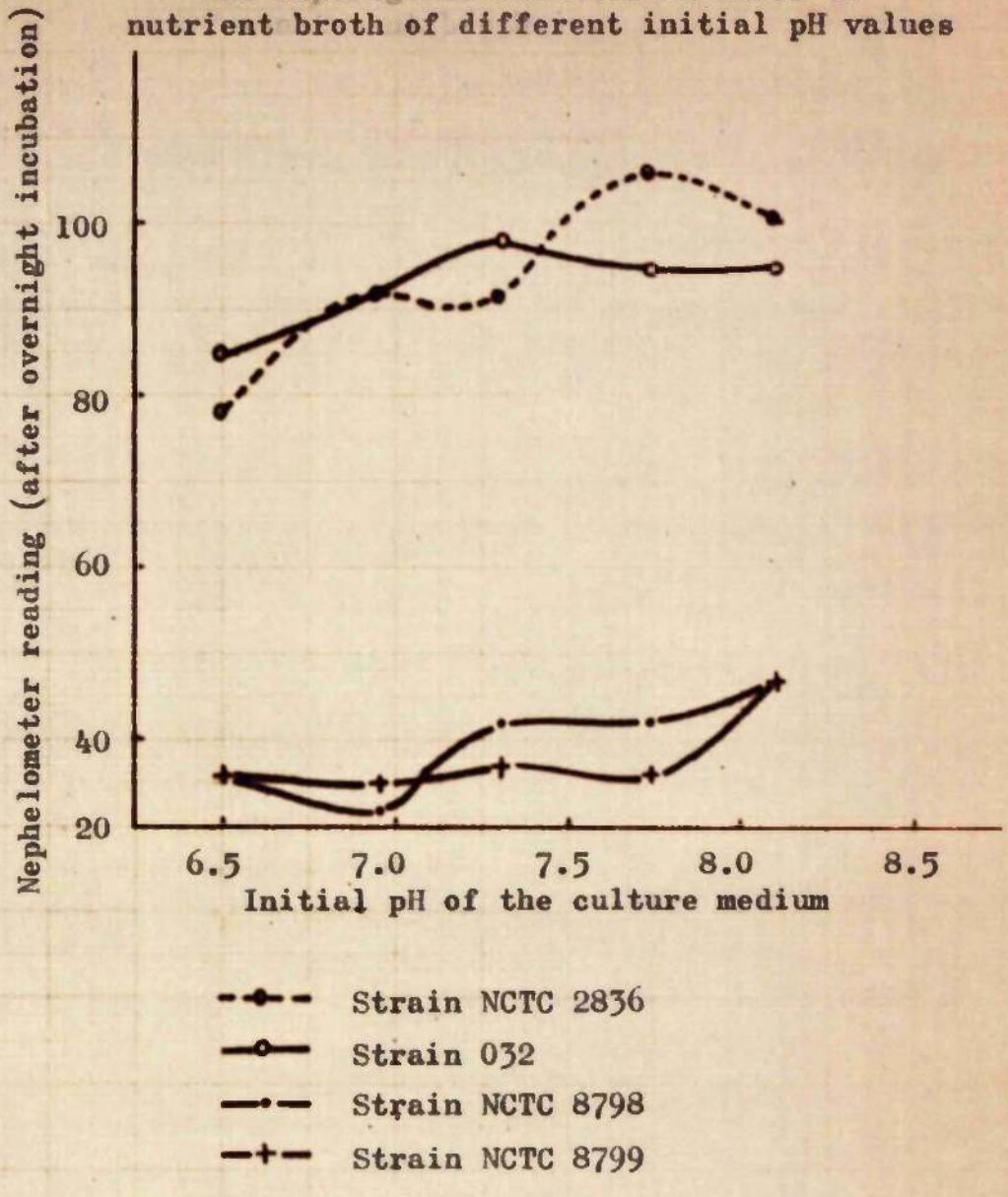
TABLE 17

The effect of growth and sporulation on the pH of Ellner's medium

Initial pH	Isolate							
	NCTC 8798		NCTC 8799		032		NCTC 2836	
	Final pH	Percentage sporulation	Final pH	Percentage sporulation	Final pH	Percentage sporulation	Final pH	Percentage sporulation
7.50	7.41	<1	7.38	<1	7.21	<1	7.20	20
7.70	7.50	<1	7.56	<1	7.30	<1	7.30	40
7.80	7.57	<1	7.59	<1	7.32	2	7.33	50
7.99	7.78	<1	7.74	<1	7.40	10	7.42	60

Fig. 7

A comparison of the growth of sporogenic and asporogenic isolates in tubes of nutrient broth of different initial pH values



Sporulation of the weakly sporing strains was not appreciably affected by small changes in the pH of Ellner's medium. With these strains sporulation was less than 1 per cent. With the two good sporing strains however, pH was an important factor in sporulation and the maximum sporulation recorded (10 per cent for strain 032 and 60 per cent for strain NCTC 2836) was obtained at the highest pH value (7.99) used. These results also show (Figure 7) that the sporing isolates produced approximately twice the amount of vegetative growth in nutrient broth than the weakly sporing strains. This was independent of pH. The lower pH produced in Ellner's medium (Table 17) by the good sporing strains is probably a reflection of the more luxuriant growth of these isolates in Ellner's medium.

5. Investigation of the components in Ellner's medium responsible for inducing sporulation

CONTENTS

Introduction

In this section a number of experiments are described in which attempts have been made to define which components of Ellner's medium are responsible for sporulation and to point out possible ways in which this medium can be improved.

In the long term this would result in the formulation of a defined sporulation medium. Further experiments with such a defined medium would eventually result in the elucidation of the biochemical steps involved in the formation of spores by this organism and would also provide a basis for experiments to determine why certain strains produce only small numbers of spores in Ellner's medium. Ellner's medium is the obvious starting point for this sort of study, as it is a comparatively simple medium in which some strains spore abundantly.

The only previous work of this nature with Cl.perfringens has been a paper by Albrycht and Trembowler (1959)(previously presented in the Review of the Literature on page 42). Briefly, these workers found that omitting the yeast extract from Ellner's medium did not affect sporulation, omitting the starch reduced the percentage sporulation of some strains, omitting the phosphates resulted in dramatic reduction in sporulation, while decreasing the peptone content to 0.2 per cent did not affect sporulation.

The first experiment in this section was carried out to determine whether Cl.perfringens would spore in water or the buffer component of

Ellner's medium, i.e. to exclude the possibility that Cl.perfringens will sporulate endotrophically. This is followed by experiments comparing the ability of media containing peptone or other recommended substrates, to promote sporulation of this organism. The effect of different commercial preparations of peptone and peptone concentration have also been studied, along with attempts to define which components of peptone are responsible for inducing sporulation.

The gross inorganic salt requirement for sporulation in a peptone medium has also been studied. The absolute inorganic salt requirement for sporulation is impossible to determine at this stage, as inorganic ions are introduced with the inoculum. Washed inocula were not used, as previous experiments have shown that washing the inoculum results in dramatic decrease in sporulation.

Materials and Methods

Throughout this section Ellner's medium and/or peptone dissolved in the buffer component of Ellner's medium has been inoculated in parallel with the media under test, as a control against asporogenous variation in the strain of Cl.perfringens used.

Organism. Strain NCTC 2836 was used throughout this section.

Media. Sporulation media were prepared by dissolving the substrate under test in buffer. The buffer system used was that in Ellner's

medium, composition:-

$Na_2HPO_4 . 12H_2O$ . . . . . 6.7 per cent (w/v)
KH_2PO_4 . . . . . 0.15per cent (w/v)
pH 7.8

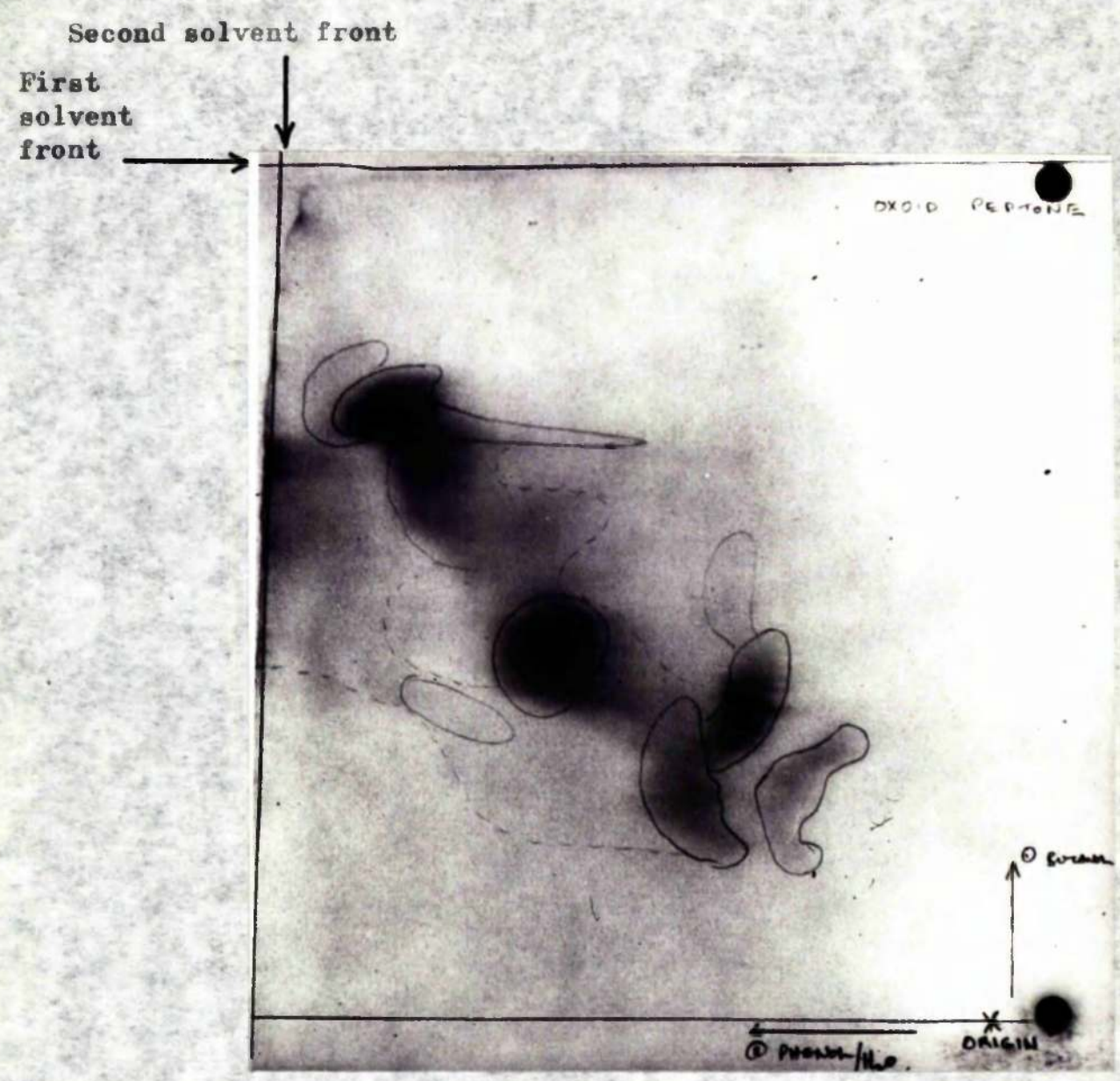
Throughout this section this is referred to as buffer. This buffer was used because Albrycht and Trembowler found that it was an essential component of Ellner's medium. Its use also ensured that the pH of the media was kept constant, so eliminating the important effect of pH on sporulation,

Unless otherwise stated Oxoid (L.37) peptone was used.

Fractionation of peptone. Five different methods for fractionating proteinaceous mixtures were used. These were: ethanol precipitation, fractionation using Sephadex (Pharmacia, Uppsala, Sweden) columns, continuous paper (curtain) electrophoresis, dialysis and a combination of ammonium sulphate precipitation and dialysis.

Paper chromatography and electrophoresis were used in an attempt to determine the effect of these fractionation methods on the composition of the fractions obtained. Unfortunately both of these valuable analytical methods gave poor separation of the components of peptone. The results of a double run chromatogram are shown in Plate 1, and of electrophoresis at the far right of Figure 10.

a. Ethanol precipitation. A 10 per cent (w/v) solution of peptone in distilled water was prepared. To 20ml of this solution four



x ½

Solvent 1. n.butanol - acetic acid - water

Solvent 2. phenol - water

PLATE 1

Double run paper chromatogram of peptone (Oxoid, L.37)

volumes of ethanol were added drop by drop from a burette (at room temperature). After standing overnight at room temperature in a closed container, the precipitate was spun down, washed with a mixture of four parts alcohol and one part water, and dried overnight at 37^{0}. The superantant was dried down under vacuum at 45^{0}, then overnight in an incubator at 37^{0}.

b. Fractionation using Sephadex columns. The method described by Bennich (1961) for the fractionation of tryptic hydrolysates of alpha-casein was used. Columns of Sephadex G-50 and G-75 were prepared as follows.

Approximately 10g Sephadex was suspended in a 1 per cent (w/v) solution of sodium chloride in water and allowed to stand overnight at 4^{0}. Fines were removed by carefully decanting and columns prepared. Each column was equilibrated with 3 volumes of $M.CH_3COOH$. 25ml of the supernatant from a 2 per cent (w/v) solution of peptone in $M.CH_3COOH$ was applied to each column and eluted with $M.CH_3COOH$. Fractions were collected at 4^{0} on a Locarte MK 6 automatic fraction collector. Fractions from the G-50 column were collected at 10 minute intervals. 65 drop fractions were collected from G-75 column. The column data are shown in the table below.

Sephadex	Column size	Flow rate
G-50	2.5 x 36cm	42ml/hour
G-75	2.5 x 50cm	15ml/hour

Fractions were scanned in quartz 1cm^2 cuvettes at 280mμ* using a Hilger and Watts H. 700 spectrophometer.

c. Continuous paper (curtain) electrophoresis. The method described by Bodman (1960) was used.

Apparatus. The leads from a Vokam constant current/constant voltage power pack (Shandon Scientific Company Limited, 65 Pound Lane, London N.W.10) were taken through the lid of a Shandon 20" glass chromatography tank and connected separately to 3" lengths of platinum. Each platinum electrode was immersed in a beaker of pyridine/acetic acid buffer (pH 5.3)** situated at each end of the tank.

Whatmann 3MM chromatography paper was cut to size (determined by preliminary experiments)(see Figure 8), soaked in pyridine/acetic acid buffer and suspended in the chromatography tank as for downward chromatography. Each paper lead was immersed in a beaker of pyridine/acetic acid buffer in which the positive or negative electrode was situated. The paper "tongue" was led to a beaker containing 2.5 per cent (w/v) peptone in pyridine/acetic acid buffer.

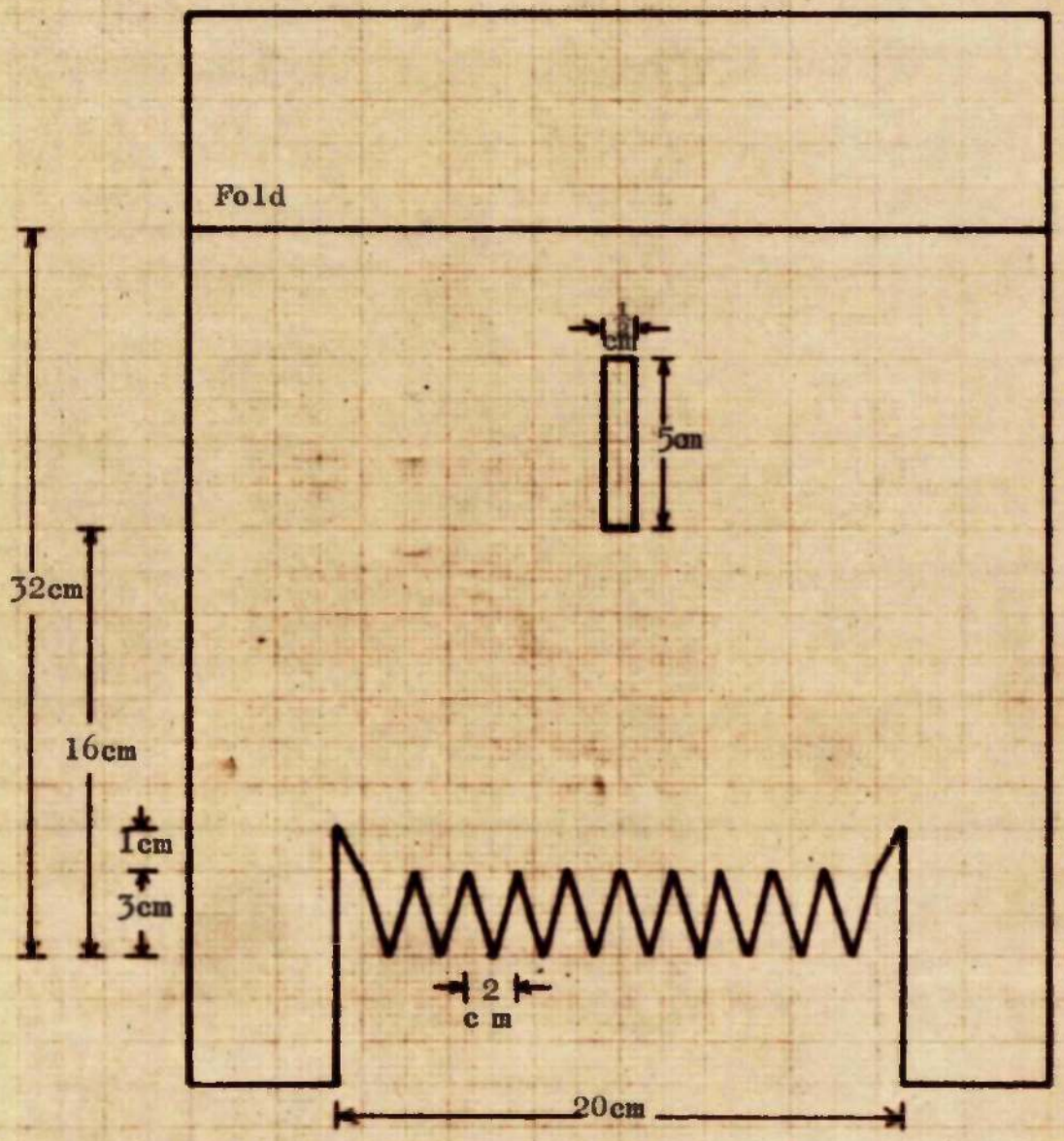
---

*This is the wavelength commonly used for locating proteins and peptides.

**Pyridine 25ml
Glacial acetic acid 10ml
Distilled water 2,465ml

Fig. 8

Dimensions and shape of paper used for continuous paper electrophoresis (not to scale)



Each collection point at the bottom of the paper was led into a 6 x ½" test tube. These tubes and the electrode compartments were supported on stainless steel stands.

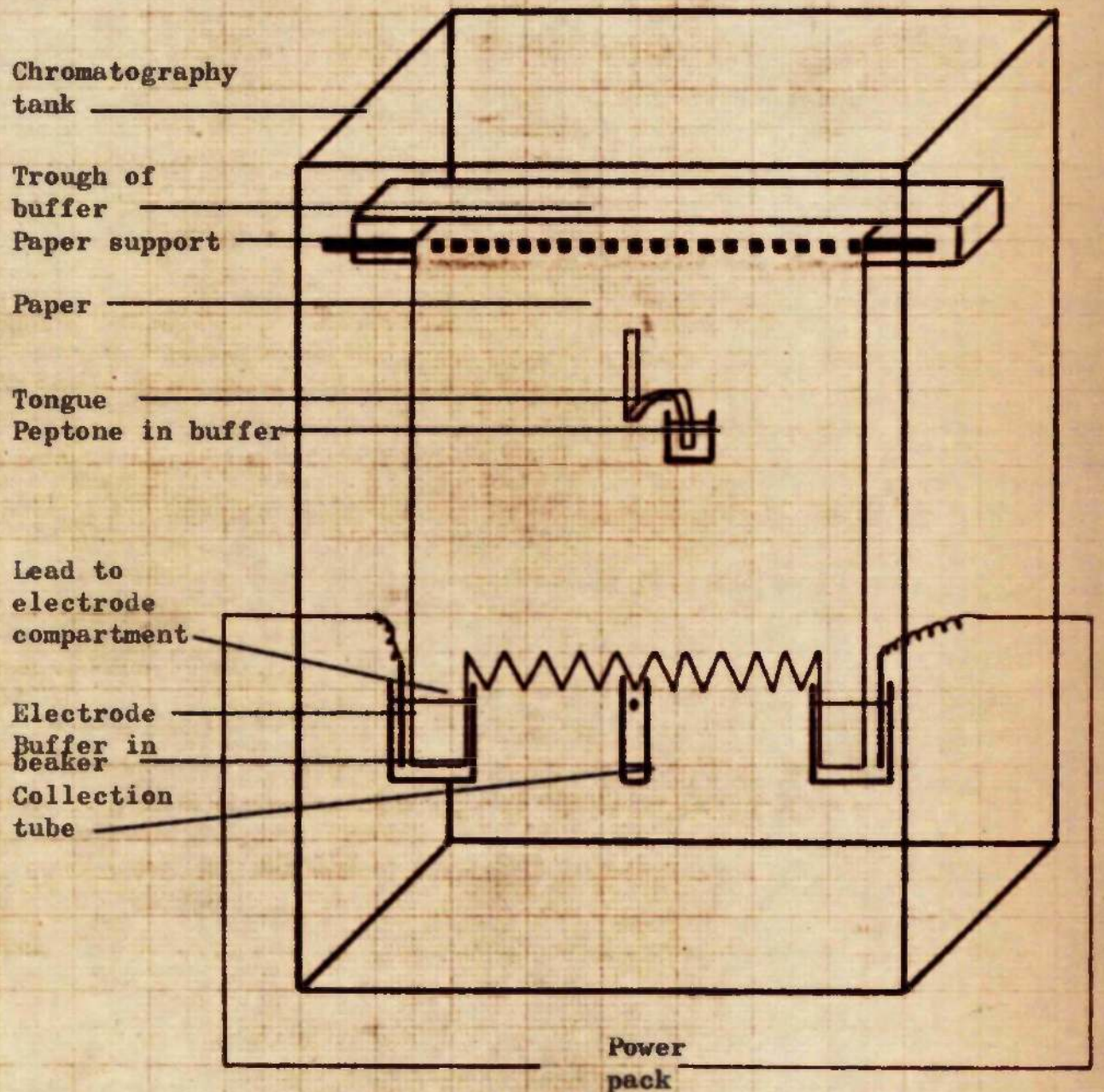
A schematic diagram of this apparatus is shown in Figure 9.

Procedure. The apparatus was set up as described above, the trough filled with pyridine/acetic acid buffer and a potential drop of 300 volts (15 volts/cm) applied. The experiment was run for 72 hours. Every 12 hours the trough was topped up with buffer and the collection tubes emptied. The effluent from each collection tube was combined and evaporated under vacuum at 45°, followed by drying overnight at 37°.

The electrophoretic mobilities of these fractions were compared at constant voltage(before drying)using the following technique:-
Whatmann number 1 paper was cut to suitable size, soaked in pyridine/acetic acid buffer and excess buffer removed by carefully blotting. The paper was placed in the electrophoresis tank with one end dipping into the anode compartment and the opposite end into the cathode compartment. Wicks cut from Whatmann 3MM paper were applied to each end of the electrophoresis strip. The apparatus was closed and allowed to equilibrate for 30 minutes. A voltage drop of 20 volts/cm length was then applied and the paper loaded with a solution of each fraction using a fine capillary. After 30 minutes the current was switched off, the paper removed from the tank, placed on a sheet of glass and dried in an oven at 55°. The paper was then run through a trough of

Fig. 9

Diagramatic representation of apparatus used for continuous paper electrophoresis



0.3 per cent (w/v) ninhydrin in 1 per cent (v/v) acetic acid in ethyl alcohol and the colour brought out by incubating at 55° overnight. The result of this experiment is shown diagrammatically in Figure 10.

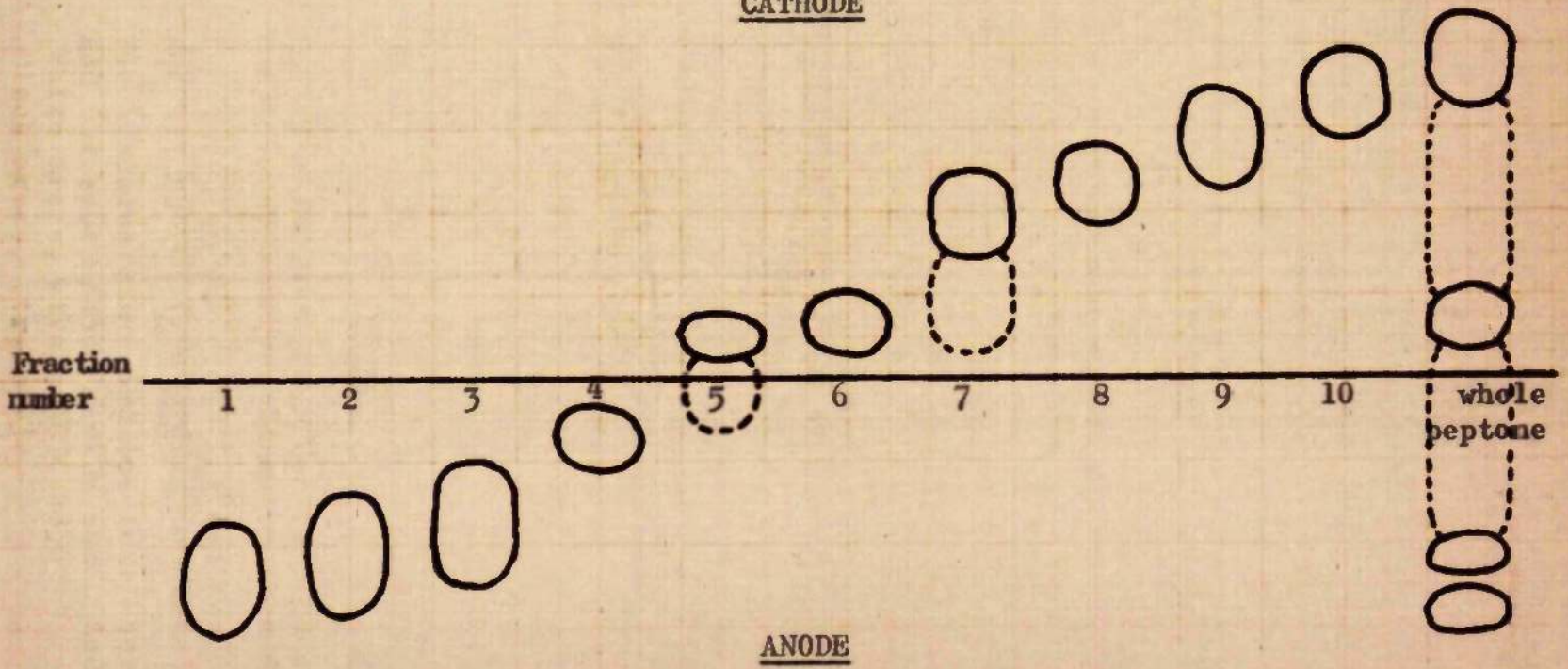
d. Dialysis. Approximately 2g peptone was dissolved in 10ml distilled water and dialysed for 24 hours against running tap water in Visking 24/32 dialysis tubing. The dialysed product was dried down under vacuum at 45° and placed in an incubator at 37° overnight.

e. Ammonium sulphate precipitation. 2g peptone was dissolved in 20ml distilled water and solid ammonium sulphate was slowly added with constant stirring at room temperature. The saturated solution was allowed to stand overnight at room temperature and the precipitate removed by centrifugation. The precipitate was then dissolved in distilled water, re-precipitated with ammonium sulphate and the precipitate centrifuged off. This second precipitate was dissolved in distilled water.

Initial attempts to remove ammonium sulphate by mixing with the deionising ion exchange resin Biodeminrolit (The Permutit Company, Permutit House, Gunnersby Avenue, London, W.4.) resulted in the almost complete removal of protein from these fractions. Ammonium sulphate was removed from both fractions by dialysing overnight against running tap water. Samples of the

Fig. 10

Electrophoresis of peptone fractions obtained by continuous paper electrophoresis (actual size)



dialysed material were shown to be sulphate-free by adding a few drops of a solution of barium chloride.

The dialysed solutions were dried down under vacuum at 45°, then overnight in an incubator at 37°.

Estimation of sporulation. In this section sporulation has been estimated predominantly by the viable spore count method (see page 52). This method was used as there was considerable clumping of spores and vegetative cells in the peptone media, which made microscopical estimation of the percentage sporulation impossible. In instances where both methods have been used (see Figures 11 and 13), the results were identical. Furthermore, when sporulation is less than 1 per cent, the viable spore count provides a more accurate comparison. In making the dilutions for these spore counts, clumps were broken down by vigorously sucking up the suspension several times into a pipette and blowing back into the tube. This procedure, which on microscopical examination was found to temporarily break down clumps, was repeated immediately before inoculating the horse blood agar plates.

Experimental

5.1 Endotrophic sporulation

The results of this experiment (see Table 18) show that

TABLE 18

Sporulation of Cl.perfringens (strain NCTC 2836) in distilled water and buffer

Medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Buffer	2.13	0.62	5.64
Distilled water	1.50	0.32	2.68
Ellner's medium	2.35×10^6	1.98×10^6	2.72×10^6

Cl.perfringens sporulates weakly, if at all, in buffer or distilled water. The difference between the spore counts in distilled water and buffer are not statistically significant.

5.2 Organic components

5.2.1. A comparison of the ability of peptone, albumin, gelatin and casein hydrolysate to promote sporulation

Each of these compounds is the chief organic component of a medium recommended to induce sporulation of Cl.perfringens (see literature review). They were compared to determine their relative efficacy as sporulation substrates.

Method. The substrates used were: peptone (Oxoid L.37), egg albumin (Light and Company, Colnbrook, England), leaf gelatin (Soc. Des Produits, Chimiques Coignet, Division Gelatines Hasselt et Vilvorde, Brussels, Belgium) and casein hydrolysate (Oxoid). Each compound was dissolved in buffer at a concentration of 1 per cent (w/v).

Results. The results of this experiment are shown in Table 19 and Figure 11. Peptone was by far the best substrate examined, producing a mean spore count of 9.25×10^4 per loopful. Only 12 spores were produced per loopful in casein hydrolysate, followed by 2 in albumin and none in gelatin.

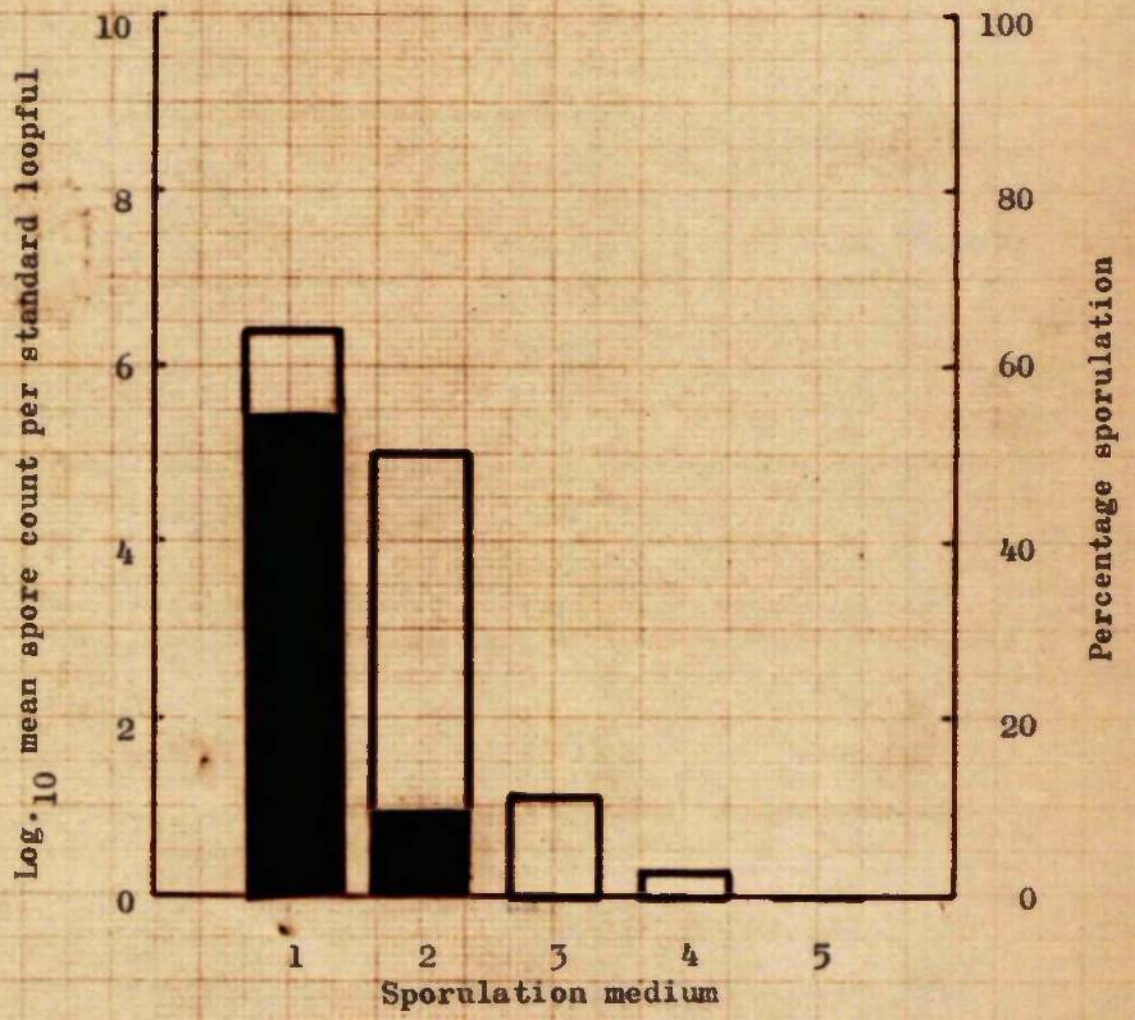
TABLE 19

Sporulation of isolate NCTC 2836 in media prepared by dissolving a number of organic substances in buffer. Substances were used which are the chief components of media recommended for the sporulation of Cl.perfringens

Sporulation medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Ellner	2.35×10^6	1.98×10^6	2.72×10^6
Peptone	9.25×10^4	8.29×10^4	10.27×10^4
Casein hydrolysate	12.00	9.10	14.90
Albumin	2.13	0.60	3.66
Gelatin	0	-	-

Fig. 11

Sporulation of strain NCTC 2836 in media prepared by dissolving peptone, casein hydrolysate, albumin and gelatin in buffer



1. Ellner's medium
2. Peptone (1 per cent (w/v) in buffer)
3. Casein hydrolysate (1 per cent (w/v) in buffer)
4. Egg albumin (1 per cent (w/v) in buffer)
5. Gelatin (1 per cent (w/v) in buffer)

White areas, log.10 spore count; black areas, percentage sporulation.

5.2.2. A comparison of spore formation on a number of commercial preparations of peptone

Previous attempts to improve sporulation of weakly sporing isolates by preparing Ellner's medium using different commercial preparations of peptone, have yielded widely varying results (see page 77). The ability of a range of peptones to induce sporulation in buffer was therefore studied.

Method. The peptones used: Oxoid (L.37), Oxoid (Tryptose), Evans (Bacteriological Peptone), B.D.H. (Bacteriological Peptone), Difco (Bacto-Peptone), Difco (Proteose Peptone) and Allen and Hanbury (Eupepton), were dissolved separately in buffer at a concentration of 1 per cent (w/v). The pH of each of these media was determined after autoclaving. The effect of pH on sporulation in buffered peptone was also studied as I have previously shown (see page 82) that this is an important factor. For this experiment 2g peptone (Oxoid L.37) was dissolved in 200ml buffer. This was divided into six 30ml batches and the pH of each batch adjusted by addition of the appropriate amount of concentrated hydrochloric acid. Solutions of sodium and potassium hydroxide were not added, as the presence of additional sodium and potassium may influence sporulation. Each batch was sterilised by autoclaving and distributed aseptically. The pH of each batch was determined after sterilisation.

Results. Sporulation in media prepared by dissolving different commercial preparations of peptone in buffer are shown in Table 20 and Figure 12. The results in Table 20 show that while there is no significant difference in the spore counts in the Oxoid Tryptose and Oxoid L.37 media, significantly higher numbers of spores (approximately 1.7×10^4) were produced in these media than in the next best medium (Allen and Hanbury's Eupepton, where the spore count was 6.1×10^3). This in turn was significantly better than Difco Proteose Peptone, which was better than Difco Bacto-Peptone. There was however no significant difference between the two worst peptones (B.D.H. and Evans, Bacteriological Peptones). The number of spores produced in media of the best and worst peptones differed by a factor of approximately 2,000.

The very important effect of pH on sporulation (see Table 21 and Figure 13) cannot explain this variation, as Oxoid L.37 peptone produced a medium of lower pH than either the Evans or B.D.H. peptones. Similarly, the pH values of the two Difco peptone media were higher than that of the Oxoid (L.37) peptone medium. In common with a previous experiment, in which the effect of pH on sporulation in Ellner's medium was studied, the greatest number of spores were produced in the most alkaline medium.

5.2.3. The effect of peptone concentration on sporulation

Method. 10g peptone (Oxoid L.37), i.e. the best of the peptones

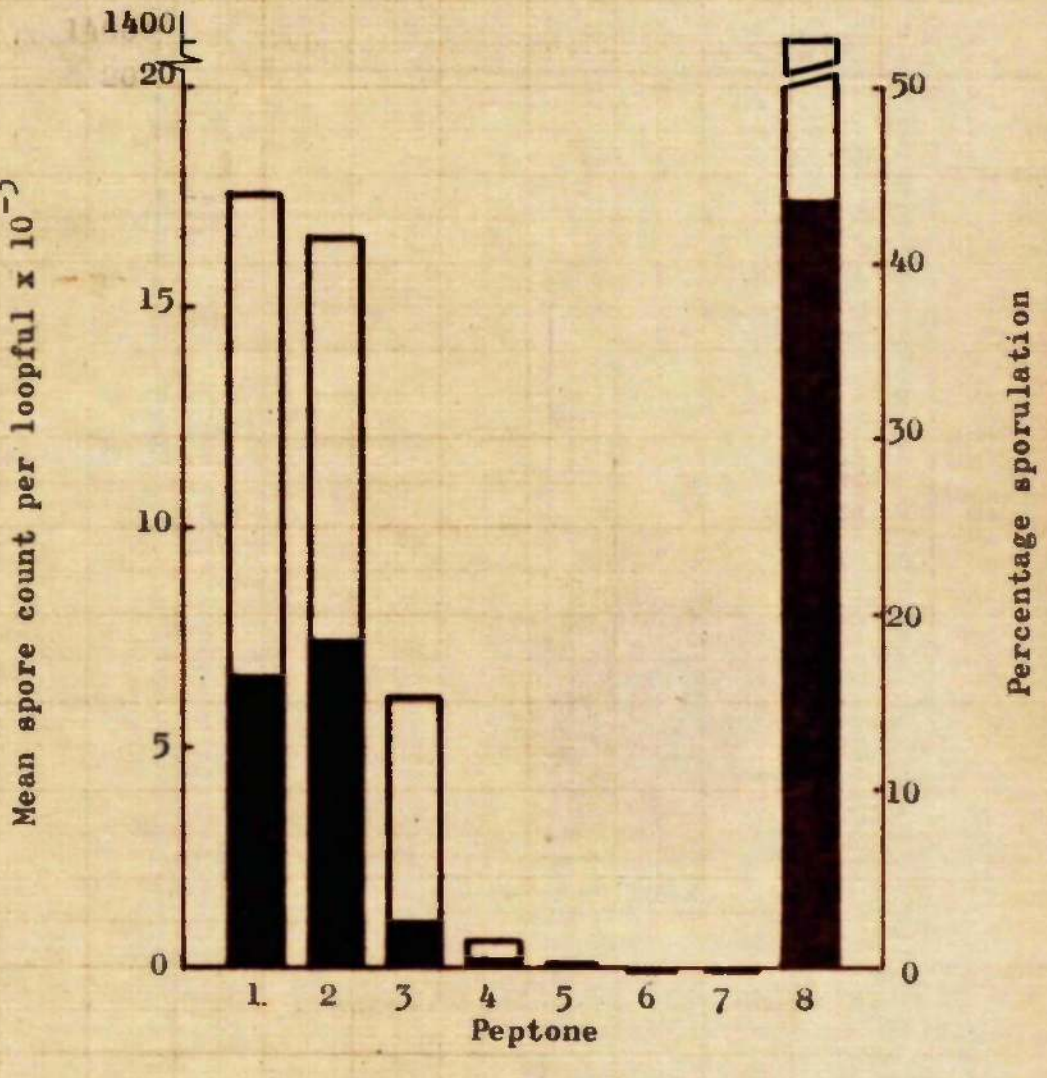
TABLE 20

Sporulation of isolate NCTC 2836 in media prepared by dissolving a number of commercial preparations of peptone in buffer

Peptone	pH	Mean spore count per loopful		
		Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Oxoid (tryptose)	7.75	1.76×10^4	1.15×10^4	2.38×10^4
Oxoid (L.37)	7.65	1.66×10^4	1.01×10^4	2.32×10^4
Allen & Hanbury	7.50	6.13×10^3	3.50×10^3	8.76×10^3
Difco (proteose)	7.75	5.39×10^2	3.08×10^2	7.70×10^2
Difco (bacto)	7.75	1.11×10^2	0.15×10^2	1.68×10^2
B.D.H.	7.70	8.63	5.31	11.95
Evans	7.70	8.38	7.20	9.56
Control (Ellner's medium)	7.80	1.54×10^6	0.78×10^6	1.90×10^6

Fig. 12

Sporulation of strain NCTC 2836 in media prepared by dissolving a number of commercial preparations of peptone in buffer



1. Oxoid (tryptose)
2. Oxoid (L.37)
3. Allen and Hanbury
4. Difco (proteose-peptone)
5. Difco (Bacto-peptone)
6. B.D.H.
7. Evans
8. Control (Ellner's medium)

White areas, mean spore count per loopful x 10^{-3}
black areas, percentage sporulation

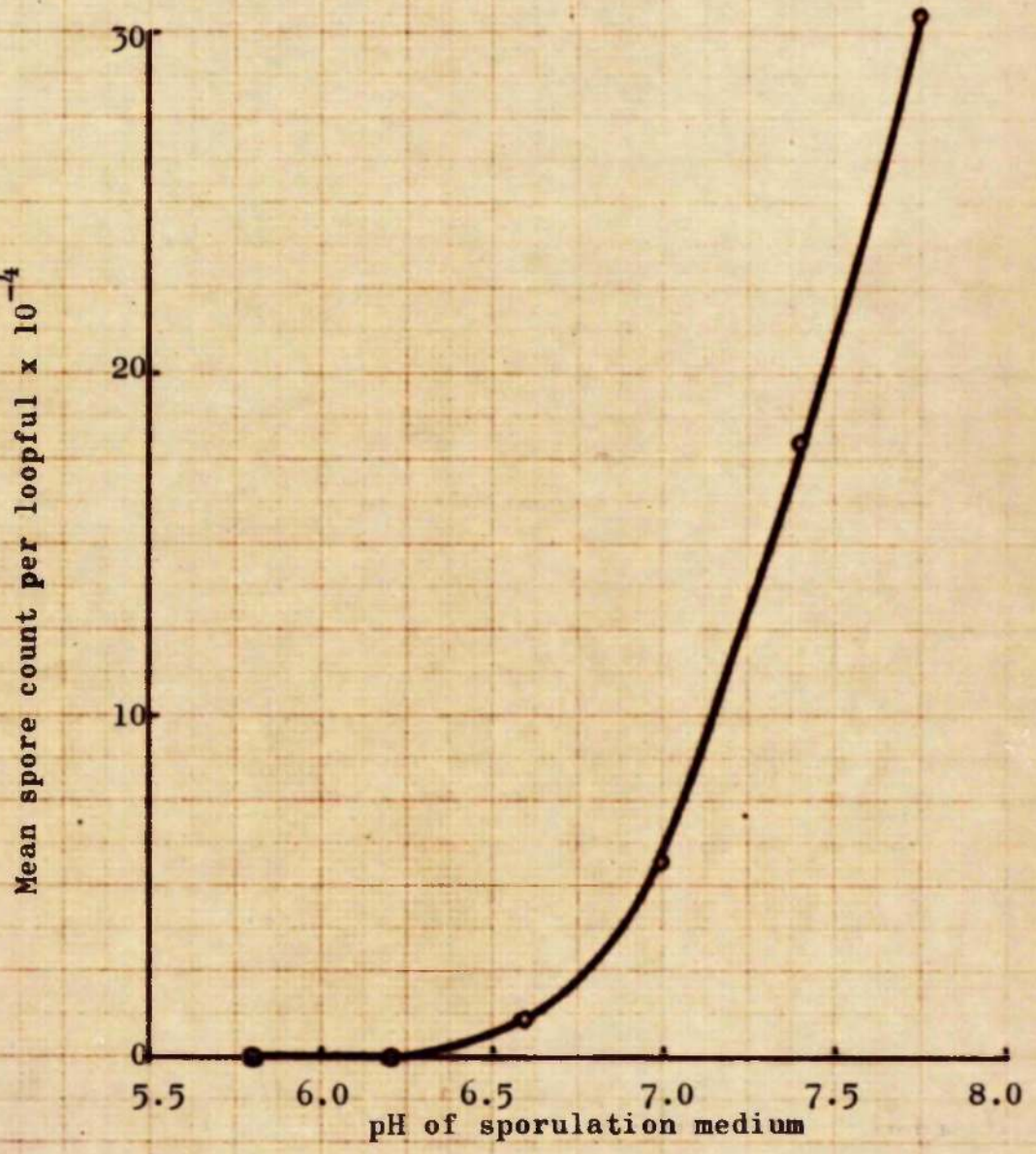
TABLE 21

The effect of pH on sporulation in a buffered peptone medium

pH of medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
7.75	3.05×10^5	2.86×10^5	3.24×10^5
7.40	1.81×10^5	0.05×10^5	3.08×10^5
7.00	5.58×10^4	4.90×10^4	6.26×10^4
6.60	1.02×10^4	0.90×10^4	1.14×10^4
6.20	3.59×10^2	3.05×10^2	4.13×10^2
5.80	2.43×10	1.63×10	3.23×10

Fig. 13

The effect of pH on sporulation of strain NCTC 2836 in a buffered peptone medium



examined in the previous experiment) was dissolved in 100ml buffer. Portions of this solution were diluted aseptically in buffer (after autoclaving) to give final peptone concentrations of 10, 5.0, 1.0, 0.5, 0.1 and 0.05 per cent (w/v).

Results. The result of this experiment is shown in Table 22 and illustrated in Figure 14.

When the log. mean spore count was plotted against log. peptone concentration; for the values of concentration of 10,5.0,1.0, 0.5,0.1 and 0.05 per cent (w/v) there was a significant departure from linearity. However, a straight line gave good fit for concentrations between 0.5 and 10 per cent (w/v). The straight line fitted by the method of least squares had the equation:-

$$N = 10^7 \times 2.834 \times C^{1.6239}$$

where N = mean number of spores per loopful

and C = peptone concentration in grams per ml.

The percentage spores formed in these cultures did not follow the same trend, and was at a maximum when the peptone concentration was approximately 5 per cent (w/v). With concentrations of peptone over 10 per cent and below 0.1 per cent, less than 1 per cent spores were formed.

5.2.4. The ability of peptone fractions to support sporulation

All the fractionation methods used, with the exception of the

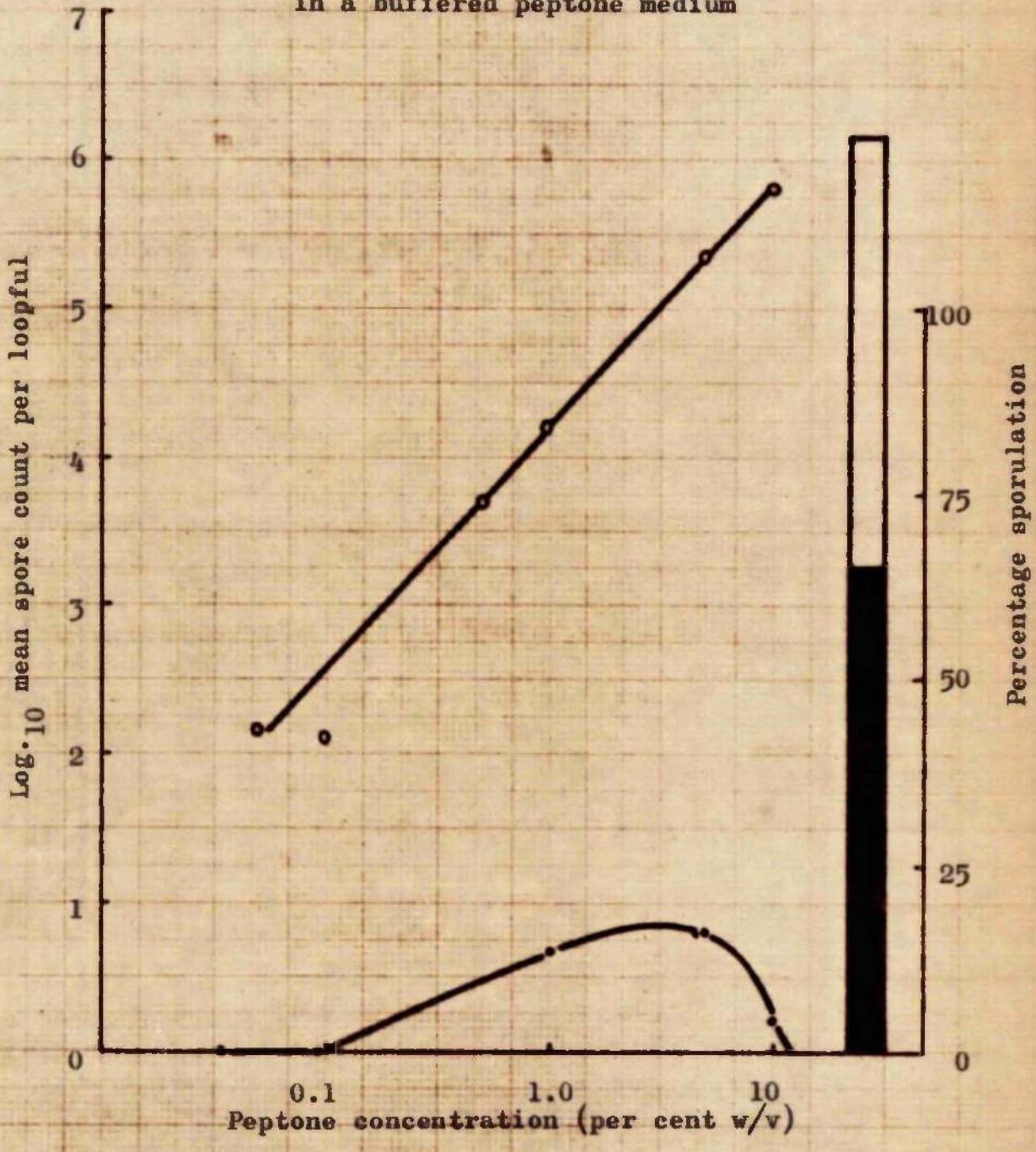
TABLE 22

The effect of peptone concentration on the sporulation of isolate NCTC 2836 in a buffered peptone medium

Peptone concentration (per cent, w/v)	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
10	6.58×10^5	4.49×10^5	8.67×10^5
5	2.20×10^5	1.89×10^5	2.51×10^5
1	1.60×10^4	1.26×10^4	1.94×10^4
0.5	5.18×10^3	4.02×10^3	6.34×10^3
0.1	1.20×10^2	0.71×10^2	1.69×10^2
0.05	1.44×10^2	0.51×10^2	2.38×10^2
Control (Ellner's medium)	1.75×10^6	1.47×10^6	2.03×10^6

Fig. 14

The effect of peptone concentration of the formation of spores by strain NCTC 2836 in a buffered peptone medium



Open circles, log.$_{10}$ mean spore count per loopful. Closed circles, percentage sporulation. The control for this experiment, Ellner's medium, is included in histogram form at the right of the figure. White area, log.$_{10}$ mean spore count per standard loopful. Black area, percentage sporulation.

experiments using Sephadex columns, yielded distinct fractions. The results obtained with Sephadex G-50 and G-75 are shown in Figures 15 and 16. As no distinct fractions were obtained in these instances, sporulation experiments were not attempted.

a. Fractions obtained by ethanol precipitation

Method. 2g peptone yielded 1.4932g supernatant and 0.3960g precipitate. The precipitate and supernatant were dissolved separately in 200ml buffer and sterilised by autoclaving. Sporulation in the two media was compared. Each component was dissolved in 200ml buffer on the assumption that the sporulation substrate initially present in the whole peptone, would now be found entirely in either the precipitate or the supernatant. Whichever of these components contained the substrate would therefore support sporulation to the same extent as a 1 per cent solution of whole peptone.

Results. The results of this experiment are shown in Table 23 and Figure 17. The hypothetical sporulation substrate(or substrates) was not confined to either fraction, although the spore counts from the precipitated fraction were significantly higher than those from the non-precipitated fraction. This indicates that the sporing factors are found predominantly in the precipitated fraction. This is even more marked when the concentrations of

Fig. 15

Fractionation of peptone on Sephadex G-50

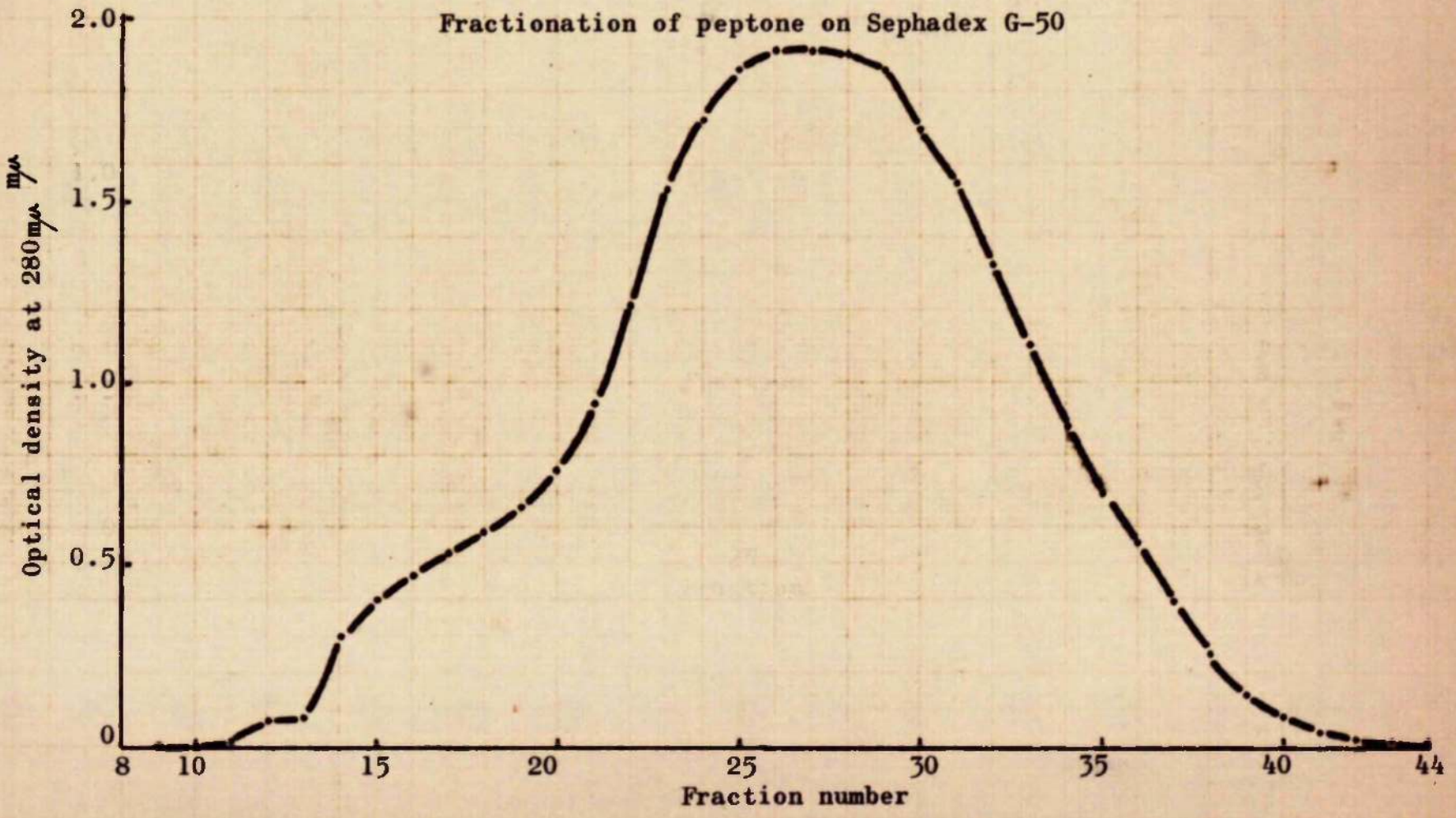


Fig. 16

Fractionation of peptone on Sephadex G-75

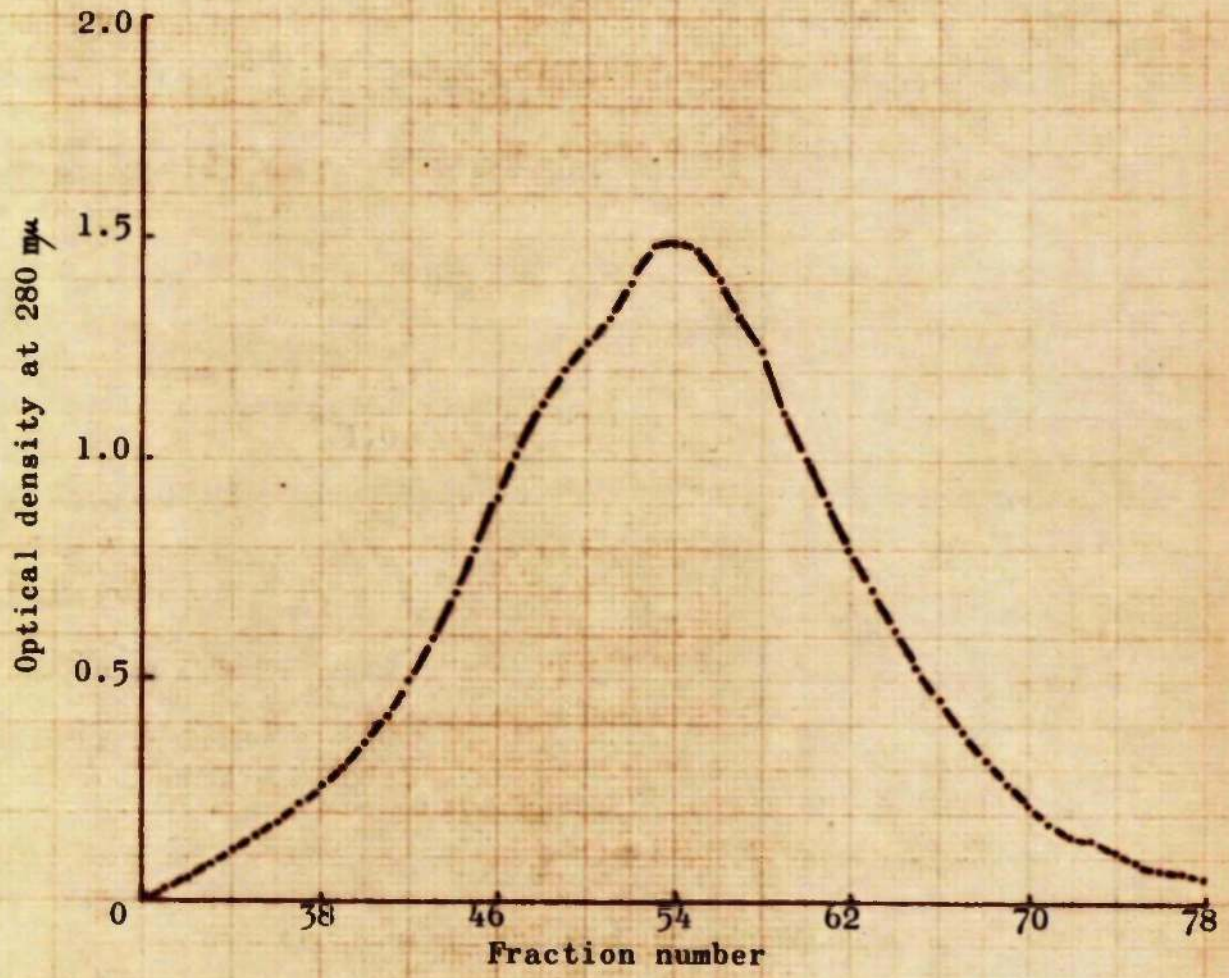


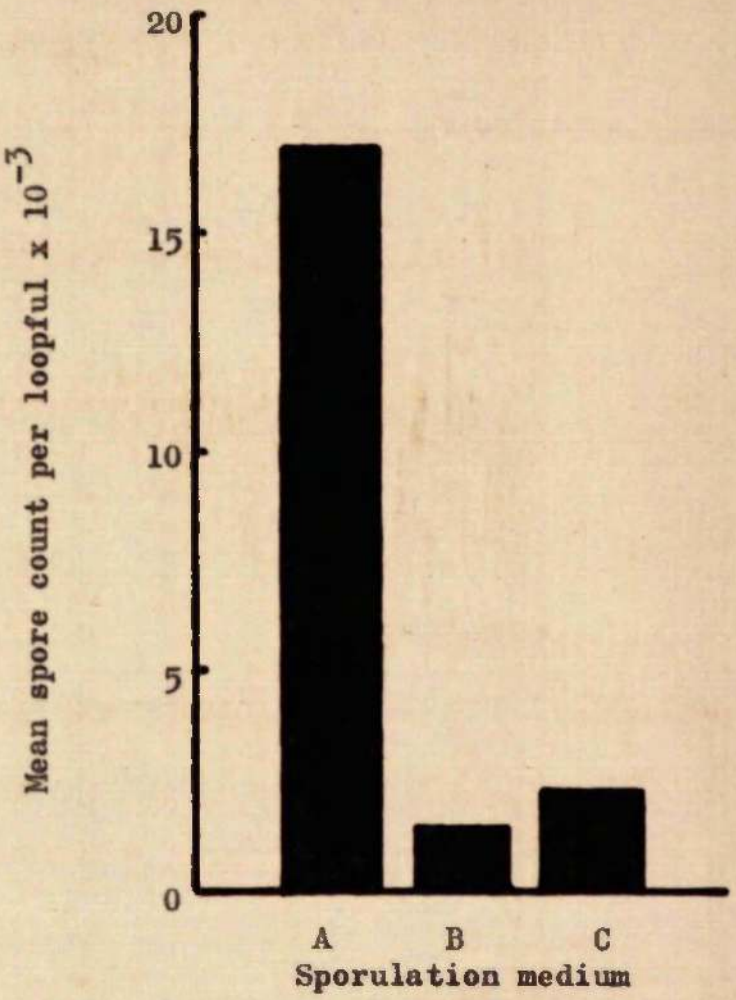
TABLE 23

Sporulation of isolate NCTC 2836 in media prepared by dissolving whole peptone and peptone fractions obtained by ethanol precipitation, in buffer

Sporulation medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Ellner	8.38×10^5	4.13×10^5	12.63×10^5
Whole peptone	1.70×10^4	0.96×10^4	2.44×10^4
Supernatant from precipitated peptone	1.50×10^3	1.02×10^3	1.98×10^3
Precipitated peptone	2.43×10^3	2.06×10^3	2.79×10^3

Fig. 17

Sporulation of strain NCTC 2836 in media prepared by dissolving peptone fractions, obtained by ethanol precipitation, in buffer



A. Whole peptone (1 per cent (w/v) in buffer)

B. Supernatant (0.747 per cent (w/v) in buffer)

C. Precipitate (0.198 per cent (w/v) in buffer)

peptone in the two media are taken into account, i.e. the concentration of peptone in the supernatant is approximately four times that in the precipitate.

b. Fractions obtained by continuous paper electrophoresis

The total weight of peptone fractionated was 0.2892g. This was distributed between the ten fractions as shown in the table below.

Fraction no.	Weight of fraction
1	0.0155g
2	0.0220g
3	0.0288g
4	0.0290g
5	0.0398g
6	0.0848g
7	0.0224g
8	0.0173g
9	0.0138g
10	0.0158g
Total	0.2892g

Method. By the reasoning expounded in the Method section of the previous experiment, each of these fractions was dissolved in 28.92ml buffer, thus maintaining the same weight of each fraction in each tube as is found in a 1 per cent solution of whole peptone.

Sporulation in these media was compared. Peptone which had been reclaimed from pyridine/acetic acid buffer by evaporation was dissolved in buffer at a concentration of 1 per cent (w/v) and used in addition to the normal controls.

Results. The results of this experiment are shown in Table 24 and Figure 18. Significantly higher spore counts were obtained in the whole peptone medium in which the peptone had been previously dissolved in pyridine/acetic acid buffer, i.e. compared with untreated peptone; although these counts were lower than those obtained in Ellner's medium. Of the ten fractions, seven produced less than five spores per loopful, two produced less than 60 spores per loopful, while the remaining fraction (fraction number 6) produced 7.19×10^3 spores per loopful. Although the concentration of peptone in fraction number 6 is greater than in any of the other fractions, the number of spores produced in this medium is greater than would be expected on the basis of concentration alone. The component (or components) of fraction number 6 - which is electrophoretically neutral at pH 5.3 in pyridine/acetic acid buffer - therefore promotes better sporulation than any of the other fractions.

c. Sporulation in dialysed peptone

Method. A 1 per cent (w/v) solution of dialysed peptone in buffer

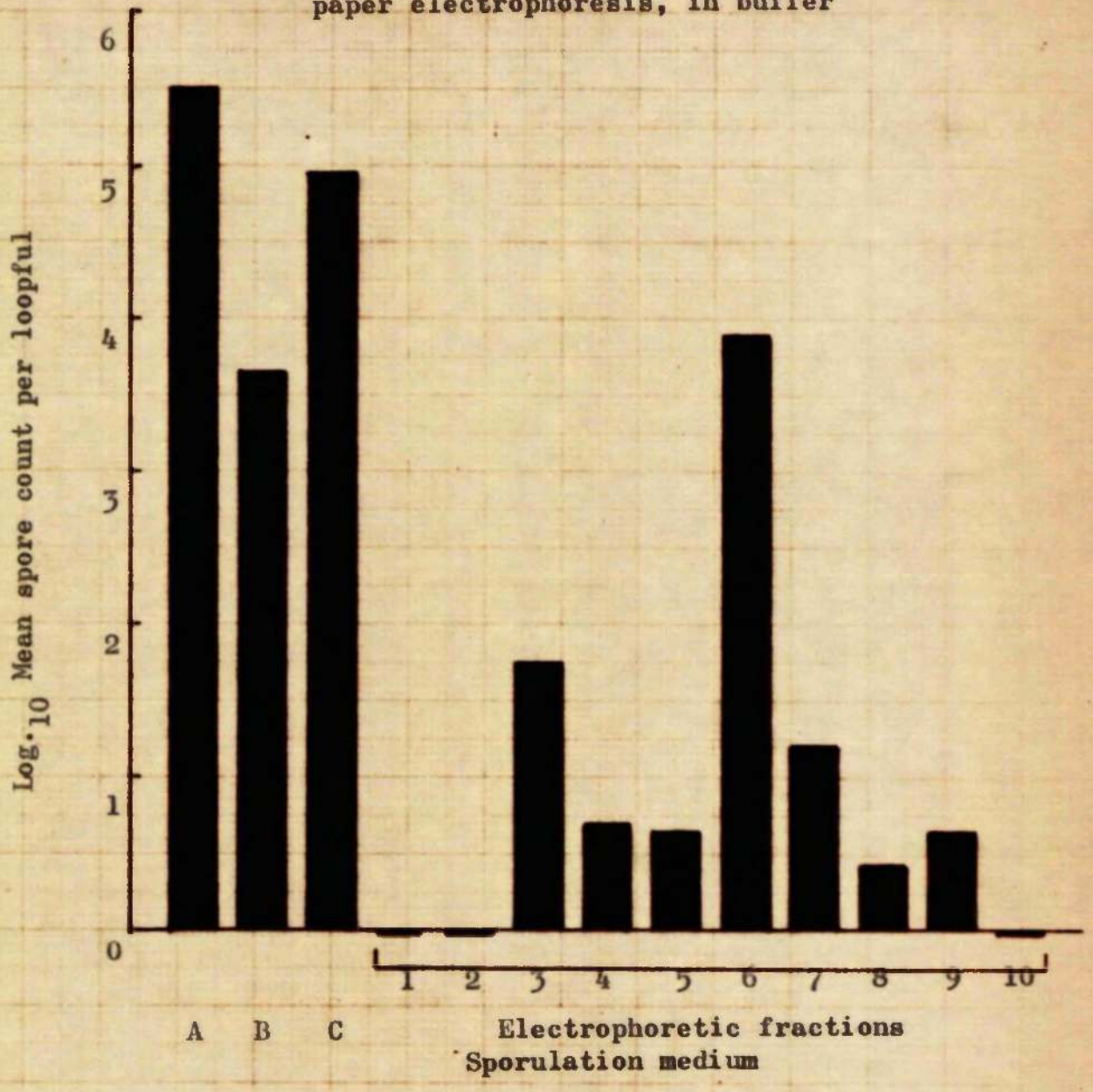
TABLE 24

Sporulation of isolate NCTC 2836 in media prepared by dissolving peptone fractions, obtained by continuous paper electrophoresis, in buffer

Sporulation medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Fraction No. 1	0.50	0	1.09
" " 2	1.00	0.49	1.51
" " 3	5.50×10	1.09×10	9.91×10
" " 4	4.88	3.67	6.09
" " 5	4.50	2.17	6.83
" " 6	7.19×10^3	5.77×10^3	8.61×10^3
" " 7	1.65×10	1.14×10	2.16×10
" " 8	2.63	1.31	3.95
" " 9	4.50	3.11	5.89
" " 10	0.63	0.05	1.21
Whole peptone	4.41×10^3	2.67×10^3	6.15×10^3
Peptone reclaimed from pyridine buffer	8.25×10^4	6.37×10^4	1.01×10^5
Control (Ellner's medium)	3.13×10^5	2.57×10^5	3.66×10^5

Fig. 18

Sporulation of strain NCTC 2836 in media prepared by dissolving peptone fractions, obtained by continuous paper electrophoresis, in buffer



A. Ellner's medium

B. Peptone (1 per cent (w/v) in buffer)

C. Peptone after first dissolving in pyridine-acetic acid buffer. (1 per cent (w/v) in buffer)

was prepared and used as sporulation medium.

Results. The pronounced effect on sporulation of dialysing the peptone is shown in Table 25 and Figure 19. It can be seen from these results that dialysing the peptone resulted in a twenty-fourfold increase in sporulation, while the number of spores produced in the dialysed medium approached that in Ellner's medium.

d. Fractions obtained by ammonium sulphate precipitation

Method. After fractionation and dialysis, 2g peptone yielded 0.0672g precipitate and 0.4686g supernatant. Thus, 2g peptone yielded 0.5358g dialysed peptone. In order to make this experiment comparable with the previous experiment (where the dialysed peptone was dissolved in buffer at a concentration of 1 per cent), each fraction was dissolved in 53.58ml buffer. These solutions were then used as sporulation media.

Results. (See Table 26 and Figure 20). Here again the ability to promote sporulation was not confined to either fractions and although the precipitate supported much better sporulation than the supernatant, the concentration of peptone in this medium was greater.

The effect of dialysis (illustrated in the previous experiment) is also apparent from these results. Thus, in media of approximately the same concentration, 15 times as many spores were produced by the

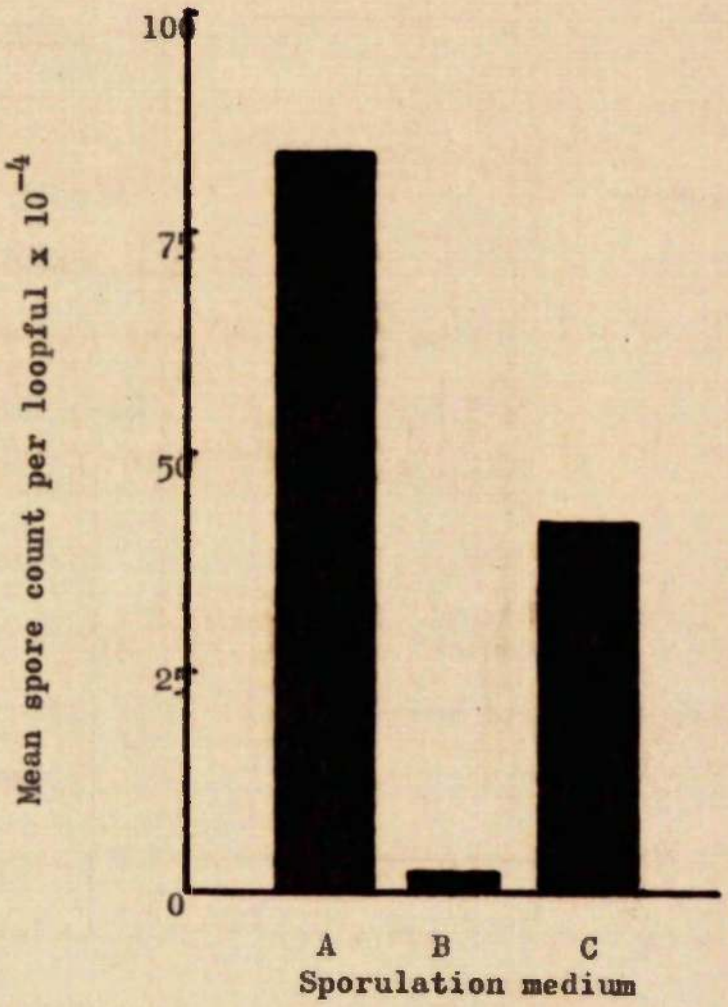
TABLE 25

Sporulation of isolate NCTC 2836 in a medium prepared by dissolving dialysed peptone in buffer

Sporulation medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Ellner	8.38×10^5	4.12×10^5	12.64×10^5
Whole peptone	1.70×10^4	0.96×10^4	2.44×10^4
Dialysed peptone	4.15×10^5	2.96×10^5	5.35×10^5

Fig. 19

Sporulation of strain NCTC 2836 in a medium prepared by dissolving dialysed peptone, in buffer



A. Ellner's medium

B. Whole peptone (1 per cent (w/v) in buffer)

C. Dialysed peptone (1 per cent (w/v) in buffer)

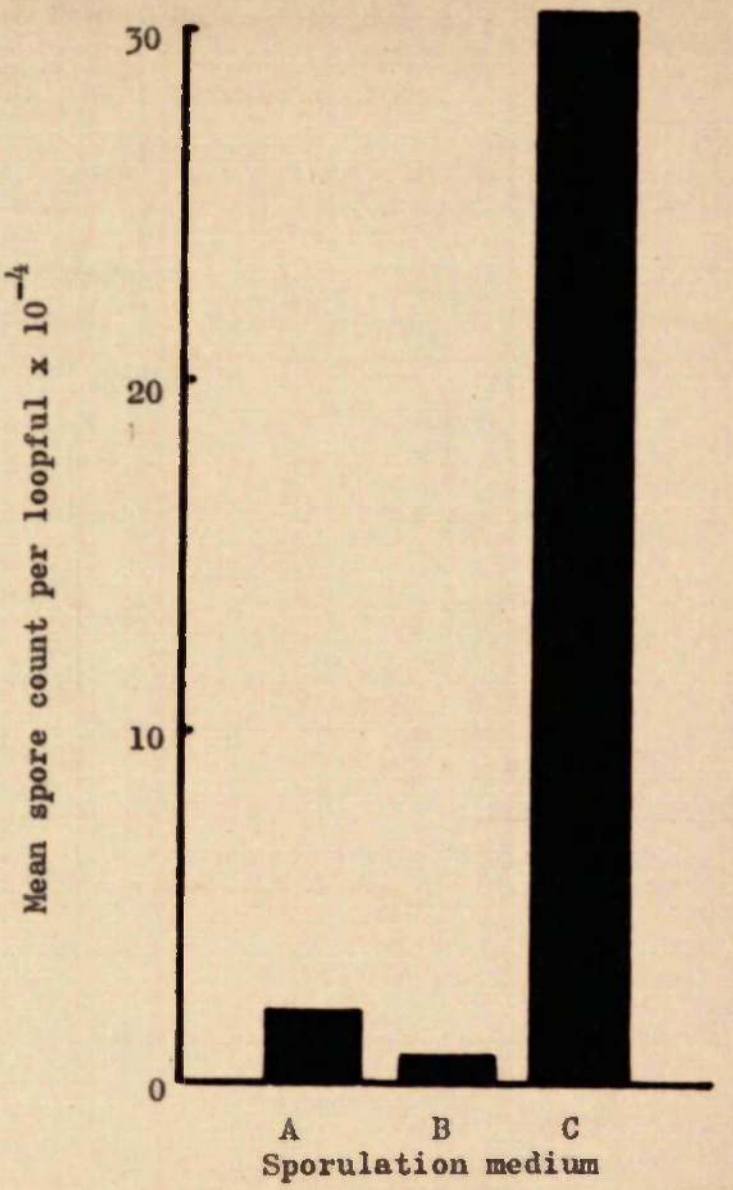
TABLE 26

Sporulation of isolate NCTC 2836 in media prepared by dissolving peptone fractions obtained by ammonium sulphate precipitation and dialysis in buffer

Sporulation medium	Mean spore count per loopful		
	Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Ellner	3.28×10^6	2.25×10^6	4.30×10^6
Whole peptone	2.15×10^4	1.16×10^4	3.14×10^4
Ammonium sulphate precipitate	8.25×10^3	4.25×10^3	12.25×10^3
Ammonium sulphate supernatant	3.06×10^5	2.31×10^5	3.82×10^5

Fig. 20

Sporulation of strain NCTC 2836 in media prepared by dissolving peptone fractions, obtained by ammonium sulphate precipitation and dialysis in buffer



A. Whole peptone (1 per cent w/v)

B. Precipitate (0.117 per cent w/v)

C. Supernatant (0.875 per cent w/v)

supernatant as by whole peptone. Similarly, although the whole peptone medium was 7 times more concentrated than that prepared from the precipitate, the numbers of spores produced in these two media did not differ significantly.

5.3 Inorganic components

5.3.1. Sporulation in peptone media buffered with different buffering agents at the same pH

The buffer in Ellner's medium contains sodium, potassium and phosphate ions. In order to compare the effect of these ions on sporulation, sporulation was compared in peptone media buffered with:-

1. Sodium, potassium and phosphate ions.
2. Sodium and phosphate ions.
3. Tris buffer.

Tris buffer provides a means of buffering the peptone without recourse to inorganic salts. It is also a chelating agent and would render any ions in the medium unavailable for sporulation.

Method. Buffer systems were prepared which provided a pH of 7.8. These are described below:-

1. KH_2PO_4 . . . . . 1.5g
   $Na_2HPO_4 . 12\ H_2O$ 67.0g
   Distilled water . . . . . 1 litre
   i.e. the buffer in Ellner's medium

2. 0.1 M $NaH_2PO_4 . 2H_2O$ 8.5ml
   0.1 M $Na_2HPO_4 . 7H_2O$ 91.5ml
   (Mackie & McCartney 1960)

3. 0.1 M Tris 50.0ml
   0.1 M HCl 32.5ml
   (Mackie & McCartney 1960)

Peptone (Oxoid L.37) was dissolved in each of these buffers at a concentration of 1 per cent (w/v) and sporulation experiments carried out.

Results. The results of this experiment are shown in Table 27 and Figure 21. Sporulation in the buffered peptone medium containing Na^+, K^+ and PO_4^{---} was thirteen times that in the medium buffered with Na^+ and PO_4^{---} only. When Tris buffer was used, sporulation was extremely weak, with a mean spore count of 1.75 per loopful. This is 4,700 times weaker than in the medium buffered with sodium and phosphate.

5.3.2. The effect of sodium and potassium on sporulation in peptone water

The previous experiment shows the effect of sodium and potassium phosphates on sporulation in peptone media. In the experiment recorded below the effects of the sodium and potassium ions have been compared. These ions were added to 1 per cent (w/v) peptone water at the same concentration as they are present in Ellner's medium.

TABLE 27

Sporulation of isolate NCTC 2836 in media prepared by dissolving peptone in different buffer systems at the same pH

Sporulation medium	pH	Mean spore count per loopful		
		Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Peptone in buffer (1)	7.8	1.10×10^5	0.39×10^5	1.81×10^5
Peptone in buffer (2)	7.8	8.25×10^3	8.14×10^3	8.36×10^3
Peptone in buffer (3)	7.8	1.75	0.46	3.04

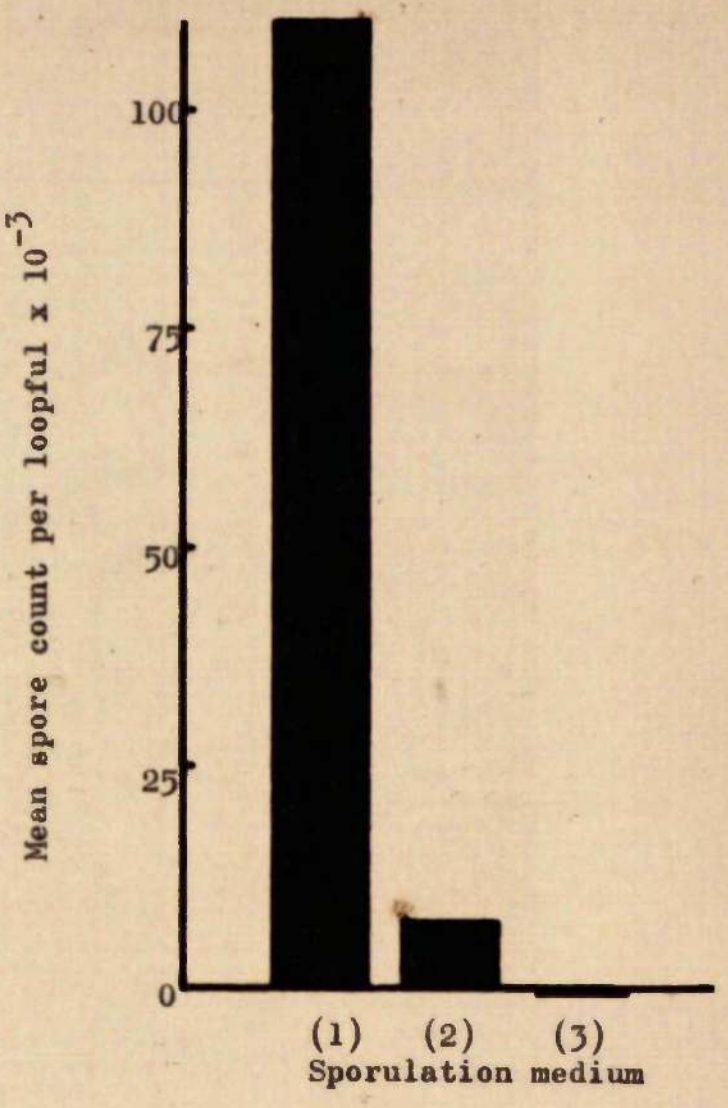
Buffer (1) contains Na^+, K^+ and PO_4^{---}

Buffer (2) contains Na^+ and PO_4^{---}

Buffer (3) contains no inorganic ions

Fig. 21

Sporulation of strain NCTC 2836 in media prepared by dissolving peptone at a concentration of 1 per cent (w/v) in different buffer systems at the same pH



(1) Peptone in buffer (pH 7.8)

(2) Peptone in sodium phosphate buffer (pH 7.8)

(3) Peptone in Tris buffer (pH 7.8)

Method. Ellner's medium contains 0.15 per cent (w/v) KH_2PO_4 and 6.7 per cent (w/v) $Na_2HPO_4 . 12H_2O$. The concentration of potassium in this medium is therefore (K^+/KH_2PO_4 x 0.15) 0.0619 per cent (w/v). The concentration of sodium is (2 $Na^+/Na_2HPO_4 . 12H_2O$ x 6.7) 1.497 per cent (w/v). These concentrations of sodium and potassium were incorporated separately and combined into a 1 per cent (w/v) solution of peptone in distilled water. This was done by weighing out pellets of sodium and potassium hydroxide, making up to a known volume with distilled water and adding an appropriate amount of each solution to the peptone water, before making up to the desired volume with distilled water. Media containing the sodium ion had a pH value of 14. The pH of these media was adjusted to 7.8 by adding a few drops of concentrated hydrochloric acid. Sporulation in these media, peptone dissolved in distilled water and peptone dissolved in buffer, was compared.

Results. (See Table 28 and Figure 22) The highest spore count was obtained in peptone water plus potassium (mean spore count per loopful = 4.41×10^4). This was 10 times higher than in peptone dissolved in buffer, which was not significantly greater than in peptone water plus sodium and potassium hydroxides. The spore count in the medium containing sodium hydroxide was the smallest obtained (mean spore count per loopful = 65) and was three times smaller

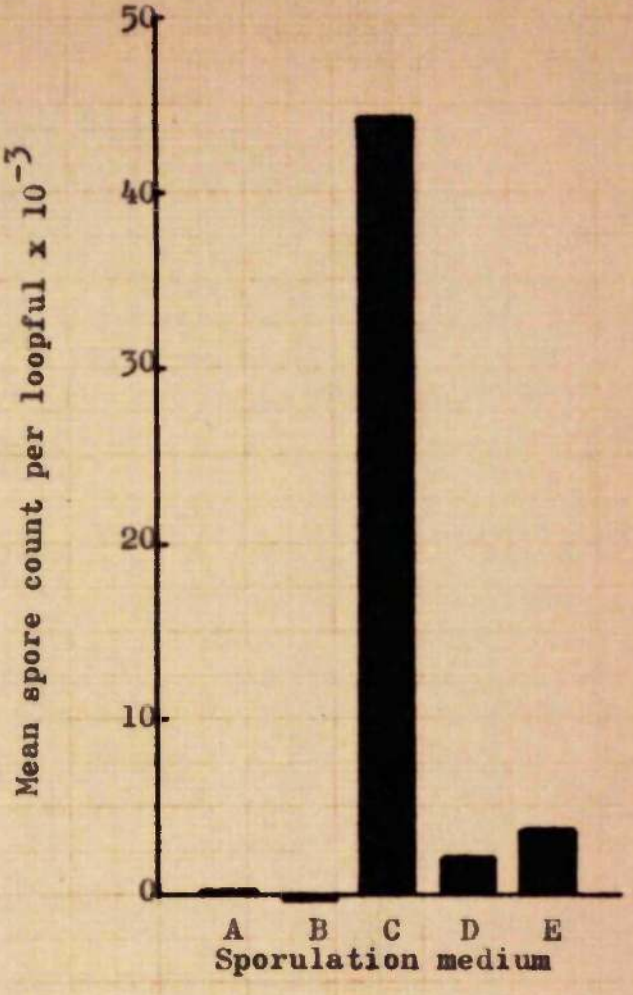
TABLE 28

The effect of Na^+ and K^+ on the sporulation of isolate NCTC 2836 in 1 per cent (w/v) peptone media

Medium	pH	Mean spore count per loopful		
		Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
1% (w/v) peptone + Na^+ (as NaOH)	7.8	6.5×10	5.93×10	7.07×10
1% (w/v) peptone + K^+ (as KOH)	7.8	4.41×10^4	3.48×10^4	5.35×10^4
1% (w/v) peptone + K^+ and Na^+	7.8	1.93×10^3	0.66×10^3	3.20×10^3
1% (w/v) peptone in buffer	7.8	3.44×10^3	1.16×10^3	5.72×10^3
1% (w/v) peptone in distilled water	6.0	2.06×10^2	1.47×10^2	2.65×10^2

Fig. 22

The effect of sodium and potassium on the sporulation of strain NCTC 2836 in 1 per cent (w/v) peptone media



A. Peptone in distilled water

B. Peptone + Na^+ (as NaOH) in distilled water

C. Peptone + K^+ (as KOH) in distilled water

D. Peptone + K^+ + Na^+ in distilled water

E. Peptone in buffer

than the next best medium, peptone dissolved in distilled water, which was of lower pH. These experiments therefore show that potassium enhances and sodium inhibits, sporulation.

5.3.3. The effect of phosphate on sporulation in peptone water containing potassium

The previous experiment has shown that potassium stimulates sporulation. The effect of phosphate cannot be determined from this experiment as sodium (which inhibits sporulation) was used whenever phosphate was used. Sporulation in media containing potassium, and potassium and phosphate was therefore compared.

Method. 1 per cent (w/v) peptone water containing potassium was prepared as described in the previous experiment. The peptone medium containing potassium phosphate was prepared by dissolving peptone at a concentration of 1 per cent (w/v), in a solution of 0.15 per cent (w/v) K_2HPO_4 in water. Even though the alkaline potassium phosphate salt was used, the pH of this medium was 7.0. Sporulation in the two media was compared.

Results. (See Table 29). Due to the variation in pH of these media, the effect of phosphate cannot be directly determined from this experiment. However, addition of phosphate did not stimulate sporulation sufficiently to overcome the effect of pH.

TABLE 29

The effect of phosphate on sporulation of isolate NCTC 2836 in an unbuffered peptone medium containing potassium

Medium	pH	Mean spore count per loopful		
		Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
1% (w/v) peptone + K^+ (as KOH)	7.8	5.94×10^4	4.73×10^4	7.14×10^4
1% (w/v) peptone + K^+ (as K_2HPO_4)	7.0	2.96×10^3	2.40×10^3	3.52×10^3

Discussion

Throughout these experiments emphasis has been laid on the production of good spore crops, rather than investigation of the numerous problems which have been found to be associated with sporulation.

In order to obtain sufficient numbers of spores for biochemical or serological analyses, it is essential to use a medium in which high percentages of spores are produced. When spores have been produced in such a medium, they must be separated from vegetative material. This is usually accomplished by lysis of the remaining vegetative cells, followed by repeated differential centrifugation, a process which invariably results in loss of spores. In Ellner's medium vegetative growth is poor, subsequently only relatively low numbers of spores are produced. It is therefore essential that if this medium is to be used for the mass production of spores, sporulation must be greater than 30 per cent.

From the present survey of sporulation of 48 isolates in this medium, it can be seen that only 8/48 (16.7 per cent) of the isolates examined produce more than 30 per cent spores. Previously reported surveys of sporulation in this medium have unfortunately been rather vague as to the percentage spores obtained. Thus, Ellner working with 138 beta-haemolytic strains found that the spore yield "frequently"

amounted to 80 per cent of the inoculum. This figure was presumably derived from viable spore counts. If it is assumed that each cell of the inoculum undergoes four divisions prior to sporulation (Cash and Collee 1962) then the sporulation in these cultures would be only 5 per cent. In the present study 10/30 (33.3 per cent) of beta-haemolytic isolates exceeded this percentage. Similarly, Yamamoto et al. (1961) claimed that spores were "readily observed" in 87.3 per cent of isolates. What this means in terms of percentage sporulation is questionable, although in the present study 64.7 per cent of the isolates produced sufficient spores to be observed in films prepared from cultures in Ellner's medium.

By far the most interesting point brought out by this survey is the observation that in Ellner's medium, beta-haemolytic isolates produce far greater numbers of spores than heat-resistant types. Similar, but less pronounced findings have been reported by Hall et al. (1963), who used a medium which they described in 1962 (Angelotti et al. 1962). This medium was used in preference to Ellner's medium, as these workers had shown in 1962 that the spores produced in Angelotti's medium were of higher heat resistance than the spores produced in Ellner's, medium although a higher percentage of spores was found in Ellner's medium. In order to compare Hall's results with those of the present study (see Table 9), Hall's 78 isolates, originally divided into three groups on the basis of strain origin,

have been divided, (as accurately as possible from the data available), into two groups, heat-resistant and beta-haemolytic (see Table 30). Comparing Tables 9 and 30 it can be seen that I detected spores in fewer instances in Ellner's medium, than Hall et al. did in Angelotti's medium. This presumably reflects differences in the sporogenicity of the strains studied. With isolates which produced sufficient spores to be detected microscopically, the results are not strictly comparable as Hall et al. did not determine sporulation percentages.

It has been shown in the present study, as well as the papers discussed above, that only a minority of strains produced large percentages of spores in Ellner's medium. It appears then, that the papers by Meisel and co-workers (1957 to 1962), Smith and Ellner (1957), Albrycht and Trembowler (1959) and Cash and Collee (1962), in which toxins have been demonstrated in spores and sporulation has been studied, have been carried out with carefully selected strains. Consultation with Dr Collee (Bacteriology Department, Edinburgh University) and Dr Willis (Bacteriology Department, Leeds University), who have worked extensively with Cl.perfringens, has confirmed this conclusion.

Whether the reason for the weak sporulation of heat-resistant strains is due to a genetic inability of these types to form spores, or to the deficiency of an essential nutrient for sporulation in Ellner's medium, is as yet unknown. The second experiment in this

TABLE 30

Sporulation of beta-haemolytic and heat-resistant Cl.perfringens isolates on a medium described by Angelotti et al. (1962) (After Hall et al. 1963)

Isolate	Percentage of isolates displaying various abilities to sporulate			
	++	+	±	−
Heat-resistant	45.5 (14/31)	38.7 (12/31)	12.9 (4/31)	3.2 (1/31)
Beta-haemolytic	87.2 (41/47)	10.6 (5/47)	2.3 (1/47)	0 (0/47)

++, Many spores observed in each microscope field

+, Many microscopic fields examined to observe a single spore

± Spores not observed in stained preparations, but detectable by means of heating (80° for ten minutes) and subculture

−, Spores not demonstrated by means of heating and subculture

series throws some light upon this problem. In this experiment the ability of a number of media to induce sporulation of heat-resistant and beta-haemolytic strains has been compared. The results show that in peptone media, beta-haemolytic strains produced far greater numbers of spores than heat-resistant strains. In other media, both types formed roughly the same number of spores. By far the best of these was the complex proteinaceous medium described by Bethge (1947-8)in which the heat-resistant strains produced far greater numbers of spores than in any of the other media examined. This finding provides some ground for believing that it may ultimately be possible to devise a defined medium which will give good sporulation of the poorly sporing heat-resistant types. The formulation of such a medium would throw considerable light on the nutritional requirements for sporulation of Cl.perfringens.

Before attempting to improve sporulation of these heat-resistant strains in Ellner's medium by slight modifications to this medium, it was decided to study the effect of the pre-sporulation medium, and a number of environmental conditions,on sporulation of a good sporing strain in unmodified Ellner's medium. Although all the factors studied were found to be important in producing good spore yields, the medium used for growing the inoculum was of prime importance. The best of the media examined was Robertson's meat broth, closely followed by serum peptone water. The most commonly used bacteriological

culture media, namely digest broth and infusion broth, were appreciably weaker, while spores could not be demonstrated in cultures from Brewer's medium. Previous workers such as Ellner (1956), Smith and Ellner (1957), Cash and Collee (1962), Hall et al. (1963) and Albrycht and Trembowler (1959), have completely overlooked this important condition but apparently fortuitously have used the better of these media.

As expected from the work of Albrycht and Trembowler (1959), who found that preparing Ellner's medium with infusion broth in place of water resulted in decreased sporulation, the present experiments showed that sporulation fell off when increasing volumes of inoculum were added to a constant volume of Ellner's medium. In this experiment the percentage sporulation was constant until the inoculum volume reached 20 per cent. In an attempt to overcome this effect and so increase the total number of spores produced in a given volume of medium, washed cells were added to Ellner's medium. This resulted in decreased sporulation which was only partially overcome by adding fresh Robertson's meat broth to the medium. Sporulation was however restored to its original level when the washed cells were reconstituted with the Seitz filtered culture supernatant. It therefore appears that factors are formed in Robertson's meat broth during the growth period which result in increased sporulation in Ellner's medium. These factors are presumably not inorganic ions, but could be either extracellular enzymes which assist in the utilisation of peptone,

metabolites produced by vegetative growth, or breakdown products of Robertson's meat broth which are utilised during sporulation.

The effect of temperature on sporulation was only studied at two temperatures (37 and 45°). These were selected as they have previously been shown to be the temperature optima for toxin production and growth respectively, of a large number of strains (Săsărman and Horodniceanu 1961). With the strain used in the present study, growth was better at 45 than at 37°, although sporulation was inhibited at the higher temperature. Possible explanations of this behaviour are not hard to find, as it is well known that the temperature at which a bacterium is grown affects the chemical composition and enzymic activities of the cell (Ingraham 1962), frequently making bacteria more nutritionally demanding at elevated temperatures. This is well illustrated by the work of Beskid and Lundgren (1959, 1961), Lundgren and Beskid (1960), Lundgren and Cooney (1962) and Cooney and Lundgren (1962). These authors used a strain of B.cereus var lacticola which spored at 28 and 37° and a number of temperature sensitive mutants which spored only at 28°. In this example sporulation of the mutants at 37° was effected by supplementing the sporulation medium with casein hydrolysate.

Aeration of cultures of Cl.perfringens in Ellner's medium resulted in complete inhibition of sporulation. This was not altogether unexpected as Leifson (1931) has shown that sporulation

of Cl.botulinum was prevented if the cells were aerated before they were "committed to sporulation." The cells becoming committed to sporulation corresponded to the appearance of forespores. The sensitivi -ty of sporulation to aeration in the present study illustrates that the cells were not committed to sporulation when Ellner's medium was inoculated. This is not surprising,as sporulation of the strain used is weak in Robertson's meat broth and a young actively growing culture was used to inoculate the sporulation medium. The stage in Ellner's medium at which the culture became resistant to aeration was not determined.

Attempts to promote abundant sporulation of weakly sporing strains in Ellner's medium by:modifying the pH, supplementing with inorganic ions or thiamine, using spent media containing disintegrated sporing cells,or different commercial preparations of peptone and yeast extract in the medium, were uniformly unsuccessful. Also unsuccessful were a number of time-consuming attempts to transform two weakly sporing strains by growing in the presence of desoxyribonucleic acid extracted from a good sporing strain. These experiments were not reported in the Experimental part of this Section, as in this type of work often a large number of strains have to be examined in order to find a single receptive strain.

Certain of the modifications to the medium had a pronounced effect on the sporulation of the sporogenous strains and it is highly likely that the sporulation of the weakly sporing strains was also

affected. However, as these strains invariably produced less than 1 per cent spores this would go undetected. With the strains which produced abundant spores, the stimulatory effect of magnesium sulphate, first reported by Ellner (1956), has been confirmed. Contrary to reports by Ellner (1956) and Albrycht and Trembowler (1959), the commercial preparation of peptone used in the preparation of the medium had a marked effect on sporulation. It is however noteworthy that Ellner routinely used polypeptone. This preparation is a mixture of pancreatic digest of casein and peptic digest of animal tissues and contains a wider range of amino acids than peptone from a single source. Unsatisfactory sporulation of at least one strain of Clostridium has been shown by Perkins and Tsuji (1962) to be attributable to the low concentration of arginine in certain peptones. For reasons to be discussed later, as well as the observation that sporulation was decreased when the peptone was replaced by a mixture of equal parts peptone and pancreatic digest of peptone, amino acid deficiency does not appear to be a suitable explanation in the present case.

When the ability of a number of substrates to induce sporulation (when dissolved in buffer at the same concentration) was compared, sporulation was far better in peptone than in any of the other proteinaceous compounds examined (gelatin, albumin and casein hydrolysate). The advantage of peptone over complex proteins for

sporulation of Cl.perfringens was first realised as early as 1908 by Hibler and there is a rapidly increasing fund of information supporting the contention that sporulation by members of the genus Clostridium is at least as good in peptone as any other media. The advantages of experimenting with peptone media are numerous, not least of these is the simplicity with which they can be prepared. They are also aparticulate, enabling the spores to be thoroughly washed for examination of chemical composition and fine structure and their composition is reasonably constant.

The number of spores formed in this peptone medium was dependent upon the concentration of peptone. Over the range 0.5 to 10.0 per cent (w/v), the $\log._{10}$ spore count per loopful was directly proportional to the $\log._{10}$ peptone concentration. The percentage sporulation however, was maximal at a peptone concentration of approximately 5 per cent. These results suggest that sporulation of Cl.perfringens in Ellner's medium might be significantly enhanced by increasing the peptone concentration. Riemann (1963) also suggested that this might be advantageous, although this worker based his conclusion on spore counts rather than percentage sporulation. Experiments with the whole medium by Albrycht and Trembowler (1959) indicate that this is not the case, as decreasing the peptone concentration from 1.0 to 0.2 per cent was without effect. Apparently a more thorough examination of this aspect of the work is necessary.

The falling off in percentage sporulation with increasing concentration of the medium has also been observed in the genus Bacillus (Knaysi 1945). In this case it was hypothesised that the increased vegetative growth in the more concentrated media resulted in oxygen starvation, which in turn inhibited sporulation. Although the necessity for a copious supply of oxygen for sporulation in the genus Bacillus is well known, this explanation can hardly be considered to hold true for anaerobic organisms. A more feasible explanation is suggested by the work of Grelet (1951, 1955 and 1957). In these papers Grelet has shown that cells spore when certain nutrients in the medium are exhausted. If this hypothesis holds true for the present example, it would be expected that there would be more vegetative growth in the more concentrated media before nutrients became limiting and sporulation was "triggered." This apparently was the case as the percentage sporulation fell off as the viable spore count rose. Moreover, the more prolific growth in the more concentrated media, would undoubtedly result in loss of synchrony in the culture, so that when the optimal conditions for sporulation were attained in the medium, a lower percentage of the cells would be in the right metabolic state to sporulate.

The experiments in which sporulation was compared in peptone fractions were designed on the assumption that all of the sporulation substrate would be confined to a single fraction. Thus,

if 1g peptone contains Xg substrate and on fractionation the substrate is confined to a single fraction, then when this quantity of substrate (possibly along with other material) is dissolved in 100ml buffer, the number of spores formed in this medium should be the same as the number of spores formed in a 1 per cent solution of whole peptone. If however, the spore count is higher in one of the fractions, then an inhibitor must have been removed. Unfortunately neither electrophoresis, ethanol precipitation, nor ammonium sulphate precipitation resulted in complete fractionation of the peptone into distinct sporogenic and asporogenic components, from which it is possible to conclude that more than one component of peptone can support sporulation.

There was however considerable variation in the number of spores formed in the different fractions, although in a number of instances this could be attributed to the difference in concentration. In order to compare sporulation in these fractions with sporulation in a whole peptone medium of the same concentration, the theoretical spore count which would be obtained in the whole peptone medium has been calculated. This was possible as it has previously been shown that the log. mean spore count was directly proportional to the log. peptone concentration. The straight line fitted to the experimental points had the equation:-

$$N = 10^7 \times 2.834 \times C^{1.6239}$$

where N = mean number of spores per loopful
and C = peptone concentration (g/ml)

This equation can be rewritten:-

$$y = 1.6239X + 7.4524$$

where $y = \log. N$

and $X = \log. C$

The standard errors for the estimated numbers of spores were obtained from the equations:-

1. $(\text{Standard error of } y)^2 = 3.3289 \times 10^{-4} + 1.5840 \times 10^{-3} (X + 1.6239)^2$

2. Standard error of N = standard error of y x N x 2.30259

The 95 per cent confidence limits for values of N are:-

N ± standard error of N.

The theoretical values of N at the different peptone concentrations, along with the actual values obtained in the different peptone fractions, are shown in Table 31. This procedure is valid for all but the electrophoresis experiment, as the spore counts in the controls for these experiments (1 per cent (w/v) peptone in buffer) did not differ significantly. In the electrophoresis experiment however, the spore count in the control medium was significantly higher than in the experiment from which the equation constants were calculated. As mentioned previously, this variation in sporulation between experiments is probably due to variation in the state of the culture at the time of inoculation of the sporulation medium.

In order to determine which of the fractionation methods yielded fractions best able to promote sporulation, the ratio:-

TABLE 31

Comparison of the number of spores formed in media prepared by dissolving peptone fractions in buffer, with the theoretical number of spores which would be produced in a whole peptone medium of the same concentration

Fractionation method	Medium	Concentration (per cent w/v)	Mean spore count per loopful			Theoretical mean spore count in whole peptone		
			Best estimate	Lower 95% confidence limit	Upper 95% confidence limit	Best estimate	Lower 95% confidence limit	Upper95% confidence limit
Ethanol precipitation	precipitate	0.198	2.43×10^3	2.06×10^3	2.79×10^3	1.15×10^3	1.03×10^3	1.27×10^3
	supernatant	0.747	1.50×10^3	1.02×10^3	1.98×10^3	9.97×10^3	9.34×10^3	1.05×10^4
Dialysis	dialysed peptone	1.000	4.15×10^5	2.96×10^5	5.35×10^5	1.60×10^4 (1.70×10^4)*	1.51×10^4 (0.96×10^4)*	1.68×10^4 (2.44×10^4)*
Ammonium sulphate precipitation followed by dialysis	precipitate	0.117	8.25×10^3	4.25×10^3	12.25×10^3	4.91×10^2	4.29×10^2	5.54×10^2
	supernatant	0.875	5.06×10^5	2.31×10^5	5.82×10^5	1.29×10^4	1.21×10^4	1.36×10^4

*Experimentally determined

$$\frac{\text{mean spore count per loopful of fractionated peptone}}{\text{theoretical mean spore count per loopful of whole peptone}} \quad (m_1/m_2)$$

was calculated. The confidence limits for this ratio were worked out as follows:-

If m_1 and m_2 are estimates of the mean spore count, with standard errors σ_1 and σ_2 respectively, then the confidence limits λ_1 and λ_2 for $\lambda = m_1/m_2$ are the roots of the equation:-

$$(m_1 - \lambda m_2) \pm 1.96\sqrt{\sigma_1^2 + \lambda^2\sigma_2^2} = 0$$

i.e. $$(m_1 - \lambda m_2)^2 = (1.96\sigma_1)^2 + \lambda^2 (1.96\sigma_2)^2$$

i.e. $$\lambda^2 \left[m_2^2 - (1.96\sigma_2)^2\right] - 2\lambda m_1 m_2 + \left[m_1^2 - (1.96\sigma_1)^2\right] = 0$$

i.e. $$\lambda = \frac{m_1 m_2 \pm \sqrt{(1.96\sigma_1)^2 m_2^2 + (1.96\sigma_2)^2 m_1^2 - (1.96\sigma_1)^2 (1.96\sigma_2)^2}}{m_2^2 - (1.96\sigma_2)^2}$$

i.e. λ_1, lower confidence limit

$$= \frac{m_1 m_2 - \sqrt{(1.96\sigma_1)^2 m_2^2 + (1.96\sigma_2)^2 m_1^2 - (1.96\sigma_1)^2 (1.96\sigma_2)^2}}{m_2^2 - (1.96\sigma_2)^2}$$

and λ_2, upper confidence limit

$$= \frac{m_1 m_2 + \sqrt{(1.96\sigma_1)^2 m_2^2 + (1.96\sigma_2)^2 m_1^2 - (1.96\sigma_1)^2 (1.96\sigma_2)^2}}{m_2^2 - (1.96\sigma_2)^2}$$

These ratios are shown in Table 32, from which it can be seen that sporulation in media prepared from the fractions obtained by methods involving dialysis, was significantly greater than in the media prepared from fractions obtained by ethanol precipitation. The ratio did not differ significantly between dialysed whole peptone and the two fractions of dialysed whole peptone obtained by ammonium sulphate precipitation. It appears then, that proteoses (high molecular weight peptides which are precipitated in saturated solutions of ammonium sulphate) are no more able to support sporulation than the lower molecular weight unprecipitated compounds, which are too large to pass through the dialysis membrane.

The maximum size of molecule which will pass through the dialysis membrane is unfortunately not known. The grade of dialysis tubing used (Visking - 24/32) is the least permeable of the grades available and insulin (M.W. = 5,733) will not pass through (Craig, King and Stracher 1957). Amino acids however, diffuse through this membrane rapidly, and in order to slow down diffusion sufficiently to compare the relative rates of passage of different amino acids, Craig and Ansevin (1963) had to reduce the pore size of the membrane by acetylation.

The low molecular weight compounds which are removed from the peptone by dialysis, as well as not supporting sporulation appear to be inhibitory. From the experimental data presented it can be seen

TABLE 32

Comparison of the number of spores produced in media prepared by dissolving peptone fractions in buffer, with the theoretical number of spores produced in a whole peptone medium of the same concentration

Fractionation method	Medium	Experimental mean spore count / Theoretical mean spore count		
		Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Ethanol precipitation	Precipitate	2.11	1.73	2.52
	Supernatant	0.15	0.10	0.20
Dialysis	Dialysed peptone	25.94	18.44	33.58
Ammonium sulphate precipitation followed by dialysis	Precipitate	16.79	8.57	25.55
	Supernatant	23.72	17.82	29.79

that 2g whole peptone yielded 0.5358g retentate (a word coined by Craig to describe the compounds which do not pass through the dialysis membrane). Therefore 1g retentate is present in 3.732g whole peptone. The theoretical mean number of spores produced per loopful in a 3.732 per cent (w/v) solution of whole peptone is 1.361×10^5 (confidence limits 1.30×10^5 to 1.42×10^5). This value is significantly lower than the number of spores actually produced in a 1 per cent (w/v) solution of retentate (4.15×10^5 with confidence limits 2.96×10^5 to 5.35×10^5). Therefore these compounds inhibit sporulation. Further evidence of this effect has previously been presented, when it was shown that replacing the peptone content of Ellner's medium with a mixture of equal parts peptone and pancreatic digest of peptone resulted in decreased sporulation. In view of these findings it is interesting to note that Schneider et al. (1963) obtained spores of a strain of Cl.perfringens by culturing in saline in dialysis sacs suspended in the sporulation medium. Although these experiments were inadequately controlled, it appears that compounds of molecular weight less than 13,000 do not invariably inhibit sporulation. The inhibitory effect observed in the present experiments could of course be due to a low molecular weight compound which was not present in the peptone used by Schneider et al.

Further experiments along these lines will undoubtedly yield

further information as to the nature of the sporulation substrate and would apparently result in the formulation of a more highly sporogenic medium than Ellner's medium.

The studies of the effect of inorganic ions on sporulation have been restricted to the sodium, potassium and phosphate ions. These were selected as they are present in large amounts in Ellner's medium (as the buffer component) and Albrycht and Trembowler (1959) found that when they were omitted from the medium, sporulation was considerably reduced. Undoubtedly biologically significant amounts of inorganic ions were present in the commercial peptone preparations used and further inorganic ions would be added to the medium with the inoculum. Washed cells were not used to inoculate the medium at this stage, as it had previously been shown (see page 69) that this procedure resulted in diminished sporulation.

In order to eliminate the pronounced effect of pH on sporulation, wherever possible media of the same pH were used. In the case of media more alkaline than pH 7.8, the pH was brought down to this value by adding hydrochloric acid. This was permissable as a number of workers have shown that the chloride ion does not affect sporulation. Media more acid than pH 7.8 were used at their natural pH. Sodium and potassium hydroxides could not be added to these media because of their pronounced effect on sporulation. In these experiments media of slightly different pH have been compared and the effect sought

has been whether the ion under consideration was able to stimulate sporulation sufficiently to overcome the difference in pH.

It is apparent from the first of these experiments that inorganic ions are essential for sporulation. Since, when Tris buffer, a chelating agent, was incorporated into the medium, sporulation was considerably reduced. Experiments not presented in this thesis have shown that this buffer has a much more pronounced effect on sporulation than on vegetative growth. Thus, although sporulation was reduced fifty-fold in the presence of 0.6 per cent (w/v) Tris, vegetative growth was not inhibited in media containing 2 per cent (w/v) Tris. In the presence of higher concentration of Tris filamentous bacillary forms were produced, although vegetative growth was not prevented until concentrations of over 5 per cent were used.

In order to compare the influence of the sodium, potassium and phosphate ions, unbuffered media were therefore used. These experiments have shown that the sodium ion inhibits sporulation. The role of the potassium ion is unfortunately more difficult to assess. Thus, although sporulation was better in media containing the potassium ion than in peptone water, this could be attributed entirely to the higher pH of the medium containing potassium. Similarly, the phosphate ion did not stimulate sporulation sufficiently to overcome the difference in pH between the medium containing potassium (as potassium hydroxide) and the medium containing potassium and phosphate

(as K_2HPO_4). In order to finalise these points, experiments could be carried out using media of the same pH. This could be done by bringing down the pH of the more alkaline media by adding an acid with an anion which does not inhibit sporulation (e.g. hydrochloric acid). This however is a purely theoretical consideration, as at these lower pH values sporulation is considerably weaker than at pH 7.8. Moreover, any improvements on Ellner's medium will have to have a pH of at least this value.

In conclusion it appears that Ellner's medium will be improved upon by:-

1. Dialysing the peptone.
2. Increasing to peptone concentration.
3. Omitting the sodium salt.

A promising outcome of the work I have described, is that it should be possible to study in some detail, the influence of inorganic ions on sporulation, a field of investigation which has not yet been seriously explored. By the use of dialysed peptone as the main ingredient of the medium the effect of controlled additions of inorganic ions can, obviously, be followed. It is hoped to persue this line in future work.

SECTION D

Variation of Sporulation Within a Single Strain, and the Association of Sporulation with Mucoid Growth, Colonial Morphology, Sensitivity to Antibacterial Substances and Proteolytic Activity

CONTENTS

Introduction

The stability of sporulation within a single strain was studied because from time to time throughout the experiments with NCTC 2836, cultures in Robertson's meat broth were obtained, which produced low percentages of spores. In retrospect, it was apparent that these cultures were always derived from a single colony, obtained by "plating out" a good sporing culture on horse blood agar. The stability of sporulation of a good sporing "race" and a weakly sporing "race" was compared by plating out each culture and comparing sporulation in nine or ten cultures of each "race."

A number of workers have correlated the ability of isolates of Cl.perfringens to form spores with colonial morphology. Unfortunately there is little agreement as to which of the three commonly found colonial types (rough, smooth and mucoid) sporulate most readily. Simmonds (1915a) and Henry (1916-17) maintained that mucoid isolates formed spores the most readily, while Stevens (1935) attributed this property to smooth strains.

Other workers have isolated variants with varying spore forming potential from single strains. McGaughey (1933) isolated non-mucoid and rough variants from the spreading granular edge of a three day old colony of a weakly sporing smooth strain. Of these, the non-mucoid variant produced numerous spores, while the rough variant did not

produce spores at all. A second, rather unusual correlation between colonial morphology and sporulation in a single strain was reported by Hobbs et al. (1953). In this instance colonies were observed which were described as "poached egg" or "pin point." These colonies were made up of sporing cells and although unstable, could be produced repeatedly from the parent culture.

From the above summary of work described at greater length in the Review of the Literature, it seems that there is no clear relationship between colonial morphology and the ability to produce spores. It therefore appeared worthwhile to examine the 48 isolates previously enumerated, with a view to comparing production of mucoid material and colonial morphology, with the ability to form spores.

Of the many other characters which could be examined for association with sporulation, only sensitivity to antibacterial substances and proteolytic activity have been tested. The sensitivity patterns of sporogenous, oligosporogenous (weakly sporing) and heat-resistant strains to a number of antibacterial substances were compared, because Slotnik (1959) has shown that only cultures of B.subtilis which are sensitive to actinomycin D will form spores. If it can be shown that these three types of Cl.perfringens have distinct sensitivity patterns, it would be possible to use antibacterial substances to select strains having a desired property, as well as providing a genetic marker for location of the spore genome on the bacterial chromosome.

The ability of sporogenous and oligosporogenous strains to produce proteolytic enzymes was compared to determine whether oligosporogenous strains do not spore well simply because they do not produce the necessary enzymes to utilise the peptone in Ellner's medium. Evidence that sporulation is stimulated by adding culture supernatants (possibly enzymic) to Ellner's medium along with the inoculum has previously been presented on page 70.

Experimental

1. Variation of sporulation within a single strain

Method. Two cultures in Robertson's meat broth were obtained, one which produced in the order of 60 per cent spores and one which produced less that 10 per cent spores. These cultures were plated on horse blood agar and separate colonies seeded into tubes of Robertson's meat broth. Sporulation of these cultures in Ellner's medium was examined.

Results. The results of this experiment are illustrated in Figures 23 and 24. The nine cultures obtained from the Robertson's meat broth which produced in the order of 60 per cent spores, all produced more than 25 per cent spores. Seven of these produced more than 55 per cent spores. In contrast, of the ten cultures derived from the weakly sporing "race", eight produced less than 15 per cent spores and one produced 26 per cent spores. The remaining culture, which produced 51 per cent spores, was the only one which showed a tendency to revert to the good sporing state. From these results it appears that the cultures tend to "breed true", although two of the nine cultures derived from the good sporing "race" produced considerably lower numbers of spores than their companions, while two of the ten cultures derived from the weakly sporing "race" produced larger numbers than their companions.

Fig. 23

Variation of sporulation within a strain. A culture in Robertson's meat broth, which produced in the order of sixty per cent spores, was plated out and nine colonies selected. The sporulation of each of these cultures was determined

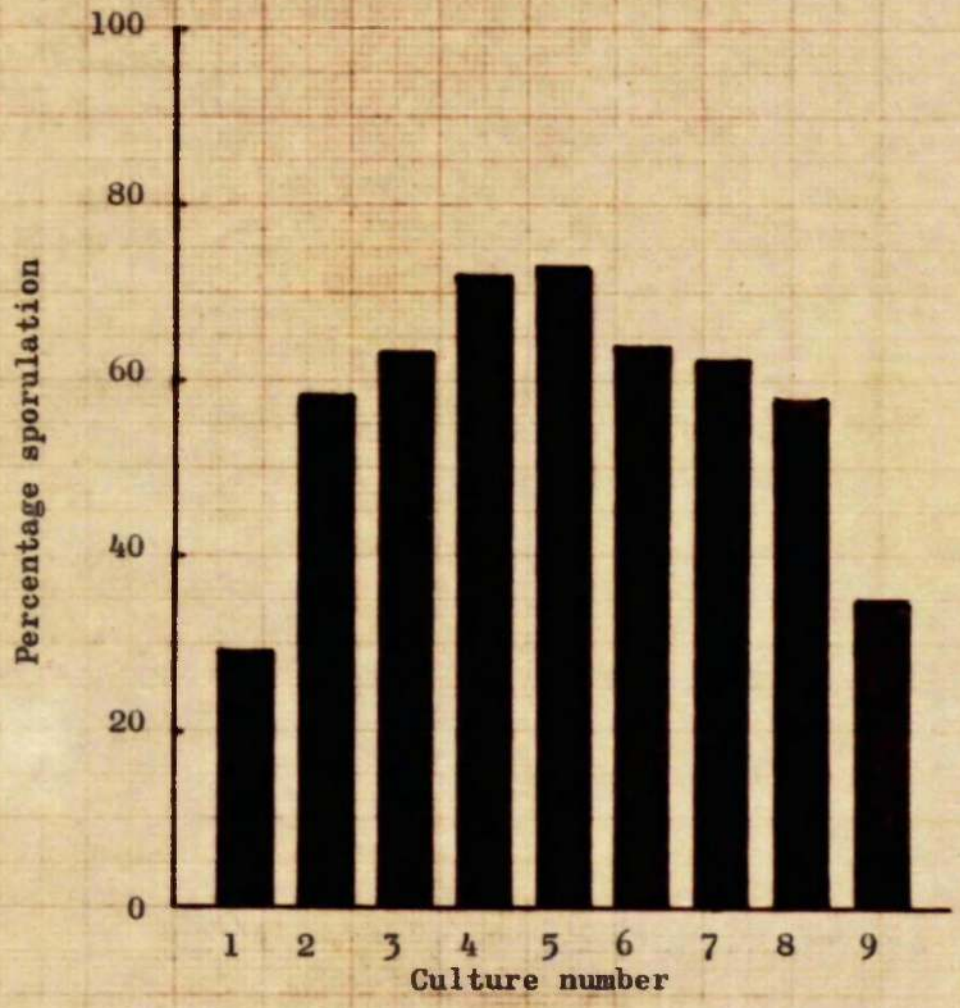
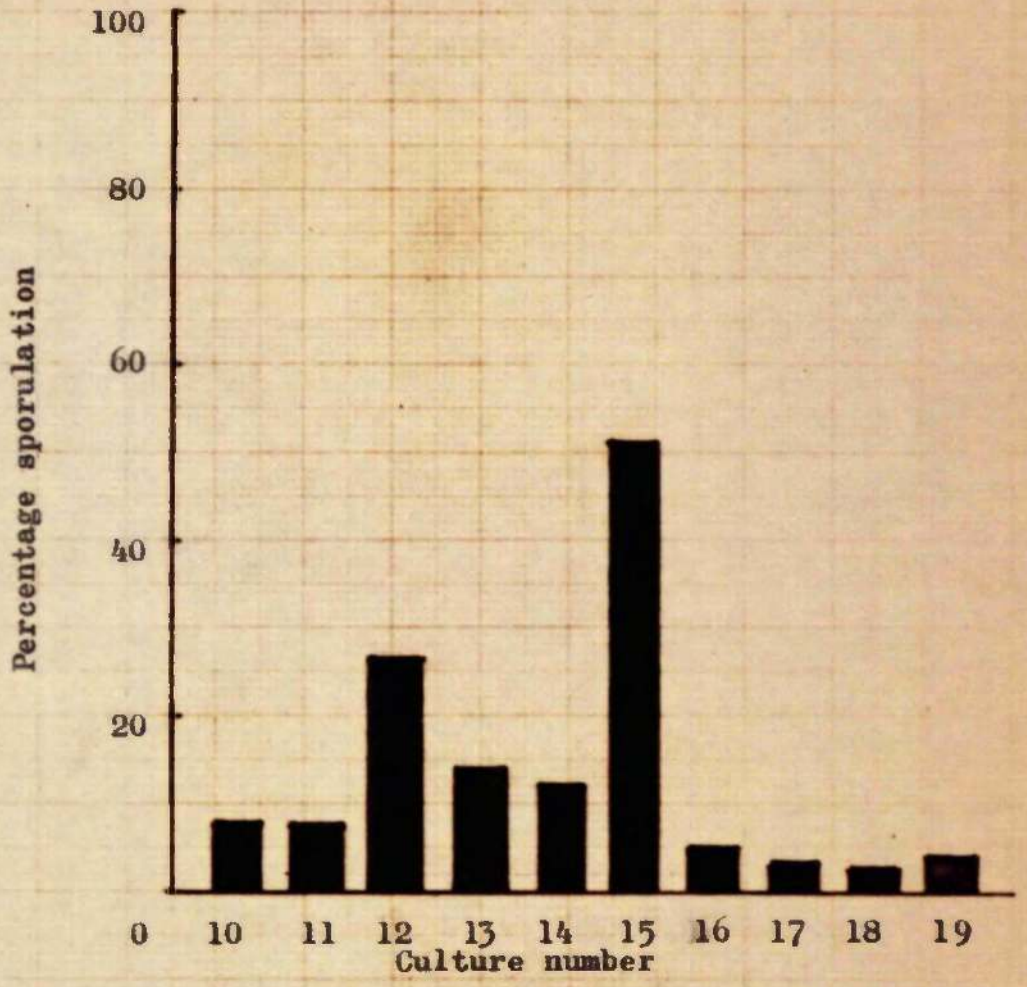


Fig. 24

Variation of sporulation within a strain. A culture in Robertson's meat broth, which produced in the order of ten per cent spores, was plated out and ten colonies selected. The sporulation of each of these cultures was determined



2. Association of colonial form and mucoid growth with ability to produce spores

Method. The colonial morphology of the strains listed in Table 6 was examined after plating on horse blood agar and incubating anaerobically at 37° overnight. Tubes of 1 per cent (w/v) glucose broth* were also inoculated with these organisms. These were incubated anaerobically overnight and examined for mucoid growth. Mucoid growth was estimated by dipping a wire loop into the culture or colony. Growth was recorded as mucoid when the culture was drawn up from the surface of the medium and non-mucoid when it was not. As attempts to stain the mucoid material were unsuccessful (see also Duguid 1951) some of these cultures were examined in the electron microscope and compared with cultures in infusion broth and Ellner's medium. For this purpose a Phillips E.M. 100 electron microscope was used. Carbon or formvar grids were loaded by dropping one drop of a dilute aqueous suspension of the culture onto the grid from a Pasteur pipette.

Results. Mucus was produced by four of the beta-haemolytic and none of the heat-resistant strains. The remaining 44 strains produced smooth

---

*Svec and McCoy (1944), Duguid (1951), Fredette and Vinet (1962) and Izumi (1962) have all found that mucus production is better in media containing glucose.

colonies. After two days incubation the edges of a high percentage of the colonies had developed rough outgrowths (see Plate 2A). On filming and staining these rough outgrowths, the vegetative cells were filamentous (Plate 2C). The smooth part of the colony was made up of typical short bacilli (Plate 2B). Subculture of these two cellular types in Robertson's meat broth yielded only the typical short celled form, which, following plating and overnight incubation on horse blood agar, gave rise to smooth colonies only. It was therefore impossible to compare the sporulation of these two cellular types due to instability of the rough form. It was however possible to maintain the rough variant by subculture on solid media (see Plate 3A).

The strains producing mucoid material were: NCTC 2836, 19, J.M.S. and S11. By referring to Table 7, it can be seen that three of these produced more than 25 per cent spores in Ellner's medium. The remaining strain, S11, spored but weakly.

When glucose broth cultures were examined in the electron microscope, the cells of both mucoid and non-mucoid strains were enveloped in an amorphous electron-dense material, through which it was only occasionally possible to make out the edge of the cell. On exposure to the electron beam this material became vacuolated and gave the appearance of boiling. Spores and cells of all the isolates examined after growth in Ellner's medium, including mucoid and non-mucoid beta-haemolytic strains and non-mucoid heat-resistant strains,

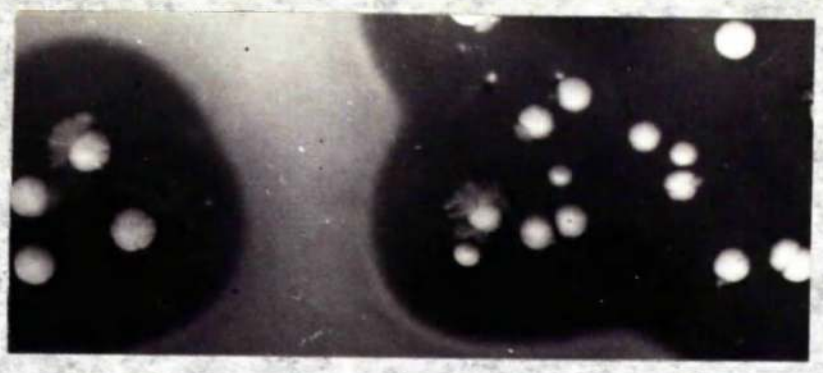
PLATE 2

A. Colonies of isolate NCTC 2836 after two days incubation on horse blood agar. A number of the colonies have granular outgrowths.

B. Gram stained film prepared from a "smooth" part of one of the colonies in A.

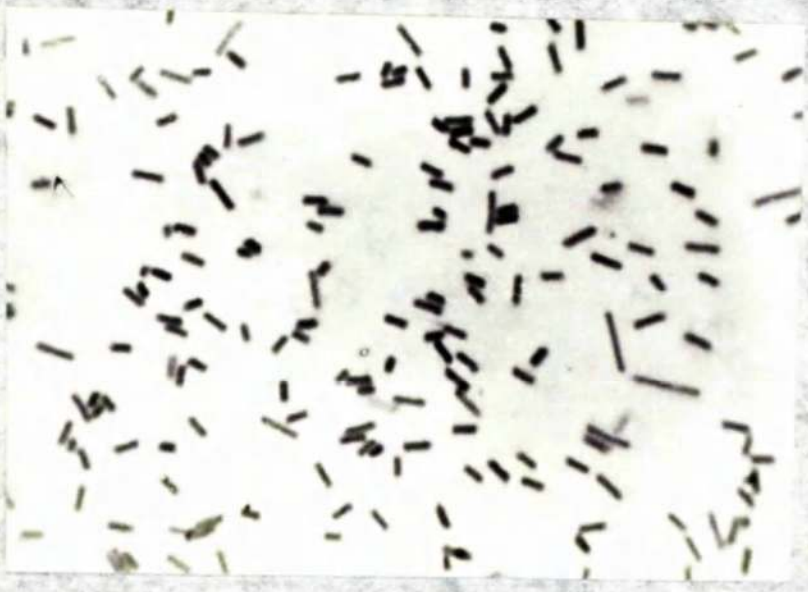
C. Gram stained film prepared from the granular edge of a colony in A.

A



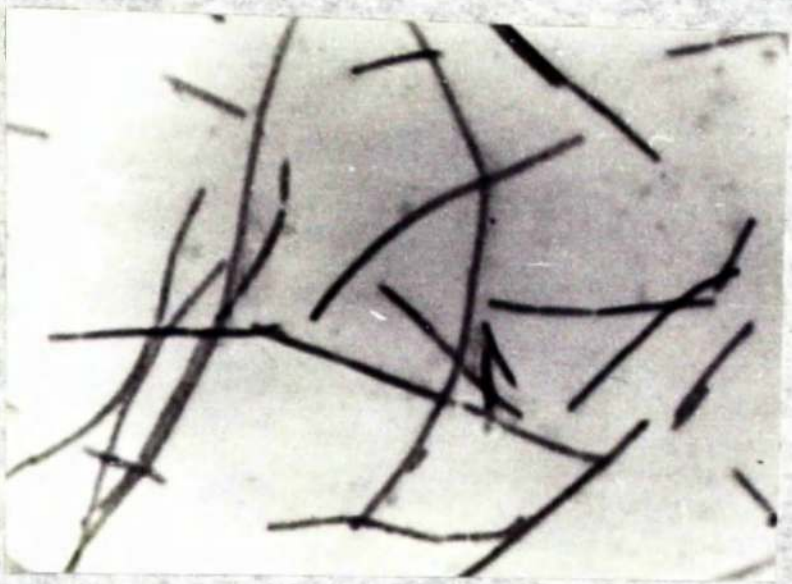
x $1\frac{1}{2}$

B



x 1200

C



x 1200

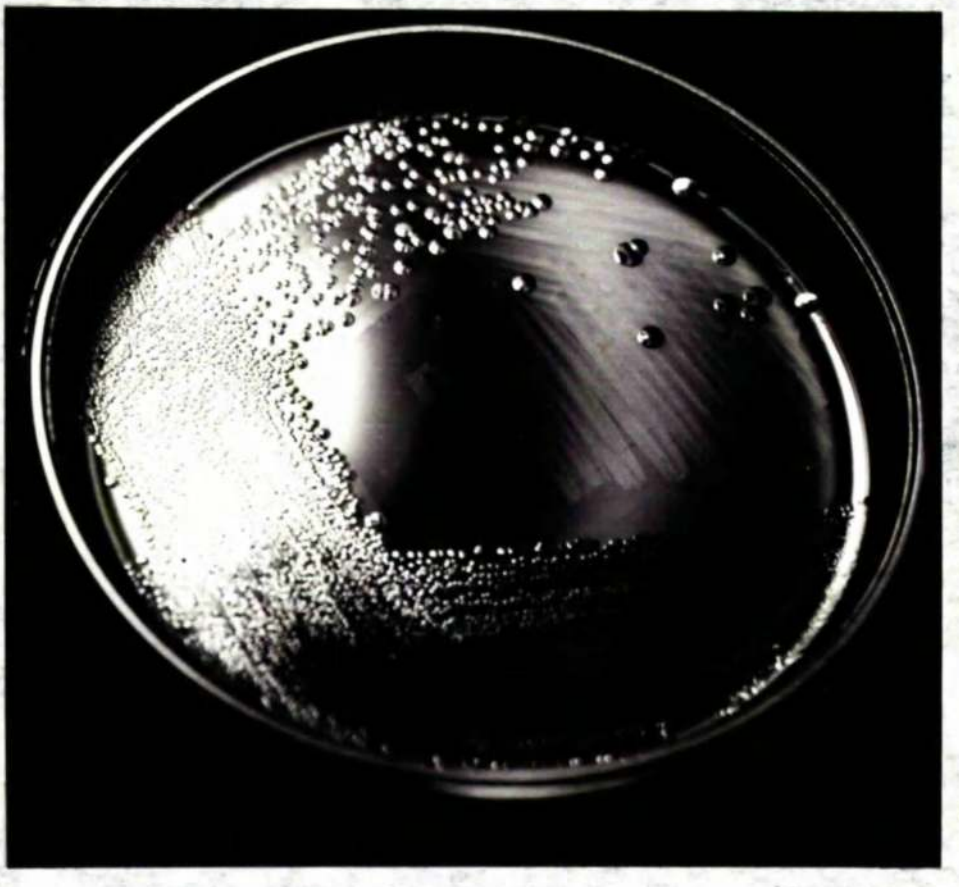
PLATE 3

A. Colonies of a "rough" variant of strain NCTC 2836 after overnight incubation on horse blood agar.

B. Colonies of the "smooth" strain NCTC 2836 after overnight incubation on horse blood agar.

A

B



x 1

were enveloped in this electron-dense material (see Plate 4). In infusion broth the cells of the mucoid isolate (NCTC 2836), which was apparently non-mucoid in this medium, were free from enveloping mucoid material and a clearly defined capsule was seen (see Plate 5). It therefore appears that mucoid and non-mucoid growth represent the two ends of the scale of mucus production and that production of large quantities of this extracellular mucoid material was not confined to strains which produced large numbers of spores.

3. Comparison of the sensitivity patterns of good sporing, weakly sporing and heat-resistant strains to a number of antibacterial substances

Method. The following organisms were used:-

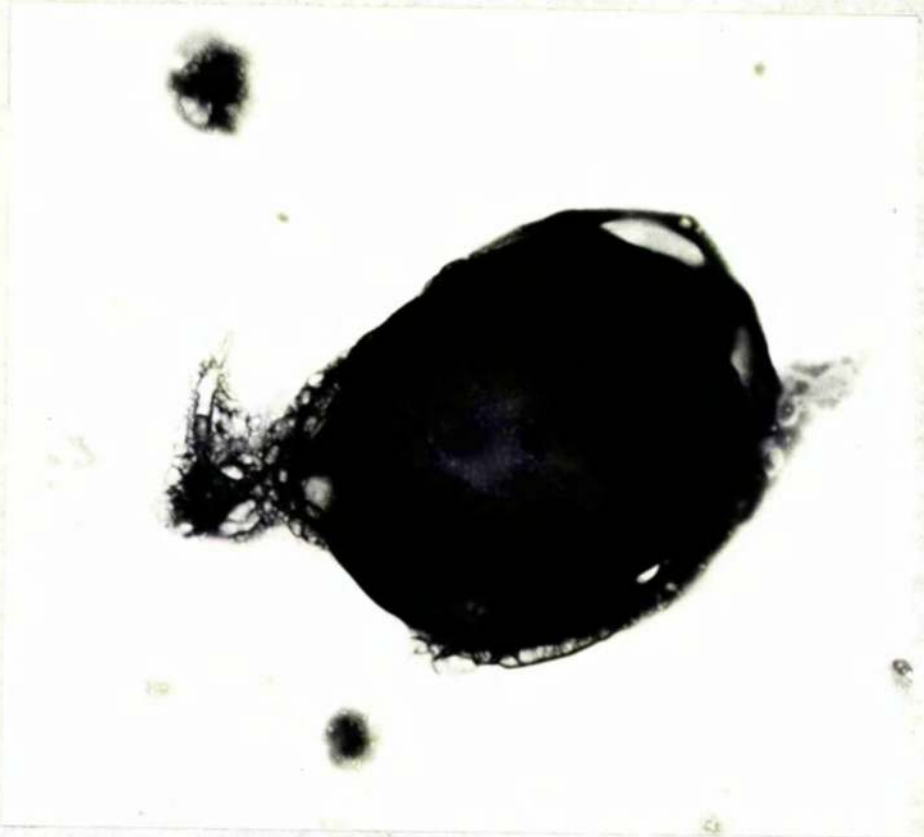
1. Heat-resistant, weakly sporing isolates NCTC 8235, NCTC 8798 and NCTC 8799.
2. Beta-heamolytic, weakly sporing isolates NCTC 2837 and NCTC 8246.
3. Beta-haemolytic, good sporing isolates 032, 19 and NCTC 2836.

Approximately 2ml of an overnight culture in Robertson's meat broth of each of these strains was used to seed the surfaces of a number of horse blood agar plates. Before seeding the plates were dried for two hours in an incubator at 37°. After seeding the plates were dried at 37° and the antibacterial substances (see Table 33) applied in disc or tablet form. Plates were read after anaerobic incubation at 37° for 18 hours.

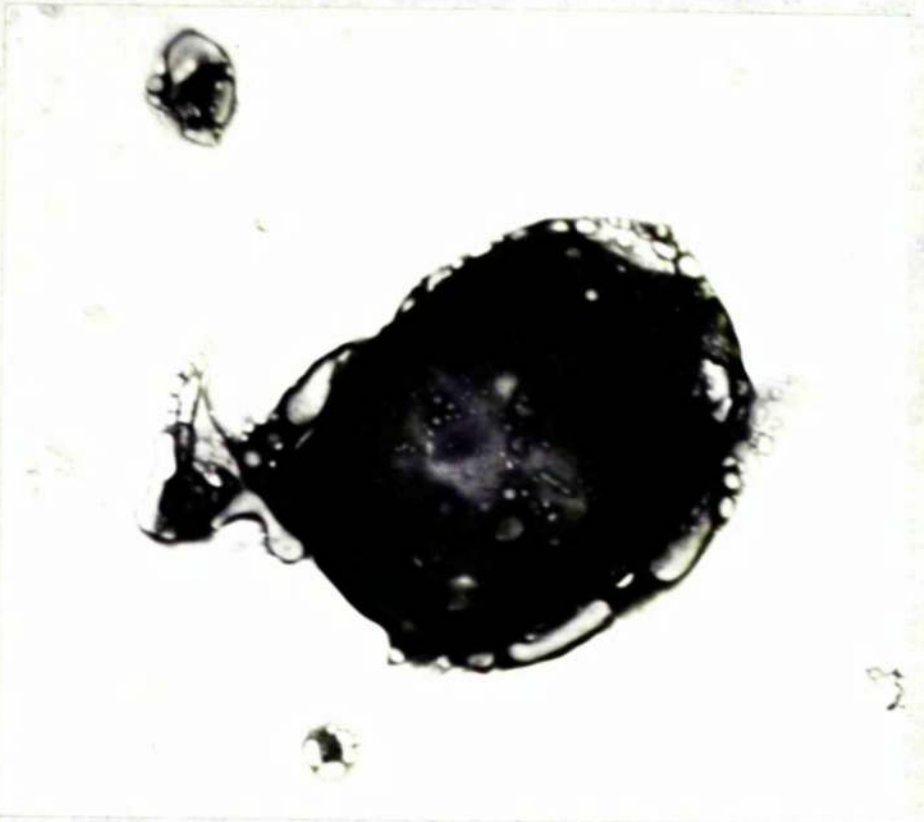
PLATE 4

Electron micrographs of a spore of isolate 032 produced in Ellner's medium showing "vacuolation" of the mucoid material surrounding the spore. Photograph A was taken as soon as the spore was visualised and photograph B a few seconds later.

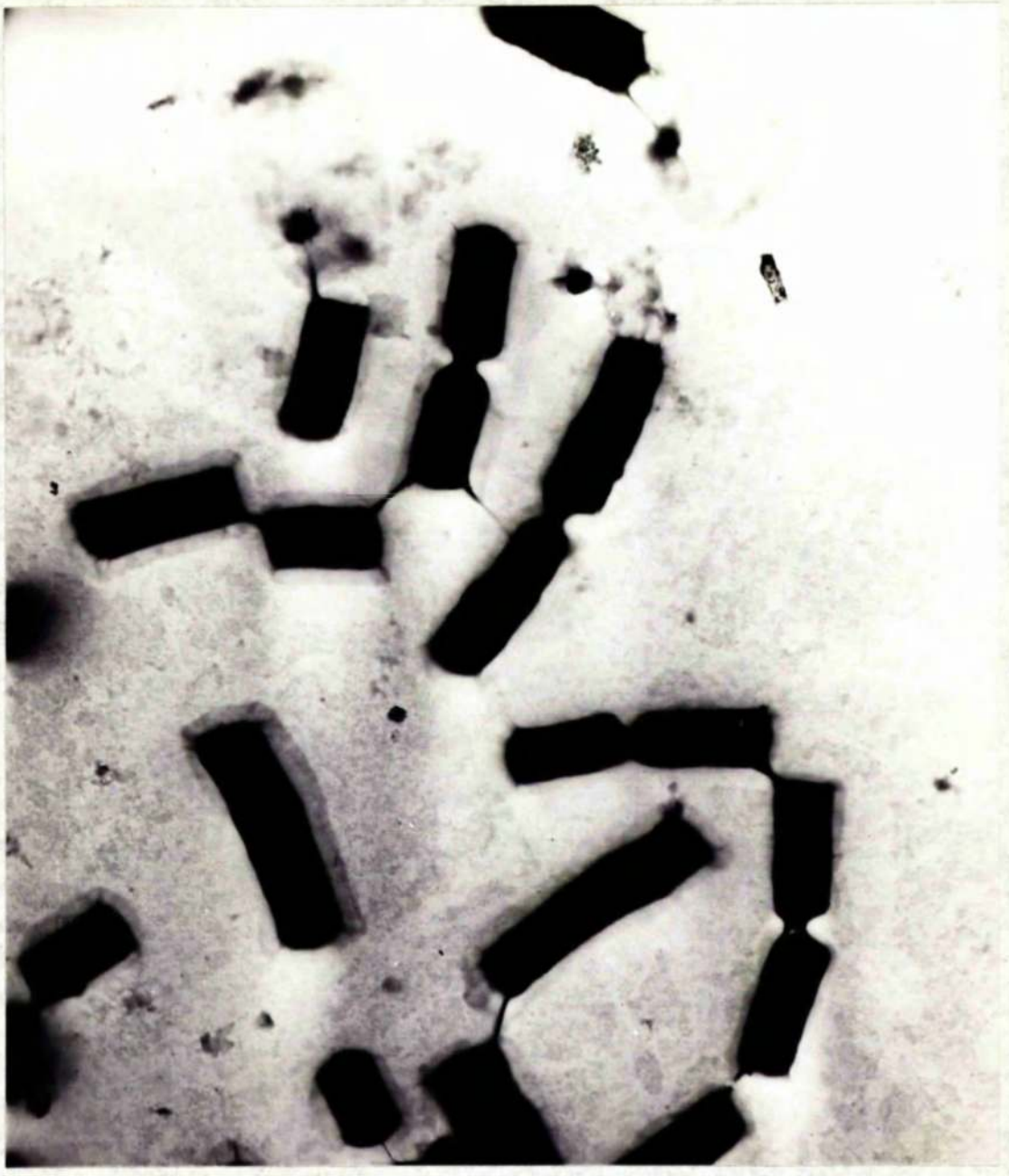
A



B



x 11400



x 7400

PLATE 5

Electron micrograph of vegetative cells of isolate NCTC 2836 grown in infusion broth. Note the presence of a well defined capsule and absence of mucoid material.

TABLE 33

Antibacterial substances

Antibacterial substance	Strength (µg)	Manufacturer
Penicillin	1.5*	1
Methicillin (Na^+ salt)	10	1
Ampicillin	10	5
Phenethicillin	5	6
Chloramphenicol	50	1
Erythromycin	10	1
Streptomycin	10	1
Neomycin	50	1
Terramycin	50	1
Polymyxin B	25	1
Polymyxin E	300	5
Methenamine mandelate	2.5	1
Sulphafurazol	500	1
Nitrofurantoin	200	1
Framycetin	50	1
Aureomycin	100	2
Oleandomycin	2	2
Novobiocin (Na^+ salt)	15	3
Albamycin T (novobiocin 15µg and tetracycline hydrochloride 15µg)	-	3
Paromomycin sulphate	30	4
Sulphamethoxypyridazine	150	4
Bacitracin	8*	5

TABLE 33 (Continued)

Antibacterial substance	Strength (μg)	Manufacturer
Kanamycin	30	5
Nystatin	50*	5
Optochin	5	5
Fucidin	10	7
Nitrofurazone	2	8
Furaltadone	50	9

*The strength of these preparations is expressed in units

1. The Oxoid Division of Oxo Limited, London, E.C.4.
2. Evans Medical Limited, Speke, Liverpool, 24.
3. The Upjohn Company, Kalamazoo, Michigan, U.S.A.
4. Park Davis and Company, Staines Road, Hounslow, Middlesex.
5. Mast Laboratories Limited, 67, London Road, Liverpool, 3.
6. Beecham Research Laboratories Limited, Brentford, England.
7. Leo Laboratories Limited, 15-18, Clipstone Street, London, W.1.
8. Menley and James Limited, London.
9. Baltimore Biological Laboratories, Baltimore, 18, Maryland, U.S.A.

Result All nine strains were sensitive to: penicillin, bacitracin, fucidin, chloramphenicol, erythromycin, terramycin, nitrofurantoin, framycetin, aureomycin, albamycin T, furaltadone and nitrofurazone. They were resistant to: kanamycin, nystatin, optochin, polymyxin B, polymyxin E, streptomycin, neomycin, sulphafurazol, sulphamethoxypyridazine, paromomycin sulphate and novobiocin. Non-uniform sensitivities were observed with methenamine mandelate, oleandomycin, methicillin and ampicillin. The sensitivities to these compounds are shown in Table 34, from which it can be seen that sporing and weakly sporing beta-haemolytic and weakly sporing heat-resistant strains cannot be differentiated by this means.

4. Comparison of the ability of good and weakly sporing strains to produce proteolytic enzymes

Methods.

Gelatin and peptone were used as substrates to compare the proteolytic activity of the sporogenous strain NCTC 2836 and the oligosporogenous strain NCTC 8798.

Gelatinase activity. Production of gelatinase by growing cultures was assayed using the macrocolony technique described by Frazier (1926). The gelatinase activity of culture supernatants was determined using a modification of this technique. The medium used for these experiments was 0.4 per cent leaf gelatin in lab-lemco agar (Oxoid).

The medium was distributed in Petrie dishes for assay by the

TABLE 34

The sensitivity of good and weakly sporing beta-haemolytic and weakly sporing heat-resistant strains, to four antibiotics against which a uniform reaction pattern was not produced

Group	Strain	Antibacterial substance			
		Methenamine mandelate	Oleandomycin	Methicillin	Ampicillin
A	NCTC 8235	+	*	+	+
	NCTC 8798	+	*	*	*
	NCTC 8799	−	−	*	*
B	032	−	−	+	+
	NCTC 2836	±	−	+	+
	19	−	−	−	*
C	NCTC 2837	−	−	*	*
	NCTC 8246	±	−	*	*

A Weakly sporing heat-resistant strains
B Good sporing beta-haemolytic strains
C Weakly sporing beta-haemolytic strains
− Resistant
+ Sensitive
± Slightly sensitive
*Resistant colonies observed in zone of inhibition

macrocolony technique. Eight Petrie dishes were used, each of which was inoculated at its centre with one loopful of a four hour culture in Robertson's meat broth. Inoculated plates were incubated anaerobically at 37° overnight.

To detect gelatinase activity in culture supernatants the medium was distributed in 12ml amounts on 3¼" x 3¼" lantern slides. Onto the lantern slides were laid antibiotic assay discs (Whatman 6mm) loaded with:-

1. Four and twenty four hour old culture supernatants of each organism in Robertson's meat broth.
2. Four hour culture supernatants of each organism in 1 per cent (w/v) glucose broth,

so that each culture supernatant was assayed in quadruplet. Lantern slides were incubated aerobically, overnight at 37°, in a humid chamber.

After incubation the unhydrolysed gelatin was precipitated by flooding the plates and lantern slides with a solution of 15 per cent (w/v) mercuric chloride in 20 per cent (v/v) hydrochloric acid. The plates and lantern slides were then washed in tap water and the diameter of each clear zone measured by taking two readings at right angles.

Peptidase activity. Previous experiments have shown that strain NCTC 2836 will sporulate in a solution of 1 per cent (w/v) peptone (Oxoid L.37) in the buffer component of Ellner's medium. Attempts were

therefore made to compare peptone degradation by sporogenous and oligosporogenous strains in this medium. The macrocolony technqiue on the medium solidified with agar was unsuccessful, as the medium would not support the necessary vegetative growth for macrocolony formation. Several attempts to demonstrate peptone degradation using the lantern slide technique were also unsuccessful. Peptone degradation was therefore followed by estimating the formation of alpha-amino groups and ammonia nitrogen using tube techniques.

Alpha-amino groups were estimated by the photometric method described by Moore and Stein (1948) and ammonia nitrogen using the technique described by Umbreit, Burris and Stauffler (1957). The estimation of alpha-amino groups was based on the knowledge that ninhydrin reacts with alpha-amino acids, converting the amino acid to the corresponding aldehyde with one less carbon atom, carbon dioxide and a compound containing the nitrogen from the amino acid. This compound is coloured between pH 5 and 7. Ninhydrin also reacts with the terminal alpha-amino groups of proteins, although with proteins neither aldehydes nor carbon dioxide are formed (Saidel 1957). Because ninhydrin reacts only with the terminal groups of peptides and proteins, the colour produced by a given concentration of protein is dependent upon the chain length. The shorter the chain the more terminal amino acids are present and the more intense the colour produced.

The colours produced in 10ml amounts of the peptone medium after

inoculating with 1ml amount of four hour old cultures of strains NCTC 2836 and NCTC 8798 in Robertson's meat broth, followed by overnight anaerobic incubation at 37°, were compared. The intensity of the colour produced in the undiluted medium was too great to be read directly and the medium had to be first diluted one-hundredfold in distilled water. This was found to give less variation between replicate samples from the same culture, than when the samples were diluted after addition of ninhydrin.

Control experiments were first carried out to determine the effect on the intensity of the colour produced, of adding to the peptone medium:-

1. Sterile Robertson's meat broth.
2. Culture supernatants from four hour cultures of strain NCTC 2836 in Robertson's meat broth.
3. Culture supernatants which had been heated at 100° for 3 minutes to destroy any enzymic activity.
4. Four hour whole cultures in Robertson's meat broth after heating at 100° for 3 minutes to kill the organisms.

Each experiment was carried out in duplicate and two samples were taken from each tube for the colour reaction. The results and analysis of this experiment are presented in Tables 35 and 36, from which it can be seen that the slight variation in optical density between the uninoculated medium and the four controls, was not significant at the 95 per cent level.

For the experiment to compare peptone degradation by good and weakly sporing strains, isolates NCTC 2836 and NCTC 8798 were used.

TABLE 35

The effect on the optical density of a peptone medium of incubating with fresh Robertson's meat broth and four hour culture supernatants of strain NCTC 2836 (optical densities were determined after addition of ninhydrin)

Inoculum	Experiment number	Sample number	Optical density	Mean optical density		
				Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Uninoculated medium	1	1	0.518	0.520	0.507	0.533
		2	0.522			
Fresh Robertson's meat broth	1	1	0.530	0.534	0.516	0.552
		2	0.550			
	2	1	0.521			
		2	0.536			
Supernatant from a four hour culture	1	1	0.530	0.534	0.516	0.552
		2	0.536			
	2	1	0.534			
		2	0.536			
As above after heating at 100° for 3 minutes	1	1	0.535	0.533	0.507	0.558
		2	0.530			

TABLE 36

The effect on the optical density of a peptone medium of incubating with a heat killed four hour culture of isolate NCTC 2836. (optical densities were determined after addition of ninhydrin)

Inoculum	Experiment number	Sample number	Optical density	Mean optical density		
				Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Uninoculated medium	1	1	0.425	0.433	0.413	0.452
		2	0.440			
Heat killed four hour culture	1	1	0.457	0.451	0.380	0.522
		2	0.442			
	2	1	0.454			
		2	0.452			

1ml amounts of a four hour culture of each organism in Robertson's meat broth were used to inoculate 10ml amounts of the peptone medium. The controls used were:-

1. Uninoculated peptone medium.
2. Peptone medium inoculated with the appropriate heat-killed cultures.

The second of these controls was used, as it has previously been shown that this gave a higher mean optical density than the uninoculated medium. Cultures were incubated anaerobically at 37° for 18 hours, when the relative amounts of amino groups in each of the cultures was determined. Each experiment was carried out in duplicate and two samples were taken from each tube for the colour reaction.

Results

A wide zone of clearing was produced on the gelatin medium by a colony of NCTC 2836, while the zone produced by NCTC 8798 was scarcely larger than the colony itself. Using the lantern slide technique and applying culture supernatants on antibiotic assay discs, the width of the zone of clearing was dependent upon the age of the culture, the culture medium and the strain used (see Table 37). Supernatants from 48 hour old cultures of both strains in Robertson's meat broth, produced zones of 10mm diameter. With supernatants from four hour cultures in Robertson's meat broth, the width of the zone was dependent upon the strain. The sporogenous strain produced a zone of

TABLE 37

Breakdown of gelatin by culture supernatants of a good sporing (NCTC 2836) and a weakly sporing (NCTC 8798) isolate

Culture supernatant	Mean diameter of zone produced by isolate	
	NCTC 2836	NCTC 8798
48 hour Robertson's meat broth	10mm	10mm
4 hour Robertson's meat broth	14mm	8mm
4 hour glucose broth	7mm	9mm

clearing of 14mm diameter, while the diameter of the zone produced by the oligosporogenous strain was only 8mm. With four hour cultures in glucose broth the position was reversed, the sporogenous strain produced a zone of 7mm, compared with 9mm by the oligosporogenous strain.

The results of the experiment comparing the formation of alpha-amino groups by a good and a weakly sporing strain in a buffered peptone medium, is shown in Table 38. Live cultures of both strains produced an increase in optical density over the controls. The increase by the good sporing strain over its heat-killed control was 15.3 per cent (0.451 to 0.520), compared with an increase of 9.4 per cent (0.467 to 0.511) by the weakly sporing strain. The significance of these values was determined using Snedecor's variance ratio test (F). The analysis of the results presented in Table 38 using this test are shown in Table 39. The value of F obtained (8.82) is significant at the 95 per cent level, showing that the difference in peptone degradation between the two strains was significant.

In the buffered peptone medium both strains produced negligible amounts of ammonia nitrogen (see Table 40).

TABLE 38

The effect on the optical density of a peptone medium of incubating and heat killed cultures of strains NCTC 2836 and NCTC 8798 (opt densities were determined after addition of ninhydrin)

Strain	Inoculum	Experiment number	Sample number	Optical density
Uninoculated medium		1	1	0.425
			2	0.440
NCTC 8798	heat killed culture	1	1	0.474
			2	0.460
		2	1	0.471
			2	0.463
	live culture	1	1	0.513
			2	0.516
		2	1	0.509
			2	0.506
NCTC 2836	heat killed culture	1	1	0.457
			2	0.442
		2	1	0.454
			2	0.452
	live culture	1	1	0.510
			2	0.515
		2	1	0.538
			2	0.516

TABLE 39

Analysis of the results presented in Table 38

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
A	0.000049	1	0.000049	0.72
B	0.012656	1	0.012656	186.10
A times B	0.000600	1	0.000600	8.82
D	0.000272	4	0.0000680	1.07
E	0.000508	8	0.0000635	

A Between strains NCTC 8798 and NCTC 2836

B Between live and boiled cultures

D Between experiments from the same inoculum

E Between samples from the same experiment

TABLE 40

Formation of ammonia nitrogen in buffered peptone by strains NCTC 2836 and NCTC 8798

Culture inoculated with:	Concentration of ammonia nitrogen (µg/ml)
Uninoculated medium	Control
NCTC 2836	< 5
NCTC 8798	< 5

Discussion

Examination of the variation in sporulation of two "races" derived from a single strain showed that there was a tendency for the variants to breed true. However, one of the ten cultures derived from the weakly sporing "race" by plating out, reverted to the good sporing state. This high frequency of reversion supports the original observation that when single colonies were selected from a horse blood agar plate, the frequency with which cultures derived from these colonies produced lower numbers of spores than the parent culture, was higher than would be expected by mutation. That sporulation is a response to environment is undoubtedly true. It is also true (see page 67) that the medium in which the organism is grown prior to inoculation of the sporulation medium has a pronounced effect on sporulation. In the present instance, it appears that a single subculture on horse blood agar can alter the metabolism of a high proportion of the cells sufficiently to reduce sporulation in cultures derived from these cells. A somewhat similar observation has previously been reported by Powell and Hunter (1955), who obtained asporogenous cultures of B.sphaericus by repeated subculture on a rich medium. These workers suggested a mechanism for this action which is in agreement with the present findings, namely, that the potential of a culture to form spores may depend on the ability of the culture to divert normal metabolic pathways operative during vegetative growth, into "abnormal" reactions operative during sporulation.

A typical example of such "abnormal" reactions is found in the formation of dipicolinic acid - a compound found only in spores. They suggested that these processes might be enhanced or lowered by adaptive (inductive) enzyme formation on different media.

Of the 48 isolates examined for colonial morphology, comprising 18 heat-resistant and 30 beta-haemolytic strains, four were mucoid and the remainder were smooth. The rough variants isolated from these strains were unstable and it was impossible to correlate sporulation with colonial morphology in these instances. The four mucoid isolates were all beta-haemolytic and three of these produced in excess of 25 per cent spores. By comparison, only five of the remaining 26 beta-haemolytic strains produced more than 25 per cent spores. These findings confirm the statement made by Simmonds (1915a) and Henry (1916-17) that mucoid strains tend to sporulate more readily than other types. However, it cannot be stated that mucoid strains produce abundant spores as this is not invariably the case. Neither was the production of mucoid material entirely confined to strains which were recorded as mucoid by macroscopical examination. Examination of representative strains of each group in the electron microscope, showed that all the strains were enveloped in an amorphous electron-dense material when grown in glucose broth or Ellner's medium. This amorphous electron-dense material is assumed to be the material

responsible for the mucoid manifestation in these cultures on morphological grounds. In addition, the observation of vacuolation in the electron beam fits in well with Svec and McCoy's (1944) finding that the antigenic property of this "capsular polysaccharide" was heat-labile. Furthermore, Ellner's medium is similar to that used by Svec and McCoy for the production of mucoid material and provides the constant alkaline pH advocated by Duguid (1951) for a good capsule producing medium.

Although the smooth to mucoid variation has been observed in apparently pure cultures (Smith 1959), as well as in mixed cultures (Prévot 1949), the strains of the present study bred true over a period of three years. Attempts were not made to induce the smooth to mucoid variation by growing in the presence of mucoid material, as Prévot found that only one of eleven smooth strains could be transformed by this means. It is unfortunate that this smooth to mucoid variation is rare, otherwise this might be a means of inducing sporulation.

Weakly sporing heat-resistant, weakly sporing beta-haemolytic and good sporing beta-haemolytic strains could not be differentiated by means of their sensitivities to antibacterial substances. Weakly sporing heat-resistant and good sporing beta-haemolytic strains did however produce different amounts of proteolytic enzymes. The sizes of the zones of gelatinase activity around paper discs loaded with

culture supernatants, were dependent upon the culture medium and the age of the culture. The largest zone was produced by a four hour culture supernatant from Robertson's meat broth of isolate NCTC 2836. This is the age of the culture routinely used to inoculate the sporulation medium and the diameter of this zone was almost twice that produced by a weakly sporing strain of the same age. Similarly, when whole cultures were used to inoculate a peptone medium, there was a significant difference in the degree of peptone degradation by the two strains.

These results, together with the observation that weakly sporing, heat-resistant strains, did not grow as well as good sporing strains in Ellner's medium, suggest that weakly sporing strains are deficient in certain proteolytic enzymes. The nature of this deficiency will be defined only when a synthetic sporulation medium, in which metabolic pathways can be traced, has been formulated.

SECTION C

Microscopical Observations of Sporulation in Slide Culture

CONTENTS

Introduction

Cytological observations of spore formation by Cl.perfringens type A have been published by Smith and Ellner (1957) and Cash and Collee (1962). These workers incubated cultures in Ellner's medium and withdrew samples at either hourly or half-hourly intervals. Smith and Ellner stained films prepared from these samples by a number of techniques, while Cash and Collee followed spore formation by the resistance of the culture to desiccation, thiomersalate and heat. From these results sporulation can be divided into three stages. During the first stage each cell of the inoculum undergoes approximately four divisions. This is followed by the appearance of a forespore and the gradual maturation of this structure. From the viable counts and turbidity readings presented by Cash and Collee, it appears that following the division phase approximately one half of the vegetative cells lysed. The remaining cells then formed spores.

Although it is obviously impossible to follow the sequence of events occuring within a single cell using this technique, the objections to these observations on whole cultures are to some extent countered by the findings of these workers that sporulation occured with a fairly high degree of synchrony. Nevertheless, it is impossible to determine from observations on whole cultures, whether the cells which lysed and the cells which formed spores, originated from the same or different mother cells. More information could be

obtained by studying sporulation in single cells. The work which is described in the following section was designed with this end in view.

The technique used was microscopic observation in slide culture using solid Ellner's medium. The difficulties which Cash and Collee encountered in obtaining high spore yields in Ellner's medium did not apply with the organism used in the present study (strain NCTC 2336). With this organism consistent and reproducible high spore yields were obtained.

The experiments using this technique fall into three groups, these are:-

1. A study of multiplication in slide culture.
2. Estimation of the proportion of cells forming spores in slide culture.
3. A study of the sequence of events in cells sporing in slide culture.

Materials and Methods

Organism. Isolate NCTC 2836 was used throughout this section.

Media. Ellner's medium was prepared as described on page 66 and solidified by adding agar to a final concentration of 2 per cent (w/v). This medium was distributed in 10ml amounts in universal containers and sterilised by autoclaving at 15lb/sq in for 15 minutes.

Photomicrography. An Edixa Model B. camera body and extension bellows were coupled with Pentax extension tubes and microscope adaptor. Adox K.B. 14 film was used. Photographs were taken using a phase contrast microscope (Baker, Series 4. Modern Research Microscope, with Trilux condenser and Projectolux illumination).

Procedure. Cells were incubated aerobically overnight in Robertson's meat broth at 37°. Approximately 2ml of this culture was used to inoculate a fresh Robertson's meat broth. After four to six hours incubation, 1ml of this culture was used to inoculate 10ml molten Ellner's medium - cooled to 45° in a water bath. A few drops of this inoculated medium was taken up in a warm, sterile, Pasteur pipette and transferred to a glass microscope slide - prewarmed on a hot plate to approximately 40°. A warm glass coverslip was laid on top of the culture and excess fluid removed by carefully squeezing the slide and coverslip together. This produced a thin film of the culture and

confined organisms to the same plane (for microscopy).

The slide culture was sealed round the edge with vaseline (to exclude oxygen) and placed in position under the oil immersion lens of the phase contrast microscope at 37°. The microscope was placed in the 37° room at least one hour before the slide was placed in position, thus ensuring that the microscope stage was at 37°.

Several series of experiments were carried out and the cultures observed at regular intervals.

Experimental

1. A study of multiplication in slide culture

Method. Cells in slide culture were observed at half-hourly intervals over the period until cell division stopped. The number of cells in each nidus was counted at each observation.

Results. In six experiments a total of forty cells, singly or in groups of two, were observed. In each instance cells had ceased to divide after 3 hours in the sporulation medium. Table 41 records these results. In this table the term clone is used to describe a group of cells derived from an original nidus of one or two cells.

It can be seen from Table 41 that the fate of any particular cell or its progeny, does not conform to a simple pattern. The following possibilities are covered:-

1. No cell division occurs.
2. Division of the cells of the nidus occurs up to three hours.
3. One of the cells of the nidus fails to divide.
4. One division of the nidus occurs, but some cells fail to undergo subsequent division.
5. After division some of the cells of the clone lyse.
6. After division all of the cells of the clone lyse.

After 3 hours in Ellner's medium the 40 cells studied had divided to form 153 cells, of which 20 had lysed. On the average then, each cell underwent two divisions and 13 per cent of the final number lysed. This

TABLE 41

Multiplication of cells of isolate NCTC 2836 in slide culture in Ellner's medium

Experiment number	Number of cells per clone after stated time interval (in hours)						
	0 (Nidus)	½	1	1½	2	2½	3
1	2	2	2	2	2	2	2
	1	1	2	2	2	2	2
	1	1	2	2	2	2	2
	1	2	2	2	2	4	4
	1	1	2	2	2	3	4
	2	2	3	3	3	4	4
2	1	1	2	2	2	4	4
	1	1	2	2	4	4	4
	1	1	2	4	4	4	4*
	1	2	2	2	2	2*	2*
	1	1	1	4	4	4	4
	2	2	4	4	4	4	7
	1	1	1	3	3	3	3*
	1	1	1	2	2	2*	2*
3	1	1	2	3	3	3	3
	1	1	2	4	4	4	4
	2	2	2	4	4	4*	4*
	1	2	2	4	4	6	6
	1	1	2	2	2	2*	2*
4	2	-	5	5	5	5	5
	2	-	5	5	5	5	5
	2	-	4	7	7	7	7
	2	-	4	8	8	8	8
5	2	-	9	-	-	-	26
	2	-	4	-	-	-	13
	2	-	4	-	-	-	16
6	1	1	2	4	4	2+2*	2+2*
	2	2	2	3	4	4	3+1*

- No observation

*Lysed cells

is a fair summation of the results, as of the twenty eight clones studied, the cells of one clone did not divide, the cells of eight clones underwent one division, the cells of fifteen clones underwent two divisions, the cells of three clones underwent three divisions and the cells of one clone underwent four divisions. Thus, the majority of cells underwent two divisions.

Furthermore, it can be seen from the results presented in Table 41, that the number of divisions undergone by each nidus varied in different experiments. Thus, in experiments 1, 2, 3, 4 and 6, the majority of cells underwent two divisions. In experiment 5 however, the cells of two nidi underwent three divisions, while those of the remaining nidus underwent four divisions. For each experiment a fresh culture in Robertson's meat broth was used to inoculate an aliquot from the same batch of Ellner's medium. Therefore, it appears that the number of cell divisions undergone in each experiment, is dependent upon the physiological state of the cells at the time of inoculation of the sporulation medium. This variation in the number of cell divisions in separate experiments will, obviously, affect the total number of spores produced in these experiments. Examples of this behaviour have been recorded earlier in the thesis.

2. Estimation of the proportion of cells forming spores in slide culture Method. An examination was made of twenty one clones grown for 18 hours

in slide culture. By this time cell division and sporulation were complete. The total number of cells in each clone and the number of spores in each clone was then counted.

Results. The ratio of the number of spores per clone to the total number of cells in the twenty one clones examined, is expressed in Table 42. Sporulation in this experiment was 60 per cent and is similar to the values obtained with this organism in liquid cultures.

3. A study of the sequence of events in cells sporing in slide culture

Repeated observations of the cells which formed spores, showed that the first indication of sporulation was the appearance of forespores. These appeared as phase-dark areas at the ends of cells after 2½ to 3 hours incubation. In the course of the next 3 hours the spores matured, with the conversion of the phase-dark areas to phase-bright spores. At the onset of forespore formation, the mother cell appeared to swell slightly and the protoplasm became less phase dense.

A photographic record of cell division and sporulation in two clones is shown in Plate 6. The clones in this series are labelled A and B and sporulation progressed as follows:-

Clone A. This clone originated from a single cell. By 2 hours four cells had been formed. By 3 hours, forespores were apparent in two of the cells (both derived from the same parent cell) and

TABLE 42

Estimation of the proportion of cells of isolate NCTC 2836 forming spores in slide culture

Number of spores per clone	Number of cells per clone
4	4
4	6
5	6
3	7
5	7
6	7
4	8
4	8
4	8
4	4
4	6
5	6
3	7
5	7
6	7
4	8
4	8
4	8
7	7
4	9
6	9
7	10
9	10
6	11
6	13
7	13
9	15
7	16
9	16
8	17
Total 124	207

PLATE 6

Sporulation of isolate NCTC 2836 in solidified Ellner's medium in slide culture.

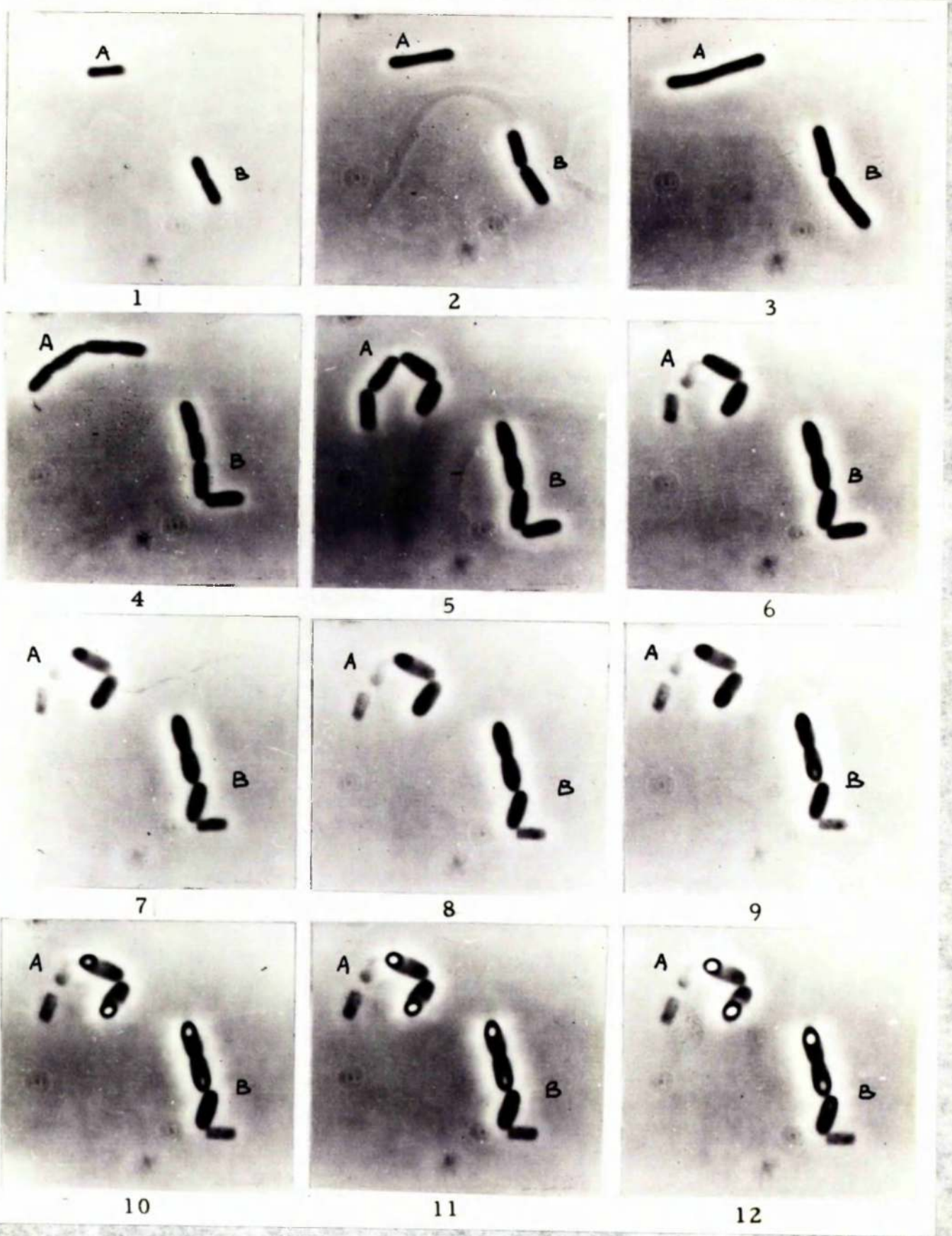
Photographs were taken at intervals of 30 minutes.

1 to 5. During this period of two hours the cells underwent cell division.

6. ($2\frac{1}{2}$ hours). Two of the cells of clone A have begun to lyse. Forespores, dark areas at the end of the cell, can be seen in the two unlysed cells of clone A.

7. (3 hours). The bottom cell of clone B has begun to lyse. Forespores can be seen in the remaining cells.

8 to 12. During this period, $3\frac{1}{2}$ to $5\frac{1}{2}$ hours after inoculation of the medium, the forespores gradually developed into mature, phase-bright, spores.



x 1600

the other two cells had lysed. The forespores, which arose terminally, at the end of the cell distant from the point of division, matured over the next 2 to $2\frac{1}{2}$ hours.

Clone B. This clone originated from a cell in the course of division. By $2\frac{1}{2}$ hours, four cells had been formed. Forespores had appeared in three of the four cells at the end of 3 hours, while the remaining cell had begun to lyse. Maturation of the spore in two of the cells (both derived from the same parent cell) was more rapid than in the third cell whose sister cell had lysed. Spores were again formed at the end of the cell distant from the point of division.

Discussion

These observations on the sporulation of Cl.perfringens in slide culture, confirm the findings of Smith and Ellner (1957) and Cash and Collee (1962), that vegetative cells in Ellner's medium divide for approximately three hours. At this time the first evidence of sporulation is indicated by the appearance of forespores. The number of divisions undergone by any one cell varied between none and four, although the majority of cells divided twice. Lysis took place between two and three hours after inoculation of the medium. The points not previously illustrated are: that approximately 50 per cent of the cells in each clone formed spores, as opposed to the possible existence of distinct sporing and non-sporing clones; similarly, lysis of cells was not confined to distinct clones.

The most striking observation is that spores were invariably formed at the end of the cell furthest away from the point of division. This is undoubtedly an important point, which has been overlooked because of the widespread use of liquid cultures for sporulation and the study of spore cytology by preparing films from these cultures. Photographs of cells sporulating in microcultures published by Knaysi (1951, 1952, 1957) and Bayne-Jones and Petrilli (1938), unfortunately do not show cells during the phase of cell division. Nevertheless a diagrammatic representation of sporulation by Knaysi (1951), shows the

spores in two daughter cells at the end of the cells furthest away from the point of division. Whether this is fortuitous or intentional is not indicated. This phenomenon is probably associated with the position of the genes controlling sporulation on the bacterial chromosome and the orientation of this body in the daughter cells following cell division. The resolution of this problem therefore awaits further knowledge of the replication and distribution of the bacterial chromosome during cell division.

SECTION D

The Morphology of Cl.perfringens Spores as Revealed by some Simple Electron Micrographs

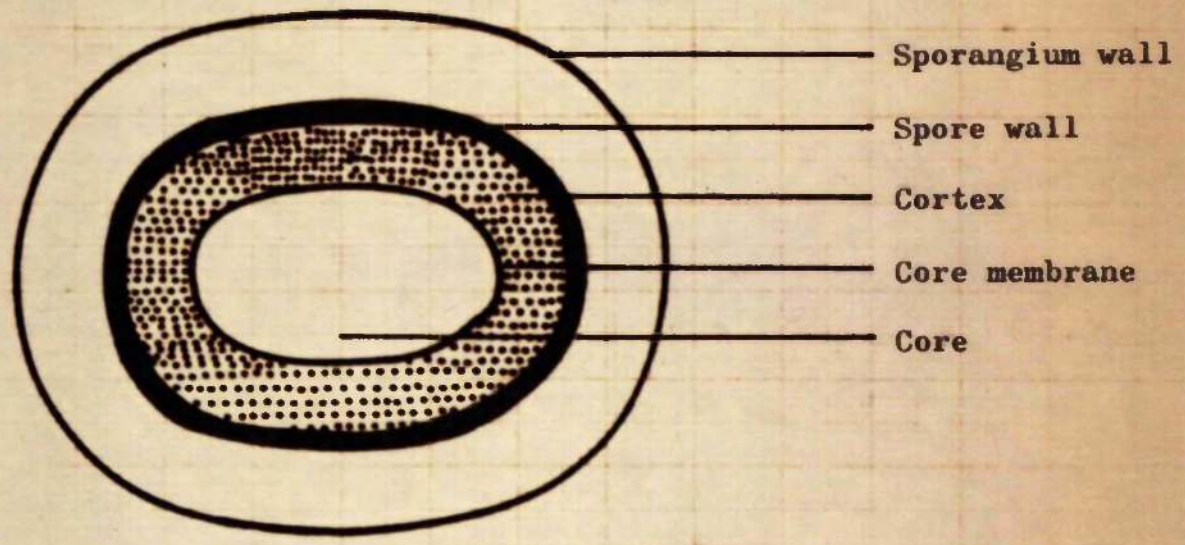
CONTENTS

Introduction

Although many workers have observed spores of Cl.perfringens, the only study of spore cytology was carried out by Smith and Ellner (1957). These workers prepared films from sporing cultures which they stained by a number of methods and observed by light microscopy. By this means they were able to define four distinct regions of the spore: the spore wall, cortex, core membrane and core (see Figure 25). While the existence of these structures is not questioned, the method of choice for such studies, is the electron microscopy of spore sections. Although it was not intended in this thesis to carry out such a detailed examination of the structure of the spore, it was thought that it might be possible to demonstrate the presence or absence of an exosporium, by examining whole spores in the electron microscope. The term exosporium is used to describe a sack-like structure found surrounding the spore, i.e. beyond the spore coat or coats but within the vegetative cell wall. Such structures have been found surrounding spores of B.cereus, B.mycoides, B.anthracis and B.thuringiensis, where the exosporium can be seen protruding beyond the poles of the spore. The exosporium was thought at one time to be the remains of the wall of the mother cell (Robinow 1960). Electron microscopy has now shown that the exosporium arises, together with the spore, from the cytoplasmic membrane of the mother cell (Chapman 1956, Young and Fitz-James 1962).

Fig. 25

Diagrammatic representation of the mature spore of Cl.perfringens (after Smith and Ellner 1957)



The term exosporium has also been used to describe a capsule-like zone on spores of B.megaterium (Tomcsik and Baumann-Grace 1959).

Materials and Methods

Organism. Spores of isolate NCTC 2836 were prepared by inoculating 100ml Ellner's medium with 10ml of a four hour culture of the organism in Robertson's meat broth. Ellner's medium was incubated anaerobically at 37° for 18 hours, when the cells were spun down and washed ten times with sterile distilled water. This spore suspension was divided into two batches. Vegetative debris was removed from one of these preparations using the technique described in Appendix Two.

Electron microscopy. A Phillips E.M. 100 electron microscope was used. Carbon or formvar grids were loaded by dropping one drop of an aqueous spore suspension onto the grid, from a Pasteur pipette. On exposure to the electron beam, the washed preparations exhibited the vacuolation phenomenon described previously. This was overcome by adding formaldehyde to the culture at a final concentration of 10 per cent (w/v) and standing overnight at room temperature. The culture was then spun down, resuspended in distilled water and grids prepared. Clean spores did not vacuolate on exposure to the electron beam.

Results

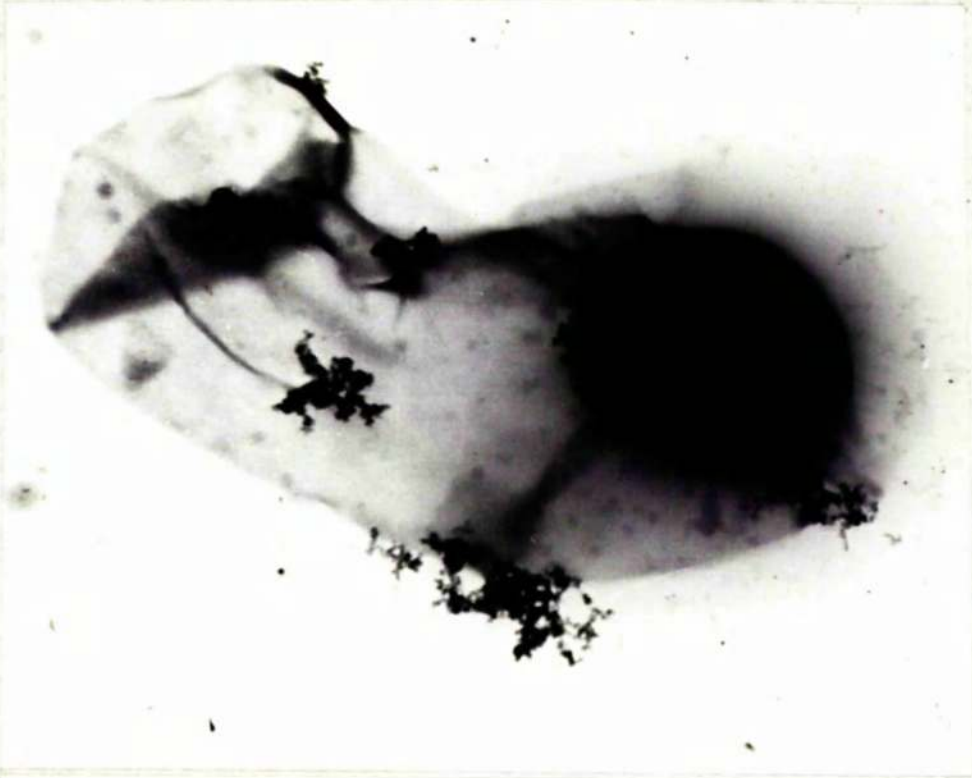
Electron micrographs of formalin-treated cells grown in Ellner's medium are shown in Plates 7, 8 and 9. In these photographs the cell wall or sporangium appears as a loose membranous sack enclosing a sub-terminal spore. The low density of the sporangium indicates that the contents have been lost, confirming the observation of loss of density of the cell during maturation of the forespore seen in the phase contrast microscope. The area of intermediate density surrounding the spore, seen in Plates 8 and 9, is the exosporium.

Removal of the sporangium and vegetative material by ultrasonic disintegration is illustrated in Plate 10. Sporangia and amorphous debris are absent from this illustration - which is typical of the preparation. Higher power views of this preparation are shown in Plates 11, 12 and 13. The spores seen in these plates are enclosed by exosporia. Occasionally a spore was seen with no exosporium (see Plate 13A). This is presumably due to removal of this structure by the cleaning procedure used. In such instances the edge of the spore is smooth, indicating that the whole of the surface of the spore is smooth.

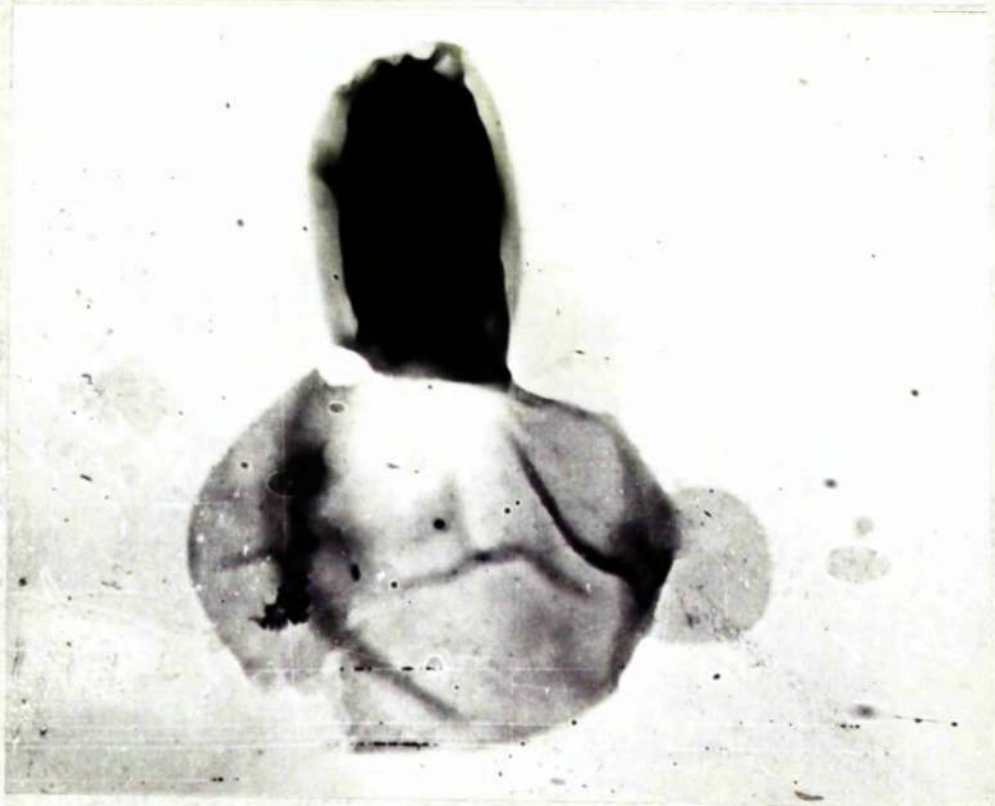
PLATE 7

Electron micrographs of two cells containing spores of isolate NCTC 2836. The spores are the dark oval areas at the right of the plate. The cell wall appears as a membranous sheath.

A



B



x 20100

x 20100

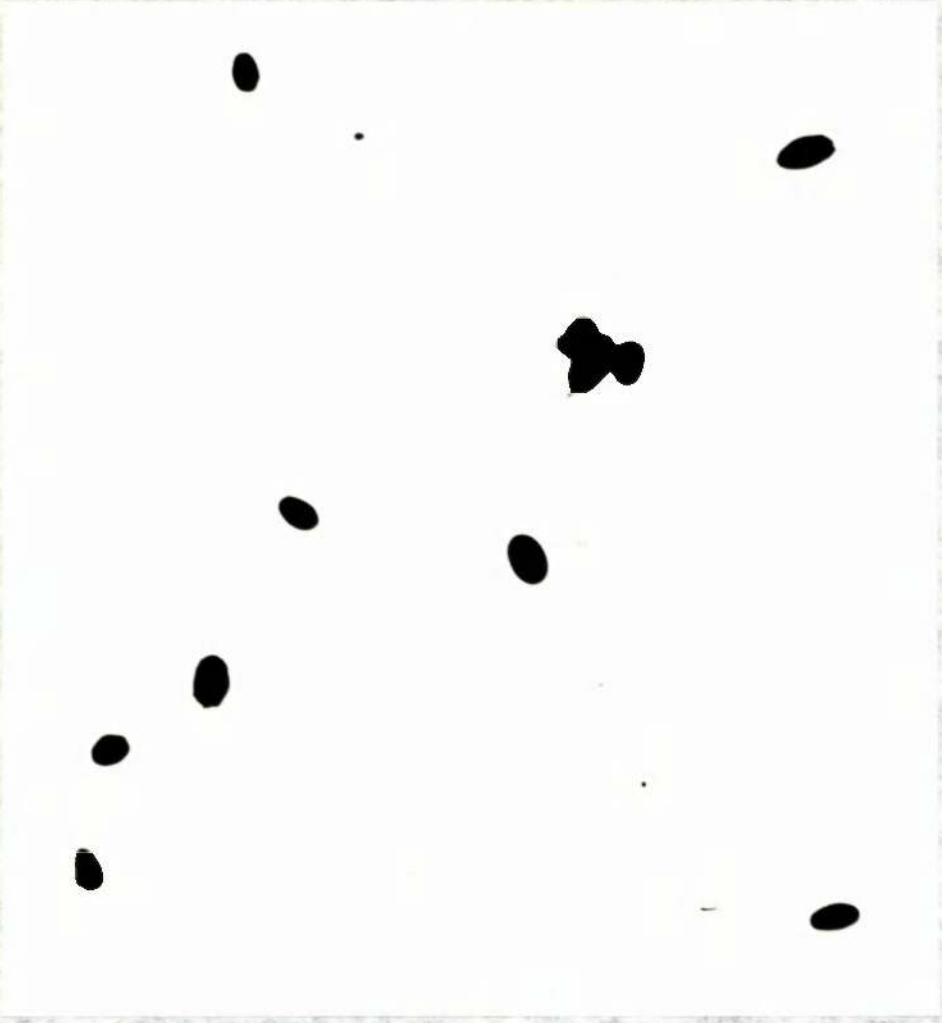
PLATE 8

Electron micrograph of a single cell from a sporing culture of NCTC 2836 grown in Ellner's medium. An exosporium is seen surrounding the spore.

x 42000

PLATE 9

High magnification electron micrograph of the cell in plate 8, showing more clearly the presence of an exosporium.



x 5400

PLATE 10

Electron micrograph of spores of isolate NCTC 2836 after removal of the sporangium by ultrasonic disintegration.

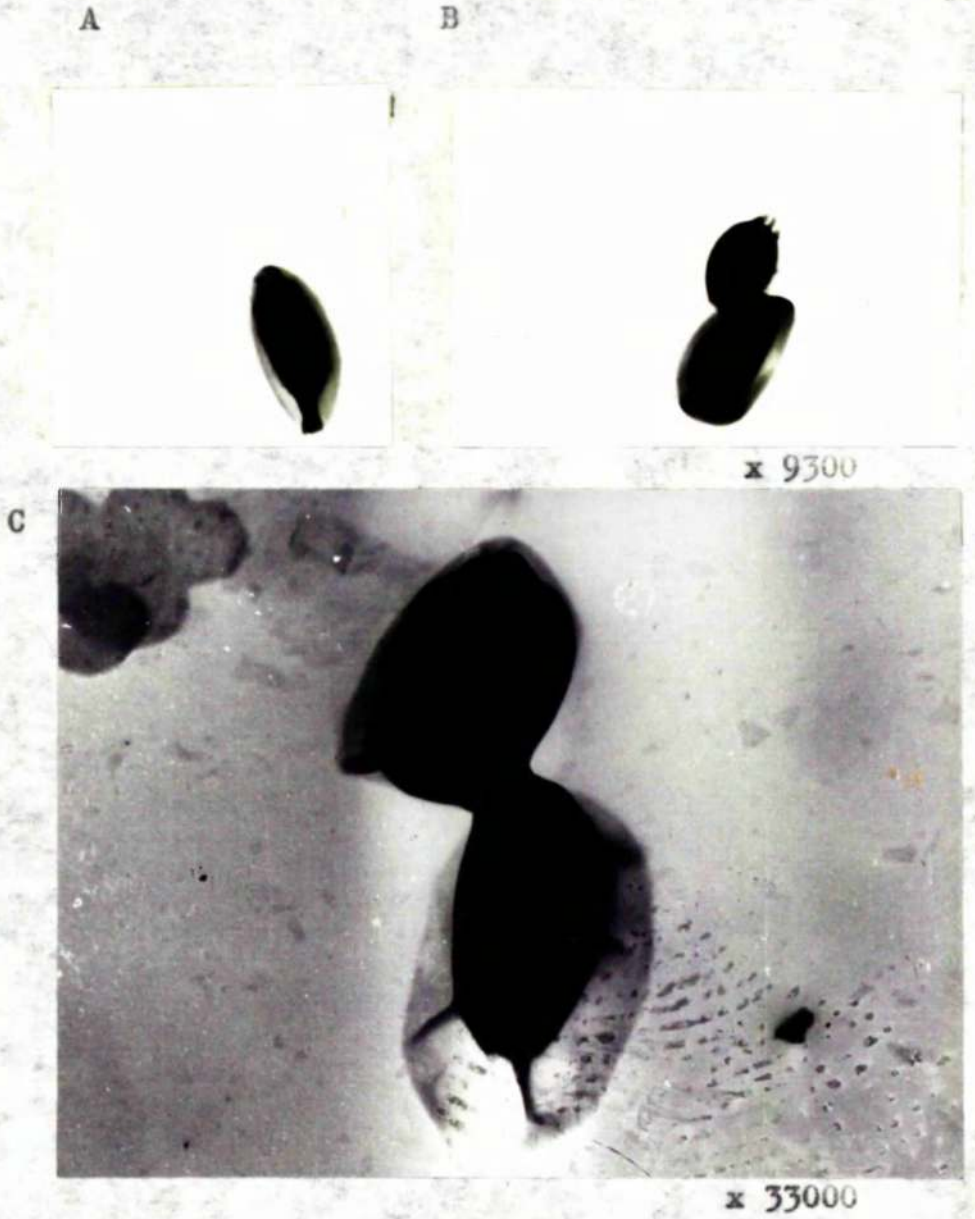


PLATE 11

Electron micrographs of five spores of isolate NCTC 2836 after removal of the sporangium by ultrasonic disintegration. Exosporia are seen surrounding the spores.

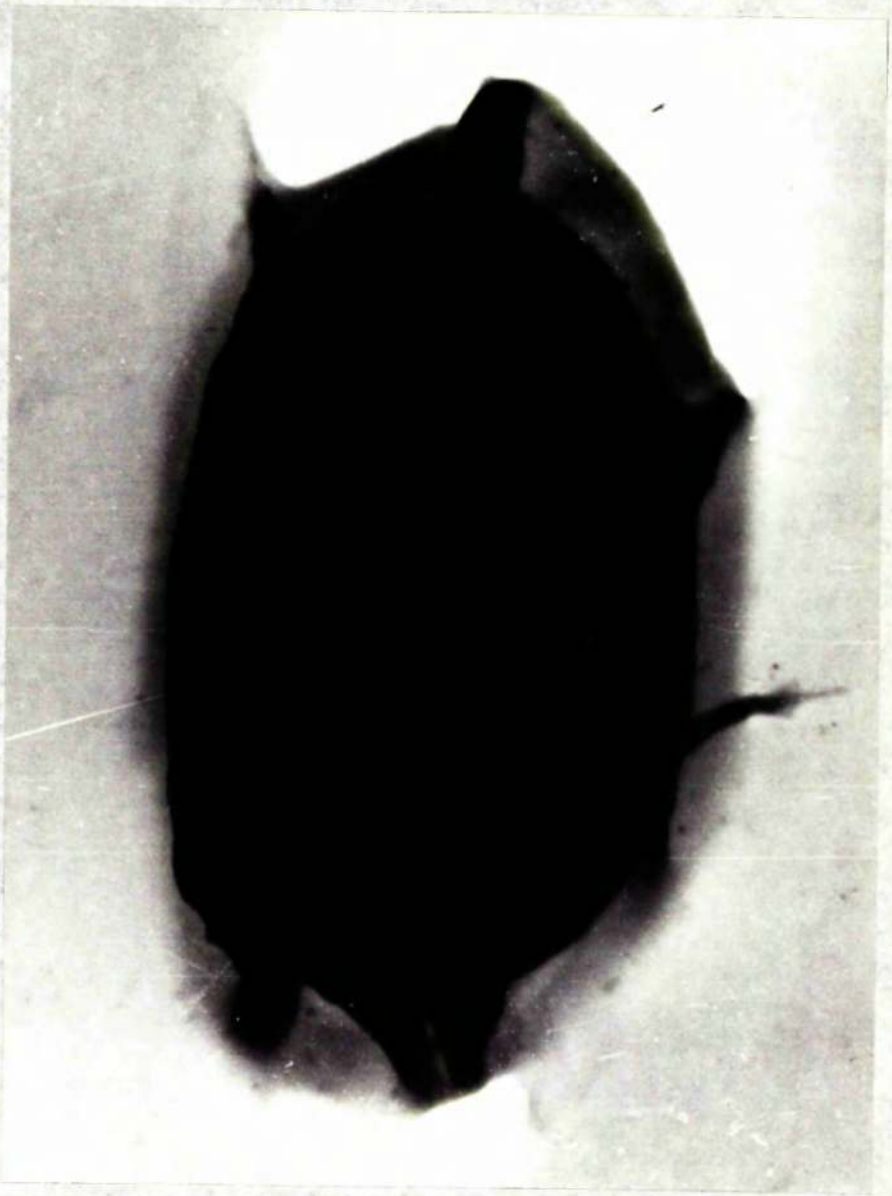
PLATE 12

Electron micrographs of a single spore of isolate NCTC 2836 at two magnifications. The sporangium has been removed by ultrasonic disintegration and a folded exosporium can be seen surrounding the spore.

A

x 20100

B

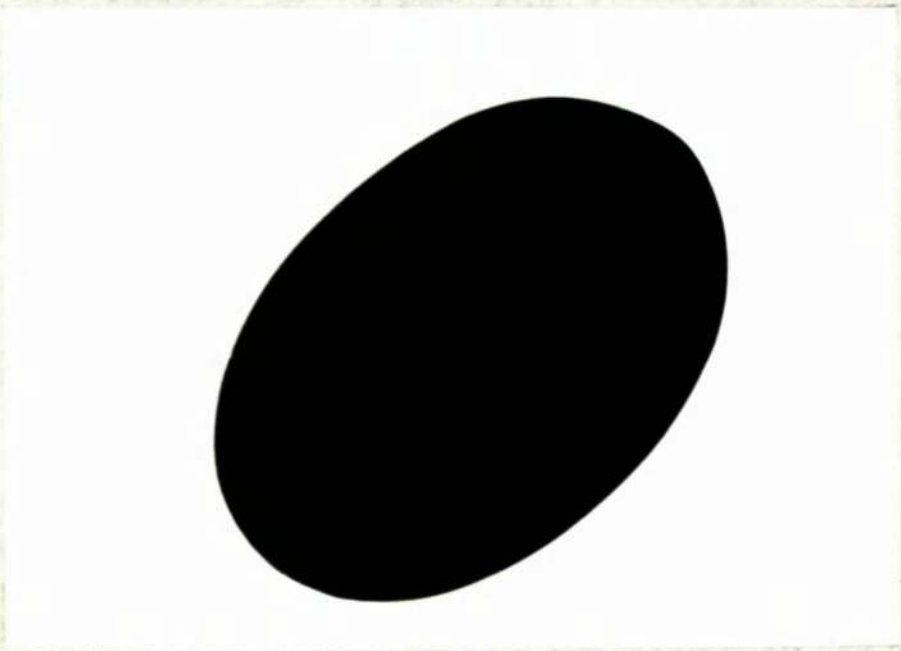


x 67800

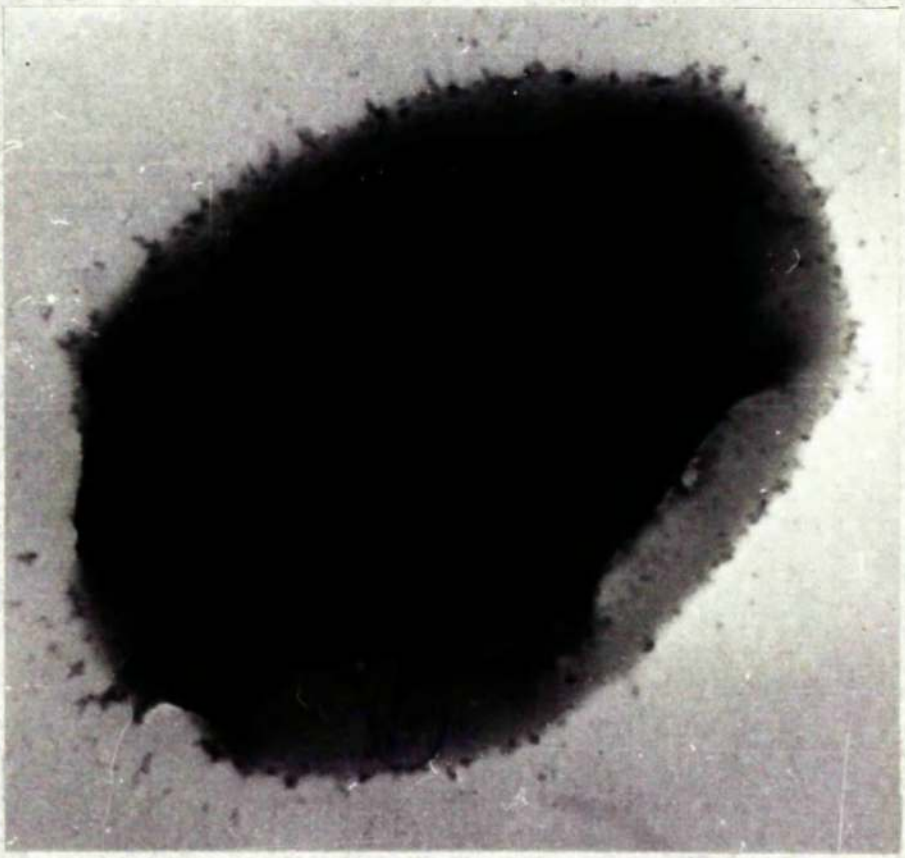
PLATE 13

Electron micrographs of two spores of isolate NCTC 2836. In photograph B ultrasonic disintegration has removed the sporangium and the spore is enclosed within the exosporium. In photograph A ultrasonic disintegration has removed both the sporangium and the exosporium leaving a "clean" spore.

A



B



x 50400

Discussion

These simple electron micrographs of unstained spore preparations have shown the presence of hitherto unreported exosporium. The only previously published electron micrograph of spores of Cl.perfringens (Franklin and Bradley, 1957) does not show this structure, probably because these workers were looking for surface sculpturing using a carbon-replica technique. Furthermore, these workers did not prepare spores free of vegetative material, which explains the rather poor quality of their electron micrographs. In common with the findings of Franklin and Bradley, the surface of the spore of strain NCTC 2836 appeared to be smooth, although the simple techniques used throughout these experiments are by no means the best for demonstrating surface features.

PART II

GERMINATION OF CLOSTRIDIUM PERFRINGENS TYPE A SPORES

CONTENTS

REVIEW OF THE LITERATURE

A vast amount of work has been published dealing with germination of the spores of aerobic spore-bearing organisms. Considerably lower numbers of papers have dealt with anaerobes, while a search of the literature over the last thirty years has revealed only one paper dealing with the germination of Cl.perfringens spores. In view of this, it is not proposed to cover in detail the work concerned with other organisms, but merely to summarise a number of important points which have emerged.

Cytological and biochemical observations of the changes occuring during the breaking of dormancy have led to wide exceptance of the definition of germination proposed by Campbell (1957). Campbell stated that spore germination may be regarded as the change from a heat-resistant spore to a heat-labile entity, which may not necessarily be a true vegetative cell. Further development of this heat-labile entity, leading to the formation of the mature vegetative cell, is called outgrowth.

Briefly, it has been shown that when dormancy is broken, the loss of refractility of the spore (as observed by phase contrast microscopy) coincides with imbibition of water, loss of heat resistance, stainability and dry weight. This decrease in dry weight has been ascribed to: loss of calcium dipicolinate, a number of amino acids and a peptide. On hydrolysis this peptide was found to contain

$\alpha\epsilon$-diaminopimelic acid, glutamic acid, alanine, acetyl glucosamine and an amino sugar.

In order to eliminate incomplete or delayed germination, a sub-lethal heat treatment is often necessary. This phenomenon, known as heat activation or heat shock, has been shown to activate several enzymes which are inactive in the dormant spore and causes spores to lose part of their dipicolinic acid content. These activated spores are less resistant to chemicals and the minimal requirements for germination are reduced. In certain cases heat activation can be replaced by ageing and even chemicals, although ageing of heat-activated spores results in de-activation. Such de-activated spores can be re-activated with a second period of heat treatment.

The specific chemical agents which promote germination and outgrowth have been widely studied, as also have germination inhibitors. The presence of compounds which inhibit germination in complex media used for spore recovery, has led many workers to add starch to these media, or to treat with absorbents such as activated charcoal.

Examination of the germinating agents cited in the literature, indicates that an amino acid (usually L.alanine), glucose and adenosine, occupy key positions. Products of hexose metabolism -pyruvate and its degradation products -nucleotides and nucleosides can also act as germinating agents. Recently however, Rode and Foster (1962a, b,c)

have produced evidence which indicates that spore germination can be "triggered" by low concentrations of inorganic ions. Thus, an isolate of B.megaterium has been found to germinate in aqueous solutions of inorganic salts. A second isolate required in addition to inorganic salts, low concentrations of L-alanine and inosine. In this instance, low concentrations of L-alanine and inosine in deionised water, were devoid of action. They were, however, active in conjunction with a wide variety of salts, and in the presence of salts could be replaced by a number of amino acids which were inactive alone - even in quite high concentrations. These workers have therefore attributed the primary role in germination to inorganic ions and believe that organic agents such as L-alanine and inosine have a secondary augmentative action.

INTRODUCTION

In the work described in the following pages, various environmental factors were selected for study. The first section deals with the influence of air on germination, the rate of germination and the ability of a variety of media - both complex and defined - to promote germination.

In the second section the effect of antibiotics on the growth of Cl.perfringens cultures from spore inocula was studied. This topic was undertaken because in a number of attempts to isolate heat-resistant strains of Cl.perfringens type A from faeces, no growth was obtained in media containing antibiotics. In this work (described in Appendix One), faeces emulsified in infusion broth were heated at 100^{o} for 30 minutes to select out the spores of heat-resistant strains, followed by enrichment culture in Robertson's meat broth containing neomycin. The neomycin medium was used for these experiments, as it was being used routinely as a selective medium for the isolation of beta-haemolytic strains from faeces (Lilly 1958, Willis and Hobbs 1959). Although it was thought that this technique would not affect the number of heat-resistant strains isolated, it can be seen from the results cited in Appendix One, that no heat-resistant isolates were obtained from the thirty six faecal specimens examined. This failure to isolate heat-resistant strains was

surprising, particularly as the carrier rate in such "institutional communities" as a hospital, has been estimated to be as high as 20 or 30 per cent (Dische and Elek 1957, Leeming,Price and Meynell 1961). This raised the possibility that the neomycin in the recovery medium was inhibiting growth from spore inocula. Preliminary experiments tended to confirm this suspicion and it was decided to investigate more fully the action of neomycin, together with streptomycin and polymyxin B, on spore germination. The last two named antibiotics were included since they have been used in media devised for the isolation of Cl.perfringens, by Willis (1957b) and Wetzler, Marshall and Cardella (1956) respectively.

MATERIALS AND METHODS

Spores. A large batch of spores of isolate NCTC 2836 was prepared by inoculating 500ml Ellner's medium with 50ml of a four hour culture in Robertson's meat broth. Inoculated Ellner's medium was incubated anaerobically overnight, when the growth was spun down in glass centrifuge tubes and washed in sterile distilled water. Sporangia and vegetative debris were removed from this material using the ultrasonic disintegration procedure described in Appendix Two. The clean spore suspension was stored for approximately two months in distilled water at 4^{o}. Immediately before use, a suitable amount of this spore suspension was heat shocked by placing in a water bath at 70^{o} for 20 minutes.

Media for germination. Each germination agent was dissolved in distilled water at the desired concentration. Difco agar was incorporated into each of these media at a concentration of 2 per cent (w/v) and the media sterilised by autoclaving. The molten media were poured into Petrie dishes, allowed to solidify and dried in the usual manner for 1 to 2 hours at 37^{o}.

Inoculation of media. Each plate was inoculated with several drops of the spore suspension using a wire loop and the inoculum spread over the centre of the plate.

Incubation. Inoculated media were incubated aerobically at 37°.

Estimation of Germination. Germination was estimated at given time intervals, by counting the number of dark spores per hundred, in impression preparations observed by phase contrast microscopy. Three hundred spores were counted for each estimation.

The following technique was used to make the impression preparations. Blocks were cut from the agar plates using a scalpel. These were inverted onto glass microscope slides and carefully removed. The impression preparations thus obtained were heat fixed (to prevent the spores lifting from the slides and complicating the counting procedure) and one drop of sterile distilled water placed over each preparation using a wire loop. A glass coverslip was placed over each preparation.

Control experiments showed that heat fixation did not cause spores to appear phase-dark.

EXPERIMENTAL

SECTION A

The Influence of Air and Substrate on the Germination of Spores of *Cl.perfringens* type A

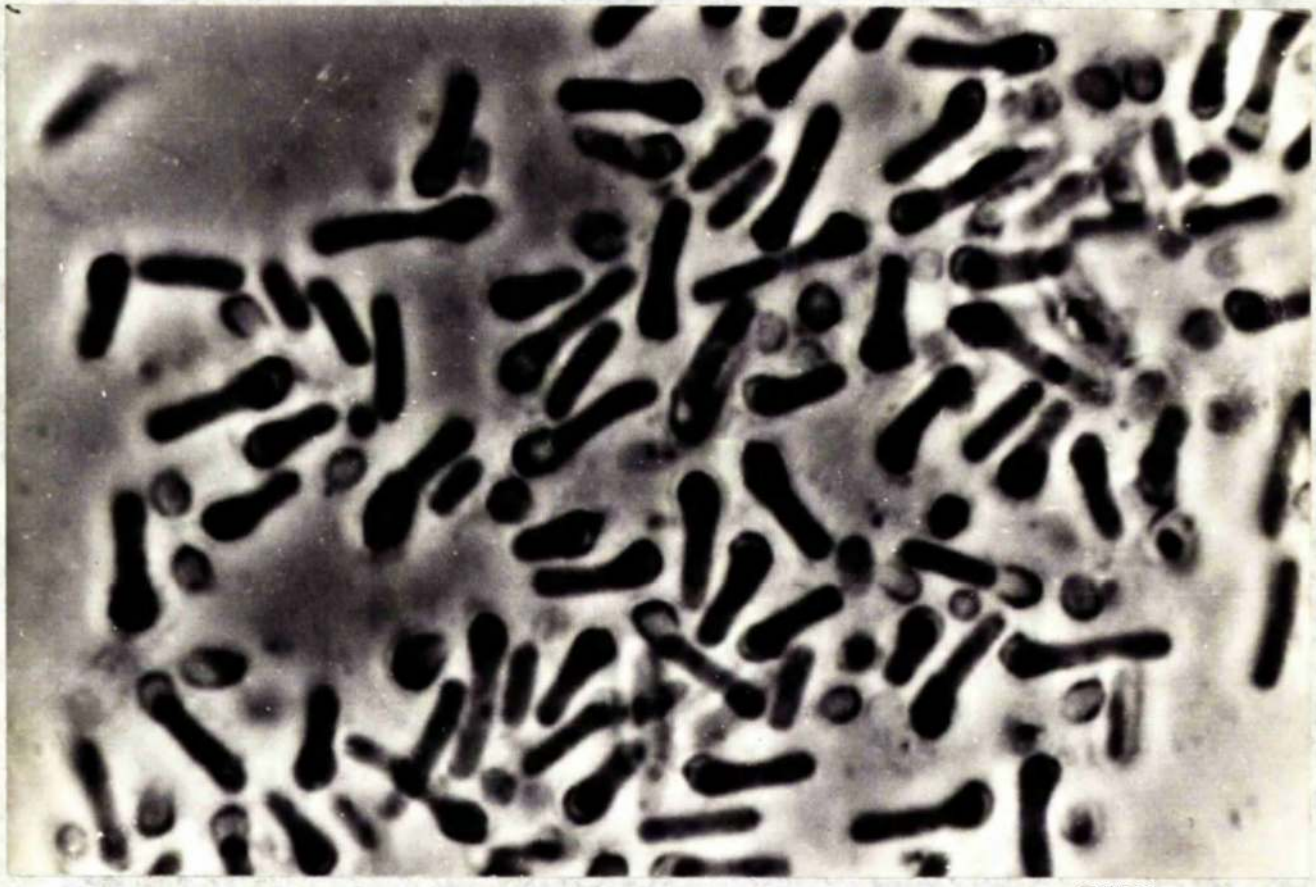
CONTENTS

1. Germination of Cl.perfringens spores in the presence of air

The purity of the clean spore suspension of strain NCTC 2836 was checked by spreading several loopfuls of the suspension over the surface of a horse blood agar plate and incubating aerobically at 37° overnight. A superficial examination of this plate indicated that there was no growth. However, when an impression preparation was made, vegetative cells were seen. The possibility that the spores were undergoing germination and outgrowth in the presence of air was investigated more closely.

Methods. Plates of horse blood agar, infusion agar and a control plate of 2 per cent agar in physiological saline, were inoculated with several loopfuls of a heat-shocked spore suspension. These plates were incubated aerobically at 37° for 18 hours, when impression preparations were made. The viability of the cells on these incubated plates was crudely determined by transferring some of the culture onto fresh horse blood agar plates using a sterile wire loop. The plates were incubated anaerobically at 37° overnight and the presence or absence of growth recorded.

Results. Vegetative cells were seen in the impression preparations made from the horse blood and infusion agar plates. A photograph of one of these preparations is shown in Plate 14. In this photograph vegetative cells and spore coats can be seen. In general each vegetative cell is associated with a spore coat, indicating that there was no cell division following germination and outgrowth.



x 2900

PLATE 14

Phase contrast photomicrograph of cells of Cl.perfringens (strain NCTC 2836) after aerobic incubation of spores for 18 hours at 37° on horse blood agar. This preparation consists almost entirely of vegetative cells, most of which are enclosed at one end by the spore coat.

The preparation from the saline agar plate contained only phase-bright spores.

When preparations from the horse blood agar and infusion agar plates were plated out onto fresh horse blood agar, there was no growth, indicating that the cells on these plates had been killed by exposure to air. In contrast, the phase bright-spores plated from the saline agar plate, yielded a heavy growth.

2. The rate of germination of Cl.perfringens spores

The unexpected finding that spores of Cl.perfringens were able to germinate aerobically, indicated that aerobic incubation would provide a convenient method for studying the ability of various media to germinate spores. As a preliminary to this study, the rate of germination and loss of heat resistance on germination was studied.

Methods. A horse blood agar plate was inoculated with the spore suspension and incubated aerobically at 37°. Impression preparations were made from this plate after 1, 20, 30, 100 and 180 minutes. The viability and ungerminated spore content of the culture after 100 minutes incubation, was examined by the following technique: A block was aseptically cut from the agar plate and placed in a 4 x ½"test tube containing approximately 2ml sterile physiological saline. The tube was shaken to dislodge the cells from the agar and the supernatant pipetted off. The total viable count per standard loopful of this

suspension was determined as follows. Ten standard loopfuls were spread over the surfaces of five horse blood agar plates, so that each loopful was spread over one half of a plate. The inoculated plates were then incubated anaerobically at 37° for 48 hours and the mean number of colonies which developed per loopful calculated. To determine the ungerminated spore count per standard loopful of this suspension, the suspension was heated at 70° for 20 minutes and the above procedure repeated.

Results. The results of this experiment are shown in Table 43. After 20 minutes incubation on horse blood agar, 76.5 per cent of the spores were phase dark, i.e. had germinated but did not show outgrowth. After 30 minutes, 92.4 per cent of the spores were phase dark, while after 100 minutes all the spores were phase dark. After 180 minutes, the impression preparations had the appearance previously illustrated in Plate 14, i.e. the germinated spores had undergone outgrowth. Development did not proceed beyond this stage.

After 100 minutes incubation the ratio: mean total viable count/ mean ungerminated spore count, was 424:1. This is in agreement with the results presented in Table 43 and shows that the phase dark-spores were heat labile. From this brief study of the rate of germination, it is apparent that inoculated media should be incubated for a minimum of 100 minutes before the percentage germination is determined.

TABLE 43

Rate of germination of Cl.perfringens spores (strain NCTC 2336) on horse blood agar plates

Incubation period (in minutes)	Mean percentage dark spores
1	1
20	76.5
30	92.4
100	100

3. The ability of a number of substrates to promote germination of Cl.perfringens spores

The previous experiments have shown that Cl.perfringens spores will germinate aerobically. This method of incubation,which is both convenient and enables serial samples to be taken without interrupting anaerobic conditions, was used to study the ability of a number of substrates to promote germination.

3.1. Casein hydrolysate, glucose and buffer

Casein hydrolysate was used to determine whether an amino acid mixture could promote germination of Cl.perfringens spores. It was used alone and combined with glucose (D-glucose). These materials were dissolved in distilled water and Hendry's buffer. Glucose dissolved in water, glucose dissolved in Hendry's buffer, and Hendry's buffer alone, were also used to determine whether these materials could promote germination in the absence of amino acids.

Methods. The following media were used:-

A. Acid hydrolysed casein (Oxoid) dissolved at a concentration of 1 per cent (w/v) in:-

1. Distilled water
2. A 1 per cent (w/v) solution of glucose in distilled water
3. Hendry's isotonic buffer (pH 7.0)
   composition:-

   35ml 0.167M $NaH_2PO_4 . 2H_2O$

   65ml 0.127M Na_2HPO_4

B. Glucose dissolved at a concentration of 1 per cent (w/v) in:-

1. Distilled water
2. Hendry's buffer

C. Hendry's buffer.

These preparations were solidified by adding agar at a concentration of 2 per cent (w/v), sterilised by autoclaving, and the molten media poured into Petrie dishes. These media, along with control plates of horse blood agar, were inoculated with the spore suspension, and the percentage germination determined after 3 and 18 hours incubation.

Results. The results of this experiment are shown in Table 44.

After three hours incubation the spores on the control plate of horse blood agar had germinated and vegetative cells were seen.

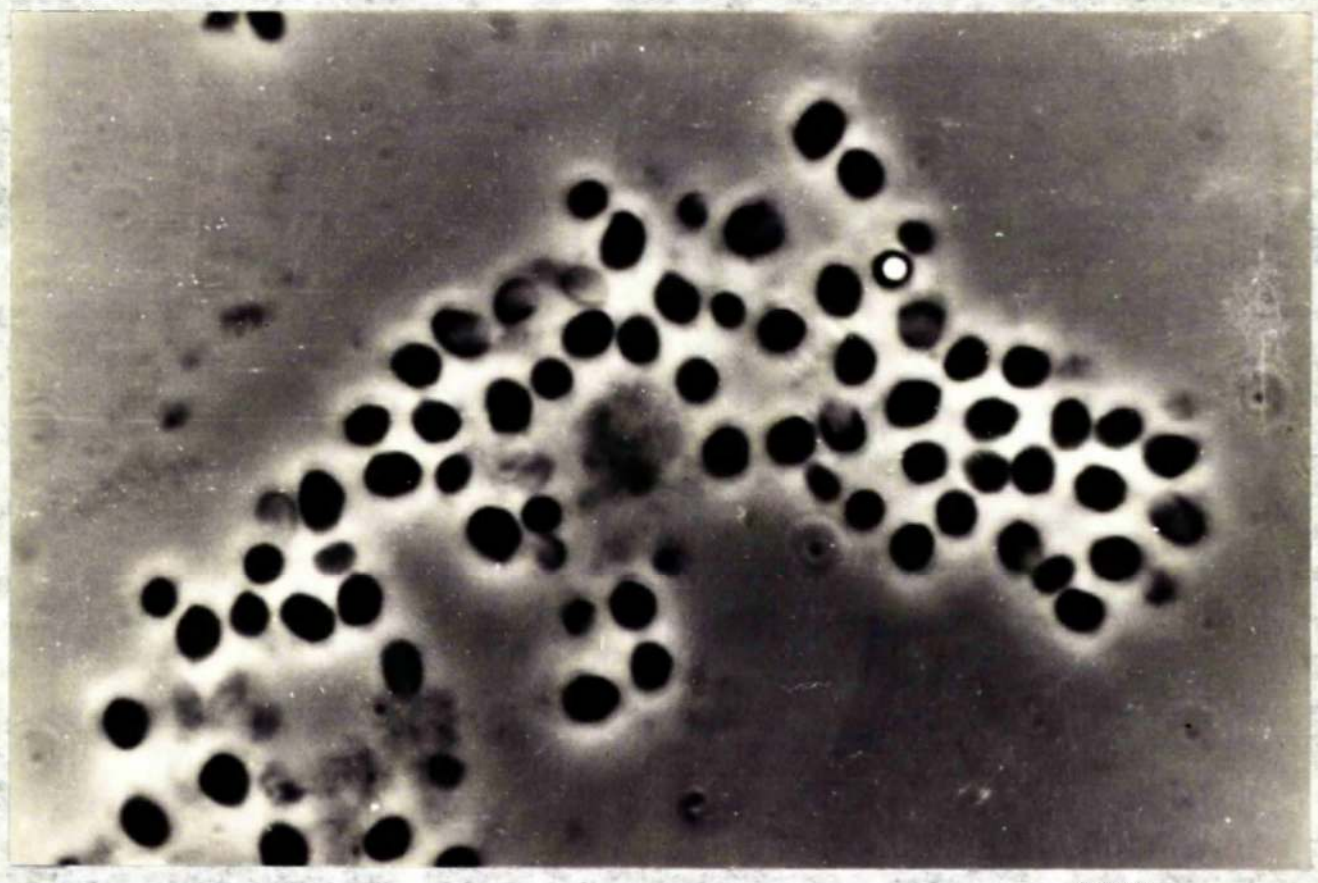
On media containing casein hydrolysate dissolved in water or buffer, the spores germinated but did not mature further. On the first of these media the spores had germinated after three hours incubation. A typical example of this preparation is shown in Plate 15. On the second medium however, germination was much slower, indicating that the buffer was inhibitory.

Spores did not germinate on media devoid of casein hydrolysate, and the presence of glucose in media containing casein hydrolysate did not affect the rate of germination.

TABLE 44

Germination of Cl.perfringens spores (strain NCTC 2836) on casein hydrolysate, glucose and buffer

Substrate	pH	Mean % germinated spores after 3 hours	Mean % germinated spores after 18 hours
Horse blood agar	7.3	98	100
Hendry's buffer	6.8	<1	<1
1% (w/v) hydrolysed casein in water	6.5	98	96
1% (w/v) hydrolysed casein in Hendry's buffer	6.6	7	24
1% (w/v) hydrolysed casein + 1% (w/v) dextrose in water	6.5	98	100
1% (w/v) dextrose in water	-	<1	<1



x 2900

PLATE 15

Phase contrast photomicrograph of germinated spores of Cl.perfringens (strain NCTC 2836). Spores were germinated by growing on a medium of 1 per cent (w/v) casein hydrolysate in 2 per cent (w/v) agar for three hours at 37°. A single phase-bright spore can be seen towards the top right of the photograph.

3.2. Amino acid and inorganic nitrogen compounds

The previous experiment has shown that a mixture of amino acids will promote germination. The following experiment was carried out to determine whether individual amino acids and sources of inorganic nitrogen were able to support germination.

Method. 25mM solutions of: L-alanine, L-asparagine, DL-cystine, glycine, DL-leucine, DL-isoleucine, DL-methionine, DL-proline, DL-serine, DL-threonine, DL-valine, potassium nitrate and ammonium sulphate, in 2 per cent (w/v) agar in distilled water were prepared. These preparations were used at their natural pH.

25mM solutions of: L-cysteine hydrochloride, DL-aspartic acid, DL-glutamic acid, DL-histidine monohydrochloride dihydrate and DL-lysine monohydrochloride, prevented gel formation at their natural pH in 2 per cent (w/v) agar. The reaction of each of these solutions was therefore adjusted (before adding agar) to pH 7.0. This was done by adding a few drops of a concentrated aqueous solution of sodium hydroxide. These preparations were sterilised by autoclaving, plates poured, inoculated and the percentage germination determined, using the techniques described previously.

Results. The results after two hours incubation are shown in Table 45. With the exception of spores incubated on the DL-valine medium,

TABLE 45

Germination of Cl.perfringens spores (strain NCTC 2836) on amino acid and simple inorganic media

Substrate (25mM)	pH	Mean % germinated spores after two hours
L-alanine	7.1	< 1
L-asparagine	6.6	4
DL-cystine	6.0	3
glycine	6.6	< 1
DL-leucine	6.9	< 1
DL-isoleucine	7.0	1
DL-methionine	6.9	< 1
DL-proline	6.9	3
DL-serine	6.9	< 1
DL-threonine	6.7	< 1
DL-valine	6.6	2
KNO_3	6.5	< 1
$(NH_4)_2SO_4$	6.8	< 1
L-cysteine HCl	7.3	97
DL-aspartic acid	7.0	< 1
DL-glutamic acid	7.0	3
DL-histidine mono HCl dihydrate	7.0	98
DL-lysine mono HCl	7.0	30

the percentages of spores which had germinated after 18 hours incubation, were identical. In the case of DL-valine the percentage of germinated spores increased from 2 per cent after two hours incubation, to 3 per cent after eighteen hours incubation.

It can be seen from these results that the only compounds which induced more than 8 per cent of the spores to germinate, were: L-cysteine hydrochloride (97 per cent), DL-histidine monohydrochloride dihydrate (98 per cent) and DL-lysine monohydrochloride (30 per cent). These compounds are all hydrochlorides and it seemed probable that the chloride ion might be supporting germination. This possibility was examined by the following experiment.

3.3. The effect of sodium chloride on germination on amino acid and inorganic nitrogenous media

Method. Sodium chloride (25mM) was incorporated into the chloride-free media used in the previous experiment. Germination on these media and in sodium chloride in 2 per cent agar, was estimated as described previously.

Results. (See Table 46). Spores did not germinate on the medium of sodium chloride in 2 per cent agar. Similarly, the mixture of sodium chloride with potassium nitrate and ammonium sulphate did not induce germination. However, a number of amino acids (DL-leucine,

TABLE 46

Germination of Cl.perfringens spores (strain NCTC 2836) on amino acid and simple inorganic media supplemented with sodium chloride

Substrate (25mM) + NaCl (25mM)	pH	Mean % germinated spores after two hours
L-alanine		<1
L-asparagine		4
DL-cystine		3
glycine		2.5
DL-leucine		98.5
DL-isoleucine		84.5
DL-methionine		99.5
DL-proline		2
DL-serine		NT
DL-threonine		99.5
DL-valine		57
KNO_3		2.5
$(NH_4)_2SO_4$		<1
NaCl (25mM)		<1
DL-aspartic acid	7.0	3
DL-glutamic acid	7.0	5

DL-isoleucine, DL-methionine, DL-threonine and DL-valine) which did not support germination alone (see Table 45), did so in the presence of sodium chloride.

Discussion

The observation that spores of Cl.perfringens were able to undergo germination and outgrowth on nutrient media in the presence of air, while remaining viable for a short period, enables considerably simplified technical procedures to be used for studying the minimal requirements for spore germination and outgrowth.

The ability of clostridial spores to germinate in the presence of air is not restricted to the species perfringens. Spores of a further species, P.A. 3679, have also been observed to germinate when incubated aerobically (Lund 1955-6). In this case Lund found that when hydrogen peroxide was added to a spore suspension, the hydrogen peroxide had the opposite to the intended effect and germination was accelerated. Nevertheless, the ability of clostridial spores to germinate in the presence of air appears to be confined to certain species, as Halvorson (1957b) found that spores of Cl.roseum and Cl.botulinum would not germinate unless the last traces of oxygen were removed from the medium. Similar findings were reported for Cl.botulinum by Wynne and Foster (1948).

The only detailed study of the effect of Eh on germination of clostridial spores was made by Knight and Fildes (1930) who used spores of Cl.tetani. In this case germination was most rapid at Eh -50mv. At progressively higher redox potentials each phase of germination was lengthened, until at +110mv, a few bacilli emerged but

did not develop further. It can be seen from these results that the upper Eh value at which spores of Cl.tetani will germinate, is higher than the upper Eh limit permitting vegetative growth. This finding is consistent with the results obtained using Cl.perfringens. In the case of Cl.perfringens however, the upper Eh value permitting vegetative growth is in the order to +250mv, while the corresponding value for germination extends to aerobic conditions. In view of these findings, it appears that the maximum redox potential at which clostridial spores will germinate is dependent upon the degree of anaerobiosis required for vegetative growth.

In the genus Bacillus the Eh requirement for spore germination appears to be less specific and spores of B.anthracis, B.subtilis, B.megaterium and B.cereus, have been found to germinate in oxygen-free media (Roth and Lively 1956, Murty and Halvorson 1957, Hyatt and Levinson 1959).

In view of the finding that spores of Cl.perfringens germinated rapidly under aerobic conditions, it is surprising that heat-resistant strains survive in uncooked foods in the spore state. This is undoubtedly the case as ungerminated (heat-resistant) spores of these strains are found in uncooked foods. Furthermore, it is thought that the exceptional heat resistance of these spores plays an important part in the aetiology of clostridial food-poisoning by enabling the bacterium to survive the cooking process. The possible explanations

of this discrepancy are:-

1. Germination initiators are not found in the food.
2. Germination inhibitors are present in the food.
3. Spores of heat-resistant strains do not germinate aerobically.
4. Spores will only germinate if they have been heat activated.

Evidence supporting the last of these possibilities has been presented by Barnes, Deepaul and Ingram (1963). These workers found that only 3 per cent of the spores of a heat-resistant strain were able to germinate without a period of heat treatment. It appears then, that heat activation plays an important role in the actiology of clostridial food-poisoning. Spores which have not been heat activated persist in foods in the ungerminated, heat-resistant state until the food is cooked, when the heat applied activates them.

The germination requirements of heat-activated spores were comparatively simple and germination was observed on a mixture of amino acids (casein hydrolysate). For outgrowth a more complex media (horse blood agar) was necessary and outgrowth was not observed on casein hydrolysate-even when supplemented with an energy source (glucose) and sodium and phosphate ions. In order to determine whether amino acids and inorganic nitrogen sources could initiate germination individually, sixteen amino acids, ammonium sulphate and potassium nitrate were examined. In these experiments the DL- form

of the amino acid was generally used. Inhibition of spore germination by the D- form of certain amino acids is known (D-alanine being particularly noteworthy), although in DL- mixtures of such compounds the tendency is for germination to be slowed down rather than completely inhibited. Of the 18 compounds examined, only the amino acids commercially available as hydrochlorides supported germination. It appeared from this experiment that the chloride ion was playing an important part in germination. Subsequent experiments, in which germination was studied in mixtures of amino acids with sodium chloride, tended to confirm this belief and although sodium chloride was inactive alone, five of the twelve amino acids which would not support germination alone, supported germination in the presence of sodium chloride.

It is unlikely that the sodium ion was influencing germination in these experiments, as it has been previously shown that germination was inhibited when sodium and phosphate ions were mixed with casein hydrolysate. It appears then, that the phosphate ion is inhibitory. The presence of high concentrations of phosphate in Ellner's medium is probably an important factor in maintaining good spore crops in this medium, by inhibiting germination and so preventing recycling.

The apparent effect of chloride and phosphate on germination of Cl.perfringens spores, is similar to that previously reported by Rode and Foster (1962a,b,c) for strains of B.megaterium. These ions

however, do not have the same effect on the spores if all the bacterial species so far studied, and just as different amino acids have been found to promote germination of spores of different species, the species specificity of inorganic ions has also been demonstrated. Thus for B.subtilis, Fleming and Ordal (1964) found that chloride was inactive, while phosphate enhanced germination.

The role of inorganic ions in promoting germination is by no means understood. The two most likely explanations are:-

1. A proper ionic environment is necessary for the optimal activity of enzymes operative during germination

2. The effect of these ions is physico-chemical rather than catalytic.

The second of these possibilities has been discussed from a purely theoretical standpoint by Fleming and Ordal (1964).

Section B

The Effect of Antibiotics on the Growth of Cultures of *Cl.perfringens* type A from Spore Inocula

CONTENTS

The effect of antibiotics on the growth of Cl.perfringens cultures from spore inocula

1 Neomycin

Method. Neomycin (Upjohn of England Limited, Fleming Way, Crawley, Sussex) was aseptically incorporated into infusion broth and 1 per cent (w/v) glucose broth at concentrations of 0, 100 and 200μg/ml. These media were distributed in 5ml amounts in 4 x $\frac{3}{4}$" test tubes. Four tubes were used for each concentration of the antibiotic. Each tube was inoculated with 0.02ml of a heat-shocked spore suspension (approximately 120,000 spores per tube) and incubated anaerobically at 37^{o} for four days, when the presence or absence of growth was estimated visually. Samples from tubes showing no growth were plated out on horse blood agar and incubated anaerobically at 37^{o} for two days.

Results. (See Table 47). The four tubes of glucose broth and the four tubes of infusion broth which did not contain neomycin, all showed growth. Of the tubes containing 100μg/ml neomycin, there was no growth in glucose broth and only one of the four tubes of infusion broth showed growth. The effect of neomycin was even more pronounced at 200μg/ml and there was no growth in these media. When samples from tubes showing no growth were plated out on horse blood agar, there was no growth on this medium. It therefore appears that the inhibitory effect of neomycin persists in antibiotic-free media.

TABLE 47

The effect of neomycin on the growth of Cl.perfringens cultures (strain NCTC 2836) from spore inocula

Concentration of neomycin (µg/ml)	Number of tubes showing growth / Number of tubes inoculated	
	1% (w/v) glucose broth	Infusion broth
0	4/4	4/4
100	0/4	1/4
200	0/4	0/4

2 The effect of the size of the inoculum on the growth of Cl.perfringens cultures from spore inocula in media containing neomycin

In the previous experiment a large number of spores were used to inoculate the media. Since the number of Cl.perfringens spores in natural environments is probably small, the effect of inoculum size on inhibition of growth from spore inocula by neomycin was studied.

Method. Neomycin was aseptically incorporated into 1 per cent (w/v) glucose broth at concentrations of 0, 62.5, 125, 250 and 500µg/ml. These media were distributed in 5ml volumes in 4 x ½" test tubes and divided into two batches. Each tube was inoculated with 0.04ml of a suitable dilution of the spore suspension, such that each tube of one batch was inoculated with approximately 12,800,000* spores and the other with approximately 68*spores. Inoculated media were incubated anaerobically at 37° for four days, when the presence or absence of growth was assessed visually.

Results. (See Table 48). In the absence of neomycin all tubes showed growth. In media containing neomycin at a concentration of 62.5µg/ml there was no growth in the five tubes inoculated with 68 spores. Even in the tubes inoculated with 12,800,000 spores considerable inhibition must have occured since only four of the five tubes showed growth. Media containing neomycin at concentrations of 125, 250 and 500µg/ml

*Calculated from viable spore count data.

TABLE 43

The effect of the size of the inoculum on the growth of Cl.perfringens cultures (strain NCTC 2876) from spore inocula, in media containing neomycin

Concentration of neomycin (μg/ml) in 1 per cent (w/v) glucose broth	Number of tubes showing growth / Number of tubes inoculated	
	Inoculum 12,800,000 spores	Inoculum 68 spores
0	5/5	5/5
62.5	4/5	0/5
125	0/5	0/5
250	0/5	0/5
500	0/5	0/5

showed no growth with either the large or small inoculum.

3 Streptomycin and polymyxin B

Having shown that neomycin inhibits growth of cultures from spore inocula, streptomycin and polymyxin B were studied to determine whether they had a similar effect.

Method. Media of 1 per cent (w/v) glucose broth were prepared containing:-

1. 0, 12.5, 25, 50, 100, 250, 500 and 1,000 µg/ml streptomycin sulphate (Glaxo Laboratories Limited, Greenford, England).
2. 0, 12.5, 25, 50, 100, 250, 500 and 1,000 units/ml polymyxin B sulphate (Burrough's Wellcome and Company, London, England).

These media were distributed in 5ml amounts in 4 x $\frac{1}{2}$" test tubes. Each tube was inoculated with 0.04ml of a spore suspension, such that each tube received approximately 68 spores. Inoculated media were incubated anaerobically at 37° for one week, when the presence of growth was estimated visually.

Results. (See Table 49). It can be seen from these results that streptomycin at a concentration of 250µg/ml completely inhibited growth from spore inocula. This is considerably lower than the strength used by Willis (1,000µg/ml) in media for isolation of Cl.perfringens. Polymyxin B however, was without effect even at a concentration of 1,000 units/ml.

TABLE 49

The effect of streptomycin and polymyxin B on the growth of Cl.perfringens cultures (strain NCTC 2836) from spore inocula

Concentration of antibiotic (μg/ml) for streptomycin; units/ml for polymyxin B	Number of tubes showing growth / Number of tubes inoculated	
	Streptomycin medium	Polymyxin B medium
0	5/5	5/5
12.5	5/5	5/5
25	5/5	5/5
50	5/5	5/5
100	5/5	5/5
250	0/5	5/5
500	0/5	5/5
1000	0/5	5/5

4 Comparison of the inhibitory effect of neomycin on growth from spore and vegetative inocula

The previous experiments have shown that growth from spore inocula is prevented by certain concentrations of neomycin and streptomycin. The use of liquid media however, gives us only a crude estimation of the number of spores inhibited by the antibiotic. In order to obtain a more accurate estimation, plate counts were carried out on media with and without neomycin. Vegetative inocula were also used in this experiment to determine the extent of inhibition of vegetative growth, if any.

Method. Molten infusion agar was cooled to 45° and horse blood added. Neomycin was added to this medium at concentrations of 0, 100 and 200µg/ml and plates poured. These plates were inoculated with standard loopfuls of dilutions in sterile saline of:-

1. A heat-shocked spore suspension.
2. An overnight culture in Robertson's meat broth (vegetative cells).

The inoculated plates were incubated anaerobically for three days at 37° and the colonies of suitable dilutions counted. From these counts the mean number of colonies developing from one loopful of the undiluted culture was calculated.

Results. (See Table 50). Inhibition of growth from spore inocula is again apparent in media containing neomycin. In the presence of

TABLE 50

Colony count data obtained when spores and vegetative cells of Cl.perfringens (strain NCTC 2836) were used to inoculate horse blood agar plates with and without neomycin

Inoculum	Concentration of neomycin (μg/ml)	Mean colony count per loopful		
		Best estimate	Lower 95% confidence limit	Upper 95% confidence limit
Spore	0	1.88×10^6	1.42×10^6	2.34×10^6
	100	4.44×10^3	3.40×10^3	5.48×10^3
	200	3.29×10^2	2.53×10^2	4.05×10^2
Vegetative	0	3.65×10^6	3.22×10^6	4.08×10^6
	100	2.75×10^6	2.14×10^6	3.36×10^6
	200	2.50×10^5	2.40×10^4	4.76×10^5

100μg/ml neomycin only 0.24 per cent of the viable spores formed colonies, while in the presence of 200μg/ml the figure was 0.018 per cent. The effect on colony formation from the vegetative inoculum was much less marked and colony formation was not significantly inhibited on media containing 100μg/ml neomycin. On media containing 200μg/ml neomycin however, only 6.85 per cent of the viable vegetative inoculum formed colonies.

5 Microscopical observations to determine which stage of the spore to replicating vegetative cell pathway is inhibited by neomycin

The preceding experiments have shown that neomycin inhibits one or more stages in the transformation of the spore to the replicating vegetative cell. Experiments were therefore carried out to determine how far germination, outgrowth and cell division progressed in the presence of neomycin. In the first of these experiments, the ability of spores to undergo germination and outgrowth on media containing neomycin, incubated aerobically, was studied. In the second, germination, outgrowth and cell division in the presence of neomycin were followed in slide culture.

5.1 Aerobic germination and outgrowth in the presence of neomycin

Method. Horse blood agar plates containing 0, 100 and 200μg/ml neomycin were inoculated with a heat-shocked spore suspension and

and incubated aerobically at 37°. Impression preparations were made after two hours incubation and examined by phase contrast microscopy.

Results. Impression preparations of all the cultures were of similar appearance. These preparations consisted entirely of vegetative cells and a spore coat was associated with each vegetative cell. In the preparations from the media containing neomycin the spore coat was attached to the vegetative cell, while in preparations from the control medium the vegetative cell was frequently separated from the spore coat.

It appears from this experiment that neither germination nor outgrowth is inhibited by neomycin. In order to determine at which of the subsequent stages development ceases, the process was followed in slide culture.

5.2 Microscopical observations of spore development in media containing neomycin in slide culture

Method. Three batches of sterile molten 1 per cent (w/v) glucose broth, containing 2 per cent (w/v) agar, were cooled to 45° in a water bath. Neomycin was then added to these media to give final concentrations of 0, 100 and 200 µg/ml. Each medium in turn was then treated as follows. Several drops of a concentrated, heat-shocked spore suspension were mixed with 5ml of the medium and one drop of the

resulting suspension transferred to a warm microscope slide using a warm, sterile Pasteur pipette. A glass coverslip was then placed on top of the culture and the preparation sealed with vaseline. This preparation was then placed on the stage of a phase contrast microscope (in a 37^{0} "hot room") and observed over a period of four hours.

Results. Spores added to the glucose agar medium germinated rapidly and the majority of spores had germinated by the time they were on the microscope stage (approximately five minutes after inoculation of the medium). Outgrowth was observed after 90 minutes and the first cell division after 2 hours.

Spores in the glucose agar media containing neomycin underwent germination and outgrowth at the same rate as the controls, but the majority of cells did not mature further. A few cells matured beyond this stage and divided normally, this was more common in the medium containing 100μg/ml neomycin than in the medium containing 200μg/ml neomycin. In the medium containing 200μg/ml neomycin filamentous cells were occasionally seen.

Discussion

A number of workers have studied the effect of antibiotics on clostridial spores (see Table 51). In these experiments antibiotics active (bactericidal or bacteriostatic) against vegetative cells were used. They were however, found to be inactive against ungerminated spores and spores germinated (estimated by loss of heat resistance) in their presence. Unfortunately, as these cultures were not examined microscopically, it was not determined whether the antibiotic exerted its effect before or after outgrowth. This point was resolved by Scheibel and Lennert-Petersen (1958) who incubated spores of Cl.tetani in slide culture in media containing: chlortetracycline, oxytetracycline, tetracycline, streptomycin, penicillin, polymyxin B, bacitracin and chloramphenicol. In the presence of these antibiotics spores germinated, but did not develop further. Similar findings were reported by Jann and Salle (1958) who studied the effect of subtilin, streptomycin and penicillin, on spores of Cl.botulinum.

Unlike the combinations of organisms and antibiotics discussed in the previous paragraph, vegetative cells of Cl.perfringens are resistant to the antibiotics they were tested against (hence their use in selective media for the isolation of this organism). In these experiments I have confirmed the finding of Lowbury and Lilly (1958), namely, that the presence of 100µg/ml neomycin in the counting medium does not significantly reduce the colony count from vegetative inocula.

Table 51

Previous reports of the action of antibiotics on Clostridial spores

Organism	Antibiotic	Reference
Cl.botulinum P.A. 3679	subtilin	Michener (1955)
Cl.botulinum	subtilin	Williams & Fleming (1952)
	subtilin	Andersen (1952)
Cl.septicum	chloramphenicol chlortetracycline penicillin streptomycin bacitracin	Taylor (1954)
Cl.tetani	penicillin	Lund (1953)
	tetracycline	Anwar and Turner (1956)
Cl.perfringens	penicillin	Wynne & Harrel (1951)

In contrast, only 0.24 per cent of the viable spores produced colonies on this medium. Lower concentrations of neomycin were also inhibitory and although accurate spore counts were not carried out on these media, it is apparent that even in the presence of 62.5μg/ml neomycin, less than 2 per cent of the spores were able to initiate vegetative growth. Streptomycin, which is closely related to neomycin, had a similar action. Polymyxin B however, even at high concentrations was without effect. It is apparent from these results that media containing neomycin or streptomycin should not be used for the enumeration or isolation of Cl.perfringens when the organism is expected to be present in the spore phase, e.g. in soil or water samples. Similarly, if Cl.perfringens is present in the vegetative phase, media containing neomycin at concentrations greater than 100μg/ml will decrease the likelihood of this organism being isolated. If, due to preponderance of other organisms, a selective medium is essential, polymyxin B would be preferable to streptomycin or neomycin.

When the effect of neomycin was studied in slide culture it was found that neither germination nor outgrowth was inhibited. The biochemical activity of the cell before the first division, must therefore be different from that of the replicating vegetative cell. A clearer understanding of this inhibitory action awaits the elucidation of the mode of action of neomycin and the mechanism of spore development.

SUMMARY

SUMMARY

PART 1

1. The ability of Ellner's medium to promote sporulation of forty eight strains of Cl.perfringens type A (comprising thirty beta-haemolytic and eighteen heat-resistant strains) was examined. Only eight strains (16.7 per cent) produced more than 30 per cent spores, these were all beta-haemolytic. Thirteen of the beta-haemolytic strains (43.3 per cent) produced more than 1 per cent spores, while none of the heat-resistant strains produced more than 1 per cent spores.

2. When sporulation was compared in Ellner's medium and a number of other media recommended to induce sporulation of Cl.perfringens, abundant sporulation of certain strains was observed only in Ellner's medium. However, sporulation was more consistent in a medium devised by Bethge (1947-8). In this medium both beta-haemolytic and heat-resistant strains formed spores to roughly the same extent, the heat-resistant strains producing more spores in this medium than in any of the other media examined.

3. The cultural conditions employed when Ellner's medium was used had a pronounced effect on the numbers of spores formed. One of the most important of these was the medium in which the cells were grown (pre-sporulation medium) prior to inoculation of Ellner's medium. The best

of the pre-sporulation media examined was Robertson's meat broth. The maximum volume of the culture in Robertson's meat broth which could be used to inoculate a fixed amount of Ellner's medium without decrease in sporulation was 20 per cent (v/v). Attempts to overcome this limitation by inoculating Ellner's medium with washed cells, resulted in decreased sporulation. The temperature at which the sporulation medium was incubated was also important, thus, although vegetative growth was better at 45 than 37°, sporulation was better at 37°. Aeration of the sporulation medium prevented sporulation.

4. Attempts to induce abundant sporulation of strains which produced only low numbers of spores in Ellner's medium, by slight modifications to this medium, were unsuccessful. Thus, different commercial preparations of peptone and yeast extract, slight variations in the pH, addition of: digest of peptone, inorganic salts, thiamine; or culturing in spent medium supplemented with disintegrated sporing cells, were all unsuccessful. Certain of these modifications had a pronounced effect on sporulation of good sporing strains. With these strains peptone preparation, inorganic salts and pH, were all important.

5. Sporulation of Cl.perfringens was compared in media prepared by dissolving a number of proteinaceous compounds in buffer. The best of the compounds examined was peptone. In peptone media the $\log_{10}$ spore count was directly proportional to the $\log_{10}$ peptone concentration. However, the highest percentage of spores was produced

at a peptone concentration of approximately 5 per cent (w/v). A number of attempts were made to fractionate peptone into sporogenic and asporogenic components using: continuous paper (curtain) electrophoresis, ethanol precipitation, dialysis and ammonium sulphate precipitation. All the fractions obtained supported sporulation, but to different extents. A marked increase in sporulation was brought about by dialysing the peptone and sporulation in the dialysed peptone medium approached that in Ellner's medium.

6. Inorganic ions were essential to sporulation. Potassium enhanced sporulation, sodium inhibited sporulation, while phosphate appeared to have no effect.

7. When a good sporing strain was "plated" on horse blood agar, sub-strains derived from single colonies tended to form spores to the same extent as the parent culture. Occasionally a sub-strain was isolated which produced lower numbers of spores than its companions. Further sub-strains derived from this culture tended to produce low numbers of spores.

8. Four of the forty eight isolates were mucoid, these were beta-haemolytic and three of the four produced abundant spores. Only ten of the remaining twenty six beta-haemolytic strains, produced more than 1 per cent spores.

9. Good sporing, weakly sporing and heat-resistant strains could not be differentiated by their sensitivities to a number of antibacterial

substances. However, good sporing strains were more able to degrade peptone.

10. In slide culture in Ellner's medium the majority of cells underwent two divisions. Some cells did not divide, while others underwent four divisions. Thirteen per cent of the total number of cells studied, lysed. In some cases this represented a whole clone, although this was not invariably the case. Cell division was complete after $2\frac{1}{2}$ hours. Forespores were first seen after $2\frac{1}{2}$ hours and over the next 3 hours forespores matured into typical phase-bright spores. Spores were always formed at the end of the cell furthest away from the site of the last cell division.

11. Electron micrographs have shown that the spore was enclosed by a structure resembling an exosporium. When this was removed, the surface of the spore appeared to be smooth.

PART II

1. Spores of Cl.perfringens underwent germination and outgrowth on horse blood agar in the presence of air. Under these conditions germination was complete after 100 minutes and outgrowth after 180 minutes. Post-germinative development did not proceed beyond the stage of outgrowth and in the presence of air the resultant cells died.

2. Glucose and inorganic nitrogen sources did not promote germination, although a number of amino acids (DL-mixtures) were able to promote

germination in the presence of chloride ions. In these instances spores germinated but did not undergo outgrowth. L-alanine did not promote germination-even in the presence of chloride ions.

3. Spore germination was inhibited in the presence of sodium phosphate buffer.

4. Vegetative growth from spore inocula was prevented by neomycin and streptomycin at concentrations used in selective media recommended for the isolation of Cl.perfringens. Polymyxin B,however, which has also been used in selective media, was without effect-even at high concentrat -ions. In the presence of neomycin spores underwent germination and outgrowth but did not develop further.

APPENDIX ONE

ISOLATION OF BETA-HAEMOLYTIC AND HEAT-RESISTANT STRAINS OF Cl.PERFRINGENS FROM FAECES

Numerous techniques have been described for the isolation of beta-haemolytic strains of Cl.perfringens type A from faeces. One of the simplest of these was recommended by Lilly (1958), who "plated" faeces on Nagler's medium containing 100µg/ml neomycin; preferably after enrichment culture in Robertson's meat broth containing neomycin.

For the isolation of heat-resistant strains of Cl.perfringens type A from faeces, Hobbs, Smith, Oakley, Warrack and Cruickshank (1953) adopted the method originally used by Hain (1949) for the isolation of Cl.perfringens type F. This method utilises the exceptional heat resistance of the spores of these organisms. In practice a portion of the stool is emulsified in a test tube containing infusion broth. The test tube is then placed in a boiling water bath for one hour and the heat-treated sample transferred to Robertson's meat broth. After twenty four hours incubation the culture is plated on horse blood agar. A variation of this technique has been recommended by McKillop (1958), who obtained heat-resistant strains from a higher percentage of samples when the emulsified faeces were heated at 100° for fifteen minutes instead of one hour.

In the experiments reported below, beta-haemolytic and heat-resistant strains of Cl.perfringens were isolated from the faeces of hospital patients, using modifications and combinations of these

techniques.

Materials and Methods

Media. Robertson's meat broth and horse blood agar were prepared as described by Mackie and McCartney (1960). These media were used with and without neomycin. Neomycin (Upjohn of England Limited, Fleming Way, Crawley, Sussex) was aseptically incorporated into these media at a concentration of 100µg/ml. In the case of the agar medium, neomycin was added at the same time as the horse blood.

Incubation. Cultures in Robertson's meat broth were incubated aerobically, plates of horse blood agar anaerobically. Inoculated media were incubated for 24 hours at 37°.

Identification of strains. Colonies typical of Cl.perfringens were subjected to the tests previously enumerated on page 46.

Isolation of beta-haemolytic strains

Attempts were made to isolate beta-haemolytic strains of Cl.perfringens from forty six faecal specimens and twenty five cultures from rectal swab specimens. The faecal specimens were examined as follows:-

1. Twenty one specimens were plated on horse blood agar containing 100µg/ml neomycin. Eleven of these specimens were also

subjected to enrichment culture in Robertson's meat broth containing 100µg/ml neomycin. After overnight incubation in this medium, horse blood agar plates containing 100µg/ml neomycin were inoculated.

2. Twenty five specimens were subjected to enrichment culture in Robertson's meat broth containing 100µg/ml neomycin. After overnight incubation, plates of horse blood agar were inoculated from these media.

The twenty five cultures from rectal swab specimens were received from Dr J. Gordon of this department. These had been grown in Robertson's meat broth and stored for some time in this medium. These cultures were treated as follows:-

1. Plated on horse blood agar containing 100µg/ml neomycin.

2. Plated as in (1) above after enrichment culture in Robertson's meat broth.

Isolation of heat-resistant strains

Thirty two of the forty six faecal samples were examined for the presence of heat-resistant strains of Cl.perfringens. A sample of the stool, approximately the size of a pea, was emulsified in 2ml infusion broth in a 4 x $\frac{1}{2}$" test tube. The faecal suspension was then placed in a boiling water bath for thirty minutes and used to inoculate 15ml Robertson's meat broth containing 100µg/ml neomycin. After twenty four hours incubation at 37° horse blood agar plates were inoculated.

Results and Discussion

Cl.perfringens was isolated from ten of the twenty one faecal samples examined by direct plating on horse blood agar containing neomycin. These were designated: 20, 21, 22, 25, S23, S24, R1, R2, R5 and R10. The eleven of these specimens subjected to enrichment culture in Robertson's meat broth containing neomycin yielded six strains, two of which were not obtained by direct plating. These were designated R6 and R11.

The twenty five faecal samples subjected to enrichment culture in Robertson's meat broth containing neomycin, followed by plating on horse blood agar, yielded eighteen strains of Cl.perfringens. Four of these (23, 24, 25 and 26) were present in pure culture on the horse blood agar plates. The remaining fourteen strains were present in mixed culture with Proteus. These were obtained in pure culture by plating on horse blood agar containing neomycin and designated: S1, S2, S3, S4, S5, S6, S8, S9, S11, S12, S14, S20, S21 and S22.

The twenty five cultures from rectal swab specimens did not yield any strains of Cl.perfringens by direct plating on horse blood agar containing neomycin. Following enrichment culture in Robertson's meat broth two strains were obtained. These were designated J8 and J12.

No growth was obtained in media inoculated with the thirty two faecal specimens subjected to heating in a boiling water bath for

thirty minutes, i.e. no heat-resistant strains were isolated. This failure to isolate heat-resistant strains from the faeces of individuals living in an "institutional community" was surprising, particularly as the carrier rate in such communities has been estimated to be as high as 20 or 30 per cent (Dische and Elek 1957, Leeming et al. 1961).

The differences between the techniques more widely used and those of the present study are:-

1. The emulsified faeces were heated for thirty minutes instead of one hour.

2. Neomycin was incorporated in the enrichment medium.

The possibility that neomycin was inhibiting some stage in the development of the vegetative cell from the spore was investigated. These experiments have been recorded on pages 185 to 194.

APPENDIX TWO

PREPARATION OF CLEAN SPORE SUSPENSIONS OF Cl.PERFRINGENS

For serological and biochemical analyses of bacterial spores, as well as the study of germination requirements (see page 170), it is essential to use spore preparations from which all vestiges of vegetative material and the sporulation medium have been removed. To minimise the cleaning procedure aparticulate sporulation media are used.

The methods which have been successfully used to promote lysis of vegetative cells fall into six categories. These are:-

1. Enzymic digestion, viz: lysozyme (Tomesik and Baumann-Grace 1959), pancreatic extract (Moussa 1959), a lytic principle - possibly enzymic - produced by B.cereus (Norris 1957), and combinations of: lysozyme and potassium chloride (Lechowich and Ordal 1962), and lysozyme, desoxyribonuclease and trypsin (Meisel and Rymkiewicz 1958a,b,c, 1959).
2. Treatment with non-enzymic lytic agents, viz: antiformin (Mellon and Anderson 1919) and thiomersalate (Norris and Wolf 1961).
3. Refrigeration, viz: refrigeration at 4^{0} (Walker, Matches and Ayres 1961), refrigeration at pH 4.0 (Harrell and Stewart 1960, cited by Grecz et al. 1962) and slow freezing and thawing (Stewart and Halvorson 1953).
4. Alkaline pH (Brown, Ordal and Halvorson 1957), potassium

hydroxide (Schlossberger 1951).

5. Alteration of the osmotic environment, viz: incubation in phosphate buffer (Tytell 1949) incubation in distilled water (Church 1954).
6. Sonic oscillation (Lundgren and Bott 1963).

In addition a combination of enzymic digestion and sonic oscillation (Grecz et al. 1962) has been used.

Having induced lysis of the vegetative material by the above methods, spores can be removed from the cellular debris by either:-

1. Various methods of centrifugation, viz: repeated washing and centrifugation (Stewart and Halvorson 1953), separation of layers of centrifuged pellets (Long and Williams 1958) or centrifugation in dense sucrose solutions (Krask and Fulk 1959, Church and Halvorson 1959).
2. Two-phase systems (Sacks and Alderton 1961).
3. Froth flotation (Black, MacDonald and Gerhardt 1958, Boyles and Lincoln 1958, Gaudin, Mular and O'Connor 1960a,b).

A number of these techniques were used in an attempt to prepare clean spore suspensions of Cl.perfringens.

Materials and Methods

A batch of spores of isolate NCTC 2836 was prepared in Ellner's medium. The culture was divided into ten portions and the growth

deposited by centrifugation. The following methods were used to separate spores from vegetative material:-

Separation of spores from vegetative material using a two-phase system

The system described by Sacks and Alderton (1961) was used. The centrifuged growth was washed twice in sterile distilled water and the deposit resuspended in the two-phase system. The system comprised: 11.18g polyethylene glycol 4,000 (L.Light and Company Limited, Colnbrook, England) in 34.1ml 3M phosphate buffer, pH 7.1 (1.76 moles K_2HPO_4 plus 1.24 moles KH_2PO_4). The mixture was homogenised by vigorous shaking in a 100ml stoppered measuring cylinder and the two phases allowed to separate at room temperature. Each phase was carefully pipetted off and examined for spores using phase contrast microscopy.

Lysis of Sporangia

Eight samples were washed once with sterile distilled water. The samples were then suspended in the following menstrua, such that one sample was suspended in one of the menstrua:-

1. Sterile distilled water.
2. Ellner's medium.
3. 25 per cent (w/v) potassium hydroxide in distilled water.
4. 25 per cent (w/v) hydrochloric acid in distilled water.
5. 200μg/ml lysozyme (Sigma Chemical Company) in phosphate buffer, pH 7.0 (Mackie and McCartney 1960).
6. Buffered pancreatin, pH 7.5 (Oxoid).

7. 1 per cent (w/v) thiomersalate in distilled water.

8. 1 per cent (w/v) thiomersalate in Ellner's medium.

Tubes were left on the bench overnight and wet films prepared from the centrifuged deposit examined by phase contrast microscopy.

Lysis of sporangia by ultrasonic disintegration

Cells grown in Ellner's medium were centrifuged in a glass centrifuge tube (24 x 110mm) and treated on a M.S.E. Mullard Ultrasonic Disintegrator. During this treatment the tube was immersed in an ice bath.

Results and Discussion

Using the two-phase system the majority of spores were found in the lower phase along with vegetative material. A few spores were found in the upper phase, these were free from vegetative material. When the experiment was repeated using a sporing culture of B.stearothermophilus the spores were confined to the upper phase and the vegetative cells to the lower phase. The distribution of cells using this technique is dependent on the nature of the cell surface, so that if the spores are enclosed within sporangia they will migrate with the vegetative phase. Similarly, if the spores are coated with vegetative debris they will accumulate at the interface along with the rest of the debris. The failure of this method to separate spores and vegetative cells of Cl.perfringens could be attributed to either of these factors.

In addition, the effect of exosporia on the distribution of spores in two-phase systems has not been reported and might be a further complicating factor.

Microscopical examination of spores suspended in: sterile distilled water, Ellner's medium, potassium hydroxide, hydrochloric acid, lysozyme, pancreatin or thiomersalate, indicated that there was no lysis of vegetative cells or cell walls and the majority of spores were enclosed by sporangia. The spores which had been suspended in 25 per cent hydrochloric acid were all phase-dark indicating loss of intracellular components.

The only method which resulted in lysis of vegetative material was ultrasonic oscillation. After 20 minutes treatment spores were observed free from sporangia in an amorphous suspension of lysed vegetative material. Spores subjected to this and prolonged treatment ($1\frac{1}{2}$ hours), were whole, phase bright and viable. Spores were readily separated from debris by repeated differential centrifugation in sterile distilled water (2,000 r.p.m. for 15 minutes). No advantage was observed by centrifugation in concentrated solutions of glucose. An electron micrograph of a spore suspension cleaned by this method is shown in Plate 10.

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A Study of the Conditions Governing the Formation and Germination of Spores of Clostridium perfringens Type A

by

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Strains of Cl.perfringens type A fall into two distinct groups. The two groups, which have been designated classical and heat resistant (or food-poisoning), are readily differentiated on the basis of spore heat resistance and variations in the extracellular "toxins" produced. The existence of these two closely related groups of organisms which produce spores of widely varying heat resistance, provides excellent material for studies of the nature of spore heat resistance. Cl.perfringens is, however notorious for its ability to produce only small numbers of spores in cultur media, although since 1956 a number of workers using a comparatively simple medium (Ellner's medium), appear to have been successful in producing large numbers of spores.

In this thesis the ability of forty eight strains to produce spores in Ellner's medium was investigated. In this medium eight strains (16.7 per cent) produce more than 30 per cent spores; these were all classical strain Thirteen of the thirty classical strains (43.3 per cent) produced more than 1 per cent spores, although none of the heat-resistant strains produced mor than 1 per cent spores. Next, the ability of media of varying composition support sporulation was studied. High percentages of spores were not forme in any of these media, although sporulation was more consistent in a medium devised by Bethge. In this medium all the strains formed spores to roughly

the same extent.

In view of the finding that only in Ellner's medium were certain strai able to produce abundant spores, the effect of cultural conditions on sporulation were studied. The important findings are summarised below:-

1. The medium in which the cells were grown(pre-sporulation medium) prior to inoculation of the sporulation medium had a pronounced effect on the number of spores produced. The best of the pre-sporulation media examined was Robertson's meat broth.
2. Although vegetative growth was better at 45° than 37° sporulation was better at 37°.
3. Aeration of the sporulation medium prevented sporulation.

Attempts to induce abundant sporulation of strains which produced only low numbers of spores in Ellner's medium, by slight modifications to this medium, were unsuccessful. However, a number of the modifications had a pronounced effect on sporulation of good sporing strains. With these strai peptone preparation, inorganic salts, and pH, affected sporulation.

The effect of the components of Ellner's medium was also studied. Attempts to fractionate peptone into sporogenic and asporogenic components showed that the ability to promote sporulation is not confined to any one compound, although the fractions obtained supported sporulation to differen extents. The greatest increase in sporulation was found when sporulation took place in a medium consisting of dialysed peptone dissolved in inorganic buffer solution. Inorganic ions are essential for sporulation; magnesium an potassium enhanced sporulation, sodium was inhibitory, while phosphate appe to have no effect.

There was no distinct morphological difference between good and weakly sporing strains and although mucoid strains tended to produce more spores th

smooth strains, this was not invariably the case. Similarly, good sporing, weakly sporing, and heat-resistant strains could not be differentiated by their sensitivities to a number of antibacterial substances. Good sporing strains were however slightly more active than weakly sporing strains in degrading peptone.

When sporulation was studied in solidified Allner's medium in slide cult it was observed that the cells divided for 2½ hours, at the end of which tim forespores were first seen. Over the next 3 hours the forespores matured into typical phase-bright spores. Only the cells which divided formed spore and following the phase of division thirteen per cent of the cells lysed. T cells which lysed and the cells which formed spores were not confined to separate clones. Spores were invariably formed at the end of the cell furthest away from the site of the last division.

Electron micrographs showed that the spore is enclosed within a strucut resembling an exosporium. When this was removed the surface of the spore appeared to be smooth.

The second part of the thesis deals with conditions promoting and inhibiting spore germination, outgrowth, and the development of cultures fro spore inocula. Spores were found to undergo germination and outgrowth on horse blood agar even when incubated aerobically. Under these conditions germination was complete after 100 minutes and outgrowth after 180 minutes. Post germinative development did not proceed beyond the stage of outgrowth and the resultant cells died. Glucose and inorganic nitrogen sources did no "trigger" germination, although a number of amino acids (DL-mixtures) were a

to promote germination. In the presence of sodium phosphate buffer germination was inhibited.

On media containing neomycin and streptomycin (at concentrations used in selective media for the isolation of Cl.perfringens) vegetative growth from spore inocula was prevented. On media containing neomycin, spores germinated but did not develop further. This finding is of considerable importance to those concerned with Cl.perfringens, since the use of antibiotic - containing selective media in the isolation of this organism has been widely recommended.