The occurrence and pathogenicity of Clostridium oedematiens in animals

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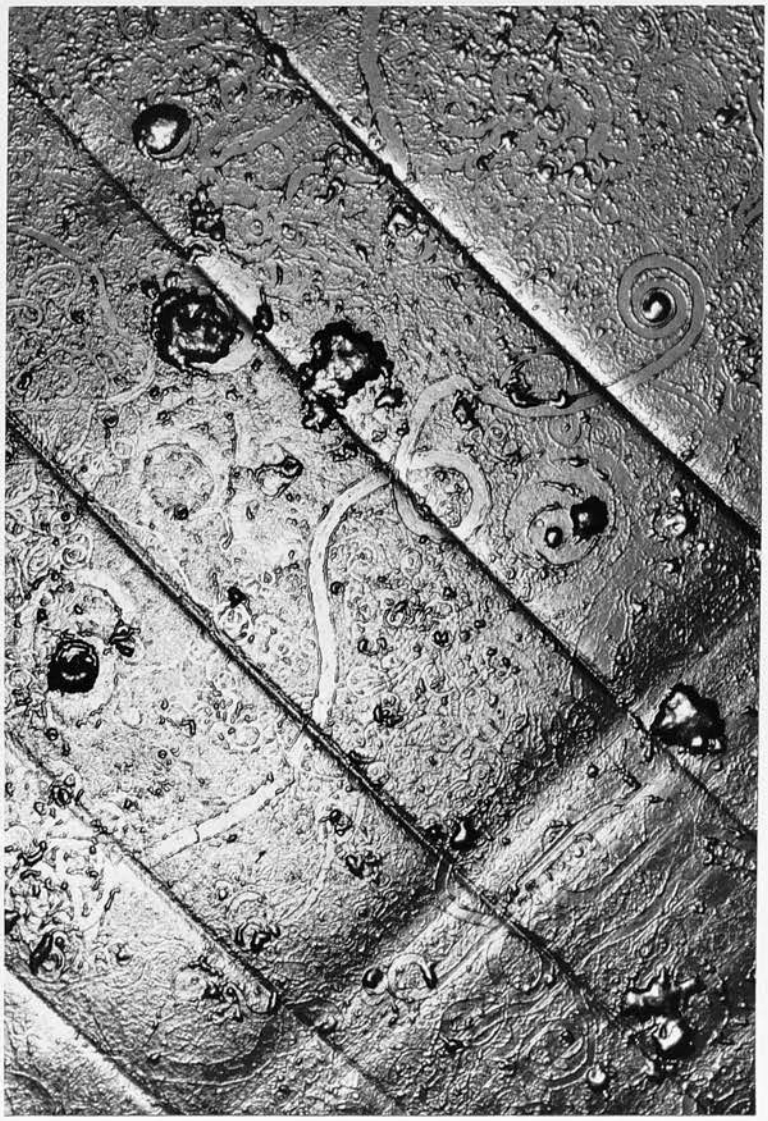

Motile colonies of Clostridium oedematiens

on human blood agar medium. X c.5.

PREFACE

Clostridium oedematiens (Clostridium novyi) is an anaerobic spore-forming bacillus. The organism is an important pathogen of sheep and cattle, and can be responsible for gas gangrene in humans. Four types designated A, B, C and D are recognised on the basis of serological neutralisation tests. Cl. oedematiens is generally regarded as being very difficult to handle in the laboratory; thus, the recent introduction of a commercially available fluorescent globulin for the identification of this organism has encouraged workers to disregard the necessity for confirmatory isolation and typing procedures.

Reliable techniques for the isolation and subculture of Cl. oedematiens are an essential prerequisite for a study of the occurrence of this organism, and my initial work soon confirmed that type B, C and D strains of Cl. oedematiens are difficult to subculture with confidence on solid media. It became clear that much preliminary laboratory work is necessary before a field investigation could yield meaningful data.

The writer considered that the irregular growth of this organism on solid media might be related to (i) failures in the anaerobic environment; (ii) a requirement for a particularly complex nutritional medium; or (iii) the viability of the inoculum; and each of these variables is carefully studied in the present work. The first part describes a reappraisal of the technique for setting up a modern

anaerobic jar. Variations in the technique are assessed, and it is concluded that the irregular growth of these strains is not necessarily related to faults in normal anaerobic procedure.

A variety of culture media are tested for the ability to support regular growth of Cl. oedematiens. The aim of these experiments is to determine whether the organism grows better on complex media than on relatively simple media; it is found that irregular growth occurs on both types of media.

The viability of a number of different inocula are studied in order to test the assumption that an inoculum containing spores should be viable. The results of these experiments are compatible with the view that consistent growth of Cl. oedematiens on solid media may be related to the spore content of the inoculum; however, it is likely that other factors are involved, and a theory is developed that a low redox potential in the microenvironment is also necessary for the successful outgrowth of viable particles.

The epidemiological distribution of this organism can be assessed only if adequate descriptions and techniques of identification are available. Thus, the characters of the Cl. oedematiens group are confirmed and extended during the present work. These studies include (i) a critical evaluation of the fluorescent staining procedure; (ii) a reappraisal of the fermentation reactions of the group; (iii) an assessment of the value of solid indicator media in the identification of this organism; (iv) an intensive study of the soluble products of Cl. oedematiens; and (v) the development of a

practical system of typing with cultures grown in cooked-meat medium.

The soluble products of the organism are investigated in various types of media, and particular attention is paid to the production of the factors that are responsible for the pathogenic effects in-vivo. Culture products of Cl. oedematiens are fractionated and it is found that the biological activities are readily separated by gel-filtration procedures. A thin-layer chromatographic technique is developed in an attempt to identify more precisely the factors that affect egg-yolk emulsion. A cytopathic effect that is produced by the soluble products of Cl. oedematiens is investigated, and is provisionally attributed to the presence of the alpha antigen.

The experimental observations are discussed in relation to our present knowledge of Cl. oedematiens and future lines of research are indicated.

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CONTENTS

SUMMARY

1. The literature regarding Clostridium oedematiens is critically reviewed and the development of our present knowledge of this organism is traced.

2. The characteristics of the organism and the distribution and properties of the soluble antigens of the group are described in detail, with particular reference to systems of classification; it is pointed out that the system of routine typing based on the soluble antigens has not hitherto been critically assessed by independent workers.

3. The microscopic and staining characters of Cl. oedematiens are confirmed. The immunofluorescent staining technique is evaluated with smears prepared from laboratory cultures and from pathological specimens. The role that the fluorescent technique should play in future studies is indicated and the necessity for confirmatory cultural and typing procedures is emphasised.

4. Preparations of Cl. oedematiens are examined in the electron microscope; the organism is non-fimbriate and possesses numerous peritrichous flagella that may aggregate to form a twisted, rope-like structure.

5. The growth of Cl. oedematiens in various laboratory media is

assessed; factors that affect successful cultures in Brewer medium are studied; cooked-meat broth medium prepared with fresh meat particles is recommended for this organism as a routine.

6. Problems associated with the isolation of Cl. oedematiens are investigated with particular reference to isolation on solid media and to the prevention of spreading growth; a selective medium is developed and an isolation procedure is described.

7. The relative ease of growth of type A strains on solid media is demonstrated; difficulties associated with reliable surface growth of strains of types B, C and D are confirmed.

8. The procedure for setting up a modern anaerobic jar is considered in detail: particular attention is paid to (i) the initial vacuum that is drawn in the jar; (ii) the indicator of anaerobiosis; (iii) the rate of production of anaerobiosis; (iv) the sources of moisture production in the jar; and (v) the use of simple manometry as an indication of catalyst activity. It is concluded that the correct operation of the anaerobic jar does not guarantee regular growth of Cl. oedematiens on solid media.

9. Many complex media are tested for the ability to support surface growth of type B, C and D strains. The presence of carbon dioxide is shown to facilitate successful surface culture, but growth is unreliable even with this precaution.

10. An attempt is made to relate the irregular growth of these strains

to the presence of spores in the inoculum. Viable counts of heated and phenol-treated inocula are performed, and the viable counts of different cultures are related to their spore content. The results are compatible with the theory that the viability of Cl. oedematiens on solid media may be related to the presence of spores in the inoculum.

11. A culture procedure is described with iron filings that allows the prompt and reliable surface growth of type B and D strains of the organism.

12. Experiments are performed with indicator dyes to measure the redox potential of different culture media. A theory is developed that a low redox potential may be necessary for the outgrowth of viable particles.

13. The fermentation reactions of Cl. oedematiens are critically evaluated. Experiments that lead to the use of cooked-meat broth as the basal medium in these tests are described, and the fermentation reactions of a limited number of strains of the organism are intensively investigated. A consistent pattern of results emerges, and the development and applications of the test system are indicated.

14. The methods for provisional typing of Cl. oedematiens on solid indicator media are assessed; the procedure is not recommended.

15. The soluble antigens of Cl. oedematiens are studied in detail with particular reference to cooked-meat broth medium. It is concluded

that the so-called lecithovitellin reaction does not provide a completely satisfactory basis for a system of provisional typing.

16. Hayward and Gray's haemolysin neutralisation test with human cells as the substrate is developed, and its incorporation in a system of provisional typing is recommended.

17. The production of haemolytic and lecithovitellin factors is investigated in different media with strains of each type of Cl. oedematiens. A haemolytic factor produced by type A strains in media that contain meat particles is described; it appears that a similar factor is formed by these strains in the presence of egg-yolk emulsion.

18. Thin-layer chromatographic analyses are performed to identify the soluble products of Cl. oedematiens that affect egg-yolk emulsion. This simple technique confirms the findings of previous workers and its promising role in future studies is indicated.

19. Production of the lethal factors of Cl. oedematiens is investigated, and particular attention is paid to the stability of the alpha antigen.

20. The soluble products of Cl. oedematiens are fractionated by ammonium sulphate precipitation and by gel-filtration techniques; the recovery of biological activity is estimated. It is found that the lethal activity of a type B culture is readily separated from the haemolytic and lecithovitellin activities. A partially-purified sample of the lethal factor is prepared and is used to produce an

antiserum that protects mice against a normally toxic challenge of Cl. oedematiens. The possible application of purified antigenic preparations in commercial vaccines is discussed.

21. The cytopathic activity of the soluble products of Cl. oedematiens is studied in detail; a powerful cytopathic effect is provisionally attributed to the presence of the alpha antigen and may be exploited in a system of provisional typing.

22. Immunodiffusion experiments are performed to investigate the precipitation lines that develop in double diffusion tests and in immunoelectrophoretic systems with preparations of Cl. oedematiens. The results indicate that many of the lines occurring in such tests are associated with antigens that are related to the bacterial cell; it is emphasised that caution is necessary in their interpretation.

23. The results obtained in the present studies are discussed in relation to the existing knowledge of Cl. oedematiens; suggestions are made for their application to future investigations.

INTRODUCTION

The definition of the Cl. oedematiens group

In 1894, Novy isolated an anaerobic bacillus from oedematous lesions in three guinea-pigs that died after inoculation with milk nuclein. He described the organism and called the new species Bacillus oedematis maligni II. Migula (1900, cited by Breed, Murray and Smith, 1957) referred to the organism as Bacillus novyi. Similar strains of the bacillus were probably reported in the following years under a variety of names, and Bergey et al. (1923, cited by Breed, Murray and Smith) confirmed the nomenclature of Migula by naming the organism B. novyi.

Weinberg and Séguin (1915) isolated a bacillus from fatal cases of gas gangrene in war victims. They showed serologically that the new isolate did not belong to the same group of organisms as Vibrion septique (now Cl. septicum), and because of the extensive oedema that was produced in both natural and artificial infections, they called the organism Bacillus oedematiens. Bergey et al. (1923) referred to Cl. oedematiens and Cl. novyi as distinct species, but Bergey et al. (1939) quoted Cl. oedematiens as a synonym of Cl. novyi. Thus, the trivial name novyi is used in the United States, whereas oedematiens has been retained in most other parts of the world.

Albiston (1927) investigated Black disease of sheep in Victoria; he isolated the causal organism and noted that it closely resembled B. oedematiens. Turner and Davesne (1927) showed that serum prepared

against B. oedematiens was completely protective against the pathogenic effect of the Black disease bacillus, and they suggested that the organism was a type of B. oedematiens. Turner (1930), in a detailed report of Black disease and the causal organism, emphasised the value of specific neutralisation tests as the final arbiter in identification.

Meanwhile in Germany, Zeissler and Rassfeld (1929) examined sheep that had died of German Bradsot. They isolated an organism which they called B. gigas and they distinguished it from B. oedematiens on a morphological and a biochemical basis. Cross-protection tests revealed no differences between the classical B. oedematiens and B. gigas (Miessner, Meyn and Schoop, 1931, cited by Turner and Eales, 1943), or between the Black disease bacillus and B. gigas (Zeissler, 1931; Turner and Eales, unpublished, cited by Turner and Eales, 1943).

Kraneveld and Djaenoedin (1933) described a bacillus that they had isolated from cases of bacillary osteomyelitis of water buffalo in the Dutch Indies; they noted that this organism gave similar results to those given by B. gigas in fermentation tests, and suggested that the two bacilli might be included in the same group as B. novyi and the bacillus of Black disease. Kraneveld (1934) observed that the bacillus that he isolated in 1933 "coincides morphologically and culturally with B. gigas except that it lacks pathogenicity."

Scott, Turner and Vawter (1934, cited by Oakley, Warrack and Clarke, 1947) divided the organisms described at that time into three groups as shown in Table I, on the basis of the source and size of the organism, fermentation of glycerol and pathogenicity.

Table I

A preliminary classification of Cl. oedematiens into three biotypes (see Oakley, Warrack and Clarke, 1947)

Type	Organisms included	Origin	Size	Fermentation of Glycerol	Experimental Pathogenicity	Toxigenicity
A	B. oedematis maligni II (Novy, 1894) B. oedematiens (Weinberg and Séguin, 1915) Cl. oedematiens Cl. novyi	Gas gangrene of man and other animals	Small 0.8 - 1 x 2.5 - 5 μ.	+	+	+
B	B. oedematiens (Albiston, 1927; Turner and Davesne, 1927) B. gigas (Zeissler and Rassfeld, 1929)	Black disease and Bradsot of sheep	Large 1.2 - 2 x 10 - 14 μ.	-	+	+
C	Bacillus of osteomyelitis bacillosa bubalorum (Kraneveld and Djaenoedin, 1933)	Bacillary osteomyelitis of buffaloes	Large 1.5 - 2 x 8 - 10 μ.	-	-	-

This classification remained until 1947, when Oakley *et al.* studied the antigenic components that were present in toxic filtrates of *Cl. oedematiens*. They identified six soluble antigens and they showed that the distribution of these factors gave a more reliable basis for typing the species than the previous criteria. They defined three types (A, B and C) and their results supported the conclusions of Scott *et al.* but provided "type-differentiating criteria of greater stringency." The authors considered that "*Cl. oedematiens* is best defined as that species of *Clostridium* possessing somatic (O) antigens OED I or II or both. It is an obligate anaerobe, usually fermenting glucose and maltose, often fermenting glycerol and not fermenting lactose, sucrose, mannitol and salicin. The species so defined is readily divisible into three types based on toxin production .... which show marked internal consistency and agree very well with those based on source, size, cultural characters and pathogenicity."

The classification devised by Oakley *et al.* has now been extended to include type D strains (see below) and is in routine use, although the soluble antigens of *Cl. oedematiens* have not been systematically studied.

The origin of Cl. oedematiens type D

Vawter and Records (1925-26) isolated an obligate anaerobic bacillus from cases of icterohaemoglobinuria of cattle in the United States. They described the clinical signs of the disease and the organism that they isolated, and they showed that it differed serologically from Cl. welchii, Cl. septicum, Cl. chauvoei and Cl. oedematiens (presumably type A). The authors suggested that the organism should be named Cl. haemolyticus bovis; Hall (1929, cited by Breed, Murray and Smith, 1957) suggested the binomial name Bacillus haemolyticus (later Cl. haemolyticum).

Records and Vawter (1945) described the organism in detail, with particular reference to its morphology, cultural characters, pathogenicity and serology. Oakley et al. (1947) presented preliminary evidence to suggest that Cl. haemolyticum is a member of the Cl. oedematiens group; then Oakley and Warrack (1959) showed that Cl. haemolyticum produces at least two soluble antigens, one of these being shared with Cl. oedematiens type B. They suggested that the organism should be referred to as Cl. oedematiens type D, and this has become standard practice.

Thus, Cl. oedematiens types A, B, C and D are now recognised. The different types produce different permutations of soluble antigens and can be identified from the results of suitable tests; type C strains are apparently non-toxigenic and do not produce any of the

major soluble antigens. The previous descriptions of Cl. oedematiens usually describe the diffusible products of the organism as toxins. The term 'toxin' is inaccurate as the individual products are not necessarily toxic. It is more precise to name these components soluble antigens, because they are found in the culture supernate, and because Oakley et al. showed that the products are antigenic.

General introduction

Cl. oedematiens is a pathogenic anaerobic bacillus that can be isolated from various infections of man and animals; its habitat is probably the soil (Breed, Murray and Smith, 1957). The species can be divided into four types designated A, B, C and D, which are based on the soluble antigens that are present in culture filtrates of the organism (Oakley et al., 1947; Oakley and Warrack, 1959).

Type A strains and occasionally type B strains are associated with gas gangrene in humans; MacLennan (1962) stated that this species is the second most important cause of gas gangrene after Cl. welchii.

Type B strains and occasionally type A strains of Cl. oedematiens cause Black disease in sheep and in cattle. Black disease (infectious necrotic hepatitis) is an acute, infectious, fatal, toxaemic disease characterised by the death of the animal after a very short illness, marked subcutaneous oedema, exudate in the serous cavities, and one or more small, sharply demarcated necrotic areas in the liver. Black disease was known to exist in Australia towards the end of the 19th century and was gradually recognised in other parts of the world including Britain (Jamieson, Thompson and Brotherston, 1948). Most sheep flocks that are now at risk are routinely vaccinated against the disease.

Type C strains of Cl. oedematiens were isolated by Kraneveld and Djaenoedin (1933) from bacillary osteomyelitis in water buffalo in

the Dutch Indies. This is an unusual sub-division of the species, because the type C strains apparently do not produce any of the soluble antigens that are characteristic of the species; the strains are non-pathogenic when inoculated into small experimental animals. The natural disease in buffalo presents as a mild or chronic lameness with intermuscular and intramuscular abscesses, and necrobiosis of the bone, sometimes with secondary complications (Kraneveld, 1934).

Cl. oedematiens type D (Cl. haemolyticum) causes bacillary haemoglobinuria (infectious icterohaemoglobinuria) of cattle. This is a rapidly fatal infectious disease characterised by a high fever, haemoglobinuria and the presence of an infarct in the liver. The disease is thought to have occurred in Nevada for many years and, in 1926, Vawter and Records isolated and identified the causal organism. The disease was first reported in Britain by Soltys and Jennings (1950).

There have been many reports of the isolation of Cl. oedematiens from several species of animals, and Keppie (1944) listed these in chronological order. He observed that Scott, Turner and Vawter (1934) divided the species into three groups (see Table I), which were based on the origin and size of the organism, the fermentation of glycerol, and the experimental pathogenicity and toxigenicity of the strain. Keppie's work confirmed that type A strains of Cl. oedematiens are smaller than type B strains, and that they ferment glycerol; in general, the type B strains did not ferment glycerol. He showed that the type A strains are easily cultivated on solid and

in liquid media. The type B strains grew poorly in his experience and he suggested that they were more oxygen-sensitive than the type A strains. The type C strains that Keppie used were as large as the type B strains, but they would not grow on solid media unless enriched with about 30 per cent. of serum; the strains did not ferment glycerol. He showed that the type A, B and C strains of Cl. oedematiens possess a common somatic 'O' antigen, but that they have a variety of 'H' antigens. This confirmed the work of Turner and Eales (1943) who observed that all of the strains of Cl. oedematiens that they examined, including representatives of each type, had one or two somatic antigens in common; however, Smith (1953) suggested that the type D strain which shared a common antigen with the other types may not have been authentic. These points are discussed in greater detail later in this section.

Probably the most significant and the most elegant contribution to our knowledge of Cl. oedematiens was provided by Oakley et al. (1947). These workers identified six antigenic components in toxic filtrates from Cl. oedematiens types A, B and C; they demonstrated that each of the soluble antigens possessed one or more biological activities. They concluded that methods based on the properties of three of these antigens gave more reproducible results than the previous criteria in the identification of the three types of Cl. oedematiens. By a similar procedure, Oakley and Warrack (1959) showed that Cl. haemolyticum produces two soluble antigens, and they suggested that the organism should be referred to as Cl. oedematiens type D. The method

of typing that was suggested by Oakley and his colleagues has been used to type the organisms that have been isolated from cattle (Marshall, 1959; Williams, 1964) and sheep (Williams, 1962).

A recent aid to the identification of Cl. oedematiens has been the development of a commercial fluorescent-labelled antiserum. Batty and Walker (1964) found that strains of Cl. oedematiens, representing all of the types, fluoresced with an antiserum prepared against a type B strain. This technique has been used to demonstrate Cl. oedematiens post mortem in cattle, sheep and pigs (Batty, Buntain and Walker, 1963, 1964; Bourne and Kerry, 1965; Hart, 1965; Corbould and Munday, 1966). Jamieson (1949) found that in areas where Black disease is present, many normal sheep and cattle harbour latent spores of Cl. oedematiens. Corbould (1966) observed that a high percentage of ovine livers collected at slaughter contain Cl. oedematiens; the organisms proliferate rapidly under suitable conditions and are then readily detected by the immunofluorescent technique. He considered that supporting evidence should be available before placing undue emphasis on the demonstration of Cl. oedematiens in post-mortem material. Batty, Kerry and Walker (1967) confirmed that they had always insisted that a diagnosis cannot be made using the flurcescent technique without considering associated relevant factors such as the clinical history, the post-mortem findings and the state of decomposition of the carcase. The development of the immunofluorescent technique is of great value in the identification of Cl. oedematiens, but in the absence of more detailed

knowledge of the distribution of somatic antigens in clostridia, it is premature to regard the procedure as infallible. Thus, it is still essential to develop methods whereby this pathogen can be readily isolated and grown reliably in artificial media.

The culture of anaerobic bacteria, with particular reference to Cl. oedematiens

(i) Environmental considerations. In diseases that are caused by certain anaerobic bacteria, the organisms grow readily in devitalised tissue; it is generally regarded that if a suitably nutritious environment is available, then the absence of oxygen is the critical factor that influences the growth of anaerobes. The reasons why anaerobic conditions must exist before growth will occur are not fully understood. It may be that anaerobic organisms form peroxide in the presence of oxygen and they lack suitable enzymes, e.g. catalase, to remove this toxic product (McLeod and Gordon, 1923). Alternatively, free oxygen may irreversibly oxidise some of the enzymes of the metabolic pathways of anaerobes. The presence of oxygen will affect the oxidation-reduction potential (redox potential) of the environment. It has been reported that the vegetative cells of some species of clostridia will not grow above a certain level of redox potential (Reed and Orr, 1943; Barnes and Ingram, 1956), although the results

are rather variable.

The redox potential may also influence the germination of spores. Knight and Fildes (1930) showed that if spores of B. tetani are subjected to different but constant levels of redox potential, then germination of the spores, as judged by a staining technique, is completely inhibited at potentials more positive than Eh = + 0.11v (pH 7.0 - 7.65). It should be borne in mind, however, that a variety of spore germination factors have now been recognised and the presence of such factors may modify the apparent inhibition that redox potential may exert on the germination of spores. The germination requirements for spores of Cl. oedematiens have not been studied, and it is not clear whether the spores of this organism are particularly demanding for certain germination factors. It is clear that the in-vitro growth of Cl. oedematiens will not occur unless suitable conditions exist in the culture medium; indeed, most workers have observed that Cl. oedematiens is a particularly strict anaerobe, and Willis (1964, p. 80) states that type D strains of the organism are probably the most fastidious anaerobes known. The terms 'fastidious' or 'demanding' are best related to the anaerobic requirements of the organism, for there is little evidence to support the view that the growth requirements of Cl. oedematiens are particularly demanding if the physical conditions are suitable for growth. However, in many cases the nutritional factors that are present in a medium also contribute to the physical environment, and it may be difficult to separate and assess the relative importance of the two functions.

(ii) Practical applications. Various methods have been devised for the cultivation of anaerobic bacteria; their development is described by Smith (1955) and Willis (1964). All of these methods are designed to exclude oxygen from the environment in which the anaerobe is growing. The nature of the organism that can be grown depends on the degree of anaerobiosis that is achieved; for example, the less demanding anaerobes may be grown in a micro-aerophilic environment, whilst the 'fastidious' or truly obligate anaerobes can be exposed only to the minimum of oxygen during subculture and subsequent growth.

Suitable conditions for the laboratory cultivation of anaerobic organisms may be achieved by using a deep fluid medium. Robertson (1915) developed cooked-meat broth (CMB) as a general purpose medium, and it is still the most useful medium of its kind in anaerobic bacteriology. CMB contains powerful reducing systems that have been studied by Lepper and Martin (1929, 1930); although the medium is complex and cannot be standardised, it supports the luxuriant growth of many demanding anaerobes.

Fluid media are used extensively in microbiology in order to study the biochemical characters of bacteria; however, a pure culture of the species must be used in such experiments. The initial procedures are frequently critical in the isolation of a pure culture of a pathogen from a mixed sample. If a mixed culture of bacteria is inoculated into a fluid medium, then the resultant growth may not reflect the ratio of the organisms that were present in the inoculum. Some

strains grow more quickly than others in such a medium, and a slowly growing pathogen may be outgrown by other organisms. Therefore, a misleading result may be obtained if a fluid medium is used for the primary isolation of an anaerobic pathogen. If the mixed culture is plated out on an agar plate which is then incubated anaerobically, the resulting growth should indicate the relative numbers of different species of bacteria that were present in the inoculum. Thus, it is important that anaerobic bacteria can be cultured readily on agar plates in order that pure cultures may be isolated and maintained, though the spreading growth that is characteristic of certain clostridial species on solid media may be a further complicating factor (q.v.).

The anaerobic incubation of plates was made possible by the introduction of the anaerobic jar and its development (see Willis, 1964, p. 5). The recognised principle for the growth of anaerobic organisms is that oxygen must be removed from the environment. The anaerobic jar functions on the basis that hydrogen and oxygen combine to form water in the presence of a suitable catalyst so that free oxygen is removed from the atmosphere. Several methods are available for producing anaerobic conditions in a sealed container (see Willis), but the McIntosh and Fildes anaerobic jar is probably the most reliable. In the early models of the jar, the catalyst in a wire-gauze capsule was artificially activated, either by heating the catalyst in a Bunsen burner flame before it was placed in the jar or by passing an electrical current through a heating element in the capsule.

Probably the most significant modification to the anaerobic jar has been the introduction of the room-temperature catalyst (Heller, 1954) which consists of pellets of alumina coated with finely divided palladium. This type of catalyst does not require heat-activation. Jars that utilise the room-temperature catalyst are commercially produced by Baird and Tatlock Ltd. and are now widely used in anaerobic bacteriology.

Although the present anaerobic jar has simplified the problem of growing anaerobic bacteria, it is well known that some organisms, e.g. Cl. oedematiens types B, C and D, are difficult to grow on the surface of plates. Smith (1955) suggests that these demanding strains can be grown on blood agar plates that are covered with a secondary thin clear layer of nutrient agar after inoculation with the anaerobe. Such plates are called 'sub-surface' plates and are designed to reduce the exposure of the organism to air. A similar principle is employed when the anaerobe is grown in a 'pour-plate', in which sterile molten agar is seeded with a fluid culture of the organism, then poured into a sterile plate and allowed to solidify. Both 'pour-plates' and 'sub-surface' plates must be incubated in an anaerobic jar, presumably because the thin layer of agar does not prevent the diffusion of oxygen into the medium. A disadvantage of both of these methods is that the organism must withstand temperatures greater than about 48° C whilst the agar is still molten.

The shake-culture or deep-agar culture is a well known method of obtaining anaerobic conditions. The nature of the medium prevents

the diffusion of oxygen into the deeper layer so that shake-cultures can be incubated under aerobic conditions. Shake-cultures were used in the early development of anaerobic bacteriology and they are still very useful; however, it is difficult to justify the extreme view of the French school of workers who use deep cultures in preference to the anaerobic jar which they regard as cumbersome and expensive (see Willis, 1964, p. 23). It may be significant that few important contributions to anaerobic bacteriology have emerged from France during the past two decades.

Brewer (1940) tried to incorporate the principle of the shake-culture with a more fluid medium. He used an infusion broth as the basal medium and added a small amount of agar, sodium thioglycollate and dextrose. The agar prevents the development of convection currents and reduces the diffusion of oxygen into the medium; sodium thioglycollate and glucose provide reducing conditions within the medium. Reed and Orr (1941) used variations of Brewer's medium in order to study the biochemical reactions of the gas gangrene anaerobes, and Keppie (1944) found that Brewer's medium was the best medium that he tried for the growth of Cl. oedematiens. The addition of dextrose was originally suggested by Brewer for its value "in promoting the growth of many organisms and prolonging the anaerobiosis." Since the majority of clostridia including all of the types of Cl. oedematiens can ferment glucose, this carbohydrate may be regarded as an enrichment factor, but the enrichment is clearly non-specific. There is little knowledge at present of the growth factors that are required by

Cl. oedematiens, and there is no obvious biochemical or metabolic character that can be exploited in a specific enrichment medium for Cl. oedematiens. Therefore the isolation of the organism from pathological material or soil is often difficult and slow.

The isolation of Cl. oedematiens

(i) Heat treatment. Willis (1964) suggests several techniques that can be employed to facilitate the isolation of Cl. oedematiens. He states that the organism is highly sensitive to free oxygen and must not be exposed to air for longer than necessary; however, there is no evidence that the non-viability of Cl. oedematiens in the laboratory is caused by death of the organism as a result of extreme oxygen sensitivity, rather than an environment that is unsuitable for the growth of vegetative organisms or the germination of spores.

The production of spores by Cl. oedematiens can be exploited to facilitate isolation of the organism. Willis (1964, p. 83) recommends that pathological material may be heated at 80 - 100° C for 10 - 15 min. before culture, the spores of Cl. oedematiens being unaffected by this treatment. Turner (1930) showed that fully mature spores of type B strains of Cl. oedematiens would resist heating at 100° C for 10 min., but were destroyed after 15 min. Williams (1962) suggested that "heating of contaminated cultures in liquid media at a

temperature of 100° C for periods of up to 3 hr can be of great value in obtaining pure cultures of Cl. oedematiens" (type B strains). However, Records and Vawter (1945) stated that most sporulating forms of type D strains of Cl. oedematiens are killed at 85° C, some survive 90° C for 15 min., but none survives 95° C; they concluded that the spores of this type are less heat resistant than those of most other anaerobes. Thus, the heating of material before culture must be carried out with reference to the organism that is suspected. Marked variation in the heat resistance of spores within a species may also be observed in Cl. welchii, cf. spores of classical type A strains and spores of typical food-poisoning strains (Hobbs et al., 1953), and Cl. botulinum, cf. spores of types A and B strains and spores of type E strains (Roberts and Ingram, 1965).

Smith (1955) recommends that the basal medium that is used to culture heated material should contain starch; he suggests that the starch inhibits the bacteriostatic action of rancid long-chain fatty acids to which heated spores are especially susceptible. He also recommends that such cultures should be incubated at 25° C and that carbon dioxide should be added to the atmosphere of the anaerobic jar.

If pathological material is heated before subculture it is probable that the resulting bacterial population is entirely derived from spores. Such a population may have certain differences compared with a population derived from vegetative organisms. For example, Nishida and Nakagawara (1965) found that sporulation and the production of toxin were inversely related in 96-hr cultures of Cl. novyi.

Non-toxigenic strains that were apparently type C strains could be produced by heat-selection from parent type A strains, and the authors concluded that sporulating potency is of prime importance in controlling the mechanism of toxigenesis in Cl. novyi. Nishida and Imaizumi (1966) investigated the toxigenicity of Cl. histolyticum; they found that the results obtained with this organism did not follow the general rule that they postulated in previous reports, but it seems advisable to consider the above observation if heating procedures are used in order to isolate anaerobic bacteria.

(ii) Selective agents. In order to isolate anaerobic bacteria from a mixed culture, the growth of many facultative anaerobes that are present may be suppressed by incorporating certain selective agents into the medium. Willis (1964, p. 38) describes various substances that are used as selective agents for clostridia, but he adds that "none of them is ideal." Narayan (1966) tested a number of agents that have been recommended for the specific isolation of anaerobes; he investigated the selective effects of each agent individually and in certain combinations. He found that no selective agent that he tried would specifically inhibit the growth of facultative anaerobes without affecting the growth of clostridia. Narayan concluded that it is difficult to choose a suitable medium that will be selective for the growth of clostridia, although it might be easier to do so if only one or two species of clostridia are taken into account. Thus, the selective agent and the amount that is

incorporated into culture media require careful consideration and, in turn, the choice of a selective culture medium should be based on a detailed knowledge of the characters of the organism.

The characters of Cl. oedematiens

Although the method of typing suggested by Oakley et al. is the most satisfactory at present, the classification and subdivision of a bacterial species may eventually depend on the distribution of a wide range of different characters. In order to judge the significance of these characters it is necessary to know as much as possible about them; there is a great deal of information about Cl. oedematiens that requires confirmation and systematic study.

(i) Microscopic appearances. Turner (1930) observed that the Black disease bacillus is usually larger than the strains of Cl. oedematiens that are associated with human disease. Table I shows that the size of the organism is one of the characters that separate type A strains from type B and C strains in the early classification. Records and Vawter (1945) found that type D strains of Cl. oedematiens are about the same size as type A strains of the organism. It is obvious that the size of a bacillus can only be used as a typing character under defined conditions.

Cl. oedematiens is generally regarded as being Gram-positive

(Breed, Murray and Smith, 1957), although Turner (1930) states that type B strains are only Gram-positive if the decolourisation is controlled, and Records and Vawter (1945) found that type D strains become Gram-negative after 12 - 16 hr growth. Spray (1936, cited by Reed and Orr, 1941) stressed that when staining clostridia with Gram's stain, it is important to decolourise with alcohol in order to avoid irregular results.

All of the types of Cl. oedematiens form spores; these are usually oval, sub-terminal and they may distend the bacillus. Keppie (1944) observed that type A, B and C strains sporulate in the presence of glucose and that a small proportion of free spores can be found in most media. Turner (1930) noted that the sporulation of type B strains was much improved by the addition of 20 per cent. serum, but that the sporulation ratio did not exceed 50 per cent. Records and Vawter (1945) stated that Cl. oedematiens type D strains spore readily at first on laboratory media but this property is gradually lost and may disappear on prolonged subculture.

All of the types of Cl. oedematiens bear numerous peritrichous flagella which can be demonstrated by suitable staining techniques, although motility may only be observed under conditions that exclude oxygen (see Wilson and Miles, 1964, p. 1073). These authors also state that Cl. oedematiens is non-capsulate; however, Keppie (1944) suggested that certain strains of the organism may possess a thin capsule.

The above description of the microscopic appearances of

Cl. oedematiens supports the observation of Willis (1964, p. 80) that there is nothing uniquely characteristic about the morphology of this organism.

(ii) Cultural appearances. Early descriptions of Cl. oedematiens reveal that the organism grows well in a nutritious liquid medium. Turner (1930) stated that type B strains fail to grow in peptone water medium, and that enrichment of broth with glucose, serum or meat particles is necessary. Vawter and Records (1925-26) observed that type D strains grow luxuriantly in peptic digest broth plus rabbit liver. Autoagglutination of most strains of Cl. oedematiens in liquid media is frequently reported (Vawter and Records, 1925-26; Turner, 1930; Keppie, 1944) and has complicated previous studies of the agglutinating antigens of the organism. Vawter and Records (1931) suggested that type D strains of Cl. oedematiens autoagglutinate when the pH of the medium reaches 5.4 - 5.6 after 24 - 30 hr incubation.

Many workers have used the shake-culture technique in order to isolate and grow Cl. oedematiens (Turner, 1930; Records and Vawter, 1945), and most descriptions of type B and D strains of the organism refer to the different colony types that occur in such media (see Breed, Murray and Smith, 1957). Although Cl. oedematiens grows readily in shake-cultures, the difficulties of growing type B, C and D strains of the organism on solid media are well recognised. Albiston (1927) stated that he was unable to grow the Black disease

bacillus as a surface culture. Turner (1930) observed that the type B strains grow as a spreading film on blood agar and he did not recommend the medium. Keppie (1944) found that if plates were inoculated directly with infected tissues they often failed to yield type B strains. He also noted that type C strains grow only on glucose blood agar plates that are enriched with a further 30 per cent. of serum. Records and Vawter (1945) stated that type D strains have exacting cultural requirements and these authors imply that the non-viability of inocula may frequently be a reason for failure to grow and maintain the organism.

Williams (1962, 1964) used blood agar plates that contained 5 per cent. of sheep blood and 3 per cent. of agar for the isolation and growth of Cl. oedematiens. He confirmed that the organism is very fastidious and that type B strains tend to spread on blood agar plates. He stated that cultures of type D strains on solid media die rapidly on exposure to air. Willis (1964, p. 80) observed that "there is a tendency for the colonies of types A and B to spread over the surface of the medium. The colonies of type D are often exceedingly small, appearing as mere pin-points after 48 hr incubation. Type B strains are more difficult to grow than type A strains whilst strains of type D are probably the most fastidious anaerobes known." Thus, it is generally agreed that type B and D strains of Cl. oedematiens are difficult to grow on solid media, particularly at primary isolation, and that type A and B strains are liable to spread on blood agar plates. However, the work of Williams above suggests that type

B strains of the organism will grow on a relatively simple medium.

Cl. oedematiens forms soluble antigens that produce characteristic effects when the organism grows on certain solid media (q.v.). Observations of these effects can be used in the preliminary identification and typing of the organism. It is clear that an essential prerequisite of such a typing procedure is the development of a solid indicator medium that supports good growth of all types of Cl. oedematiens and limits spreading of the organism.

(iii) Biochemical characters. The biochemical characters of Cl. oedematiens have been extensively studied in the past, and there are many conflicting reports. The pattern of results that has evolved is shown in Table II which has been compiled from the results quoted by Breed, Murray and Smith (1957).

Turner (1930) noted the wide discrepancies in the recorded results of the fermentation reactions of Cl. oedematiens despite the use of apparently pure cultures by various workers. He concluded that there were too many variables involved in the tests, including (a) the basal media and indicators, (b) the method of sterilisation and the amount of heat to which the sugar was exposed, (c) the chemical purity of the carbohydrates, (d) the final concentration of the sugar, and (e) the method of obtaining anaerobiosis. To this list may be added the duration of incubation of the test medium.

Zeissler and Rassfeld (1929) found that B. gigas strains ferment glucose and fructose but not maltose; galactose fermentation was

Table II

The various biochemical reactions of Cl. oedematiens

Test		Reaction of Cl. oedematiens of TYPE			
		A	B	C	D
The fermentation of:	Glucose	+	+	NR	+
	Maltose	v	v		-
	Fructose	+	+		+
	Galactose	NR	+		-
	Glycerol	v	v		+*
	Lactose	-	-		-
	Sucrose	-	-		-
	Raffinose	NR	NR		-
	Arabinose	NR	NR		-
	Xylose	NR	NR		-
	Inulin	NR	-		-
	Salicin	-	-		-
	Mannitol	-	-		-
	Dulcitol	NR	-		-
	Mannose	NR	NR		-
	Dextrin	NR	NR		-
The formation of:	Indole	-	NR	NR	+
	Hydrogen sulphide	NR	NR		+
Methyl red test		NR	NR		-
Voges-Proskauer reaction		NR	NR		-
Detection of nitrites from nitrates		-	NR		-
Gelatin liquefaction		+	+		+
Reaction in milk medium		A; NC	v		A; SC
Liquefaction of coagulated albumen		-	-		-
Liquefaction of blood serum		-	NR		-
Blackening and digestion of brain medium		-	NR		-

+ = positive result; - = negative result; NR = not recorded;
v = variable result; A = acid produced; NC = no coagulation;
SC = slow coagulation, clot not digested.
*Records and Vawter (1945) state that glycerin was not fermented.

doubtful. Turner (1930) reported that type A strains of Cl. oedematiens strongly ferment glucose, maltose and glycerin; galactose was weakly fermented and fructose fermentation was doubtful. Type B strains of the organism ferment glucose, maltose, fructose and galactose according to Turner.

Kraneveld and Djaenoedin (1933) examined 17 strains of B. gigas and 6 strains of an anaerobic bacillus that they isolated from osteomyelitis of buffalo, (type C strains). The English summary of their paper states that "all strains showed to behave themselves in quite a similar manner opposite ten different carbohydrates," although from the text it appears that the type C strains ferment maltose. Keppie (1944) observed that some type B strains apparently ferment glycerol; he also confirmed that type C strains ferment glucose, maltose and fructose.

Oakley et al. (1947) tested the fermentation reactions of 59 strains of Cl. oedematiens that they typed as A or B strains on the basis of their soluble antigens. All of the strains fermented glucose; in general, the type A strains also fermented maltose and glycerol, but many of the type B strains fermented glycerol and only a few of the latter failed to ferment maltose. Oakley et al. stress that "the organism must grow well in the fermentation medium, or false negatives will be found, as was often the case with type B strains." They analysed their results in terms of typical and atypical strains using the criteria of Scott et al. According to Scott et al., however, the fermentation of glycerol differentiates type A strains

from type B strains, and it does not appear that this criterion was precisely applied by Oakley et al. Nevertheless, it is clear that there is considerable variation and the authors concluded that "we are therefore disinclined to rely on fermentation reactions alone for identifying and classifying Cl. oedematiens."

Jamieson (1949) observed that all of the type B strains of Cl. oedematiens that he isolated did not affect glycerol. He pointed out that those strains that were freshly isolated from Black disease of sheep (Turner, 1930; Jamieson, 1949) failed to ferment glycerol.

Apart from the fermentation reactions, other biochemical characters of Cl. oedematiens have been sporadically studied. The demonstration of the formation of indole from tryptophan is widely used as an aid in the identification of a bacterial species. Reed and Orr (1941) showed that type A strains of Cl. oedematiens fail to produce indole in a semi-solid basal medium, but Reed (1942) demonstrated that the type A strains of the organism utilise indole as rapidly as it is formed; furthermore, if indole is added to the medium then it disappears. Thus, the qualitative detection of indole will depend on the ratio of the rates of its formation and breakdown. Records and Vawter (1945) reported that type D strains of Cl. oedematiens have a requirement for tryptophan in the culture medium; they noted that these strains produce indole after 16 - 18 hr incubation, but that the property of indole formation was decreased after prolonged subculture. Willis (1964, p. 59) states that the indole test is not of great value in dealing with the commonly occurring anaerobes.

Many bacterial species are able to produce hydrogen sulphide from a suitable substrate in a culture medium. The test can be poised at various levels and Reed and Orr (1941) developed a technique that would give a positive reaction with large or moderate yields of hydrogen sulphide; they observed that type A strains of Cl. oedematiens gave a positive result. Records and Vawter (1945) noted that type D strains of the organism produce large amounts of hydrogen sulphide. Willis (1964, p. 59) states that the majority of commonly occurring anaerobes produce hydrogen sulphide, therefore the test is of limited value in routine work.

Certain species of bacteria can reduce nitrates to nitrites, or even further to ammonia or nitrogen. The detection of nitrites in a suitable medium is regarded as an indication of nitrate reduction. Reed and Orr (1941) found that on the basis of this test, type A strains of Cl. oedematiens fail to reduce nitrate; however, Reed (1942) showed that the type A strains do reduce nitrate rapidly and that nitrite is not detectable. Records and Vawter (1945) reported that nitrites are not produced from nitrates by type D strains of the organism.

Records and Vawter also observed that the type D strains give a negative reaction in the methyl red test, and that they do not produce acetylmethylcarbinol.

It is generally agreed that all of the types of Cl. oedematiens liquefy gelatin but do not attack more complex protein substrates such as milk, serum or meat particles. The organism is usually described

as being saccharolytic and non-proteolytic. Several clostridia can liquefy gelatin but have no effect on more complex protein substrates. Grant and Alburn (1959) noted that in most proteolytic systems the substrate is more readily attacked if it is denatured. They stated that gelatin is severely denatured collagen and that it lacks the ordered structure of the parent collagen molecule; thus, gelatin is attacked by a variety of endopeptidases of bacterial and mammalian origin, whereas the action of collagenase is a highly specific proteolytic activity.

Several authors state that Cl. oedematiens does not grow in nutrient gelatin unless the medium is enriched (Zeissler and Rassfeld, 1929; Willis and Hobbs, 1959). Turner (1930) made the curious observation that Cl. oedematiens type B strains will grow well in nutrient gelatin and in glucose-gelatin, but that gelatin is liquefied in the latter medium only. Records and Vawter (1945) noted that type D strains of the organism liquefy gelatin and that prolonged subculture of a strain may reduce its potency in this respect.

The variable results that have been recorded in biochemical tests such as those described above may be partly related to differences in culture media and procedures that are not standardised. It is clear that Cl. oedematiens should not be classified and identified on fermentation reactions alone, and that the other biochemical characters are of little value at present. However, a system of typing that is based solely on the detection of soluble antigens may founder when strains that do not produce key antigens are encountered. The type C

category of so-called non-toxigenic strains of Cl. oedematiens is a classical example of this problem.

The antigens of Cl. oedematiens

(i) The somatic and flagellar antigens

The 'H' and 'O' antigens of Cl. oedematiens have been studied by Kreuzer (1939, cited by Turner and Eales, 1943), by Turner and Eales (1943) and by Keppie (1944). Turner and Eales investigated 5 type A strains, 22 type B strains and 6 type C strains (classified after Scott et al., 1934) as well as 2 strains of Cl. haemolyticum. They obtained evidence that all of these strains share two 'O' antigens in various proportions, but they concluded that more work was necessary to clarify the relationship of Cl. haemolyticum to other members of the Cl. oedematiens group. Smith (1953) suggested that one of the strains of Cl. haemolyticum that Turner and Eales used was classified incorrectly, and he stated that strains of Cl. haemolyticum and Cl. oedematiens possess no common somatic antigens; he later (Smith, 1955) described Cl. haemolyticum as a species distinct from Cl. oedematiens.

The inclusion of Cl. haemolyticum as Cl. oedematiens type D was suggested by Oakley and Warrack (1959); these authors showed that the major soluble antigen that is produced by Cl. haemolyticum is

serologically indistinguishable from one of the antigens that is produced by type B strains of Cl. oedematiens. Further evidence was provided by Batty and Walker (1964), who found that a fluorescent-labelled antiserum that was prepared against the vegetative antigens of a type B strain of Cl. oedematiens stains type D (Cl. haemolyticum) strains. On the basis of these findings it is reasonable to consider Cl. haemolyticum as a type of Cl. oedematiens.

Although type D strains of the organism apparently share somatic antigens with other members of the group, there is evidence (Kreuzer, 1939, cited by Turner and Eales, 1943; Turner and Eales, 1943) that type D strains also possess one or two type-specific somatic antigens. The fluorescent technique has so far been unable to distinguish type D strains of Cl. oedematiens from the other types (Batty, Buntain and Walker, 1964).

(ii) The characterisation of the soluble antigens

Early descriptions of Cl. oedematiens refer to several biological effects that are attributed to culture filtrates of the organism; these effects include lethal activity, a dermonecrotic reaction (Turner, 1930; Records and Vawter, 1945), haemolysis of red blood cells and the production of opalescence in egg-yolk emulsion. The significant contributions to our knowledge of the above effects were discussed by Oakley et al. (1947) and by Oakley and Warrack (1959).

Oakley and his colleagues used an immunological approach in order

to study the biological effects of Cl. oedematiens culture filtrates. They prepared antisera against all of the types of the organism, so that soluble antigens that were present in different proportions produced specific antibodies in varying ratios. Standard tests were devised for the demonstration of several biological activities and the end-point of each test was read as a "standard indicating effect." Dilutions of the prepared antisera were then used to neutralise the effects of culture filtrates of Cl. oedematiens in the standard tests and the authors found that the results of the neutralisation tests varied according to the concentrations of the soluble antigens and the respective antibodies that were present. Thus, the biological effects of the soluble antigens were identified and qualitative and quantitative differences were revealed. Seven antigens were detected in culture filtrates of Cl. oedematiens by this technique (see Table III), some of which produce a single demonstrable biological effect, whilst others possess more than one activity.

The basis of the neutralisation tests that were described by Oakley and his colleagues is as follows. A single bacterial product X may produce several demonstrable biological effects; for example, a lecithinase X may be haemolytic and it may or may not be lethal to laboratory animals. These activities can be detected in appropriate systems and it is well known that the sensitivities of the systems may be different. However, if the single component X is not contaminated with other biologically active substances, then the results of assays performed with the different systems will always be in a constant

Table III

The soluble antigens of the Cl. oedematiens group
(after Oakley and Warrack, 1959)

Activity	Designation	Presence in filtrates from Cl. oedematiens type			
		A	B	C	D
Necrotising, lethal	alpha	++	+++	-	-
Haemolytic, necrotising, lethal lecithinase	beta	-	+	-	++
Haemolytic, necrotising lecithinase	gamma	+	-	? -	-
Oxygen-labile haemolysin	delta	+	-	-	-
Opalescence in egg-yolk emulsion: ? pearly layer	epsilon	+	-	-	-
Haemolysin	zeta	-	+	-	-
Opalescence in egg-yolk emulsion: ? lipase	theta	-	tr	-	+

+, ++, +++ is taken to denote increasing amounts; - = none detected; ? = doubtful result;
tr = trace amount detected.

ratio for a 'pure' preparation of X. It follows that if different neutralising antitoxins contain anti-X activity they will neutralise preparations of X in proportion to the anti-X content of the sera. But, if the sera are tested against products that possess opalescent factor Y or haemolysin Z in addition to X, then the results of assays performed with these two systems will depart from the expected ratios. In Oakley's work, discrepancies between the serum values in haemolytic and lecithinase tests for example were attributed to the presence of a second component having one activity but not the other. Oakley et al. used several biological indicators in order to test many culture products and antitoxic sera that were derived from all of the types of Cl. oedematiens; thus they were able to characterise seven antigens that are present in culture filtrates of Cl. oedematiens.

The lethal activity (alpha and beta antigens). The alpha antigen of Cl. oedematiens is present in culture filtrates of type A and B strains and is the classical 'lethal toxin'; it is also dermonecrotic. Walbum and Reymann (1937) stated that the lethal factor of Cl. oedematiens is highly labile, has optimum stability at about pH 6.1, and is inactivated by heating at 50° C for 30 min. or by precipitation with alcohol and ammonium sulphate. 'Coli-fermented broth' was the best medium for production of the lethal factor and the addition of glucose to the medium markedly reduced the toxin yield.

Nishida and Nakagawara (1964) found that 'coli-fermented broth' gave a poor yield of the lethal factor and they described a medium

that contained phosphate, meat particles, peptone and a fermentable substrate which gave a potent yield of the alpha antigen. The fermentable substrate that is added to this medium appears to be critical; the authors found that the incorporation of maltose gave the best yield of lethal factor; the yield was reduced with glucose, and the addition of fructose gave a very low yield of the lethal antigen.

The lethal activity of Cl. oedematiens filtrates is usually determined by estimating the minimum lethal dose (MLD) for mice. However, little is known about the mode of action of the alpha antigen. The physiological effects of crude culture filtrates of Cl. oedematiens have been studied in different systems by Aub, Zamecnik and Nathanson, 1947; Krayer et al., 1947; Miles and Miles, 1952; Elder and Miles, 1957; Aikat and Dible, 1960; and Cotran, 1967. Aub et al. and Krayer et al. investigated the effect of Cl. oedematiens culture filtrates in dogs. Elder and Miles studied the action of type B filtrate antigens on capillary permeability. These authors used a serological technique similar to that described by Oakley et al. (1947) and showed that the alpha antigen is the principal "permeability factor" in the filtrate. They confirmed the observation of Miles and Miles (1952) that Cl. oedematiens culture filtrates induce a peculiarly long-lasting increase in vascular permeability, but Elder and Miles also showed that substances other than the antigenic toxins may cause misleading results in the tests that they used. The latter workers infer that the preparation of pure samples of single antigens would be desirable, and they conclude that the alpha

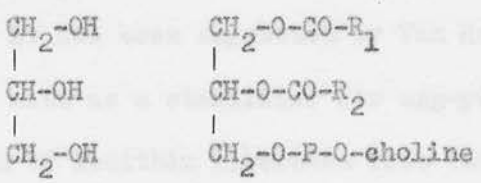
antigen deserves further study as a unique tool for exploring the nature of pathological increases in capillary permeability.

There has been one attempt (Phillips and Batty, 1963) to purify a concentrated culture supernate of Cl. oedematiens. The alpha antigen was not detectable after electrophoresis of the crude concentrate on starch-gel, or after passing the concentrate through cellulose columns. A column of Sephadex G-100 was used in order to separate the alpha antigen from an amylase that was also present in the starting material; 90 per cent. of the lethal activity was recovered and the eluted product was purified 80-fold on the basis of total nitrogen content. The separation of other biological effects was not reported.

The lethal factor that is present in cultures of type D strains of Cl. oedematiens is not the alpha antigen. Vawter and Records (1925-26) demonstrated a profound fall in the erythrocyte count of cattle that were experimentally infected with Cl. haemolyticum and attributed the lethal activity of such cultures to the presence of a "haemolytic toxin." Oakley and Warrack (1959) showed serologically that cultures of type D strains of Cl. oedematiens contain no detectable alpha antigen but that a high concentration of the beta component is present. They found that the beta antigen is a lethal, necrotising, haemolytic lecithinase and it now became clear why many authors had observed that Cl. oedematiens type A [illegible] antisera fail to protect against the pathogenic effects of type D cultures.

Factors that produce effects in egg-yolk preparations (beta, gamma, epsilon and theta antigens). Crook (1942) first observed that opalescence develops in a mixture of Cl. oedematiens "toxin" and egg-yolk emulsion that is incubated at 37° C; then MacFarlane (1942) briefly reported the presence of a lecithinase in occasional preparations of Cl. oedematiens. These findings were explained by Oakley et al. (1947) and by Oakley and Warrack (1959) who showed that the beta and gamma antigens of Cl. oedematiens are haemolytic lecithinases. MacFarlane (1948, 1950a) demonstrated that both of these antigens behave as lecithinase-C enzymes and split lecithin into phosphoryl choline and a diglyceride.

The chemical composition of lecithin varies and is influenced by its source, but in general lecithin can be regarded as a glycerol molecule that is esterified through the hydroxyl groups.



glycerol lecithin - R_1 = saturated fatty acid;

R_2 = unsaturated fatty acid.

Enzymes that are termed phospholipases (or lecithinases) hydrolyse specific bonds in the lecithin molecule. Phospholipase-A hydrolyses lecithin to yield lysolecithin plus an unsaturated fatty acid ion and it is well known that lysolecithin is powerfully haemolytic.

Phospholipase-B splits lysolecithin into non-haemolytic products whilst phospholipase-D removes the choline moiety from lecithin. The beta and gamma antigens of Cl. oedematiens and the alpha antigen of Cl. welchii all function as phospholipase-C enzymes, but each factor is immunologically distinct and its activity is neutralised by specific antisera only.

If culture filtrates that contain the above antigens are incubated with egg-yolk emulsion then an opalescence develops in the mixture; this is known as a positive lecithovitellin (LV) reaction, and a filtrate that is rich in enzyme activity may produce a precipitate or curd in the emulsion. The physical explanation for the visible manifestation of the LV reaction has not been fully explained; it appears that the opalescence is due mainly to the aggregation of finely divided free fat, but a small part is due to the liberation of fat probably from lipoproteins (MacFarlane, Oakley and Anderson, 1941). It has been suggested by Van Heyningen (p. 31, 1950) that lecithin acts as a stabiliser for egg-yolk emulsion and that the breakdown of lecithin liberates free fat. However, Van Heyningen points out that phospholipase A derived from snake venom does not cause an opalescence to develop in egg-yolk emulsion. Various agents may give a positive LV reaction and the value of this test in clostridial research lies in the specific neutralisation that can be demonstrated with suitable antitoxic sera.

The detection of the epsilon antigen provided an explanation for the so-called "pearly-layer" effect that was described by Nagler

(1944, 1945). He observed that when type A strains of Cl. oedematiens are cultured on blood-egg-yolk-agar plates, the colonies and the underlying haemolytic zone are covered by an opaque film with a pearl-like lustre. Nagler found that certain other clostridia also show the pearly-layer effect but that type B, C and D strains of Cl. oedematiens are not in this category. McClung, Heidenreich and Toabe (1946) stated that the pearly layer is produced on a medium that does not contain blood and this was confirmed by Oakley et al. (1947), who also showed that culture filtrates of type A strains of the organism contain gamma and epsilon antigens, both of which cause opalescence in egg-yolk emulsion. These observations were clarified when MacFarlane (1948) identified two lipolytic enzymes in Cl. oedematiens type A culture filtrates, a lecithinase (the gamma antigen) and a lipase that splits off both saturated and unsaturated fatty acids either from the diglyceride that is produced by the action of lecithinase on lecithin or possibly from lecithin itself. She suggested that it seems reasonable to suppose that the action of the epsilon antigen is that of a lipase, forming a pearly layer of fatty acid upon the colony.

Later, MacFarlane (1950a) found that type D culture filtrates of Cl. oedematiens may contain, in addition to the major beta antigen, traces of a lipase whose action becomes apparent if the incubation period of the test is prolonged. Oakley and Warrack (1959) confirmed that type D culture filtrates contain a factor that causes opalescence in egg-yolk emulsion but is non-haemolytic; they suggested that it

should be named the theta antigen and that it might be the lipase that was described by MacFarlane. Oakley and Warrack also state that "type B most probably produces a little theta, as type B antisera often have antibody to it." It is interesting to note that only type A strains produce a pearly layer on egg-yolk agar yet according to the above description type B and D strains also produce indications of lipase activity. However, the lipase concentration in type B and D cultures may be rather low. The lipolytic activity of anaerobic bacteria on solid media is discussed by Willis (1960; 1964, p. 55).

Oakley and Warrack also suggested that there may be an antigenic relationship between the beta antigen of Cl. oedematiens and the alpha antigen of Cl. welchii, but they considered it improbable that a close relationship exists.

It is clear that the visible manifestation of the LV reaction in tests with egg-yolk emulsion cannot differentiate between two enzymes that apparently cause the same effect. A more elegant test would help to resolve the present confused situation.

Haemolysins (beta, gamma, delta and zeta antigens). The haemolytic activity of the beta and gamma antigens of Cl. oedematiens can be attributed to the presence of lecithin in the red cell envelope; lecithinase decomposes lecithin and the structural integrity of the cell is destroyed. Oakley et al. pointed out that different species of red cell seem to vary in their sensitivity towards these antigens and that the lysis exhibits a so-called "hot-cold" effect.

MacFarlane (1950b) showed by chemical measurement that the rates of hydrolysis of phospholipid in intact red cells by enzymically equipotent amounts of different lecithinases can vary according to the kind of lecithinase and the species of red cell. She observed that haemolysis was always preceded by the decomposition of phospholipid and concluded that these differences appear to be sufficient to account for the known differences in sensitivity of various species of red cell to haemolysis by immunologically specific lecithinases. MacFarlane also explained that the species of red cell affects the manifestation of "hot-cold" lysis by the Cl. welchii alpha antigen, and that certain species of cells are more susceptible to this phenomenon than others.

The marked haemolytic effect that is produced by cultures of type D strains of Cl. oedematiens is reflected in the name that was originally given to these strains (Cl. haemolyticum). Vawter and Records (1925-26) found that the pathogenicity of type D strains depends on the presence of an 'unstable haemolytic toxin.' Records and Vawter (1945) then suggested that there were two toxic fractions in cultures of Cl. haemolyticum; an unstable, haemolytic component that appears at 8 - 10 hr and disappears after 30 - 36 hr, and a necrotising fraction that persists for a week or longer. They stated that the pathogenicity of 'toxic cultures' after injection into rabbits, guinea-pigs, mice and cattle is dependent on a high concentration of haemolytic 'toxin'. Jasmin (1947) showed that culture filtrates of type D strains of Cl. oedematiens were lethal and haemolytic after storage

for 6 mth at refrigerator temperature, and that there was little evidence of the necrotising fraction when the lethal and haemolytic activity were lost. Jasmin also demonstrated that these culture filtrates gave parallel results in tests that detect lethality in mice, haemolysis of rabbit erythrocytes, and opalescence in human serum or LV solution; the effects were neutralised by the addition of specific antisera, and the neutralising ratio of antigen to antibody was similar in each system.

Bard and McClung (1948) described a series of experiments in which they investigated the lethal, haemolytic and lecithinase activities of Cl. oedematiens cultures. They showed that a crude lecithinase-B preparation reduced the haemolytic activity of both type B and D culture products. These authors presented evidence that lysolecithin may be an important haemolytic component of Cl. oedematiens cultures, and they also suggested that some of the lethal activity of type B and type D strains appears to be attributable to the action of lysolecithin. They assumed that lysolecithin could occur in the form of an antigenic protein complex, but no serological evidence was presented. Oakley and Warrack (1959) pointed out that no quantitative work was reported by Bard and McClung, and that the lecithinase-B was a crude preparation that may have contained other active material, e.g. proteases.

The delta and zeta antigens of Cl. oedematiens were described by Oakley et al. (1947). The delta antigen was detected in type A filtrates from peptic digest media. These filtrates caused immediate

haemolysis of horse cells at 37° C and the authors state that "the haemolysin (delta) is oxygen-labile and is to some extent neutralised by heterologous sera. .... serum values against this haemolysin (diluent phosphate buffer pH 6.5 + M/25 sodium thiolacetate, indicator 0.5 ml. 6 per cent. washed horse cells in saline) show no relationship to the alpha, gamma or epsilon values ..." No further information is given. The zeta antigen "though not definitely oxygen-labile was neutralised to some extent by heterologous sera, and .... is distinct from the alpha, beta, gamma, delta and epsilon antigens." It is clear that the haemolytic components of Cl. oedematiens culture filtrates require further characterisation and definition.

Factors that produce effects on other biological substrates. Several other activities have been attributed to culture filtrates of Cl. oedematiens but they are not as well characterised as those described previously. McClean et al. (1943) showed that 7 of 15 strains of Cl. oedematiens produced hyaluronidase when they were incubated in the presence of hyaluronic acid. The positive strains were shown to be type B strains by Keppie (1944) who also demonstrated that no type A strains tested by him produced the enzyme.

Evans (1947) reported that no enzyme that attacks hide powder (collagenase) was demonstrable in cultures of Cl. oedematiens.

MacFarlane (1955) detected the enzyme tropomyosinase in culture filtrates of type B and D strains of Cl. oedematiens. Tropomyosinase is an antigenic sulphydryl-activated enzyme that decomposes

tropomyosin. MacFarlane partially characterised the enzyme and its action and named it the eta antigen of Cl. oedematiens.

It has also been observed that culture filtrates of Cl. oedematiens produce a cytopathic effect in tissue culture cells (Penso and Vicari, 1957; Veto, Backhausz and Horvath, 1957; Zemskov, 1964). Veto et al. noted that "antitoxin solution can neutralise the toxins specifically and in accordance with the law of multiplicity." Zemskov found that Cl. oedematiens exhibits a definite and specific cytotoxic effect in a culture of trypsinised chick-embryo tissue. He suggested that the effect could be observed in 6 hr and that the technique is 60 times more sensitive than a parallel titration in white mice.

The hyaluronidase activity and the cytopathic effect of culture filtrates of Cl. oedematiens are not generally included in schemes that refer to the biological effects of the organism.

Typing of Cl. oedematiens by the detection of soluble antigens

When Oakley et al. (1947) demonstrated the distribution of the soluble antigens and their biological effects in type A, B and C strains of Cl. oedematiens many of the previous confusing observations could be explained. It was now obvious why Turner and Davesne (1927) were able to neutralise the lethal activity of type B culture filtrates with type A antisera, and it was possible to understand why Keppie (1944) found that type A antisera failed to neutralise the haemolytic activity of type B culture filtrates. The system devised by Oakley and his colleagues provided a working basis for the identification of the individual antigens and the typing of Cl. oedematiens, but it demanded a wide range of antisera and a detailed knowledge of their antitoxic components. Such antisera are not readily available, and, although a detailed procedure was necessary in order to understand the complexities of the system, a less comprehensive analysis may be performed for the routine typing of the organism. Oakley et al. proposed that methods based on the properties of the beta, gamma and epsilon components of culture filtrates of Cl. oedematiens in Brewer's medium are sufficient to differentiate type A, B and C strains of the organism. They stressed that type-specific antisera are essential for typing experiments because heterologous antitoxins produce equivocal results in neutralisation tests. Oakley and Warrack (1959) showed that type D culture filtrates contain a strong

beta component, and they suggested that the identification scheme should include a neutralisation test that contained a high level of anti-beta activity in order to neutralise the LV effect of such filtrates. Oakley and Warrack differentiated type B from type D strains by showing that the necrotising activity of a type D filtrate was completely neutralised by type D antisera that contained no detectable anti-alpha component.

Jamieson (1949) used the LV test in order to type Cl. oedematiens strains that he isolated from Black disease in sheep, but the final criterion of identification of the beta antigen is not clear from his description. He found that these freshly-isolated type B strains gave a poor LV reaction after growth in Brewer's medium and variations of lecithinase production in different media proved troublesome. He tested a variety of media and concluded that the differences observed were mainly due to growth conditions and not to strain variation.

Williams (1962, 1964) demonstrated the presence of preformed LV and necrotising activity in ground-up liver lesions and peritoneal fluid of sheep and cattle that were naturally infected with Cl. oedematiens. In LV neutralisation tests he identified the beta antigen in 57 of 69 liver lesion macerates.

The characteristic effects that are produced by a culture of Cl. oedematiens on egg-yolk agar plates were described by Hayward (1941), Nagler (1945) and McClung, Heidenreich and Toabe (1946). Oakley et al. (1947) suggested that although the LV tube test is satisfactory, it is more convenient to type Cl. oedematiens on egg-

yolk-VF agar plates, and it does not appear that these authors had any difficulty in growing type B strains of the organism on this medium. Willis and Hobbs (1958) found that type B, C and D strains of Cl. oedematiens fail to grow on egg-yolk agar unless 0.1 per cent. of sodium thioglycollate is added; they later (1959) described a selective indicator medium for clostridia that contained lactose, egg-yolk, milk, neomycin sulphate and agar, and confirmed the difficulty of growing Cl. oedematiens strains.

It is clear from the above description that different permutations of the soluble antigens are present in culture filtrates of type A, B and D strains of Cl. oedematiens, and that the four types are recognised by the characteristic biological effects that are produced by the antigens. The present method of typing the organism relies mainly upon the demonstration and neutralisation of LV activity in culture filtrates; this system apparently gives satisfactory results but has not been critically evaluated.

MATERIALS AND METHODS

Strains. Preliminary experiments in the present work were performed with 11 strains of Cl. oedematiens from the collection of Dr Nancy J. Hayward and held in this laboratory for some years. These strains were derived from various sources, and LV neutralisation tests with CMB cultures of each strain suggested that they were type A strains.

Twenty strains of Cl. oedematiens representing at least one strain of each type of the organism were kindly provided by Mr J. R. Hepple formerly of Glaxo Laboratories Ltd. A number of these strains originated from the N.C.T.C; others were isolated at the Aberystwyth Veterinary Investigation Centre (see Appendix I). The strains obtained from Hepple are designated by a number and the type letter preceded by the letters GR.

Ten strains of Cl. oedematiens were isolated in the present work from infected animal tissues kindly provided by Dr J. A. A. Watt of the Edinburgh Veterinary Investigation Centre. These strains are referred to by the letter R followed by one or two numbers; the first number represents the specimen and the second number is the isolate; thus, R3/1 and R3/2 are two isolates obtained from the third liver specimen.

All of the above strains were lyophilised as soon as their purity was confirmed; thereafter, the strains were subcultured in cooked-meat broth. Frequent checks of purity were made on aerobic and anaerobic blood agar plates; each strain was tested periodically with the immunofluorescent staining procedure, and in haemolytic and LV

neutralisation tests with appropriate antisera.

Anaerobic jar procedure. Anaerobic jars with a room-temperature catalyst (Baird and Tatlock) were employed. The procedure for setting up these jars varied during the present work, and the routine that was finally adopted is given in Appendix 2. A liquid indicator of anaerobiosis was usually included in each jar during incubation.

Culture media were incubated as a routine at 37° C with 10 per cent. of carbon dioxide added to the anaerobic environment.

Culture media. Cooked-meat broth was used as a routine in most of these studies; nutrient broth (Oxoid) replaced peptone infusion broth in the preparation of this medium (Cruickshank, 1965, p. 755). Other media prepared from Oxoid constituents included reinforced clostridial medium and nutrient broth; blood agar medium was prepared from blood agar base (Oxoid) with added citrated human, defibrinated horse or defibrinated sheep blood. Blood agar plates with 20 per cent. of added human blood were used extensively in the present studies. Standard meat infusion broth, horse flesh digest medium and peptone water were prepared as described by Cruickshank (1965); sheep liver infusion broth was prepared with 500 g. of fresh defatted sheep liver in place of the lean meat. Brewer medium (Brewer, 1940) was used in several experiments and modifications to this medium are described in the text. Heated ('chocolate') blood agar was prepared by heating blood agar medium at 80° C for 10 min. before pouring the plates.

Sugar solutions were prepared as 10 per cent. solutions and sterilised by Tyndallisation; an appropriate volume of each solution was added to the sterile basal medium in order to give a final concentration of 1 per cent. of the added substrate.

Calcium gelatin saline ('cagsal'). This solution was used extensively as a diluent in the present work; it consists of 227.5 ml. of 1 per cent. calcium chloride, 45g. of sodium chloride, 200 ml. of 5 per cent. gelatin and 5g. of phenol; these ingredients are made up to 5 litres with distilled water and sterilised by autoclaving at 10 lb pressure for 10 min. (Brooks, Sterne and Warrack, 1957).

Buffers. Michaelis buffer (barbital sodium/sodium acetate/hydrochloric acid, pH 6.8, see Documenta Geigy, 1962, p. 314) was used in most of the present work. Other buffers were prepared as described by Cruickshank (1965).

Red cell suspensions. Citrated human group-O blood was obtained from the Blood Transfusion Department, Edinburgh Royal Infirmary, and was stored at 4° C. A sample of the blood was washed three times by centrifugation with physiological saline and the washed cells were made up as a 2 per cent. (v/v) suspension in cagsal. The cell suspensions may be stored for about 4 days at 4° C before lysis of the red cells occurs. Suspensions of defibrinated horse blood (Wellcome Laboratories) and defibrinated sheep blood (Oxoid) were prepared in a

similar manner.

Haemolysin (HL) tests. Serial doubling dilutions of 0.5 ml. of the test material were made in 3 x $\frac{1}{2}$ in. tubes with 0.5 ml. of Michaelis buffer (pH 6.8). One volume (0.5 ml.) of the red cell suspension was added and the mixtures were shaken and then incubated in a water bath at 37° C; readings were made after 1 hr, then the tubes were chilled overnight at 4° C before final estimates of haemolysis were made. The end-point of the titrations was generally regarded as 25 per cent. haemolysis of the test mixtures; a control tube was prepared by a 4-fold dilution of a completely lysed test mixture and a visual comparison of the test series was made. A negative control tube was included for comparison in each test. In some tests, perspex WHO plates were used to hold the reaction mixtures. This method of testing conserves material but the end-point of the test is difficult to assess; it was concluded that given sufficient material, the tube test is preferable to the plate test.

The inclusion of different buffers in the test system, such as acetate buffer, phosphate buffer (0.2 M and 0.02 M) and phosphate-buffered saline had little effect on the haemolytic titre of culture products of Cl. oedematiens with human cells; it was observed that ageing red cells are more fragile than fresh cells in phosphate buffer. The haemolytic titre of cultures of the organism is reduced as the substrate concentration increased from 1 - 10 per cent; a 2 per cent. suspension of red cells was chosen for the present studies.

Tests to demonstrate oxygen-labile haemolytic activity. Attempts to detect the oxygen-labile haemolysin in cultures of Cl. oedematiens were performed as described by Gadalla (1965). Culture supernates were oxidised with fresh hydrogen peroxide solution and subsequent reduction was with a neutral solution of thioglycollic acid. In later tests, the cultures were oxidised for 1 hr at 37° C with a final concentration of 0.1 per cent. of hydrogen peroxide solution (100 vols); reduction was for 30 min. at 37° C with a 0.2 M solution of sodium thioglycollate (1 ml. to 2 ml. culture). Cultures of type A strains of the organism were grown in cooked-meat broth, cooked-meat broth plus glucose, nutrient broth and glucose broth, and these were tested against human cells; tests were also done with horse and sheep red cells.

Lecithovitellin (LV) suspensions. The lecithovitellin substrate was either a commercially available egg-yolk emulsion (Oxoid) or a suspension prepared from a fresh egg yolk in borate-buffered saline; the latter emulsion was clarified by filtration through Whatman's No.1 filter paper, sterilised by Seitz filtration and held at -27° C until required for use. The emulsion was held as a routine at 4° C.

Titration of factors that cause opalescence in egg-yolk emulsion (described in the present work as LV factors or LV activity). Serial doubling dilutions of 0.5 ml. of the test material were made in 0.5 ml. of Michaelis buffer (pH 6.8); one volume (0.5 ml.) of LV solution was

added. The mixtures were shaken and then incubated for 1 hr at 37° C; readings were taken and then the mixtures were chilled overnight at 4° C before final readings were made. The end-point was regarded as a slight opalescence when compared with a negative control tube. Preliminary experiments indicated that a more clear-cut end point was obtained in titrations of LV factors with fresh egg-yolk emulsion than with the commercial product; in addition, a 10 per cent. solution of the substrate was rather more sensitive then undiluted emulsion. In most of the tests the substrate was prepared from fresh egg yolk and was diluted with cagsal to give a 10 per cent. solution (but see Crook, 1942).

The procedure described by Oakley et al. (1947) with 1-ml. volumes of undiluted test materials and undiluted LV solution was also used; these tests confirmed that type B strains of the organism occasionally fail to give a positive LV reaction (q.v.).

Neutralisation of haemolytic and LV activities. The test procedure developed during the present studies employed one volume (0.5 ml.) of a 1 in 2 dilution of the culture or culture supernate in Michaelis buffer; the antitoxin (usually 0.02 ml.) was added to the toxin, to avoid the Danysz phenomenon. After 30 min. to allow neutralisation, 0.5 ml. of the substrate was added to the test mixtures and the tests were incubated for 1 hr at 37° C and then chilled overnight at 4° C. The results were estimated visually from + (25 per cent. lysis) to 3+ (complete lysis).

Tests to demonstrate the neutralisation of LV activity with commercial Cl. oedematiens diagnostic antisera gave cross-neutralisation reactions with cultures of type A and B strains of the organism; it was necessary to dilute the antisera 1 in 10 (as recommended by the manufacturers) in order to obtain the expected neutralisation pattern. Tests to demonstrate the neutralisation of haemolytic activity of cultures of Cl. oedematiens gave the appropriate results with 0.02 ml. of commercial antisera.

Thin-layer chromatography. Concentrated bacterial culture products (usually 0.5-ml. volumes) were held at 37° C for 16 hr with 1-ml. volumes of undiluted egg-yolk emulsion. The total lipid was extracted from the reaction mixture with 25 ml. of chloroform/methanol (2 : 1 v/v) plus 5 ml. of 0.017 N sulphuric acid. The organic phase was removed and concentrated by evaporating to dryness in vacuo; the extracted lipid was then resuspended in a small volume of chloroform. Glass plates (9 x $2\frac{5}{8}$ x $\frac{1}{8}$ in.) were coated with a thin layer of silica gel G (Kieselgel G nach Stahl, E. Merck AG, supplied by Anderman and Co. Ltd., London) about 1/32 in. in depth and spread by means of a metal spreading device obtained from Dr. G. S. Boyd. The thin-layer plates were activated in an oven for 60 min. at 100° C, then about 0.04 ml. of the lipid extract was loaded at one end of the plates in the form of a spot. After a few minutes to allow air drying the plates were placed vertically in a chamber containing a suitable solvent. The solvent advances up the silica gel and carries fractions

of the lipid mixture with it; the distance moved by each class of lipid depends upon the charge of the molecule and the solvent system that is used. Thus, the lipid mixture is fractionated into components that are located at different levels on the chromatograph. Iodine vapour was used as the locating agent in the present work; the plates were allowed to dry in air after removal from the solvent chamber and then were held for periods of 5 - 10 min. in a sealed jar that contained iodine crystals.

Demonstration of lethal activity. The soluble lethal factors in culture products were estimated by intravenous injection of 0.1-ml. volumes of suitable dilutions of the material in saline into male white mice that weighed approximately 20 g. The results were recorded after 48 hr. Occasionally, the intraperitoneal route was employed. Tests to demonstrate neutralisation of the soluble lethal factors were performed by allowing the antiserum to neutralise the test material for 30 min. before injection of 0.1 ml. of the mixture into mice.

The mice were restrained in a small, cylindrical wire cage during the injection procedure, and warm water was used as a vasodilator; injections were made into the tail vein with a 1-ml. tuberculin syringe with a Luer fitting and 25G x $\frac{5}{8}$ (No. 20) Gillette needles.

Demonstration of dermonecrotic activity. Intradermal injections of 0.2 ml. of the test material were made into shaven guinea-pig skin; a depilating paste (Cruickshank, 1965, p. 1011) was the most effective

method of removing the hair. The animals were observed daily and visible lesions were usually well-developed after 48 - 72 hr.

Antitoxic sera. (i) Experimental sera. Three experimental Cl. oedematiens antisera were used in the present work: EX1552, EX1546 and EX1321. These sera were kindly gifted to Dr Collee by Dr G. Harriet Warrack and their various antitoxic potencies were known. (see table IV).

(ii) Commercial sera. Diagnostic Cl. oedematiens antisera were obtained from the Wellcome Laboratories; details of the antitoxic components of these antisera were kindly provided by Mr H.B.G. Epps (see table IV).

(iii) Sera prepared in this study. Two antitoxic sera were prepared in rabbits in the present work; the antigens were (a) a crude culture concentrate of a type B strain of Cl. oedematiens, and (b) a partially-purified fraction obtained after gel-filtration of a type B concentrate. The soluble products were detoxified with formalin (0.4 per cent. of formaldehyde) for 48 hr at 37° C. The antisera were prepared in male white rabbits (approx. 3.5 kg.). A sample of control serum was removed from the marginal ear vein of each rabbit, then 1 ml. of toxoid was injected intravenously; after 5 min., 1.6 ml. of a mixture of equal volumes of toxoid and Freund's incomplete adjuvant were injected subcutaneously. Twelve days later, 20 ml. of blood were removed from the ear vein of each rabbit and a booster dose of 1 ml. of toxin emulsified with adjuvant was administered by the

Table IV

Details of the antitoxic sera that were used in the present studies

Cl. oedematiens antiserum	Antitoxic activity in units per ml. against the soluble antigens of Cl. oedematiens		
	alpha	beta	gamma
EX 1552	450	< 10	495
EX 1546	315	280	< 10
EX 1321	< 0.1	4800	< 10
Commercial type A	100 - 150	< 2	700 - 1000
Commercial type B	510	400	/

/ = not stated .

subcutaneous route. Eight days after the booster injection, 40 ml. of blood were removed from each of the rabbits. The blood was collected in 1-oz vials coated internally with a thin layer of paraffin-wax; after standing overnight at 4° C the serum can be readily removed.

The preparation of concentrated culture products. Strains of Cl. oedematiens were grown for an appropriate period in 500-ml. amounts of different culture media (see text). The bacterial cells were removed by centrifugation at 6800 g for 60 min. in a M.S.E. ground centrifuge. In my initial studies, the protein content of the culture supernate was precipitated by adding varying amounts of ammonium sulphate (see Dixon and Webb, 1964, p. 40); precipitation was allowed to occur at 4° C then the precipitate was removed by centrifugation (see above), dissolved in the minimum volume of distilled water and dialysed extensively in cellophan sacs (Visking) against distilled water at 4° C to remove the ammonium sulphate. The solution was concentrated by placing the cellophan sacs in plastic pails and adding polyethylene glycol (M.W. 6000, Koch-Light Laboratories Ltd); the concentrated solution was finally dialysed for a short period in the cold against distilled water or a buffer solution. The pH was not controlled during these procedures; pH measurements indicated that the pH was about 5.8 after the addition of ammonium sulphate.

In later work, the culture supernate was concentrated directly with polyethylene glycol, omitting the precipitation step. The final

pH of most of the concentrated products was about 6.0. An antibacterial agent was often added to the concentrated products; sodium azide (0.02 per cent.) and Hibitane (chlorhexidine, I.C.I., 0.002 per cent.) were used. In several cases, the concentrated products were filtered with a membrane filter; this resulted in a slight loss of biological activity. The concentrated products were held as a routine at 4° C or deep-frozen at -27° C.

Gel-filtration studies. Sephadex G-200 and Sephadex G-100 were used in gel-fractionation experiments during the present studies. The column bed was prepared as indicated by the manufacturers (Pharmacia G.B. Ltd, London). Phosphate-buffered saline (pH 6.8, 0.02 M) was generally used as the eluting fluid and was fed to the column by gravity from a double reservoir system that minimised changes in the hydrostatic pressure head during the experiment. The eluted material was passed through a Uvicord monitoring apparatus (LKB Produkter) and the fractions were collected in 5 x $\frac{5}{8}$ in. tubes in a Locarte fraction collector. The protein content of the eluate was recorded as a continuous trace, and fraction changes were also indicated. Appropriate fractions were tested for biological activity. In some experiments, the individual fractions were pooled and concentrated with polyethylene glycol to give partially-purified products.

Protein estimations. The protein content of various solutions was estimated by measuring optical extinctions at 260 and 280 mμ in a

SP500 spectrophotometer (Unicam Instruments Ltd). Silica cuvettes with light paths of 1 cm. or 0.2 cm. were used. The protein content of the solution was estimated from the two readings by reference to a standard nomogram.

Counting methods. Total counts of bacteria were performed in a Helber bacterial counting chamber (Hawksley Ltd) with a Thoma ruling and 0.02 mm. depth; at least 400 bacteria were counted and the count was repeated at least once. Counts were made with phase-contrast microscopy and the number of spores was estimated during the total count. The spore content of cultures was checked by preparing stained smears in which the free spores were more readily visible. It was found that Ashby's modification of the malachite green stain for spores (Cruickshank, 1965, p. 657) was the most satisfactory stain that was used.

Viable counts were performed with the shake-culture, pour-plate or spread-plate methods. Dilutions of the bacterial culture were prepared with the supernate of pre-steamed tubes of cooked-meat broth as the diluent. An appropriate volume of each dilution was added to the culture medium and the number of colonies that appeared were counted after incubation.

Immunofluorescent staining. A commercially available Cl. oedematiens fluorescent globulin (Wellcome Laboratories) was employed; the staining procedure was similar to that recommended by the manufacturers

with the following modifications: a) gentle heat-fixation was used instead of acetone-fixation, and b) the smears were not blotted; excess buffer was allowed to drain from the slide and then the surrounding area was dried before mounting. The smears were examined with a Leitz microscope with a UV light source and with dark-background illumination.

Electronmicroscopic observations were made on bacilli from (i) fluid cultures that were fixed with glutaraldehyde (see Cruickshank, 1965, p. 639), and (ii) cultures on blood agar plates. The specimens from solid media were prepared by placing a drop of distilled water on the grid, then transferring a fraction of a colony to the distilled water with the tip of a platinum bacteriological loop; the preparation was not fixed. The grids were allowed to dry in a desiccator overnight, and then the specimens were shadow-cast with gold-palladium alloy (60 : 40) at an angle of 15°. They were examined in the electron microscope (Associated Electrical Industries, type EM6).

Ultrasonic disintegrates. Bacterial suspensions were held in small ice-cooled glass tubes; disintegration by the 6-mm. probe of a M.S.E.-Mullard ultrasonic disintegrator was allowed to proceed for 15 min.

Immunodiffusion procedures. Double diffusion experiments in plates were performed as described by Cruickshank (1965, p. 948); the precipitation bands were allowed to develop at room temperature (18°C)

or at 4° C. The immunoelectrophoresis procedure is described by Cruickshank (1965, p. 952). Phosphate buffer (pH 6.8, 0.15M) was diluted 10-fold with distilled water and was used in the electrophoresis system. The slides were coated twice with the supporting medium; the first layer of agar was allowed to dry before the second layer was applied. Electrophoresis of the antigen was performed for 90 - 120 min. at a constant voltage of 250 V. The precipitin bands in these experiments were usually stained with naphthalene black, and it was observed that more lines may be visible after the staining procedure.

Tissue culture experiments. Monolayers of chick-embryo fibroblasts were prepared from 11 - 12-day-old chick embryos (see Appendix 3). In my early experiments, 5×10^5 cells per ml. were used to seed each tube but the cells did not grow out satisfactorily. Later, 1×10^6 cells per ml. were used to prepare each monolayer; these cells grew out within 2 - 3 days and formed a complete cell sheet. The cytopathic activity of test materials was assessed by adding 0.1 ml. of appropriate dilutions to the monolayers; immediately before this, 0.9 ml. of fresh medium was added to each tube. The monolayers were rolled at 37° C, and the cytopathic effect was usually obvious after overnight incubation.

It was observed during the present studies that the chick fibroblasts become granular and slightly shrunken after exposure to cagsal; this may be due to the presence of a small concentration of phenol in

cagsal. Subsequently, saline or phosphate-buffered saline (pH 7.1 0.02M) was used as the diluent in these experiments.

BHK and MK cells used in the present work were kindly provided by the Virology Unit of this department. BHK cells (MacPherson and Stoker, 1962) were obtained from the Animal Disease Institute, Moredun, Edinburgh, and were propagated in Eagle's medium. MK cells were obtained from the MRC Laboratory, Holly Hill, London, and were propagated in '199' medium.

Redox indicator dyes. The following indicator dyes were used in the redox experiments: 2 : 6 dichlorophenol indophenol, thionine, methylene blue, Nile blue, phenosafranine, and neutral red. Data concerning the percentage oxidation and colours of these dyes at different levels of redox potential were obtained from Hewitt (1950, pp. 23 - 24).

Abbreviations. The following abbreviations are used in the text: BHK = baby hamster kidney; BTL = Baird and Tatlock Ltd; CMB = cooked-meat broth; CMB/G = cooked-meat broth plus 1 per cent. of glucose; CPE = cytopathic effect; DCA = desoxycholate citrate agar; Eh = oxidation-reduction potential; g. = grammes; g = acceleration due to gravity; HDB = horse flesh digest broth; Hg = mercury; HL = haemolysin; IB = infusion broth; LV = lecithovitellin; MK = monkey kidney; NB = nutrient broth; NB/G = nutrient broth plus 1 per cent. glucose; PBS = phosphate-buffered saline; RCM = reinforced clostridial medium; and TLC = thin-layer chromatography.

EXPERIMENTAL OBSERVATIONS

The anaerobic jar procedure

The degree of evacuation

My preliminary observations rapidly confirmed that strains of Cl. oedematiens of types B, C and D do not grow readily on solid media. Previous descriptions have stressed that the organism is a demanding anaerobe and a reappraisal of the anaerobic jar procedure was indicated.

The present model of the Baird and Tatlock (BTL) anaerobic jar is supplied with a leaflet that recommends evacuation of the jar to a pressure of approximately 100 mm. Hg before hydrogen is admitted. Other authorities have different views; Willis (1964, p. 10) states that "the jar is evacuated to a negative pressure of 300 mm. of mercury," and Cruickshank (1965, p. 798) describes a similar procedure for jars that contain Wright's capsule implying that this is suitable for the BTL jar. Thus, the latter references suggest that 2/5 of the air should be exhausted from the jar and the manufacturers recommend that 6/7 of the air should be removed. It is conceivable that a surface inoculum of a demanding anaerobe may be influenced by exposure to oxygen during the period of gaseous reaction prior to development of anaerobiosis, and it was necessary to evaluate the effect of the degree of evacuation on the anaerobic environment that is produced and also to assess its influence on the growth of Cl. oedematiens.

The accepted method of setting up an anaerobic jar is as follows. The jar is evacuated to a pressure of about 100 mm. Hg and then filled with hydrogen that is run through water in a Woulff bottle so that the flow of gas is visible. If the catalyst is active, hydrogen will continue to flow into the jar as the reaction with oxygen takes place and this is indicated by gas bubbling in the Woulff bottle. The internal pressure changes that occur in a gas-tight jar equipped with an active catalyst after the admission of hydrogen can be demonstrated on a suitable manometer; in the present work, this system was preferred to the use of a wash-bottle which may introduce infection into the jar and adds to the moisture content of the admitted gas.

Catalyst activity is readily evident in a jar that is filled with hydrogen after evacuation to 100 mm. Hg; an internal vacuum develops as the hydrogen and oxygen combine and a negative pressure can be recorded within minutes on a mercury manometer. Considerable pressure changes are involved if only 2/5 of the air is removed from the jar as a greater volume of oxygen is available for combination with hydrogen, and the internal vacuum of such jars can be registered on a 'Speedivac' vacuum gauge; this instrument is usually insensitive to the smaller negative pressure that results from the reaction of hydrogen with oxygen in a jar that is initially evacuated to 100 mm. Hg.

Experiments showed that considerable pressure variations occur in jars containing mixtures of hydrogen and oxygen over a 5 day period. A negative pressure develops even in jars that do not contain a catalyst in a sachet; it is possible that the metal surface of these jars

may assist the combination of hydrogen with oxygen. Results of a typical experiment in which 2 equivalent jars were used with and without a catalyst are given in table V. The jars were evacuated to 100 mm. of mercury then equilibrated with hydrogen. The jars were held at 37° C and the pressure within each jar was recorded over 5 days on a simple mercury manometer.

The positive pressure initially recorded within the jars reflects the pressure of the gas supply via the rubber bladder. The internal pressure of the jar containing a catalyst decreased by 10 mm. within 15 min; no change was observed in the other jar for at least 90 min. These observations were confirmed in a series of tests over a period of several months. It was found that the initial positive pressure in the jar may vary from 0 - 30 mm. of mercury depending on the expansion of the hydrogen bladder; in general, 10 - 15 min. after the admission of hydrogen a decrease in pressure of at least 10 mm. of mercury occurs if the catalyst is active. The internal pressure of a jar may still be negative after incubating cultures of Cl. oedematiens, but in many cases a positive pressure is recorded. This is often related to the evolution of gas from fermentable substrates in fluid media, but it can also occur in the absence of such obvious sources and may be partly caused by gaseous expansion resulting from the heat of the incubator.

It is clear that with suitable precautions the rapid development of a demonstrable vacuum within an anaerobic jar can be used as evidence of catalyst activity. The significance of the detailed results

Table V

Internal pressure changes that occur within empty BTL jars set up with and without a catalyst;

(the jars were evacuated to 100 mm. of mercury then filled with hydrogen at 15 mm. Hg above atmospheric pressure).

Time (hr)	Jar I (catalyst removed) readings in mm.	Jar II (catalyst present) readings in mm.
0	+ 15	+ 15
$\frac{1}{4}$	+ 15	+ 5
$\frac{1}{2}$	+ 15	0
1	+ 15	- 5
$1\frac{1}{2}$	+ 15	- 7.5
2	+ 12.5	- 7.5
7	+ 10	- 5
22	- 5	- 10
48	- 11	- 8
72	- 25	- 12.5
96	- 30	- 12.5
120	- 50	- 25

\+ = positive pressure;

\- = negative pressure.

of these experiments is discussed later (see Discussion).

The evacuation procedure and the growth of Cl. oedematiens. The effect of the degree of evacuation of the anaerobic jar on the growth of Cl. oedematiens on agar plates was extensively studied during the present work. The organism grew irregularly in jars that were evacuated to each of the levels described and the results of many experiments indicate that the degree of evacuation has no obvious effect on the subsequent growth of these strains. An indicator of anaerobiosis (q.v.) was present in each jar during these trials and the jar environment was apparently anaerobic. It was therefore concluded that additional factors account for the fastidious nature of Cl. oedematiens.

The indicator of anaerobiosis

The most effective method of checking that anaerobic incubation has occurred is to include an indicator of anaerobiosis in the jar. The external semi-solid indicator supplied with the BTL jar is of limited value; it may function efficiently once or twice but I have experienced many difficulties with this indicator and preferred to use an internal liquid indicator during the present work. The obvious disadvantage of the internal indicator is that it is not visible during incubation.

Most anaerobic indicators incorporate methylene blue, a dye that is reduced and decolourised in the absence of oxygen or by heating. The alkaline-glucose-methylene blue indicator (see Cruickshank 1965, p. 798) is satisfactory but soon becomes irreversibly oxidised on exposure to air; when this occurs the solution cannot be completely reduced and remains coloured. It is therefore essential to heat the anaerobic indicator before it is placed in the jar and to prepare a fresh solution if necessary.

In the present work a simple indicator was used that consists of methylene blue and a reducing agent. Equal volumes of an aqueous 0.015 per cent. solution of methylene blue and a 10 per cent. solution of sodium thioglycollate were mixed and the resulting solution is completely decolourised after heating for 2 - 3 min. in a boiling water bath or after anaerobic incubation. The indicator becomes dark blue when oxidised and old solutions eventually become irreversibly pale blue and should be discarded. Subsequent work showed that the sensitivity of this indicator may be related to the batch of sodium thioglycollate that is used and the amount of heating to which it is exposed; it may be necessary to decrease the concentration of thioglycollate. The indicator functioned satisfactorily in the present experiments but further testing is clearly needed before it could be accepted for routine use.

The methylene blue-thioglycollate (MBT) indicator was used to estimate the delay before anaerobiosis is attained in the BTL jar. The results of these experiments suggested that an anaerobic

environment occurs slightly sooner if 6/7 of the air is evacuated from the jar; if the jar is gas-tight, anaerobic conditions are produced within 2 - 3 hr of the admission of hydrogen whether 2/5 or 6/7 of the air is removed from the jar.

The room-temperature catalyst and the formation of moisture in a BTL jar

The catalyst in the BTL jar is active at temperatures above 0° C; it is inactivated by contact with chlorine, sulphur dioxide, hydrogen sulphide, carbon monoxide and by exposure to water or any other solution (see Instruction leaflet). It was observed in the present work that if large volumes of fluid cultures of Cl. oedematiens are incubated in anaerobic jars the catalyst becomes inactive after 2 or 3 incubations. Presumably, excessive amounts of gases such as hydrogen sulphide are produced in such cultures and it is essential to check that the catalyst is active at each incubation.

Strenuous attempts have been made in this study to avoid the formation of moisture in the anaerobic jar. A humid environment in the jar may contribute to catalyst inactivity and it may enhance the spreading growth of Cl. oedematiens (q.v.). The moisture in an anaerobic jar may be derived from the following sources:

(i) the catalytic reaction of hydrogen with oxygen,

(ii) passing the hydrogen through a wash bottle,

(iii) extraction of water from plates during evacuation and incubation.

The amount of water resulting from the catalytic reaction of hydrogen with oxygen is less than 1 ml. with each of the two evacuation procedures; some of the water condenses on the cool metal surface of the jar in the vicinity of the catalyst. The problems associated with the introduction of hydrogen via a wash bottle can be avoided by using a manometer to indicate catalyst activity.

The extraction of moisture from agar plates was studied by incorporating drying agents such as calcium chloride and silica gel in the jars. Containers of calcium chloride were weighed before and after incubation with plates; it was observed that the crystals of calcium chloride became liquid during incubation in the presence of normal agar plates, and that if the agar concentration was doubled the water formation was reduced by almost 50 per cent. It was possible to show by weighing the agar plates that the drying agent was actually extracting water from the plates, and it was noted that a much smaller amount of moisture was lost from the plates in the absence of calcium chloride. Later experiments suggested that the water loss from the plates depends upon the initial agar concentration and the degree of pre-drying of the plates. It was also observed that more moisture condenses on the under-surface of the lids of plates near the top of an anaerobic jar, and that these plates are particularly liable to show spreading growth.

It seems that the extraction of water from the plates by a

powerful hygroscopic agent *in situ* paradoxically results in the presence of more moisture at the agar surface and a greater humidity in the atmosphere of the jar. I concluded that the drying of plates with calcium chloride during or prior to anaerobic incubation is of no advantage; it does not prevent spreading of *Cl. oedematiens*, and it appears to extract a considerable amount of water from plates which in several experiments were heavily contaminated. The active drying of plates is best achieved by pre-heating (q.v.).

The laboratory culture of Cl. oedematiens

Explanatory note. The growth of Cl. oedematiens was studied in a wide range of culture media during the present work. The organism was grown at 37° C as a routine in these experiments although type A strains grew equally well at 30° C and at 45° C. Strains of the other types grew at 30° C but failed to grow at 45° C in Brewer medium (q.v.) incubated under aerobic conditions.

The isolation of Cl. oedematiens

The isolation of a pure culture of Cl. oedematiens from pathological material may be laborious and time-consuming. Heat-treatment of specimens or growth on a selective medium, e.g. Willis and Hobbs medium, is often recommended and both techniques were used in the present work.

Attempts were made to isolate Cl. oedematiens from sheep that had died of suspected Black disease; a typical necrotic lesion is usually present in the liver of such an animal. The lesion was removed aseptically and impression smears were made from the cut surfaces. The tissue was then macerated with a small volume of sterile saline or nutrient broth. Impression smears prepared from the lesion usually revealed an apparently pure population of Cl. oedematiens; smears prepared from the macerate showed several different types of organism.

If Cl. oedematiens is present in the lesion, the organisms fluoresce well with a commercially-prepared fluorescent antiserum (q.v.). After Gram-staining, the organisms appear stouter than organisms from artificial culture and oval, subterminal spores may be present.

If portions of the lesion or the macerate are subcultured into CMB or onto human blood agar plates, Cl. oedematiens is often outgrown by other organisms that are present in the liver-substance. The majority of successful isolations in the present work followed differential heating of the inoculum or subculture on a selective medium. Cl. oedematiens grows more rapidly in liquid media than on solid media and this fact should be borne in mind if prompt type-identification is necessary. The organism was isolated after heating specimens at 80° C for 10 min. and at 100° C for 5 min. However, if type D strains are suspected in the material, heating at a lower temperature, e.g. 65° C, may be necessary.

It is clearly advantageous to isolate a pathogenic organism on a solid medium, but this may not be easy if the organism grows slowly and spreads readily. In the present work, only type A strains of Cl. oedematiens grew on Willis and Hobbs medium and growth invariably occurred as a spreading film; thus, the value of this selective indicator medium with Cl. oedematiens is limited. Willis and Hobbs medium incorporates 250 µg. per ml. of neomycin sulphate as a selective agent; Cruickshank (1965, p. 322) recommends that only 70 - 100 µg. per ml. of neomycin should be used in media for clostridia. Tests with increasing amounts of neomycin sulphate in culture media

showed that the growth of type A strains of Cl. oedematiens was inhibited in the presence of the antibiotic at a concentration of 50 µg. per ml; strains of types B and D were inhibited on solid media by 25 µg. neomycin per ml. There was some evidence to suggest that neomycin sulphate is more inhibitory alone than if it is combined with other selective agents. Type A strains of Cl. oedematiens were also inhibited on solid media in the presence of penicillin (1.5 units), tetracycline (10 µg.), chloramphenicol (10 µg.) and erythromycin (10 µg.).

A selective medium that was designed to restrict spreading growth was developed in the present study. The medium contained nutrient agar base with added agar (4 - 6 per cent. W/V), human blood (20 per cent. V/V), sulphadimidine (1 in 4000), neomycin sulphate (10 µg. per ml.), and crystal violet (1 in 100,000). The plates were incubated for 48 - 72 hr so that the individual colonies became sufficiently large for subculture. Subsequent work suggested that the crystal violet concentration in the above medium should be reduced to 1 in 500,000 or 1 in 750,000 to prevent a slight inhibition of the growth of Cl. oedematiens. Sodium azide (0.02 per cent. V/V) was also used as a selective agent for Cl. oedematiens, but restricted haemolysis of strains of types B and D was observed in the presence of this agent with plates that contain human blood.

Preliminary identification of Cl. oedematiens

Provisional typing tests with half-antitoxin egg-yolk agar plates gave variable results in the present study. On occasions, the Nagler reaction was clear-cut after 48 hr incubation with a classical neutralisation in the presence of antitoxin. More often, however, the results were disappointing. Strains of types B, C and D usually failed to grow on the medium; spreading often occurred that masked the neutralisation pattern; and on several occasions growth was inhibited in the presence of antitoxin. The enriched egg-yolk medium of McChung and Toabe (1947) did not give consistent results, and a medium that incorporated 10 per cent. of egg-yolk emulsion and 5 per cent. of blood was the most satisfactory medium that contained egg-yolk. The neutralisation pattern with type A strains on this medium is similar to that in fig. 6 (p. 82d) with human blood agar. It should be borne in mind that the haemolytic or LV factors that may produce an effect in the well of indicator plates can be derived from the liquid inoculum; the effect is not necessarily associated with bacterial growth on the plate.

The results of the present study suggest that the typing of Cl. oedematiens by direct culture on indicator plates gives inconsistent results and is not recommended. Tube tests of CMB culture supernates are greatly superior (q.v.). LV tube tests were also performed on portions of the liver macerate from infected sheep. These gave negative results although Cl. oedematiens was subsequently isolated from the tissues.

Growth of Cl. oedematiens in fluid media

A number of fluid media were tested for their ability to support the growth of Cl. oedematiens. All types of the organism grew consistently well in cooked-meat broth (CMB) and this medium was used routinely in most of the work. Strains of types A, B and C often autoagglutinate after overnight growth in CMB and a flocculent deposit settles on top of the meat particles. Cultures of strain GRID do not autoagglutinate during incubation but may settle after the tubes have stood on the bench for some time. It appears that autoagglutination is a stable property of Cl. oedematiens because the strains autoagglutinate consistently and in different fluid media.

The meat particles of CMB are not digested during the growth of Cl. oedematiens; slight reddening of the particles may occur as a result of saccharolytic activity. Gas is usually formed in the depths of the medium during the growth of all strains in CMB.

Type A strains of Cl. oedematiens grow irregularly in tubes that contain 10 ml. of nutrient broth; the other types have not grown in tubes of this medium during the present study. The addition of glucose or sodium thioglycollate to nutrient broth allows consistent growth of type A strains only. During the latter part of this work, sheep liver infusion broth was prepared and tubes of this highly nutritious medium usually support good growth of all types of Cl. oedematiens.

Strains of types A, B and D grow readily in pre-steamed 100-ml. volumes of CMB, horse-digest broth, infusion broth and nutrient broth,

although growth may be delayed until 48 hr in the latter medium. The type C strain of Cl. oedematiens has proved particularly difficult to grow in fluid media and CMB gives the most consistent results.

These observations suggest that a suitable physico-chemical environment is essential for the growth of Cl. oedematiens in fluid media, and that this is in part provided by meat particles, reducing substances or large volumes of freshly steamed media.

Growth of Cl. oedematiens in semi-solid media

Brewer (1940) suggested that anaerobic organisms were overlooked in many laboratories because of procedural difficulties and he described a medium that was suitable for the aerobic incubation of anaerobic organisms.

(i) The agar concentration in Brewer medium. Brewer incorporated 0.05 per cent. of agar in his medium and this presumably reduces convection currents and delays aeration of the medium from the surface. I found that a medium that contains 0.05 per cent. of agar is semi-solid, and that the agar comes out of suspension to form a deposit at the bottom of uninoculated tubes that stand on the bench for several days. The medium becomes in effect a liquid medium and it may not support the growth of a demanding anaerobe. Cl. oedematiens often shows 2 distinct zones of growth in Brewer medium, an upper zone of diffuse growth and a lower zone of discrete colonies. I concluded

that the agar tends to form a deposit during incubation and that this influences the colonial morphology of a culture.

If the agar concentration is increased to 0.1 per cent. the medium becomes almost solid and no deposit forms on standing; this amount of agar was the most satisfactory in the present work. Later results suggested that the optimum concentration of agar in the medium may be related to the creation of a suitable local environment for growth of Cl. oedematiens and these observations merit further study.

If agar is omitted from Brewer medium then only type A strains of Cl. oedematiens grow readily.

(ii) Sodium thioglycollate in Brewer medium. Brewer incorporated 0.1 per cent. of sodium thioglycollate in his medium in an attempt to create a suitable redox potential for the growth of anaerobic bacteria. Sodium thioglycollate may become toxic to micro-organisms particularly if it is not fresh (Willis, 1964), therefore type A strains of Cl. oedematiens were tested for inhibition of growth in the presence of increasing concentrations of thioglycollate. It was found that 1.0 and 0.5 per cent. of thioglycollate markedly inhibited the growth of 3 of 4 test strains, and 0.1 per cent. caused slight inhibition of growth; lower concentrations of thioglycollate had no obvious effect. The results were correlated with measurements of the depth of the zone of coloured methylene blue at the surface of the medium and it was found that the higher concentrations of thioglycollate effectively delay oxidation of the medium. Type A strains were used in these

tests because they grew well in the test media; thioglycollate was incorporated in solid media for the growth of other types in later work (q.v.).

(iii) Other factors that affect growth in Brewer medium. Type A strains of Cl. oedematiens grow well in Brewer medium with nutrient broth as the peptone base; strains of the other types require a more nutritious base, e.g. 2 per cent. of proteose peptone. Brewer medium that has stood on the bench for several weeks still supports growth of the organism if it is steamed for 20 min. prior to inoculation. It should be borne in mind that the depth of Brewer medium in the tube must exceed 7 cm. in order that growth may be initiated from a small inoculum (Brewer, 1940; Hayward and Gray, 1946). Brewer suggested that a suitable internal environment for the growth of anaerobic organisms is produced in tubes of his medium despite incubation of the tubes in an aerobic external environment. In the present work, only type A strains of Cl. oedematiens grew consistently in Brewer medium that was incubated aerobically; the other types grew more reliably with anaerobic incubation and the addition of 10 per cent. of carbon dioxide to the atmosphere.

Growth of Cl. oedematiens on solid media

(i) Control of spreading growth. Spreading growth of surface

cultures is well-recognised in clostridial work and the problem was frequently encountered during the present study. Spreading growth is undesirable because (i) it is difficult to isolate a pure culture of the organism on solid media, (ii) the Nagler reaction on egg-yolk agar plates is affected, and (iii) the visible neutralisation of the Nagler effect with specific antisera is masked by spreading growth.

Every strain of Cl. oedematiens has grown as a spreading film at some stage of this investigation, but it was noted that strains of types A and D do not spread as readily as the other strains. Spreading growth appears to be related to a humid environment, yet the inclusion of calcium chloride in the anaerobic jar is of no advantage (see p. 72). Various substances have been added to culture media in order to suppress the spreading growth of different organisms, e.g. sodium azide with chloral hydrate (Wetzler, Marshall and Cardella, 1956), sulphonamides (Holman, 1957) and activated charcoal (Alwen and Smith, 1967); these agents were not entirely satisfactory in the present work. Prolonged drying of normal agar plates is not consistently effective as spreading was observed on plates that were dried for 35 min. at 60° C.

The most successful method of preventing spreading growth of Cl. oedematiens in the present study was to increase the agar concentration of the medium (Hayward and Miles, 1943) in conjunction with prolonged drying of the plates. The maximum concentration of agar that can be poured readily is 6 per cent. (New Zealand agar W/V), and it is difficult to prepare plates of this medium that have a smooth

surface. Satisfactory plates can be prepared if the agar content is reduced to 4 per cent., but spreading growth of Cl. oedematiens may occur on this medium and it is necessary to dry the 4 per cent. agar plates for 30 min. at 60° C to be reasonably certain of obtaining discrete colonies. However, in parallel tests, some strains of Cl. oedematiens failed to grow on 4 per cent. agar plates yet grew as a spreading film on 1.5 per cent. agar plates. Presumably, the diffusion of nutrients is diminished from the concentrated agar plate and the advantage of obtaining discrete colonies must be set against the possible disadvantage of restricted growth. This is a difficult choice.

(ii) Growth of type A strains. Type A strains of Cl. oedematiens grow consistently well on simple, solid media providing that the atmosphere of incubation is truly anaerobic. These strains form discrete, raised, opaque colonies, 1 mm. in diameter after overnight incubation on blood agar plates; the diameter of the colonies increases to 2 - 3 mm. after 48 hr incubation (figs. 1 and 2, p. 82a). The colonial morphology varies depending on the strain, the medium and the environmental conditions; type A strains often tend to spread (fig. 3, p. 82b), and large, mucoid, creamy colonies are formed in the presence of 1 per cent. of glucose.

Two zones of haemolysis are produced on 10 per cent. horse blood agar plates; there is a narrow inner zone of beta-haemolysis and an outer zone of partial haemolysis that extends beyond the edge of the

The colonial morphology of Cl. oedematiens on blood agar plates

FIG. 1. - Type A strain 223. X 3.

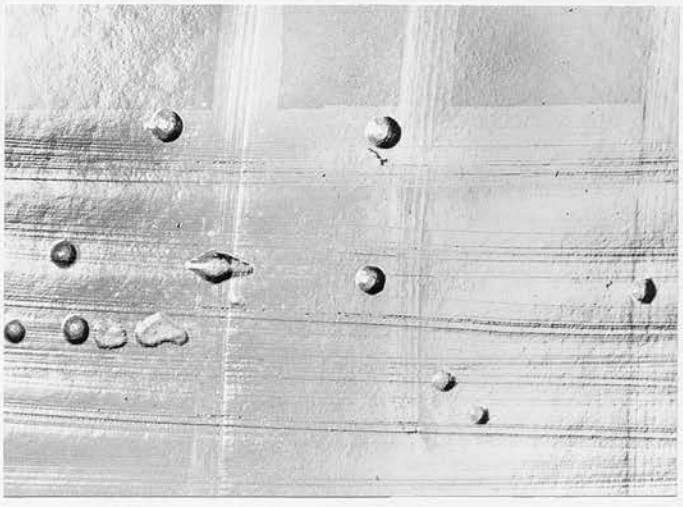


FIG. 2. - Type A strain GR1A. X 3.

The colonial morphology of Cl. oedematiens on blood agar plates

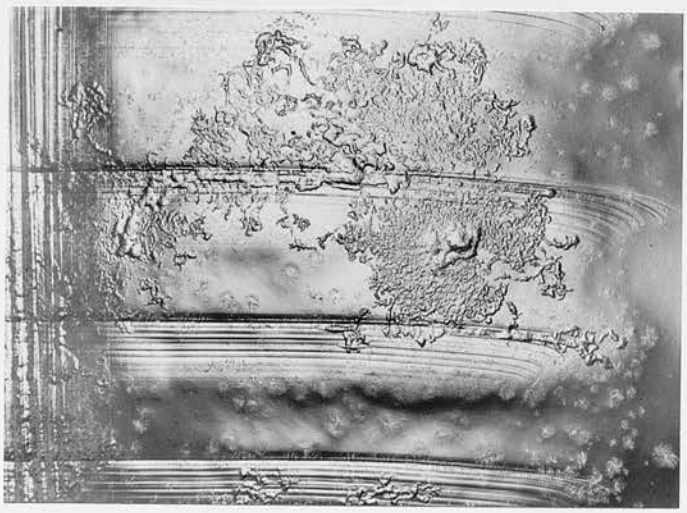


FIG. 3. - Type A strain GR2A. X 3.

FIG. 4. - Type B strain GR2B. X 3.

The colonial morphology of Cl. oedematiens on blood agar plates

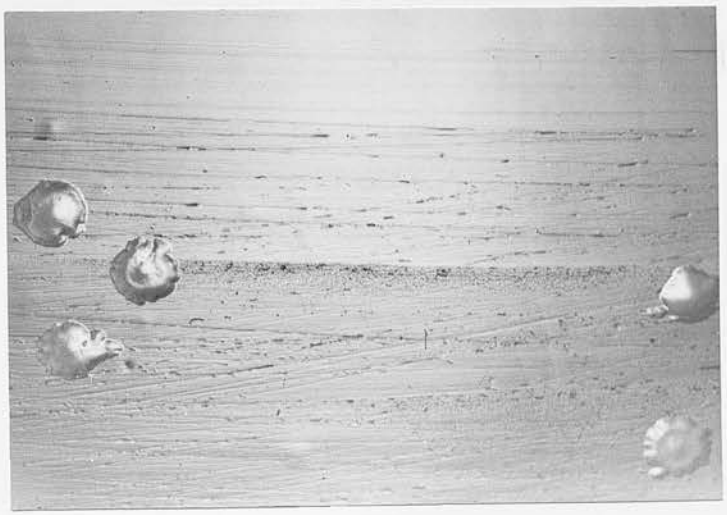


FIG. 5. - Type D strain GR1D. X 3.

Neutralisation of the haemolytic activity of Cl. oedematiens

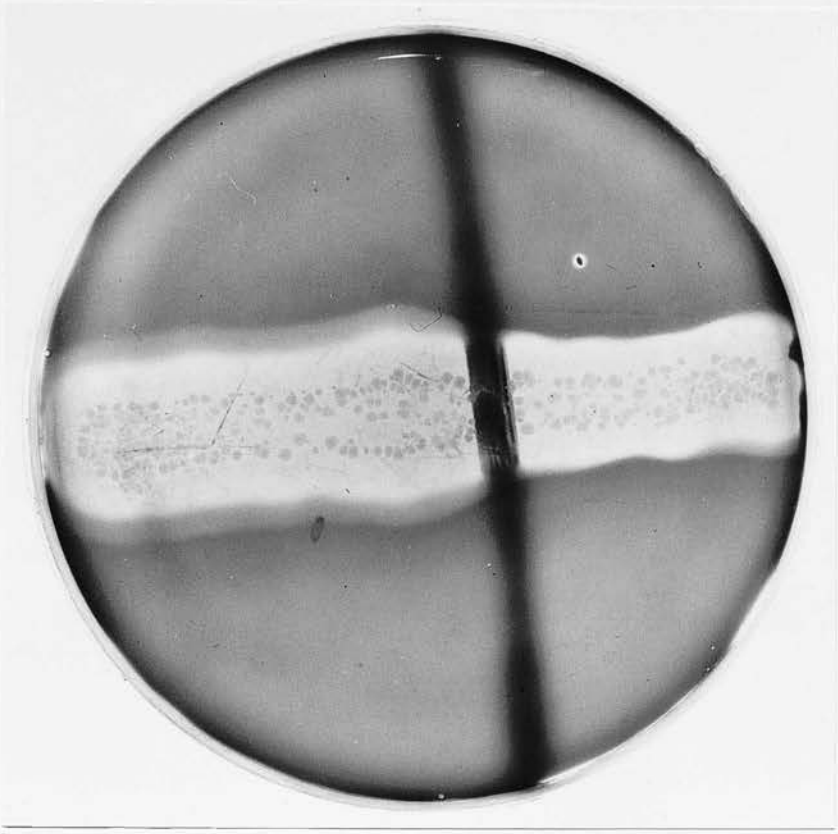


FIG. 6. - Strain GR2A after 48 hr anaerobic incubation on a 10 per cent. human blood agar plate; the right half of the medium is spread with 50 anti-gamma units of homologous antitoxin EX1552. X 1.

colony. The pattern of haemolysis varies according to the species of blood that is used; on human blood agar plates the beta-haemolysis is more extensive, and on sheep blood agar the lysis is markedly reduced. The haemolytic zones may be partially neutralised by covering half of the medium with homologous antitoxin. Strain GR2A was grown for 48 hr on human blood agar to demonstrate the two zones of lysis (fig. 6, p. 82d); half of the medium was spread with 50 anti-gamma units of Cl. oedematiens type A antitoxin; the outer zone of haemolysis is clearly inhibited by the antitoxin and the inner zone of lysis is slightly reduced in width.

When Cl. oedematiens type A strains grow as a spreading film on blood agar the pattern of lysis is obscured and the whole plate is haemolysed to a degree that depends upon the species of blood. If the agar content of the medium is increased to 3 or 4 per cent., the zones of haemolysis are restricted.

Type A strains of Cl. oedematiens have grown on most laboratory media in the present work including blood agar, egg-yolk agar, chocolate agar and RCM agar; smaller and fewer colonies were obtained on nutrient agar, serum agar and plasma agar, and the strains failed to grow on MacConkey agar or DCA medium. A weak Nagler reaction is produced on serum agar, but the zone of turbidity and the pearly layer are seen more readily on egg-yolk agar; homologous antitoxin neutralises the zone of turbidity only. Type A strains often spread on egg-yolk agar and produce a diffuse turbidity in the medium; under these conditions the pearly layer may not be visible and antitoxin

fails to inhibit the generalised turbidity.

(iii) Growth of type B, C and D strains. Type B, C and D strains of Cl. oedematiens grew inconsistently on solid media during the present study. Initially, it seemed that the problem of subculturing these strains might be related to particularly stringent nutritional requirements; many different permutations of solid media were assessed during this work with parallel tests from time to time in deep agar cultures. Additions to a basal medium of nutrient agar (Oxoid) have included varying concentrations of glucose, egg-yolk emulsion, sodium thioglycollate, blood of different species, peptone, yeast extract, casein-hydrolysed amino acids, sodium hydrosulphite, cobalt nitrate, liver extract, inositol, ascorbic acid, ammonium sulphate, sodium nitrate, pyruvic acid and fumaric acid. Promising results were obtained with certain combinations of constituents in a complex medium (see tables VI & VII), but a quantitative assessment of growth is impossible when spreading occurs. On many occasions the strains grew readily on 10 per cent. human blood agar plates and it was concluded that a complex medium is not essential for the subculture of these strains of Cl. oedematiens; a complex medium does not support growth more consistently than a simple medium that contains added blood.

The following observations regarding the growth of Cl. oedematiens on solid media are based on tests performed throughout the present study.

Table VI

The growth of type B, C and D strains of Cl. oedematiens on different solid media

Strain of Cl. oedematiens	Degree or type of growth obtained with an inoculum derived from				
	24 hr CMB/G			28 day CMB	
	on Medium I*	on Medium II*	on Medium III*	on Medium II	on Medium III
GR1B	Spreading	1 colony	++	-	+
GR2B	++	-	+++	-	++
GR1C	-	-	4 colonies	-	++
GR1D	+	+	+++	-	+

+, ++, +++ = increasing amounts of growth;

\- = no growth;

*Medium I = 30 per cent. human blood agar;

Medium II = 10 per cent. human blood agar plus 10 per cent. egg-yolk emulsion;

Medium III = Medium II plus 0.1 per cent. sodium thioglycollate.

Table VII

The growth of type B, C and D strains of Cl. oedematiens on different solid media

Strain of Cl. oedematiens	Degree or type of growth obtained with an inoculum derived from 24 hr CMB/G on				
	Medium I*	Medium II*	Medium III*	Medium IV*	Medium V*
GR1B	2 colonies	3 colonies	Spreading	3 colonies	-
GR2B	+	⊥	Spreading	+	-
GR1C	-	-	Spreading	-	-
GR1D	7 colonies	3 colonies	7 colonies	⊥	-

⊥, + = increasing amounts of growth;

\- = no growth;

*Medium I = 10 per cent. human blood agar;

Medium II = Medium I plus 1 per cent. glucose and 5 per cent. egg-yolk emulsion;

Medium III = 30 per cent. human blood plus 1 per cent. glucose and 5 per cent. egg-yolk emulsion;

Medium IV = Medium III plus 0.1 per cent. sodium thioglycollate;

Medium V = Medium III plus 0.5 per cent. sodium thioglycollate.

a) Human blood appears to support the growth of type B, C and D strains of Cl. oedematiens more consistently than horse or sheep blood. Larger colonies are formed if 30 per cent. of blood is incorporated in the medium, but this may encourage spreading growth. Chocolate agar plates prepared from 10 per cent. of human blood often supported satisfactory growth of these strains; no visible effects, e.g. clearing or bleaching, were produced by the organism on this medium.

b) Freshly-poured medium is desirable but not essential for the growth of Cl. oedematiens; strains of types B and D grew on plates previously held at 4° C for 7 days.

c) The addition of 0.1 per cent. of sterile sodium thioglycollate solution to the medium may assist the growth of these strains, and membrane-filtered thioglycollate solution appears to be more satisfactory than autoclaved solutions. The results given in table VII indicate that 0.1 per cent. of sodium thioglycollate does not affect the growth of type B and D strains of Cl. oedematiens; the addition of 0.5 per cent. of thioglycollate to the medium appears to inhibit these strains. The findings follow the pattern of the observations obtained in tests with type A strains of the organism (see p. 79).

d) The addition of 10 per cent. of carbon dioxide to the anaerobic environment is usually beneficial. Type A strains may form a mixture of mucoid and non-mucoid colonies in the presence of carbon dioxide

and this gives the impression of a mixed culture.

e) The results of a limited number of attempts to grow Cl. oedematiens beneath a clear nutrient agar overlay were not satisfactory. Contaminant growth on the overlay caused difficulties that were reduced but not always overcome by the addition of inhibitory agents to the overlay.

f) Type B, C and D strains of the organism required at least 48 hr for adequate growth on the media that were used in this study. Tiny colonies of type B and D strains surrounded by a large zone of haemolysis may be present on blood agar after overnight incubation and the colonies may increase in size and number if incubation is continued. Large colonies are formed on a suitable medium after uninterrupted incubation for 72 hr. Spreading growth usually appears after 24 hr incubation, and it is unwise to discard plates until at least 48 hr after inoculation.

g) Type B strains and the type C strain spread readily on solid media. Type B strains usually grow as a flat spreading film, but they may form discrete, raised, grey colonies with an irregular outline (fig. 4, p. 82b). The type C strain grew as a spreading film on almost every occasion, but the film was not flat; raised, grey, irregular growth was produced and motile colonies were well shown with this strain (see frontispiece). Motile colonies were observed

to a lesser degree with type A and B strains. The type D strains that were used in this study formed raised, almost circular colonies (fig. 5, p. 82c) that varied in size according to the nature of the medium; occasionally, spreading growth was observed.

h) Type B strains and especially type D strains of Cl. oedematiens produce large zones of haemolysis on blood agar plates; the haemolytic zones are larger than those produced by type A strains, but the pattern of haemolysis is similar. The type C strain is generally regarded as being non-haemolytic, but a faint haemolysis was occasionally observed with this strain on blood agar plates.

i) Egg-yolk agar usually fails to support adequate growth of type B, C and D strains of Cl. oedematiens. If growth does occur, the type D strains produce a larger zone of turbidity than type B strains; the zone diameter varies with different type B strains. A pearly layer is not produced by either of the types.

j) The addition of 1 per cent. of glucose to the medium may affect the development of the effects that are described above. The haemolytic zones of type B and D strains were absent on a batch of glucose/horse blood agar plates, although type A strains were haemolytic on the same medium; also the pearly layer was not present with certain type A strains on plates that contained glucose and egg-yolk solution. These observations were not investigated further.

k) Some of the results in this series of experiments suggested that growth on solid media was obtained more consistently in the older model of anaerobic jar that contains a catalyst activated by passing an electrical current through a heating element in the capsule. Subsequent work did not confirm this observation although in several cases it appeared that failures in growth could only be attributed to conditions that were apparently insufficiently anaerobic.

The viability of the inoculum

At this point in the study it appeared that the problem of subculturing Cl. oedematiens was not related to a failure of anaerobiosis or to a requirement for a particularly complex medium; other factors that might be significant were therefore considered. Experimental results suggested that the source of the inoculum is a critical factor in the subculture of type B, C and D strains on solid media. It seemed that in general, these strains can be subcultured more readily from CMB than from CMB plus 1 per cent. of glucose (CMB/G). Cultures grown in CMB and then held for several weeks on the bench often yielded successful subcultures on solid media. An obvious difference between the various inocula might be the ratio of spores to vegetative organisms and the significance of this factor was investigated more fully.

In order to assess the potential viability of different cultures

it was necessary to perform viable counts. Preliminary experiments indicated that the Miles and Misra (1938) technique is not suitable for viable counts of Cl. oedematiens because strains of types B, C and D fail to grow consistently on solid media and because spreading growth often occurs. The pour-plate and shake-culture procedures were applied to serial dilutions of a test culture of Cl. oedematiens and gave comparable results. The pour-plate technique is the more accurate method as it is difficult to count a large number of colonies in shake-cultures; in addition, gas is often produced in shake-cultures and the medium may be completely disrupted. An advantage of shake-cultures however is that the cultures can be incubated aerobically whereas pour-plates require anaerobic incubation in BTL jars; a number of anaerobic jars are thus necessary if several counts of each dilution are made. The spread-plate technique was also used in the present work. The method often gave a higher viable count than parallel deep agar techniques; this may be due to inactivation of viable particles at the temperature of molten agar.

In order to gain experience of enumeration techniques for Cl. oedematiens a growth curve experiment was performed with a type A strain in 150 ml. of CMB/G medium. Viable counts were performed every 2 hr from 2 - 12 hr and from 24 - 28 hr with the pour-plate technique. The results suggested that the lag phase of this culture lasted for at least 12 hr; there was a substantial increase in the viable count between 12 and 24 hr and at 24 hr the culture appeared to have reached the stationary phase of growth. The viable count was not measured

between 12 and 24 hr and a complete growth curve was not constructed.

Growth from heated inocula. The effect of pasteurising the inoculum was studied by growing a strain of each type of the organism in CMB for 18 hr, and then holding a sample of the cultures at 80 - 85° C for 20 min; plates of RCM media were spread with 0.03 ml. of the heated and unheated culture and incubated for 72 hr. The type A strain grew considerably better than the other strains and the type C strain failed to grow. Strains of types A and B grew from the heated and the unheated inocula, but the type D strain grew from the unheated inoculum only. The results also indicated that the type B strain grew more readily from the heated inoculum. It seemed reasonable to conclude that the viable particles in the heated inocula were probably spores, and that the type D strain failed to grow after heating because the spores of this type are more susceptible to heat-treatment. On the basis of these results, and bearing in mind that the vegetative cells of Cl. oedematiens are by repute highly sensitive to air or oxygen, it is conceivable that the growth of Cl. oedematiens on solid media may be initiated solely from spores; thus, the problem associated with subculturing the organism on solid media might be related to the degree of sporulation in the inoculum. In order to test this hypothesis, viable counts were performed on an 18-hr CMB culture and a 4-day CMB culture of strain GR1A; samples of each culture were counted after heating for 20 min. at 80° C and for 5 min. at 100° C. The cultures were diluted in pre-steamed nutrient broth and 0.03 ml.

of each dilution was spread on the surface of plates that contained RCM enriched with agar to 3 per cent. and 20 per cent. of human blood. The counts were performed in duplicate and the plates were incubated for 18 hr. The initial results were promising (see table VIII).

It is interesting to compare the viable counts of an 18-hr culture after heating. The sample that was heated at 100° C for 5 min., then diluted 4-fold, grew as a spreading film over the whole plate; the sample that was heated at 80° C for 20 min. contained only 4 viable particles. These results suggest that spreading growth may be initiated from a limited number of viable particles.

Spreading growth occurred on many of the plates in spite of the increased agar content; the degree of growth cannot be assessed quantitatively on these plates. The numerical results indicate that the 4-day culture contained about 10 times more viable particles than the 18-hr culture; heating the inoculum at 80° C or 100° C did not markedly reduce the viable count. The results following heating should be interpreted with caution, because the heating procedure not only inactivates vegetative organisms but may also activate spores so that the viable count may be artificially increased.

The results confirmed quantitatively that heated inocula of a type A strain are almost as viable as unheated inocula, but the error associated with heat-activation of spores must be considered. A treatment was required that would inactivate vegetative organisms without affecting the spores: exposure of the culture to 10 per cent. of phenol was chosen.

Table VIII

A quantitative estimate of the viability of 2 cultures of Cl. oedematiens type A before and after heating

Dilution of sample	Degree or nature of growth obtained with an inoculum from											
	18 hr CMB culture						4 dy CMB culture					
	Unheated		80° C for 20 min.		100° C for 5 min.		Unheated		80° C for 20 min.		100° C for 5 min.	
	No. of colonies	Viable count	No. of colonies	Viable count	No. of colonies	Viable count	No. of colonies	Viable count	No. of colonies	Viable count	No. of colonies	Viable count
Neat culture	Spreading		Spreading		Spreading		Spreading		Spreading		Spreading	
-1	Spreading		Spreading		Spreading		Spreading		Spreading		Spreading	
-2	Spreading		184	$6.1_5 \times 10^5$	Spreading		Spreading		Spreading		Spreading	
-3	23	$7.7_5 \times 10^5$	Spreading		Spreading		310	$1.0_7 \times 10^7$	252	$8.4_6 \times 10^6$	245	$8.2_6 \times 10^6$
-4	1	$3.3_5 \times 10^5$	4	$1.7_6 \times 10^6$	Spreading		Spreading		62	$2.1_7 \times 10^7$	31	$1.0_7 \times 10^7$

The viability of a phenol-treated inoculum. An overnight CMB culture of strain GR1A was used as the test culture and a total count was performed in a standard Thoma counting chamber. Samples of the culture were treated for 3 hr with an equal volume of sterile saline or 10 per cent. phenol solution; the cells were thoroughly washed and viable counts were performed in shake-cultures and on spread-plates of RCM agar (see table IX).

Table IX shows that 1 in 75 of the total number of cells in the culture is viable on the spread-plates, and 1 in 8 of the viable particles is phenol-resistant. If spores alone are viable on solid media, then the calculated degree of sporulation in the overnight CMB culture should be 1 - 2 per cent; more than 10 per cent. of the spores resist the phenol treatment. Alternatively, if both vegetative cells and spores are viable on solid media, and assuming that the vegetative forms are inactivated by phenol without activation of spores, the degree of sporulation in the culture should be more than 10 per cent. These deductions are based on the simplest explanation of the above figures and do not apply if different proportions of the viable particles are affected by phenol.

The correlation between spore counts and viable counts. It is difficult to design a suitable experiment to prove that vegetative organisms do not initiate growth on solid media. The following results represent further attempts to correlate the spore content of different cultures with the viable counts. Strains of Cl. oedematiens

Table IX

The viability of strain GR1A of Cl. oedematiens before and after phenol-treatment

Total Count	Viable counts obtained with inocula from					
	Untreated culture		Saline-diluted suspension		Phenol-treated suspension	
	Shaken	Spread	Shaken	Spread	Shaken	Spread
2.5×10^7 cells per ml.	1.1×10^5	3.0×10^5	1.2×10^5	3.8×10^5	1.1×10^4	4.9×10^4

types A and B were grown in 100-ml. amounts of medium and samples of the cultures were withdrawn at intervals. The total count was estimated in a standard counting chamber with phase contrast microscopy; the percentage of phase-bright spores was calculated and this was compared with observations on parallel smears stained by Gram's method and Ashby's modified spore stain (see Cruickshank, 1965, p. 657). Triplicate viable counts were performed on the cultures with the pour-plate technique and occasionally the spread-plate method; the medium consisted of RCM agar plus 5 per cent. of human blood to assist colony differentiation. The results of these experiments are summarised in table X.

The results given in table X indicate that type A strains of Cl. oedematiens sporulate more readily than type B strains. An overnight CMB culture of strain GR1A contained 1 - 2 per cent. of spores and similar cultures of strains R3/2 and GR1B contained less than 1 per cent. of spores. Predictably, the degree of sporulation increased as the culture aged. The addition of glucose to unbuffered CMB markedly reduced the sporulation of strain GR1A. The viable count of strain GR1A in CMB showed a good correlation with the estimated spore content of the culture; in the presence of glucose the viable count of this strain was greatly reduced although the total cell count was higher than that of the corresponding CMB culture.

The viable counts of overnight CMB cultures of strains R3/2 and GR1B were extraordinarily low. There was a significant increase in the viable count and the degree of sporulation of strain R3/2 between

Table X

The total counts, spore counts and viable counts of different cultures of Cl. oedematiens

Culture and age	Total Count per ml.	Spore count (per cent.)			Spore estimate (by phase-contrast) per ml.	Viable count per ml. (pour-plate)	Viable count per ml. (spread-plate)
		Phase	Gram	Spore stain			
18 hr GR1A CMB	1.0×10^7	1.5	2	2	2.0×10^5	2.7×10^5	2.1×10^5
4 day GR1A CMB	6.8×10^6	66	65	60	4.5×10^6	1.8×10^6	-
18 hr GR1A CMB/G	2.8×10^8	0.2	1	0.2	1.4×10^5	7.4×10^3	-
4 day GR1A* CMB/G	2.8×10^8	<0.02	<1	<1	$< 5.6 \times 10^4$	8.2×10^3	-
18 hr R3/2 CMB	4.7×10^6	<1	<1	<1	$< 4.7 \times 10^4$	2.8×10^1	-
4 day R3/2 CMB	1.5×10^7	28	29	34	4.3×10^6	1.7×10^4	2.8×10^4
7 day R3/2 CMB	1.1×10^7	44	52	57	4.8×10^6	1.4×10^4	-
7 + 1 R3/2+ CMB	Not done	-	49	64	-	1.7×10^4	-
7 + 7 R3/2 CMB	Not done	-	62	64	-	1.7×10^4	-
7 + 11 R3/2 CMB	Not done	-	58	68	-	1.8×10^4	-
18 hr GR1B IB	2.6×10^8	<0.1	-	-	$< 2.6 \times 10^5$	3.5×10^2	-

*This culture was spun gently, then a smear was made from the cell deposit and stained with Ashby's modified spore stain; an occasional spore was observed among a large number of vegetative organisms.

+R3/2 culture was incubated for 7 days then held on the bench for 11 days; readings were made after 1, 7 and 11 days.

- = not done.

1 and 4 days, but the ratio between the viable count and the estimated spore content of the culture is 1 to 150. The degree of sporulation of this strain increases after prolonged incubation and standing; the viable count does not alter during this period. The implications of these results are discussed below (see Discussion).

Studies on the initiation of growth of Cl. oedematiens in relation to the redox potential of the medium

Preliminary studies were made of the initial redox potential in various media that support the growth of this demanding organism. A number of oxidation-reduction potential indicator dyes (see Methods) were prepared in 0.1 per cent. (w/v) solutions and were sterilised by membrane filtration. A small volume (0.5 ml.) of each dye was added to different media, and the degree of decolourisation of the dye was regarded as an indication of the redox potential in the medium (see Hewitt, 1950, pp. 23 - 24). Control experiments showed that the growth of Cl. oedematiens was not inhibited at the dye concentration described above.

It appears that the addition of reducing agents such as sodium thioglycollate (0.1 per cent.) or ascorbic acid (0.1 per cent.) to nutrient agar shake-cultures does not lower the Eh of the medium below that obtained with freshly prepared vials of nutrient agar alone; however, the depth of colour at the top of the vial (i.e. oxidised

dye) is less in the presence of the reducing agents. Sodium hydrosulphite (0.1 per cent.) is a more effective reducing agent than thioglycollate or ascorbic acid according to the results of similar experiments, but later work showed that hydrosulphite may inhibit the growth of Cl. oedematiens. The most effective reducing agent in the present work was 6 in. of heated iron wire in the form of a coil; the results of experiments with the indicator dyes suggested that the Eh in the vicinity of the coil was less than -0.15V. RCM agar has an Eh that approaches -0.1V; this value is slightly lower than that obtained with nutrient agar as RCM agar contains several reducing agents.

Type A strains of Cl. oedematiens grow well in all of the above media. The Eh of a glucose-agar shake-culture is -0.05V initially; after overnight culture of strain GR1A in this medium the Eh falls to -0.35V, then 48 hr later the Eh has risen to -0.2V.

Type B, C and D strains of Cl. oedematiens may grow in the lower half of nutrient agar shake-cultures after 48 hr incubation provided that a moderately large inoculum (0.5 ml.) is used; growth is a little heavier, occurs earlier and extends nearer to the surface of the vial in the presence of added reducing agents. These strains grow better in RCM agar than in nutrient agar, and it may be significant that the Eh of RCM agar is rather lower than that of nutrient agar.

Cl. oedematiens grew consistently well in CMB during the present studies. Experiments with indicator dyes suggest that an Eh of at least -0.15V is attained in pre-steamed tubes of this medium; after

18 hr anaerobic incubation of uninoculated tubes the Eh fell to -0.35V.

At this point in the project, all of the resources of the laboratory were directed towards attempts to improve the reliability of surface growth of type B and D strains on solid media. The reliable growth that was obtained in CMB led to the incorporation of meat particles into agar plates; a type B strain of Cl. oedematiens grew as a spreading film in close association with the meat particles of this medium. It was considered that the particulate matter might be contributing either actively or passively to the initiation of growth of the organism. Passive participation, for example a particle adsorption effect, was investigated by spreading inert material such as powdered glass and carbon particles onto the surface of the medium; neither of these substances enhanced the growth properties of the medium. The powerful reducing systems that are present in the meat particles may assist in the initiation of growth of the organism on agar plates, and this was investigated by spreading iron filings onto the surface of the medium. It was found that after incubation of the organism on human blood agar plates with iron filings, growth is much heavier and more rapid than on control plates without the iron. A large number of colonies are present in close relation to the iron filings after overnight incubation, and growth extends over the whole plate after prolonged incubation.

Further work has indicated that other metals such as zinc, chromium, and manganese do not have similar properties in this respect; nickel and platinised asbestos have a similar but much less marked

effect after overnight incubation of the plates (Coleshill, 1968). The effect appears to be confined to type B and D strains of Cl. oedematiens, and the incorporation of iron filings on a suitable medium ensures reliable subculture of these organisms. Further studies are in progress to elucidate the above observations, and the implications of these findings are considered below (see Discussion).

The characters of Cl. oedematiens

Microscopic morphology and staining characteristics

The microscopic morphology of Cl. oedematiens varies depending on the culture medium in which the strain is grown. In general, the bacilli have straight sides and rounded ends; strains of types A and D are shorter than type B and C strains; type B and D bacilli are stouter than the others. The cells of type A and B strains occur singly or in pairs, but type B strains may form longer chains of bacilli. The type C strain usually shows long filamentous forms and type D strains tend to be rather pleomorphic (fig. 7, p. 103a).

In the present study, acetone was used as the decolourising agent in Gram staining. Smears of Cl. oedematiens prepared from cultures and stained by Gram's method are almost invariably Gram-negative, although Gram-positive forms are sometimes seen. Gram-positive bacilli are frequently seen in direct smears from pathological material, and Gram-positive granules are often present in Gram-negative bacilli in these smears.

Spores are readily demonstrated in suitable smears of Cl. oedematiens. Gram-positive forespores are often present in Gram-negative bacilli, and oval, unstained, mature spores can usually be recognised after Gram staining; however, many bacilli stain irregularly and contain unstained areas that may be difficult to differentiate from

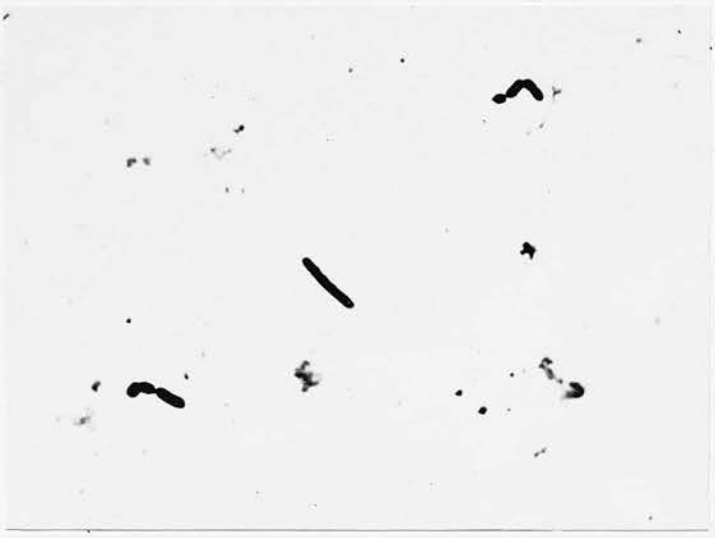


FIG. 7. - The microscopic morphology of Cl. oedematiens strain GR1D grown for 24 hr in cooked-meat broth. X 1200.

spores. A specific spore stain such as the modified Ziehl-Neelsen acid-fast stain or Ashby's modification of the malachite green stain (see Cruickshank, 1965, p. 657) gives more reliable results. Both of these techniques produce true staining of spores that occur within the bacilli. The central portion of a free spore is almost invariably unstained and there is a halo of stain round the periphery; these relatively unstained spores may also occur as endospores and they then appear larger than stained endospores. It is assumed that the relatively unstained spores are mature spores. Phase-contrast microscopy of wet films reveals phase-bright spores within phase-dark cells. Free spores are readily visible by phase-contrast microscopy, but various artefacts often simulate spores.

The spores of Cl. oedematiens are usually oval, subterminal and do not bulge the bacillus; exceptions to this description are occasionally seen. Type A strains of the organism sporulate more readily than the other types, particularly on solid media. However, a 72-hr culture in CMB of each of the strains tested usually contains spores. Good spore crops were obtained on occasions from cultures in media with added plasma and in modified CMB (Nakagawara and Nishida, 1963), but markedly increased spore yields were not a consistent feature of cultures grown in subsequent batches of these media.

The bacilli of Cl. oedematiens in smears prepared from CMB/G cultures contain reddish-brown granules after staining by Albert's method. The granules are clearly not volutin granules and it seems that they might represent stores of reserve glycogen that stains with the iodine

component of Albert's stain. Subsequent work confirmed that the granules stain with Gram's iodine. Several bacilli in each microscopic field usually contain a granule although more than one granule may be present in each cell. The granule is large, often terminal and does not bulge the cell; a granule and a spore may be present in the same organism. The production of granules does not appear to be specifically related to culture in the presence of added fermentable substrate; granules were present on occasions in cells from overnight CMB cultures of all strains of Cl. oedematiens.

Intracellular lipid granules were demonstrated with Sudan black (see Cruickshank, 1965, p. 661) in strains of Cl. oedematiens during the present work. The granules are small and up to 4 granules may be present in each bacillus. The reserve granules described above may account in part for the irregular staining that is frequently seen with Cl. oedematiens.

The present investigation confirmed that strains of Cl. oedematiens do not possess a capsule. A wet India ink film examined with phase-contrast microscopy is the most satisfactory method of demonstrating the presence of a capsule; a suitable wet film shows that Cl. oedematiens does not form a capsule during culture in a liquid medium with or without added carbohydrate. Dry India ink films and wet films that contain insufficient India ink may produce artefacts that simulate a micro-capsule. India ink controls are essential; in the present work a capsulate bacillus was present in one preparation of India ink.

The immunofluorescent staining procedure

A direct immunofluorescent staining technique was used with Cl. oedematiens during the present work. Smears were prepared from pathological specimens and from cultures in artificial media, and were stained with a commercially available fluorescent anti-Cl. oedematiens globulin (Wellcome Laboratories).

The actual staining procedure is simple to perform, but the recommended method of fixation of the smears with commercial acetone was inadequate. Subsequent work showed that gentle heat-fixation gave superior results and this was used as a routine in later work. Heat-fixation is quicker than acetone-fixation and does not appear to affect the subsequent staining of the smear. Two important points of technique are noted in the instruction leaflet provided with the commercial stain: (i) that the stain should not dry on the slide, and (ii) that a reliable and correctly operated optical system is essential. Early batches of the fluorescent globulin became turbid and granular; they were heavily contaminated despite aseptic precautions during use. The changes in the stain did not detract from the staining potential, but caused anxiety regarding the shelf-life of the stain and also interfered with microscopy (see below, p.180).

The following general observations regarding the fluorescent procedure were obtained after intensive investigations during the present study. Smears prepared for fluorescent staining should be thin and well-emulsified; if the smear is too thick, a fluorescent haze is

present during microscopy that may obscure the organisms. The smear should not be blotted during the staining procedure. The degree of fluorescence obtained in a preparation depends on the age of the culture, the nature of the culture medium and the type of Cl. oedematiens that is present. Smears prepared from overnight cultures of Cl. oedematiens fluoresce better than smears from older cultures; there is much fluorescent debris in the latter smears. Direct smears from pathological material, and organisms cultured in CMB fluoresce consistently well; smears prepared from solid media are more variable, but it should be borne in mind that the latter cultures were incubated for 48 hr. Cultures in CMB/G often produce swollen cells particularly with type A strains; these forms usually stain well with the fluorescent globulin. The smaller more classical forms of Cl. oedematiens fluoresce better than the longer and filamentous forms; banded bacilli were observed on several occasions. Consistently good staining results were obtained with strains of types A and B; smears of type C and D strains frequently contained poorly-stained or unstained bacilli that were readily visible with the dark-background condenser if the light source was changed from ultra-violet to white light. A smear prepared from a type B strain of Cl. oedematiens and stained with the fluorescent globulin is shown in fig. 8 (p. 107a).

Appearance in the electron-microscope

The electron-microscopic appearances of Cl. oedematiens are shown

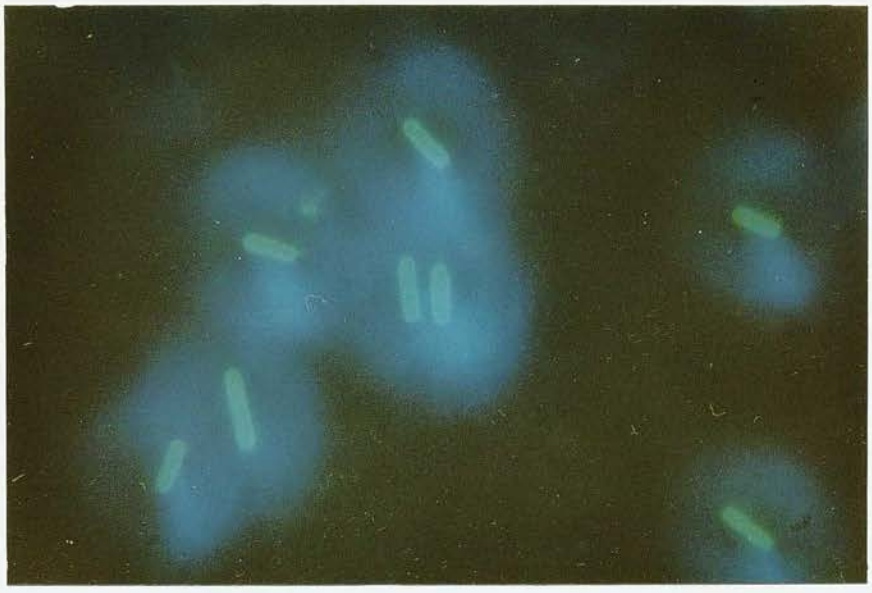


FIG. 8. - A type B strain of Cl. oedematiens stained with fluorescent globulin.
X 1250.

in figs. 9 - 12 (p. 108a-d). It is clear that the bacilli possess long, peritrichous flagella and do not have fimbriae. The flagella are closely adherent in many of the specimens and often appear as a rope or as a hank of wool. There is an aggregation of small irregular particles round the periphery of the bacilli in many of the specimens prepared from solid media. These may represent artefacts associated with the medium as they were not present in grids prepared from liquid media. It is possible that the aggregation of flagella may be responsible for the phenomena of auto-agglutination and motile colonies that are observed in culture media.

Fermentation reactions

The descriptions of earlier workers make it clear that differences exist in the biochemical properties of the 4 types of Cl. oedematiens. The reported fermentation reactions of the organism are not consistent and a critical comparative investigation does not seem to have been made.

My initial problem was to find a basal medium in which the fermentation reactions of the organism could be tested. Cl. oedematiens does not grow in a simple peptone water medium and the addition of a fermentable substrate or an iron nail did not adequately enhance the growth properties of the medium. After many unsuccessful attempts to find a suitable liquid basal medium, a modified Brewer medium that contained 0.1 per cent. of agar was chosen. This medium was not

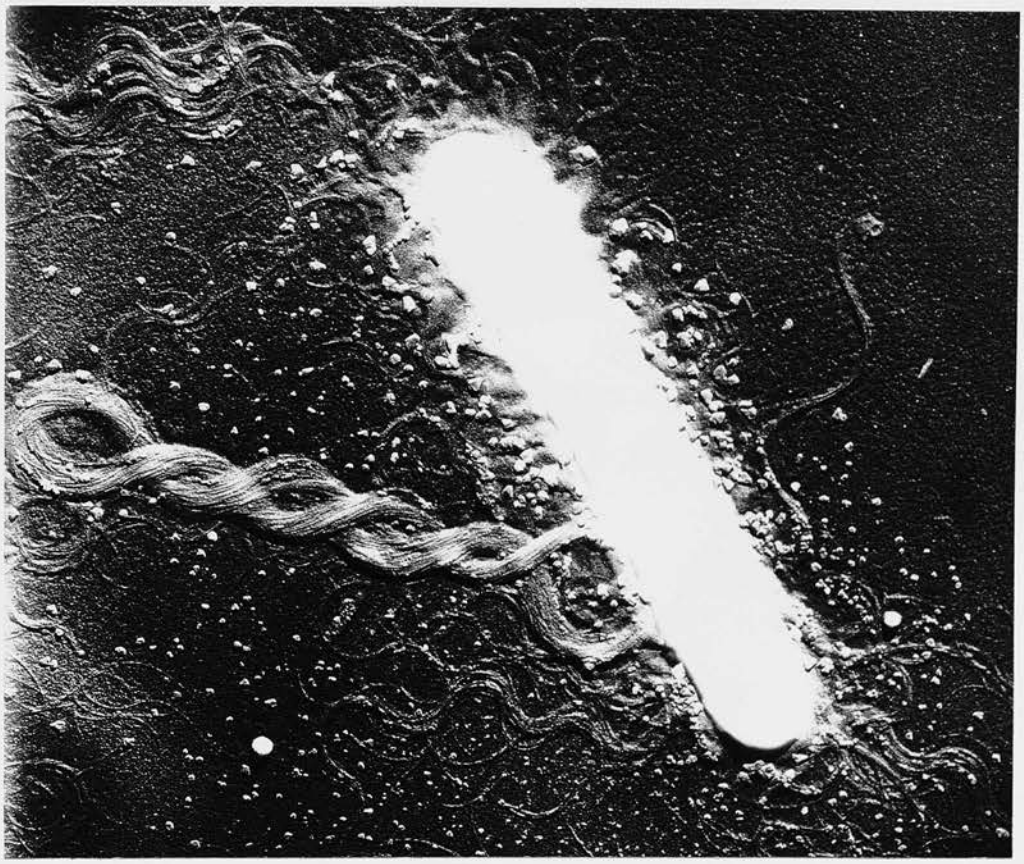


FIG. 9. - Cl. oedematiens strain GR2B. X 12,000.

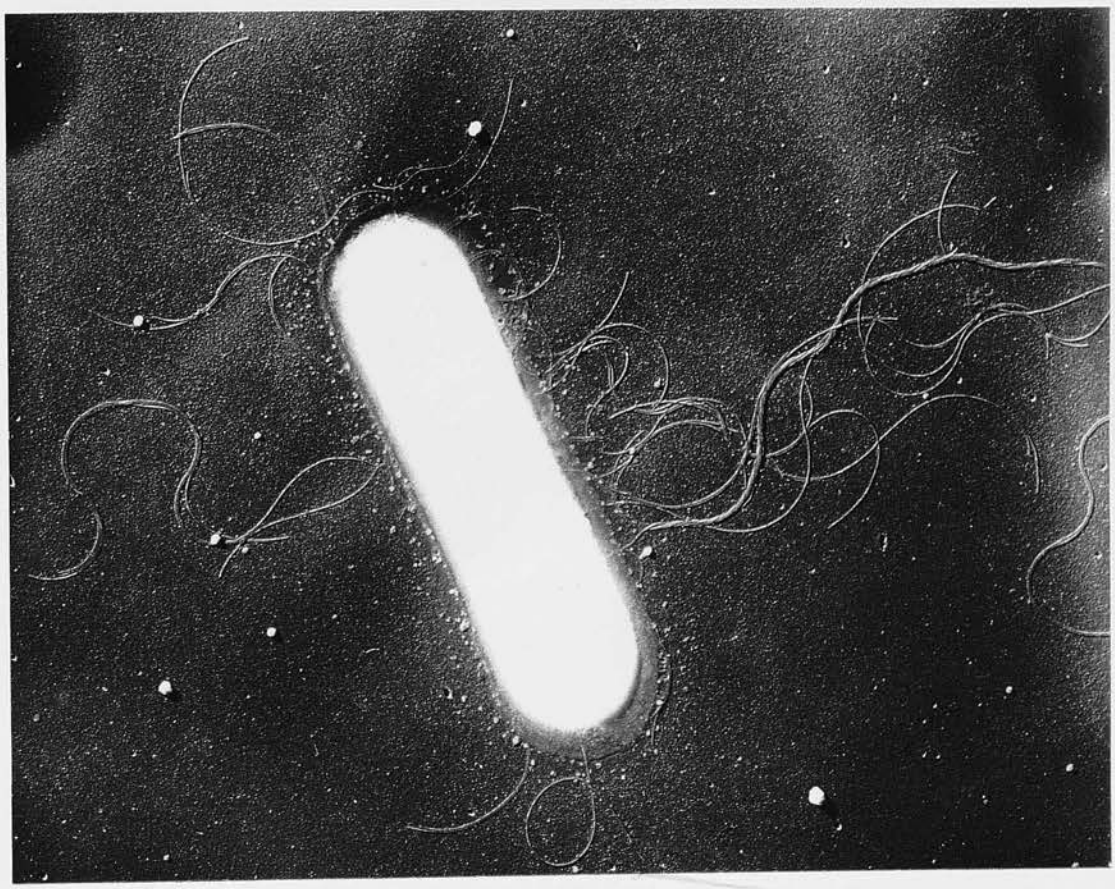


FIG. 10. - Cl. oedematiens strain R3/2. X 16,000.

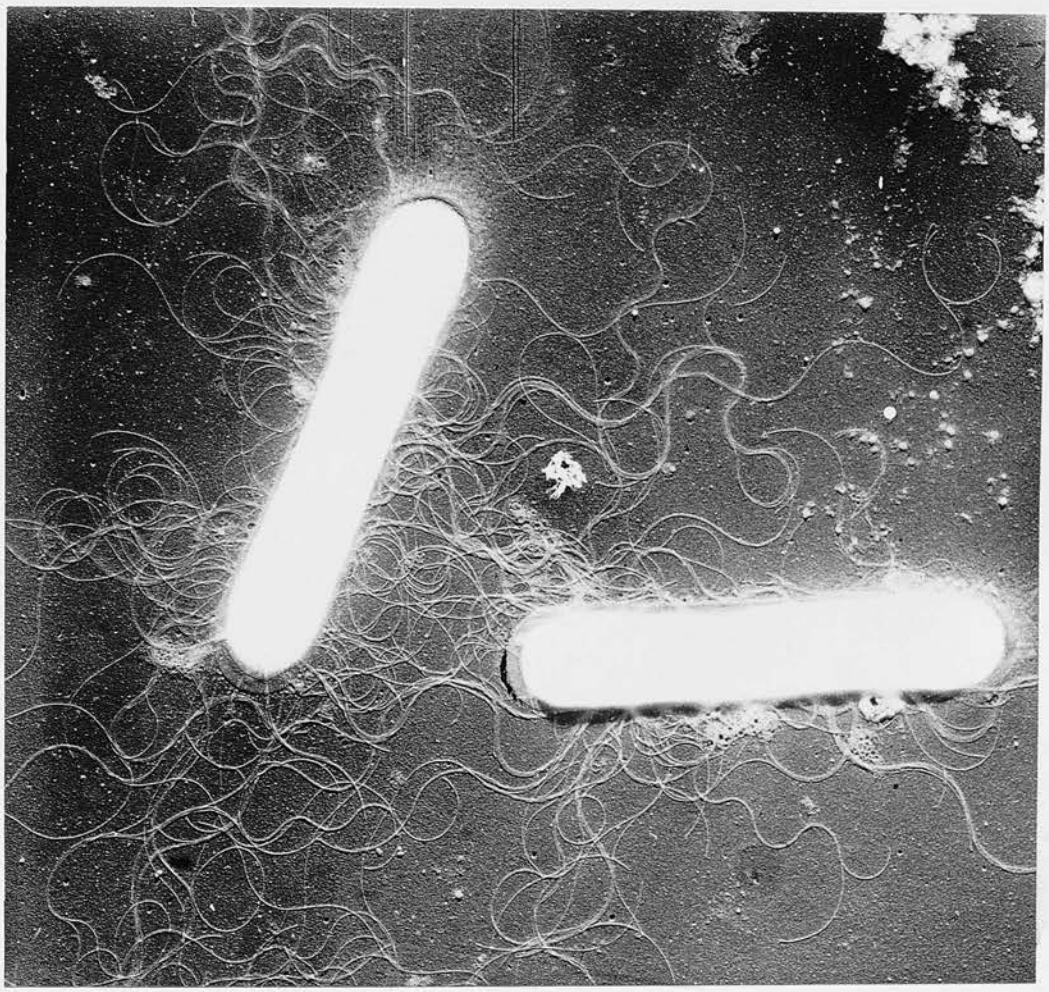


FIG. 11. - Cl. oedematiens strain R3/2. X 8000.

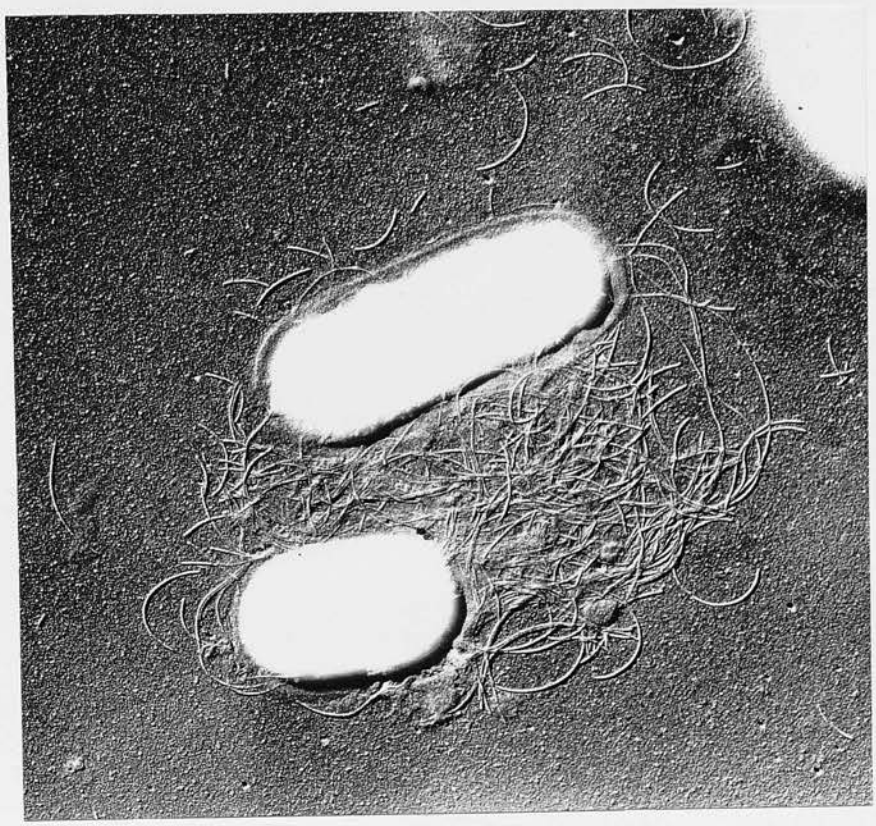


FIG. 12. - Cl. oedematiens strain GR1D. X 16,000.

completely satisfactory because strains of types B, C and D failed to grow on occasions; however, it was the best medium that was tested.

The formation of acid and gas from fermentable substrates is usually detected by incorporating a pH indicator and a Durham tube in the medium. In tests with anaerobic organisms the indicator may be reduced and decolourised by powerful reducing substances that are produced during bacterial growth. In addition, many anaerobic organisms form gas from peptone. During the present work, gas production was not regarded critically; the pH of cultures in Brewer medium was checked by spot tests on a white tile with bromothymol blue.

Preliminary experiments showed that 5 type A strains of Cl. oedematiens fermented glucose, maltose and glycerol after 24 hr incubation in Brewer medium; the strains grew better in the presence of these sugars. Lactose, sucrose, fructose and salicin were not fermented and these substrates did not stimulate growth. The observations were extended to include strains GR1A, GR2A, GR1B, GR2B, GR1C and GR1D and the following sugars:

a) Monosaccharides:

(i) Pentoses - XYLOSE,

(ii) Hexoses - GLUCOSE, FRUCTOSE, MANNOSE and GALACTOSE;

b) Disaccharides:

SUCROSE, MALTOSE and LACTOSE;

c) Polysaccharide:

DEXTRIN;

d) Sugar alcohols:

GLYCEROL, MANNITOL, DULCITOL, SORBITOL and INOSITOL;

e) Glycoside:

SALICIN.

The tests were incubated aerobically at 37° C and the results are summarised in table XI. The experiment was repeated at 30° C and observations with strain GR1C at 30° C are included in table XI because the series at 37° C became contaminated.

It was found that type A strains of Cl. oedematiens grow better than the other strains in the sugar-free media; all of the strains produced a slight fall in pH when grown in the control tubes. Table XI shows that:

(i) the substrate may not be fermented after 24 hr incubation; testing at 48 hr gives more reliable results but it may be necessary to incubate the tests for 7 days to detect late positive reactions;

(ii) acid was produced from glucose, maltose and glycerol by the type A strains; a large amount of gas was formed in the presence of mannose and dulcitol;

(iii) the type B strains produced acid from glucose and mannose; growth of these strains was improved in the presence of most of the carbohydrates;

(iv) the type C strain grown at 30° C produced acid from glucose and mannose; and

(v) the type D strain fermented glucose; this strain failed to

Table XI

The fermentation reactions of Cl. oedematiens in Brewer medium

Brewer medium enriched with 1 per cent. of	Effect on growth and fermentation reactions of test substrate with Cl. oedematiens of strain													
	GR1A			GR2A			GR1B		GR2B		GR1C		GR1D	
	*Growth	**Acid	Gas	Growth	Acid	Gas	Growth	Acid	Growth	Acid	Growth	Acid	Growth	Acid
...	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	⊥	-	⊥	-	-	-	-	-
Glucose	+	+(1)	+	+	+(1)	+	+	+(2)	+	+(2)	+	+(2)	+	+(2)
Fructose	+	-	-	+	-	-	+	⊥	+	-	+	-	-	-
Galactose	+	-	-	+	-	-	⊥	-	+	-	-	-	+	-
Mannose	+	-	+	+	-	+	+	+(7)	+	+(2)	+	+(7)	-	⊥
Maltose	+	+(7)	-	+	+(2)	-	+	-	+	⊥	-	-	+	-
Lactose	-	-	-	-	-	-	⊥	-	+	-	-	-	-	-
Sucrose	-	-	-	-	-	-	⊥	-	⊥	-	-	-	None	...
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	None	...
Glycerol	+	+(2)	-	+	+(1)	-	+	-	+	-	-	-	-	-
Dextrin	+	-	-	+	⊥	-	+	-	+	-	+	-	+	-
Mannitol	-	-	-	-	-	-	+	-	+	-	⊥	-	None	...
Dulcitol	+	-	+	+	-	+	-	-	-	-	+	-	+	-
Inositol	-	-	-	-	-	-	⊥	-	+	-	+	⊥	-	-
Sorbitol	-	-	-	-	-	-	+	-	+	-	+	-	-	-

*Growth: - = no stimulation; + = stimulation;

** Acid production: - = no acid; + = acid at (-) days;

⊥ = doubtful result.

grow in the presence of sucrose, salicin and mannitol.

Tests performed at 30° C gave similar results to those described above, except that strain GR1B fermented glucose, mannose, maltose and inositol, and strain GR1D fermented inositol in addition to glucose. Gas was produced at 30° C by type A strains in the presence of xylose, glucose, mannose, maltose and dextrin; more gas was formed at 30° C than at 37° C.

It was observed that cultures in Brewer medium became less turbid between 24 and 48 hr at 37° C; this was particularly evident with type A strains; slight clearing occurred at 30° C with all strains except GR1C.

Tests with fresh isolates. If sugar fermentation reactions are to prove useful in the provisional typing of Cl. oedematiens, clear-cut results should be obtained with freshly-isolated strains of the organism. Ten strains of Cl. oedematiens were isolated from sheep that died of suspected Black disease; the strains were identified by the immunofluorescent technique and were assumed to be type B strains (but see p. 200). The organisms were tested for the ability to ferment fructose, glycerol, maltose and mannose, as these sugars may be significant in the type-differentiation of Cl. oedematiens. The strains did not grow well after 3 days' incubation in the test medium, and 14 of 40 cultures failed to grow. Glycerol and maltose were not fermented and the results with fructose and mannose were variable. The results showed that the test procedure in Brewer medium is of

little value when dealing with freshly-isolated strains of this demanding organism.

Fermentation tests in modified cooked-meat broth medium. It seemed reasonable to assume that the indifferent growth of Cl. oedematiens in the basal medium was contributing to the variable results in fermentation reactions. It has already been shown that the organism grew most reliably in CMB, and I decided to use CMB as the basal medium for fermentation tests. To compare the influence of inherent fermentable substances in the medium, an uninoculated control and a substrate-free control were included in each test. Strains were grown in 100-ml. amounts of CMB with and without added substrate. A sample was withdrawn aseptically from each culture and the opacity was measured by comparison with Brown's opacity test tubes. The bacterial cells were removed by centrifugation and the pH of the clear supernate was measured with a Pye Dynacap pH meter that had an automatic temperature compensator and a Pye Ingold combined glass and reference electrode. The results of tests with one strain of each type and selected substrates are recorded in table XII. It should be noted that the pH measurements in these experiments were combined with readings of biological activity in the culture supernates. Some of the results in table XII are the same as those given in tables on pp. 128-9 and separately recorded here for convenience.

The results of these experiments showed that the pH of uninoculated CMB may fall by 0.4 unit during anaerobic incubation; the addition

Table XII

The fermentation reactions of Cl. oedematiens with CMB as basal medium

Strain of Cl. oedematiens	CMB medium plus 1 per cent. of	Initial pH of medium	Readings of opacity* and pH at day							
			1		2		3		5	
			Opacity	pH	Opacity	pH	Opacity	pH	Opacity	pH
GR1A	...	6.7	2	6.3	2	6.3	2	6.2	-	-
	Glucose		>6	5.0	>6	4.9	>6	4.9	-	-
	Maltose		>6	5.4	>6	4.9	>6	4.9	-	-
	Fructose		1	6.3	1	6.2	1	6.2	-	-
	Glycerol		5	5.2	>6	5.2	>6	5.2	-	-
	Lactose		1	6.1	2	6.2	-	-	1	6.1
	Sucrose		2	6.2	2/3	6.1	-	-	2	6.1
GR1B	...	7.1	2	6.5	2	6.1	-	-	2	6.2
	Glucose		2	6.5	2	5.9	-	-	3	5.0
	Maltose		3/4	6.0	4	5.5	-	-	4	5.1
	Fructose		2/3	6.2	3	6.2	-	-	3	6.1
	Glycerol		2	6.5	3	6.0	-	-	2	6.3
	Mannose		3	6.1	5	5.3	-	-	5	5.1
	Inositol		2/3	6.0	5	5.2	-	-	4	5.1
	...	6.9	3	6.2	3	6.4	-	-	3	6.5
	Lactose		2/3	6.2	3	6.4	-	-	3	6.4
	Sucrose		2/3	6.2	3	6.4	-	-	3	6.4

Table XII (continued)

GR1C	...	6.8	3	6.3	-	-	4	6.4	-	-
	Glucose		4	5.5	-	-	5	5.4	-	-
	Maltose		3	6.2	-	-	3	6.3	-	-
	Fructose		4	6.3	-	-	4	6.5	-	-
	Glycerol		3	6.2	-	-	3	6.3	-	-
	Mannose		3/4	6.1	-	-	4	6.1	-	-
	Inositol		4	5.9	-	-	4/5	5.5	-	-
	Lactose	6.9	2	6.3	2/3	6.4	-	-	2	6.4
	Sucrose		2	6.2	2/3	6.5	-	-	-	-
GR1D	...	7.1	3	6.2	2	6.4	-	-	2/3	6.5
	Glucose		1/2	6.4	2	6.4	-	-	2/3	4.8
	Maltose		3	6.3	2	6.1	-	-	3/4	6.2
	Fructose		3/4	6.0	5	5.4	-	-	5	5.1
	Glycerol		1	6.6	2	6.2	-	-	1/2	6.4
	Mannose		3/4	6.0	5	5.0	-	-	5	4.9
	Inositol		3	5.7	4	5.9	-	-	4	5.9
	...	6.9	3	5.9	3/4	6.0	-	-	3/4	6.0
	Lactose		3	5.9	3/4	6.0	-	-	3/4	6.0
	Sucrose		2	5.9	3	6.0	-	-	2/3	6.0

- = not recorded;

*degrees of opacity recorded by naked-eye comparison with Brown's opacity tubes; 1 - 6 indicate increasing degrees of opacity.

of carbohydrates does not affect the pH of the medium. The pH of CMB medium inoculated with Cl. oedematiens falls by 0.5 - 1.0 unit depending on the strain. If the added substrate is fermented by the organism, the pH usually falls during incubation by a further 1.0 unit, although it may be necessary to prolong incubation for up to 5 days in order to detect this. Stimulation of growth is usually associated with fermentation of the substrate.

In order to assess the CMB fermentation test as a practical routine procedure, 6 of the 10 fresh isolates were screened with the CMB tests described above. The results after 7 days' incubation are given in table XIII. All of the strains fermented glucose, maltose, mannose and inositol; 3 of 6 strains fermented fructose. Tests with 3 strains in the presence of fructose and 5 of 6 strains with glycerol gave results that are difficult to interpret.

The tests that gave the doubtful reactions in tables XII and XIII were repeated. It was found that strain GR1C fermented mannose after 5 days' incubation, and strains GR1D and GR2D fermented inositol after 7 days. Tests with 14-ml. amounts of CMB medium in tubes gave similar results to those recorded above. Strain R3/3 fermented fructose in the tube tests, but the pH values in tests of this strain with glycerol and in tests of strain R4/1 with fructose and glycerol were 5.8 - 6.0.

The results of fermentation tests in Brewer medium and in CMB confirm that Cl. oedematiens grows consistently in CMB. The production of acid from fermentable substrates is usually clear-cut in CMB

Table XIII

The fermentation reactions of freshly-isolated strains of Cl. oedematiens

Strain of Cl. oedematiens	Readings of opacity* and pH after 7 days' incubation of Cl. oedematiens in CMB medium, pH 6.9, plus 1 per cent. of															
	...		Glucose		Maltose		Fructose		Galactose		Mannose		Inositol		Glycerol	
	Opacity	pH	Opacity	pH	Opacity	pH	Opacity	pH	Opacity	pH	Opacity	pH	Opacity	pH	Opacity	pH
R1	2/3	6.5	5/6	5.0	4	5.3	3/4	5.4	1/2	6.5	3/4	5.4	3	5.5	2	6.3
R3/1	2	6.5	2/3	5.0	5	4.9	2	6.1	1/2	6.4	3/4	5.1	3/4	5.4	1/2	5.9
R3/2	2	6.3	4/5	5.0	4	5.1	4	5.9	1/2	6.4	3/4	5.1	3	5.4	2	5.9
R3/3	2	6.3	2	5.3	5	5.1	4/5	5.5	1	6.2	6	5.1	2/3	5.3	2	5.8
R3/4	3	6.6	4	5.1	6	5.0	5/6	5.5	2/3	6.4	6	5.1	5	5.2	3	5.9
R4/1	2	6.5	3	5.2	4	5.0	5/6	5.7	1	6.5	5/6	5.0	3	5.2	2/3	5.8

*degrees of opacity recorded by naked-eye comparison with Brown's opacity tubes;

1 - 6 indicate increasing degrees of opacity.

but variable and doubtful results may occur. It does not appear that the delayed or doubtful results are associated with inadequate growth of the organism in the basal medium. The results of tests with a limited number of strains of Cl. oedematiens in the present study are compared with the recognised fermentation reactions of Cl. oedematiens (Breed, Murray and Smith, 1957) in table XIV.

Other biochemical reactions of Cl. oedematiens

(i) Gelatinase activity. It is generally regarded that all strains of Cl. oedematiens liquefy gelatin. In the present work, only type A strains of Cl. oedematiens grew in glucose-gelatin medium and gelatin was liquefied after overnight incubation. Gelatinase production by strains of types B and D of the organism was demonstrated in a medium consisting of sheep liver infusion broth plus 10 per cent. of gelatin. After overnight incubation, good growth of these strains was obtained and the gelatin was liquefied. Gelatinase production by the type C strain has not been confirmed in the present study because this strain failed to grow in the basal medium with added gelatin. These observations show that if a suitable basal medium is used, strains of types A, B and D of Cl. oedematiens liquefy gelatin.

(ii) Indole formation. Variable results were obtained in tests

Table XIV

The fermentation reactions of Cl. oedematiens

SUGAR	Results obtained in fermentation reactions with Cl. oedematiens of type							
	A		B		C		D	
	Bergey	Rutter	Bergey	Rutter	Bergey	Rutter	Bergey	Rutter
Glucose	+	+	+	+	NR	+	+	+
Maltose	v	+	v	+	NR	-	-	-
Lactose	-	-	-	-	NR	-	-	-
Sucrose	-	-	-	-	NR	-	-	-
Xylose	NR	-	NR	-	NR	-	-	-
Fructose	+	-	+	v	NR	-	+	+
Galactose	NR	-	+	-	NR	-	-	-
Mannose	NR	-	NR	+	NR	+	NR	+
Salicin	-	-	-	-	NR	-	-	-
Glycerol	v	+	v	±	NR	-	+	-
Dextrin	NR	-	NR	-	NR	-	NR	-
Mannitol	-	-	-	-	NR	-	-	-
Dulcitol	NR	-	-	-	NR	-	-	-
Inositol	NR	-	NR	+	NR	+	NR	+
Sorbitol	NR	-	NR	-	NR	-	NR	-
Raffinose	NR	NR	NR	NR	NR	NR	-	NR
Arabinose	NR	NR	NR	NR	NR	NR	-	NR
Inulin	NR	NR	-	NR	NR	NR	-	NR
Rhamnose	NR	NR	-	NR	NR	NR	NR	NR

+ = positive result; - = negative result;

± = doubtful result; v = variable result;

NR = not recorded;

Bergey = Breed, Murray and Smith (1957).

for indole production during the present work. Cl. oedematiens does not grow in peptone water medium and Brewer medium was usually used as the basal medium. Indole was not detected after 48 hr incubation at 37° C, but 2 of 5 type A strains gave strong positive reactions after 5 days' incubation; the colour faded rapidly on standing and disappeared after 5 min. A type D strain and 1 of 2 type B strains gave a positive indole reaction after 8 days' incubation at 30° C. Strain GR1C produced indole after 24 hr incubation in media with added meat particles; traces of indole were detectable in CMB cultures of this strain, and the reaction was very strong in cultures of HDB plus meat particles. Tests of the uninoculated control media gave negative results, and cultures of strain GR1C in nutrient broth enriched with 5 per cent. of peptone were also negative. On the basis of these observations it appears that strains of all types of Cl. oedematiens may produce indole under suitable conditions.

(iii) Hydrogen sulphide production. Hydrogen sulphide formation was detected by retaining lead acetate-impregnated paper strips above CMB cultures during incubation. Hydrogen sulphide production was demonstrable with strains of types A, B and C by this method, but some strains gave negative results in the test.

The soluble antigens of Cl. oedematiens

Oakley and his colleagues (1947, 1959) showed that at least seven soluble antigens exist in culture filtrates of Cl. oedematiens. On the basis of their results, these workers identified four types of the organism, and they implied that the LV reaction attributable to the gamma or beta antigens affords a sufficient basis for the provisional typing of unknown strains. In their critical work, Oakley et al. used concentrated culture filtrates of Cl. oedematiens prepared from 1 - 4-litre amounts of a digest medium; for routine typing they cultured the organism in Brewer medium and on egg-yolk agar plates.

The LV reaction of Cl. oedematiens

In my preliminary experiments with type A strains grown in Brewer medium, the LV test was unsatisfactory; several strains gave a negative reaction and a medium that contains 0.1 per cent. of agar presents practical difficulties in the test. It should be borne in mind that type B, C and D strains of the organism sometimes fail to grow in Brewer medium. CMB is the medium of choice for the routine culture of Cl. oedematiens and it was necessary to evaluate the method of typing described by Oakley et al. in relation to this medium.

Overnight CMB cultures of type A strains of Cl. oedematiens consistently give a positive LV reaction; the reaction is readily

neutralised by an appropriate volume of homologous antiserum that contains anti-gamma activity.

CMB cultures of type B strains of Cl. oedematiens often give a negative LV reaction; some apparently negative reactions may become positive if the incubation period of the test is increased from 1 to 4 hr. The LV reaction of type B cultures is readily neutralised by Cl. oedematiens antisera in relation to their anti-beta content.

Type D strains of Cl. oedematiens usually produce a potent LV factor in CMB cultures; the reaction is neutralised by antisera that contain a sufficiently high level of anti-beta activity.

CMB cultures of the type C strain always gave a negative LV reaction in the present work.

Factors that affect the LV reaction

Initial experiments indicated that the LV activity of concentrated cultures of type A and D strains was detectable in tests performed over a pH range of 2.6 to 9.4, with an optimum pH for activity at 4.6 and 5.6 respectively. A curd was often produced on the surface of the test mixtures, and the depth of curd was maximal between pH 3.6 and 7.6.

Keppie (1944) noted that type B strains of Cl. oedematiens often produce a negative LV reaction; he suggested that glucose should be present in the culture medium if these strains are to produce a detectable LV factor. In the present work, results obtained with

the addition of 1 per cent. of glucose to CMB did not confirm Keppie's observation. Tests with CMB/G culture supernates of type A strains, however, showed that an LV factor is produced in this medium that is not neutralised by the same amount of homologous antiserum that neutralises the LV reaction of the same strains when cultured in CMB. The LV factor in CMB/G culture is of low potency, and it was considered that it might represent the epsilon antigen; it should be noted however, that the reaction occurs at 37° C, and Oakley et al. (1947) stated that the epsilon effect with LV solution usually occurs after cooling the reaction mixture at 4° C.

The above results suggested the LV test with strains of Cl. oedematiens cultured in CMB or CMB/G is not entirely satisfactory for the rapid, routine typing of the organism and it was necessary to investigate the other reactions of CMB culture filtrates. Hayward and Gray (1946) described a haemolysin (HL) neutralisation test with human cells as the substrate for the identification of type A strains of Cl. oedematiens; they noted that CMB cultures of the organism contained a haemolytic factor that was not neutralised in the test and they concluded that CMB medium was not suitable for the test system. Oakley et al. (1947) stated that LV tests with the soluble products of Cl. oedematiens are much more sensitive than HL tests with horse cells as the substrate. Clearly, more information is necessary regarding the haemolytic activity of cultures of Cl. oedematiens.

The haemolytic activity of Cl. oedematiens

Preliminary experiments indicated that human and horse erythrocytes do not lyse when held in isotonic solution within the pH range 5.6 - 8.6, and that the cells are more stable in cagsal diluent than in saline. A concentrated type A culture of the organism showed maximum HL activity in tests performed at pH 5.6 to 6.8; a concentrated type D culture showed a similar pH optimum with human cells, and there was a marked hot-cold effect at pH 8.6.

Three species of red cell were employed as the substrate in titrations of haemolytic activity with concentrated culture supernates of Cl. oedematiens; the results of these tests are given in table XV

Table XV indicates that human cells are considerably more sensitive than horse or sheep cells to lysis by the soluble products of all types of Cl. oedematiens. Horse cells are slightly more sensitive than sheep cells to type A cultures, but the reverse holds for type B and D cultures. Sheep cells are most susceptible to 'hot-cold' lysis, and type A cultures produce less of a 'hot-cold' effect than type B and D cultures. HL tests with horse and sheep cells may not give clear-cut results after 1 hr at 37° C; red cells of these species do not settle as rapidly as human cells and there is a marked 'hot-cold' effect with cultures of type B and D strains.

The results of the previous experiments suggest that human cells are a satisfactory substrate for the haemolysins of all types of Cl. oedematiens. In several tests there was evidence of haemagglutina-

Table XV

The HL titres of different culture concentrates of Cl. oedematiens with three species of red cell

Concentrated culture fraction from Cl. oedematiens of strain	Haemolytic activity demonstrable against different red cells with fraction in dilution up to 1 in *		
	Human cells	Horse cells	Sheep cells
GR1A	320 : 640	160 : 160	10 : 80
GR2A	80 : 160	10 : 20	< 10 : 10
GR1B	320 : 1280	< 10 : 40	20 : 160
GR1D	1280 : 10,240	20 : 2560	10 : 5120

*The first reading was taken immediately after the incubation period of 1 hr at 37° C; the second reading refers to observations made after overnight cooling at 4° C.

tion of human cells by cultures of type B and D strains; the haemagglutination appeared to be neutralised by corresponding antisera. The significance of this observation was not further investigated.

Applications to routine typing

HL neutralisation tests with the soluble products of cultures grown in CMB and tested against human cells gave encouraging results. A comparative experiment was performed with 19 strains of Cl. oedematiens and the results are given in table XVI. With this test system, the HL neutralisation test provides a more sensitive and consistent method of typing Cl. oedematiens than the LV test, although type A strains of the organism produce a haemolytic factor in CMB that is not neutralised after cooling the test mixtures. On the basis of these results, strain GR5B is a type A strain, and strain GR9B may be a type D strain. The results obtained with strain GR1D suggest that the HL test is a more sensitive indication of the beta antigen than the LV reaction, and it is clearly unwise to differentiate strains of types B and D solely on the relative amounts of the beta antigen that can be detected.

HL and LV neutralisation tests were then used for the provisional typing of 10 freshly-isolated strains of Cl. oedematiens. All of the HL tests and 6 of 10 LV tests performed on overnight CMB cultures of these strains gave positive results; the reactions were neutralised

Table XVI

Results of HL and LV neutralisation tests with culture products of different types of Cl. oedematiens tested against experimental type-specific antisera

Strain of Cl. oedematiens	Results of HL and LV tests in the presence of a test dose of culture and given doses of the following antisera																			
	... (positive control)				Cl. oedematiens anti-D (109 anti-beta units)				Cl. oedematiens anti-B (10 anti-beta units)				Cl. oedematiens anti-A (20 anti-gamma units)				Cl. welchii anti-A (about 2 Cl. oedematiens anti-beta units)			
	HL		LV		HL		LV		HL		LV		HL		LV		HL		LV	
	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C
1A	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	⊥
2A	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	-
3A	+	+	-	+	+	+	-	⊥	+	+	-	⊥	-	+	-	-	+	+	-	-
4A	+	+	-	+	+	+	-	⊥	+	+	-	⊥	-	+	-	-	+	+	-	-
1B	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	⊥	+	-	-
2B	+	+	-	⊥	-	-	-	-	-	-	-	-	+	+	-	⊥	⊥	+	-	⊥
3B	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	⊥	+	⊥	⊥
4B	+	+	-	⊥	-	-	-	-	-	-	-	⊥	+	+	-	+	⊥	+	-	⊥
5B	+	+	+	+	+	+	+	+	+	+	+	+	-	⊥	-	-	+	+	-	⊥
7B	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-
8B	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-
9B	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
10B	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-
11B	+	+	⊥	+	-	-	-	-	-	-	-	-	+	+	⊥	⊥	⊥	+	-	-
12B	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	⊥	+	-	-
13B	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-
1C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1D	+	+	+	+	-	-	-	-	⊥	+	-	-	+	+	+	+	+	+	-	-
2D	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+

+ = positive result;
⊥ = doubtful result;
- = negative result.

by antisera that contained anti-beta activity. These results confirm that the HL test gives consistently positive results in the test system developed in the present work; with a similar test dose of material derived from type B strains, the LV test frequently gives negative results.

HL and LV factors of cultures grown in different media

At this point in the investigation it seemed that neither the HL test nor the LV test is entirely satisfactory for the provisional typing of CMB cultures of Cl. oedematiens; the haemolytic activities of type A cultures may not be neutralised in the HL test with homologous antiserum, and type B cultures of the organism often fail to produce a positive LV reaction. The following experiments were performed to investigate the HL and LV factors produced by Cl. oedematiens in various media and the neutralisation of these effects with appropriate antisera. Centrifuged supernates were prepared from cultures of Cl. oedematiens grown in 100-ml. amounts of different media for periods of 1 - 5 days. Titrations of HL and LV activities were performed on the supernates together with pH readings and estimates of growth; the results of these tests are given in tables XVII and XVIII.

Results with a type A strain. In general, the titres obtained in HL and LV tests with nutrient broth cultures of type A strains of

Table XVII

The HL and LV titres of a type A strain of Cl. oedematiens in different media

*Medium	Strain of Cl. oedematiens	DAY 1				DAY 2				DAY 3			
		Degree of growth (opacity)*	pH	Cold+ titre HL	Cold+ titre LV	Degree of growth (opacity)	pH	Cold+ titre HL	Cold+ titre LV	Degree of growth (opacity)*	pH	Cold+ titre HL	Cold+ titre LV
NB pH 7.5	GR1A	< 1	7.4	<2	<2	2	6.7	16	8	1	6.7	16	8
NB plus 1% of:													
glucose		4	5.1	4	4	>6	5.0	2	4	>6	5.0	2	4
Maltose		< 1	7.3	<2	<2	< 1	7.3	<2	<2	< 1	7.2	<2	<2
fructose		1	6.4	8	4	1	6.5	8	4	1	6.6	8	8
glycerol		< 1	6.7	<2	<2	>6	5.0	4	4	>6	5.0	2	4
HDB pH 6.8		2	6.1	128	16	3	6.1	32	8	4	6.3	16	8
HDB plus 1% of:													
glucose		4	5.1	8	4	>6	5.1	4	4	>6	5.1	4	4
maltose		3	5.4	64	16	> 6	5.2	8	4	> 6	5.2	8	4
fructose		2	6.0	128	16	2	6.1	32	8	2	6.1	16	4
glycerol		4	5.3	8	4	> 6	5.3	4	4	> 6	5.3	2	2
CMB pH 6.7		2	6.3	32	8	2	6.3	16	8	2	6.2	8	8
CMB plus 1% of:													
glucose		>6	5.0	4	4	> 6	4.9	4	2	> 6	4.9	4	<2
maltose		>6	5.4	32	16	> 6	4.9	4	4	> 6	4.9	4	2
fructose		1	6.3	32	8	1	6.2	8	8	1	6.2	8	4
glycerol		5	5.2	8	4	> 6	5.2	4	4	> 6	5.2	2	2
lactose		1	6.1	32	4	2	6.2	16	4	1	6.1	8	4
sucrose		2	6.2	64	4	2/3	6.1	16	4	2	6.1	8	4

*NB = nutrient broth; HDB = horse digest broth with added meat particles; CMB = cooked-meat broth.

+ = readings after overnight cooling at 4° C;

*1 - 6 represent increasing amounts of growth as judged by visual comparison with Brown's opacity test tubes.

Table XVIII

The HL and LV titres of a type B strain and a type D strain of Cl. oedematiens in different media

Medium[x]	Strain of Cl. oedematiens	DAY 1				DAY 2				DAY 5			
		Degree of growth (opacity)*	pH	Cold titre* HL	Cold titre* LV	Degree of growth (opacity)*	pH	Cold titre HL	Cold titre LV	Degree of growth (opacity)*	pH	Cold titre HL	Cold titre LV
NB at pH 7.6	GR1B	< 1	7.0	< 2	< 2	< 1	6.9	< 2	< 2	1	6.6	2	< 2
IB at pH 7.3		3	6.3	16	< 2	1	6.4	4	< 2	1	6.5	2	< 2
HDB at pH 7.5		3	6.4	32	< 2	1	6.6	< 2	< 2	1	6.7	< 2	< 2
CMB at pH 7.1		3	6.2	4	< 2	2	6.4	4	< 2	2/3	6.5	2	< 2
CMB plus 1% of:													
glucose		2	6.5	4	< 2	2	5.9	8	< 2	3	5.0	4	< 2
maltose		3/4	6.0	8	< 2	4	5.5	4	< 2	4	5.1	2	< 2
mannose		3	6.1	32	< 2	5	5.3	4	< 2	5	5.1	2	< 2
fructose		2/3	6.2	4	< 2	3	6.2	4	< 2	3	6.1	2	< 2
inositol		2/3	6.0	8	< 2	5	5.2	4	< 2	4	5.1	4	< 2
glycerol		2	6.5	4	< 2	3	6.0	8	< 2	2	6.3	< 2	< 2
lactose		2/3	6.2	8	< 2	3	6.4	< 2	< 2	3	6.4	< 2	< 2
sucrose		2/3	6.2	4	< 2	3	6.4	< 2	< 2	3	6.4	< 2	< 2
NB at pH 7.6	GR1D	< 1	7.1	2	< 2	< 1	7.2	4	< 2	< 1	7.2	2	< 2
IB at pH 7.3		< 1	6.8	2	< 2	< 1	6.9	4	< 2	2	6.1	256	16
HDB at pH 7.5		2/3	6.8	> 64	32	2	6.4	512	64	2/3	6.5	32	2
CMB at pH 7.1		2	6.5	16	4	2	6.1	128	8	2	6.2	32	4
CMB plus 1% of:													
glucose		1/2	6.4	16	4	2	5.4	32	8	2/3	4.8	4	< 2
maltose		3	6.3	16	8	2	6.1	128	16	3/4	6.2	64	4
mannose		3/4	6.0	16	8	5	5.0	16	2	5	4.9	4	< 2
fructose		3/4	6.0	16	8	5	5.4	64	8	5	5.1	2	< 2
inositol		3	5.7	16	8	4	5.9	4	< 2	4	5.9	4	< 2
glycerol		1	6.6	8	2	2	6.2	128	8	1/2	6.4	32	4
lactose		3	5.9	512	32	3/4	6.0	128	16	3/4	6.0	128	8
sucrose		2	5.9	256	32	3	6.0	128	16	2/3	6.0	64	4

[x] NB = nutrient broth; IB = infusion broth; HDB = horse digest broth; CMB = cooked-meat broth;

* = readings after overnight cooling at 4° C;

*1 - 6 represents increasing amounts of growth as judged by visual comparison with Brown's opacity test tubes.

Cl. oedematiens are lower than the titres obtained with cultures grown in the presence of added meat particles. The yield of HL activity in cultures grown in nutrient broth is a little higher than the yield of LV activity, and the activities remain fairly constant during 3 days' incubation. In cultures with added meat, the HL activity is more potent than the LV activity but falls off more rapidly; it should be borne in mind that low yields of factors giving the HL and LV reactions may be obtained with cultures grown in some batches of CMB. The absence of carbon dioxide from the anaerobic environment does not affect the yields of these soluble products.

In the presence of certain fermentable substrates such as glucose and glycerol the yields are low and remain so, but the HL and LV activities of the 48 hr culture supernates grown in the presence of these substrates are not neutralised by a volume of antiserum that neutralises the same activities in sugar-free culture supernates. In cultures with added maltose, which is also fermented by type A strains, the initial HL activity falls off rapidly; the activity present on day 1 was not neutralised by a type A antiserum.

Results with a type B strain. Strain GR1B produced a low level of HL activity, but failed to produce detectable LV activity in any of the media that were tested; the HL activity was readily neutralised by a homologous antiserum. The low level of HL activity and the apparent absence of LV activity was not parallelled by the lethal activity of the CMB culture; on the contrary, there was potent lethal activity (q.v.).

Results with a type C strain. CMB culture supernates of strain GR1C have no recognised biological activity. However, strong HL activity in the absence of LV activity was detected in overnight cultures of this strain grown in NB and in nutrient broth plus 5 per cent. of peptone. The HL reaction showed no hot-cold effect; its potency diminished rapidly after storage of the supernate at 4° C and after prolonged incubation of the culture; the reaction was neutralised by a volume of experimental type A antiserum that contained 50 anti-gamma units. The titre obtained in the HL test was unaltered after treatment with a solution containing 2 vol. hydrogen peroxide, therefore the HL activity does not appear to be truly oxygen-labile. Further studies of this haemolysin were frustrated because the type C strain usually fails to grow in the absence of meat particles.

Results with a type D strain. Cultures of strain GR1D contain potent HL and LV activities that may not develop until adequate growth occurs in the medium. HL activity is usually considerably lower after culture in the presence of an added fermentable substrate. The HL and LV activities of sugar-free cultures and cultures with added glucose and inositol were neutralised by Cl. oedematiens type B and type D experimental antisera.

Conclusions. The results of this series of experiments suggest that in the presence of meat particles the HL activity of a type A strain of Cl. oedematiens is more labile than the LV activity; both

activities are equally labile with a type D strain of the organism. The HL and LV activities of Cl. oedematiens are diminished when culture is performed in the presence of added fermentable substrates, and in such media the activities of type A strains may not be neutralised by homologous antisera. There is some evidence that the HL and LV factors may be reduced in potency as the cultures become more acid.

During these experiments it was also observed that an opalescence develops after cooling in LV tests with supernates from cultures of strain GR1C grown in CMB plus glucose and inositol. Subsequent work with cultures grown in sheep-liver infusion broth showed that the production and neutralisation of HL and LV factors by all types of the organism may be rather variable; the type C strain produced strong haemolytic activity without LV activity in sheep-liver infusion broth, and the HL reactions of type B and D strains were not neutralised by the homologous experimental antisera.

The above results indicate that the production of HL and LV factors in cultures of Cl. oedematiens varies markedly depending on the culture medium that is used. These observations may help to explain the occasional inconsistencies in behaviour of homologous antisera in neutralisation tests (see Discussion). The system of provisional typing suggested by Oakley et al. (1947) is successful with CMB cultures of the organism; but, in order to avoid equivocal results with cultures of type B strains it is preferable to include in addition to the LV test system, a HL neutralisation test with human cells as the substrate. It is clear that the addition of fermentable carbohydrate

to cultures destined for analysis of their soluble products may carry disadvantages in relation to the production of haemolytic and LV factors that are not neutralised by homologous antisera. The addition of glucose to CMB is not essential for the growth of Cl. oedematiens, and it may be better to avoid this addition in routine work.

The HL reaction of type A cultures of Cl. oedematiens

If the HL neutralisation test is to be recommended as a standard procedure with CMB cultures of Cl. oedematiens, we require more knowledge regarding the HL factors that are produced in this medium by type A strains of the organism. The HL and LV factors that these strains produce in NB are readily neutralised by 10 anti-gamma units of a type A experimental antiserum. The LV reaction of the same strains in CMB is completely neutralised by the same amount of antitoxin, and the HL activity of a 5-fold dilution of the HL test dose is completely neutralised by 10 anti-gamma units. If 0.5 ml. of undiluted CMB culture is used as the test dose in HL neutralisation tests, the HL activity is not neutralised by 250 anti-gamma units of antitoxin; lysis in these tests occurs after 10 min. incubation at 37° C, and may be delayed for up to 40 min. in the presence of antiserum. If the test dose of the culture is diluted 1 in 2, the HL reaction may be partially neutralised by 20 or 50 anti-gamma units after 1 hr at 37° C, but lysis occurs after cooling at 4° C. Thus, it seems

that the haemolytic activity of the test dose is largely attributable to a neutralisable (gamma) fraction, but a weak haemolytic component exists that is apparently not neutralised by the experimental antiserum; the effect of this latter component can be readily diluted out. Horse and sheep cells gave similar results to human cells in the HL neutralisation tests.

Further work suggested that the haemolytic activity of CMB cultures can be partially neutralised after prolonged incubation or after centrifugation of the cultures. Accordingly, 100-ml. amounts of identical media with or without added meat particles were inoculated with a type A strain of Cl. oedematiens. Samples of each culture were removed at intervals and the culture or supernate obtained after centrifugation was titrated for haemolytic and LV activity; neutralisation tests were performed with 10 anti-gamma units of experimental antiserum and the results are shown in table XIX. It appears that a haemolytic factor is formed in the presence of meat particles that cannot be neutralised by the amount of antitoxin employed in the tests; nutrient broth cultures that are more potent than the CMB cultures in terms of haemolytic activity are readily neutralised in the test system. Centrifugation of the cultures may reduce the titres slightly, and it may allow complete or partial neutralisation of the haemolytic effect; there is no evidence that the haemolytic activity of older cultures can be neutralised more easily than that of overnight cultures.

An obvious conclusion at this stage was that the 'non-neutralisable'

Table XIX

The effect of culture in the presence of meat particles on the yields of HL and LV activities of a type A strain of Cl. oedematiens in different media, and the results of neutralisation tests with a test dose of 0.5 ml. of culture or supernate diluted 1 in 2 and 10 anti-gamma units of a Cl. oedematiens type A antiserum

Duration of incubation of the culture in hr	Test system (HL or LV)	Culture (C) or Supernate (S)	Tests performed with the soluble products of a culture grown in									
			NB*		CMB* (NB plus meat)		HDB*		HDB* plus meat		IB*	
			Titre	Neutralisation	Titre	Neutralisation	Titre	Neutralisation	Titre	Neutralisation	Titre	Neutralisation
24	HL	C	< 2	...	8:32	- -	<2:4	+ +	32:64	- -	2:4	+ +
		S	< 2	...	8:32	- -	<2:2	+ +	32:128	- -	2:8	+ +
	LV	C	< 2	...	2:4	+ +	/	...	/	...	<2:2	+ +
		S	< 2	...	4:4	+ +	/	...	/	...	<2:2	+ +
72	HL	C	32:64	+ +	8:32	- -	8:16	+ +	16:64	- -	2:8	+ +
		S	16:64	+ +	4:16	+ -	4:16	+ +	8:32	+ +	2:8	+ +
	LV	C	8:16	+ -	2:4	+ +	2:4	+ +	4:8	+ -	2:4	+ -
		S	8:8	+ +	2:4	+ +	2:4	+ +	4:8	+ +	2:4	+ +
216	HL	C	8:32	+ +	4:8	- -	4:8	+ -	4:16	- -	/	...
	LV	C	2:4	+ +	<2:2	+ +	4:8	- -	2:4	+ +	/	...

NB = nutrient broth; CMB = cooked-meat broth; HDB = horse digest broth; IB = infusion broth; first reading after incubation at 37° C; second reading after cooling at 4° C;
+ = neutralisation; - = partial neutralisation; - = no neutralisation.

haemolytic factor might be the delta antigen; however, it was not possible to demonstrate that CMB cultures of type A strains of Cl. oedematiens contain a truly oxygen-labile haemolysin. The haemolytic titre of these cultures was often reduced after treatment with hydrogen peroxide, but the residual haemolytic activity did not increase after reduction with sodium thioglycollate. In addition, the haemolytic reaction was not neutralised by combining homologous antiserum with anti-streptolysin serum. Of course, the above observations do not exclude the presence of the delta antigen in these cultures.

Interesting results were obtained in an investigation of the heat stability of the haemolytic and LV activities in CMB cultures of type A strains of the organism. The titres obtained in haemolytic and LV tests were slightly reduced after holding cultures at 56° C for 60 min. Heating at 60° C reduced the haemolytic activity more than the LV activity; after heating cultures at 60° C for 60 min. haemolytic activity was not detectable but a weak LV reaction remained. It would be of interest to determine whether the epsilon antigen is the heat stable LV factor in these cultures.

The partial neutralisation of haemolytic activity in culture supernates of Cl. oedematiens suggested that a haemolytic factor might be associated with the bacterial cell, and tests were performed with washed bacterial cell suspensions before and after disruption of the cells by ultrasonic treatment. The results of these tests showed that a trace of haemolytic activity may be associated with disintegrated bacterial cells but the activity is too low to account for the

non-neutralisable haemolytic factor in CMB.

The present results suggest that the haemolytic activity of CMB cultures of type A strains of Cl. oedematiens is due to the gamma antigen plus a haemolytic factor that is associated with the meat particles of the medium. If this is so, it may be possible to adsorb the gamma antigen during treatment of a culture with egg-yolk emulsion, and the haemolytic factor might then be detectable in the HL test system. A concentrated CMB culture of a type A strain of Cl. oedematiens was mixed with egg-yolk emulsion and incubated at 37° C for 30 min; after centrifugation of the mixture, the supernate was tested for haemolytic activity against human cells; it was found that the haemolytic activity increased during the above treatment. Similar tests with unconcentrated cultures showed that the egg-yolk treatment removed all of the haemolytic activity that was present.

Type A strains of the organism were then cultured in media with added egg-yolk emulsion; HL titrations and neutralisation tests were performed on the cultures (see table XX). The results of these experiments suggest that a haemolytic factor is formed (i) when a concentrated culture of a type A strain of Cl. oedematiens is incubated in the presence of egg-yolk emulsion, and (ii) when type A strains of the organism are cultured in the presence of the egg-yolk emulsion. The haemolytic factor is not neutralised by an experimental homologous antiserum. The haemolytic activity of the NB + egg-yolk emulsion culture was not neutralised after titration of the culture and subsequent addition of a fixed volume of antiserum to each dilution; thus,

Table XX

The HL titre and HL neutralisation

in cultures with added egg-yolk emulsion

Strain GR1A cultured in *	HL titre	Neutralisation of HL activity with with 10 anti-gamma units of *Cl. oedematiens* type A antiserum at a dilution of the test dose of 1 in	
		2	10
CMB	10 : 40	...	+ : +
CMB + EY	40 : 80	...	- : -
NB	8 : 16	+ : +	...
NB + EY	32 : 64	- : -	- : -

*CMB = cooked-meat broth; EY = egg-yolk emulsion;
NB = nutrient broth;
first reading after incubation at 37° C;
second reading after overnight cooling at 4° C;
+ = neutralisation; - = no neutralisation.

it appears that the experimental antiserum possesses no neutralising antibodies for this haemolytic factor (but see below).

Similar tests were performed with concentrated cultures prepared from type B, C and D strains of Cl. oedematiens. The haemolytic activity of these strains does not increase, and may be reduced after treatment with egg-yolk emulsion and after culture in the presence of this substrate.

HL and LV neutralisation tests with other antisera

The occasional incomplete neutralisation patterns obtained in tests with cultures of type A strains of Cl. oedematiens and the homologous experimental antiserum are described above. It appears that a haemolytic factor in addition to the gamma antigen may be formed in the presence of meat particles, and that this factor is not neutralised by the experimental antiserum. However, in subsequent neutralisation tests, the haemolytic activity of CMB cultures of type A strains of the organism was completely neutralised by a commercial Cl. oedematiens antiserum that contained about 8 anti-gamma units in the neutralising dose of antiserum. In addition, this antiserum

partially neutralised the haemolytic activity of a type A strain cultured in CMB with added egg-yolk emulsion. These results suggest that neutralising components exist in the commercial antiserum for haemolytic factors that are produced in CMB when type A strains of the organism are cultured in this medium. Clearly, it would now be valuable to perform tests similar to those described by Oakley et al. (1947) in order to determine whether any relationship exists between the haemolytic factor described above and the delta antigen of these strains of Cl. oedematiens (Oakley et al., 1947).

Evidence was obtained in the present work that the haemolytic and LV activities of Cl. oedematiens may be partially neutralised by heterologous antisera. Apparent cross-neutralisation effects may occur if the antisera are not species- or type-specific, and it may be necessary to dilute the antisera in order to obtain the appropriate neutralisation patterns. In the present studies, however, the haemolytic activity of a diluted type A culture concentrate was partially neutralised by an antiserum that was prepared in a rabbit against a type B culture concentrate; the rabbit was not previously exposed to injections of the other antigens of Cl. oedematiens. Thus, there may be a partial neutralisation by heterologous antisera of the haemolytic and LV activities of diluted test materials. These observations should be borne in mind when a neutralisation test system is designed.

The present studies have indicated that methods suggested for the provisional typing of Cl. oedematiens are rather delicately poised, and that variations in the test system may affect the results obtained. It appears that the HL test with human red cells as the substrate is rather more sensitive than LV tests with the same test materials. In the present work, HL and LV neutralisation tests were always performed in parallel with CMB cultures of Cl. oedematiens, and this procedure is recommended for the provisional typing of unknown strains of the organism.

Thin-layer chromatography experiments

Explanatory note. If culture products of Cl. oedematiens are incubated with egg-yolk emulsion at 37° C, a visible reaction usually occurs; this may appear as an opalescence, a turbidity or a curd that rises to the surface of the mixture. Oakley and his colleagues (1947, 1959) and MacFarlane (1948, 1950a) showed that culture filtrates of Cl. oedematiens possess lecithinase-C activity and that, in addition, type A strains have lipase activity. It is usually considered that the lecithinase of Cl. oedematiens is responsible for the development of turbidity around colonies on egg-yolk agar and that the lipase causes a pearly layer to develop on the surface of the colonies.

Recent advances in lipid chemistry enable mixtures of lipids to be analysed simply and rapidly, and the different classes of lipid are readily separated. Whilst the present work was in progress,

Scrimgeour, Keaneand Alder (1967) utilised thin-layer chromatography to differentiate the end products extracted from reaction mixtures of egg-yolk emulsion with cultures of Staphylococcus aureus or Clostridium welchii. Cultures of Staph. aureus possess lipase activity, and Cl. welchii produces a lecithinase-C enzyme; both organisms give a similar opacity when grown in media that contain egg-yolk. Scrimgeour et al. demonstrated a decrease in the triglyceride or phospholipid content of the reaction mixtures described above if the culture possessed lipase or lecithinase activity respectively. This technique has an obvious application to the elucidation of the lecithinase and lipase activities of Cl. oedematiens; it was hoped that the procedure might be of value in identifying the haemolytic component that appears when type A strains are incubated with egg-yolk emulsion.

Procedure. The solvent system in my early experiments was a mixture of chloroform and glacial acetic acid (95 : 5 v/v). This solvent separates the so-called neutral lipids; complex lipids such as phospholipids do not move from the origin. The distance travelled by individual components of the mixture can be expressed as a fraction (RF) of the distance moved by the solvent front. An RF value can thus be obtained for each lipid component in that particular system, and the RF values given by Scrimgeour et al. are shown in table XXI.

In the present work, concentrated culture products of Cl. oedematiens were incubated overnight at 37° C with egg-yolk emulsion; the total lipid fraction was extracted from the reaction mixtures (see

Table XXI

The RF values of certain neutral lipids in a defined solvent (see Scrimgeour et al., 1967)

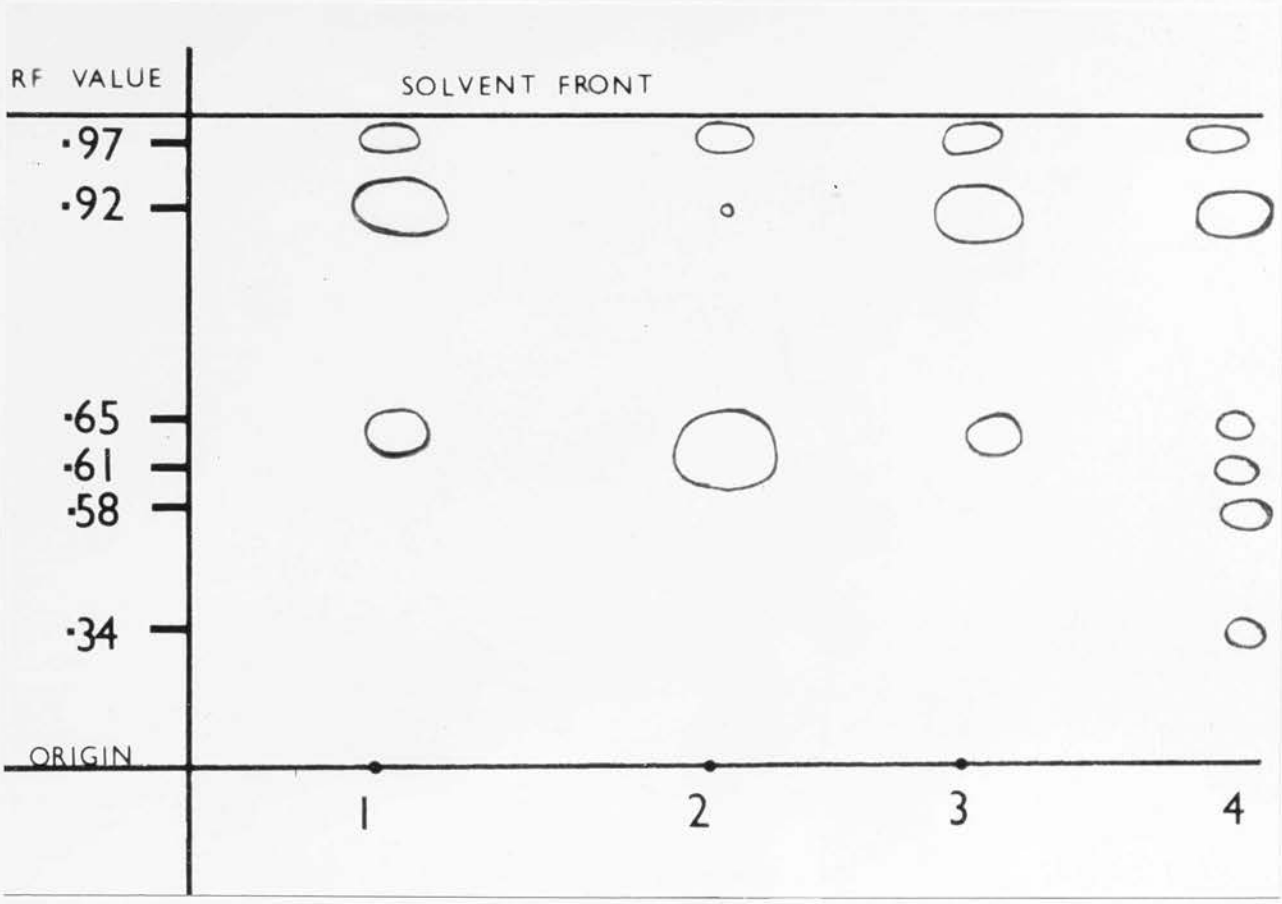
Class of lipid	RF value in chloroform/ glacial acetic acid (95:5)
Cholesteryl esters	1.0
Triglycerides	0.95
Fatty acids	0.55
Diglycerides	0.50
Cholesterol	0.49
Phospholipid	0.00

Methods) and investigated by thin-layer chromatography. Experiments with chloroform/glacial acetic acid (95 : 5) as the solvent gave the following results (see fig. 13, p.144a) after locating the separated components of the mixtures with iodine vapour. On the basis of the RF values quoted in table XXI it seemed reasonable to conclude that the type A concentrate contained a lipase that was breaking down triglycerides. The type B concentrate slightly reduced the phospholipid spot; the type D concentrate almost obliterated the phospholipid spot and a new spot appeared at RF value 0.34. Tests with a type A concentrate grown in NB gave a similar indication of lipase activity, but concentrates prepared from CMB/G and NB/G produced no apparent change in the lipid content of the egg-yolk emulsion, although the CMB/G reaction mixture contained a surface curd.

It seemed at this point that only lipase activity could be detected in test mixtures containing concentrated products of type A strains. Clearly, it was necessary to investigate the action of culture concentrates of the organism on the phospholipid fraction of egg-yolk emulsion. A solvent consisting of chloroform, methanol, acetic acid and water (65 : 25 : 8 : 4) was chosen for these experiments. This system does not separate the neutral lipids which run with the advancing solvent front; however, the solvent separates certain complex lipids including lecithin.

Tests with 0.1-ml. amounts of culture concentrates and 1.0 ml. of egg-yolk emulsion did not give consistently positive results, and 0.5 ml. of concentrate was used in the subsequent reaction mixtures.

Thin-layer chromatography studies



1 = egg-yolk emulsion control;

2 = egg-yolk emulsion plus CMB concentrate of Cl. oedematiens type A strain;

3 = egg-yolk emulsion plus IB concentrate of Cl. oedematiens type B strain;

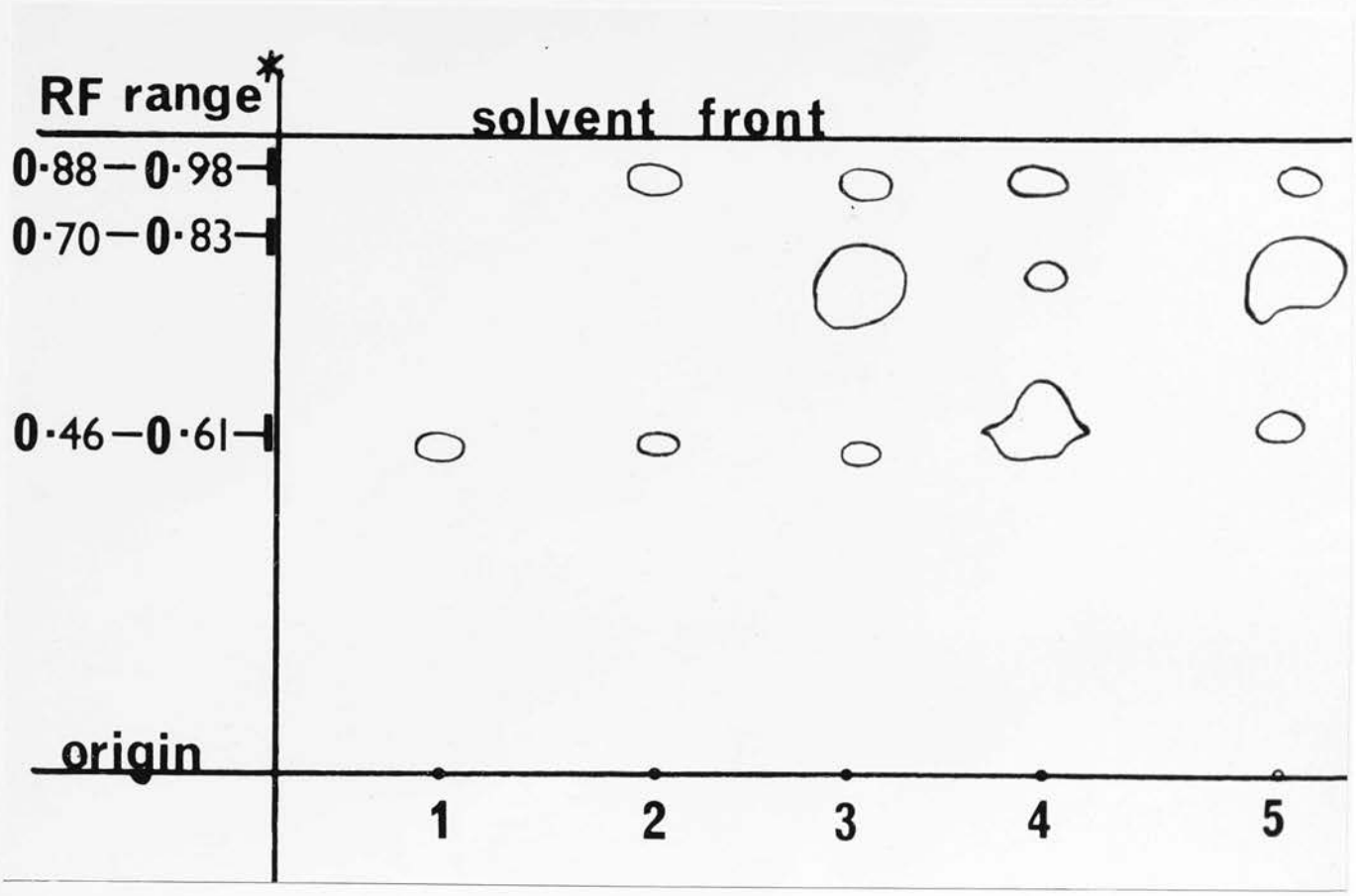
4 = egg-yolk emulsion plus IB concentrate of Cl. oedematiens type D strain.

FIG. 13. - A diagram of the results obtained in thin-layer chromatographic analyses of test reaction mixtures with chloroform/glacial acetic acid solvent.

With the chloroform, methanol, acetic acid and water solvent, culture concentrates of type B and D strains removed a spot that was assumed to represent lecithin; the same spot was markedly reduced in the presence of type A culture concentrates prepared from CMB and NB, and slightly reduced by CMB/G and NB/G concentrates of the type A strain.

Provisional identification of some of the spots was the next step, and semi-standard preparations were kindly provided by Dr. G. S. Boyd. These included lecithin, triglyceride (olive oil), cholesterol, cholesterol ester (cholesterol laurate), fatty acid (lauric acid), monoglyceride (glycerol monostearate) and phospholipid. Preliminary experiments revealed that impurities were present in most of these standards; further purification was not attempted.

Comparative experiments were performed with some of the standards and with reaction mixtures of Cl. oedematiens of types A and D (see figs 14 & 15, p. 145a & b). The results of these experiments suggest that the egg-yolk emulsion contains lecithin, cephalin, triglycerides, cholesterol and cholesterol esters. Incubation of egg-yolk emulsion with the concentrated type A product reduced the triglyceride spot, and incubation with a type D product removed the lecithin spot at RF 0.41. I was unable to identify the spot that increased in size after treating the egg-yolk emulsion with a type A concentrate. Experiments with the cholesterol and fatty acid standards showed that these components are not easily distinguished on the basis of RF values; in addition, diglycerides have a similar RF value to cholesterol in the chloroform/acetic acid solvent. It seems reasonable to suggest that



1 = cholesterol;

2 = cholesterol ester;

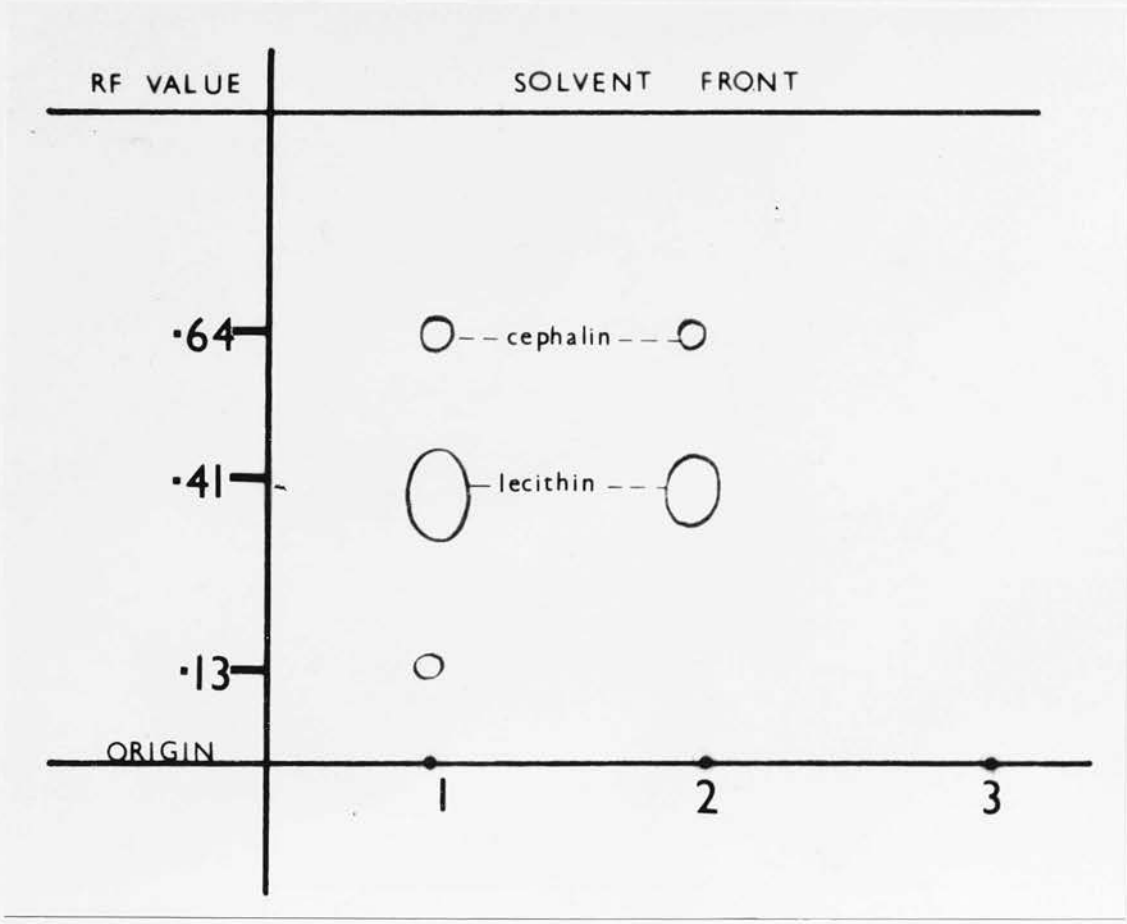
3 = egg-yolk emulsion control;

4 = egg-yolk emulsion plus CMB concentrate of Cl. oedematiens type A strain;

5 = triglyceride (olive oil).

*The RF values varied in these experiments; however, the pattern of results was consistent, and the spots were provisionally identified by reference to standard preparations.

FIG. 14. - A diagram of the results obtained in thin-layer chromatographic analyses of (i) standard preparations, and (ii) a test reaction mixture with chloroform/glacial acetic acid solvent.



1 = Lecithin;

2 = egg-yolk emulsion control;

3 = egg-yolk emulsion plus IB concentrate of Cl. oedematiens type D strain.

FIG. 15. - A diagram of the results obtained in thin-layer chromatographic analyses of (i) standards, and (ii) a test reaction mixture with chloroform/methanol/acetic acid/water solvent.

the enlarged spot may represent a coalescence of two spots: (i) cholesterol that is already present in the emulsion and (ii) diglycerides that result from the lipase activity of the culture concentrate. Scrimgeour et al. interpreted a similar spot as being free fatty acids, apparently on the basis of the RF value.

It should be borne in mind that the experiments described above can be regarded as semi-quantitative only; in this limited series of tests the following effects have been provisionally identified in mixtures of concentrated cultures of Cl. oedematiens with egg-yolk emulsion (see table XXII).

The results in table XXII indicate that the visible reaction that is produced by culture concentrates of Cl. oedematiens in egg-yolk emulsion appears to be associated with the breakdown of lecithin. The effects of the type A CMB concentrate and the type D IB concentrate are almost completely neutralised in tests with homologous antisera; the chromatographic results of the partially neutralised mixtures show a reduction in the breakdown of triglycerides and lecithin; thus, neutralising antibodies for each reaction appear to be present in the respective antisera.

In addition to the effects noted in table XXII, the following changes have been tentatively identified in some of the egg-yolk reaction mixtures: (i) a decrease in cephalin in the presence of type B and D IB culture concentrates; and (ii) an increase in diglycerides with type B and D culture concentrates.

No biochemically recognised haemolytic factor was detected in

Table XXII

The effect of concentrated cultures of

Cl. oedematiens on egg-yolk emulsion

Cl. oedematiens of type	Culture medium from which concentrate was prepared*	Visible effect on egg-yolk emulsion	TLC detection of breakdown of	
			Triglyceride	Lecithin
A	CMB	curd ++	+++	+
	NB	curd ++	+++	+
	CMB/G	curd ⊥	-	⊥
	NB/G	precipitate tr.	-	⊥
	IB	-	-	-
B	CMB	curd ++	-	+++
	IB	curd ++	-	+++
D	CMB	curd +++	-	+++
	IB	curd +++	-	+++

*CMB = cooked-meat broth; NB = nutrient broth;

G = plus 1 per cent. of glucose; IB = infusion broth;

+, ++, +++ = increasing amounts of activity;

⊥ = doubtful reaction;

tr. = trace detected;

- = negative reaction .

reaction mixtures of egg-yolk emulsion with type A culture concentrates in the present work other than the lecithinase enzyme described by Oakley and his colleagues (1947).

The soluble lethal factors in Cl. oedematiens cultures

Oakley and his colleagues demonstrated that the lethal factor in cultures of type A and B strains of Cl. oedematiens is the alpha antigen, and that the beta antigen is responsible for the lethal activity of type D cultures of the organism. There is little information regarding the optimum conditions for the production of these soluble products, and the following experiments were necessary to obtain potently active starting materials for fractionation studies (q.v.). The results of some of the tests are summarised in table XXIII, and the following conclusions were drawn from the comparative data.

(i) Soluble products of cultures of type A strains of Cl. oedematiens are less lethal in mouse MLD tests than cultures of type B strains. On several occasions a frequently subcultured type A strain produced no detectable lethal factor in CMB; a loss of lethality was not observed with cultures of type B strains. Cultures of strain GR1C are not lethal in mice. In the present work, cultures of two type D strains of Cl. oedematiens were not lethal following intravenous inoculation into mice.

(ii) Type A strains of the organism produce a low level of lethal

Table XXIII

The biological activities of

Cl. oedematiens in different media

Strain of Cl. oedematiens	Medium*	Day	HL titre+	LV titre+	Lethality titre*	final pH of Medium
GR1A	CMB	1	16 : 32	4 : 8	4	6.3
		2	2 : 8	2 : 4	2	6.2
		5	<2 : 2	<2 : <2	2	6.2
GR1A	CMB/G	1	4 : 8	2 : 4	<2	5.1
		2	2 : 4	2 : 4	2	4.8
		5	2 : 4	2 : 4	<2	4.8
GR1A	CMB (Nishida & Nakagawara, 1964)	1	8 : 32	8 : 16	8	7.0
		2	<2 : 2	2 : 4	8	5.3
		5	<2 : 2	2 : 4	4	5.4
GR1A	IB	1	4 : 8	<2 : <2	8	6.0
		2	2 : 4	<2 : <2	8	6.0
		5	4 : 8	<2 : <2	16	6.0
GR1B	IB	1	16 : 64	4 : 8	40	5.9
		2	<2 : 2	<2 : <2	80	6.2
		5	<2 : <2	<2 : <2	40	6.4
GR1B	CMB	1	4 : 8	<2 : <2	80	6.2
		2	4 : 8	<2 : <2	80	6.4
		5	<2 : 2	<2 : <2	80	6.5
GR1B	CMB/G	1	4 : 4	<2 : <2	8	6.5
		2	4 : 8	<2 : <2	8	5.9
		5	2 : 4	<2 : <2	/	5.0
GR1B	HDB	1	16 : 32	<2 : 4	8	6.4
GR2B	IB	1	<2 : 16	<2 : 2	400	/
GR1D	CMB	1	16 : 16	4 : 4	<2	6.5
		2	128 : 128	4 : 8	<2	6.1
		5	32 : 32	4 : 4	<2	6.2

*CMB = cooked-meat broth; IB = infusion broth;

HDB = horse digest broth; G = plus 1 per cent. of glucose;

+ = first reading after 1 hr incubation at 37° C; second reading after cooling overnight at 4° C;

* = test dose of 0.1 ml. injected into mice.

activity in CMB and in IB; cultures grown in the modified CMB medium recommended by Nishida and Nakagawara (1964) did not contain potent lethal activity in the present investigation. CMB medium was more satisfactory than IB for the production of adequate amounts of haemolytic, LV and lethal activities.

(iii) Type B strains of Cl. oedematiens produce large amounts of the lethal factor in cultures grown in IB and CMB; the yield in IB is usually greater than in CMB; cultures in HDB do not yield potent lethal activity. There is a marked strain variation in the potency of the lethal factor; and the lethal activity in cultures of type B strains of the organism bears no relation to the level of HL and LV activities.

(iv) The lethal activity in cultures with 1 per cent. of added glucose is markedly reduced compared with the sugar-free medium; the results obtained with the modified CMB of Nishida and Nakagawara (1964) suggest that the lethal factor is not particularly sensitive to acid pH values.

(v) The lethal potency of a culture does not alter markedly during prolonged incubation provided that initially there is satisfactory growth in the medium.

(vi) Subsequent tests with culture products of strain GRlD showed that if the test material is lethal, then potent haemolytic and LV activities are present (see table XXIV). The lethal activity of the concentrated product was neutralised by an experimental homologous antiserum.

Table XXIV

The levels of biological activities in a culture of Cl. oedematiens of type D

Strain of Cl. oedematiens	Medium	Nature of test material	HL titre*	LV titre*	Lethal effect of 0.1 ml. dose i/v in mice
GR1D	IB	culture	320:320	32:64	-
		supernate	320:320	32:64	-
		concentrated supernate	5120:5120	320:640	+*

*first reading after 1 hr at 37° C;
second reading after cooling overnight at 4° C;

*a 0.1 ml. dose of dilutions of the concentrate up to 1 in 10 was lethal.

Studies on the lethal factor of Cl. oedematiens type B

(i) Effect of pH. Volumes of a concentrated type B culture diluted to contain 160 mouse LD were allowed to stand with equal volumes of Michaelis buffer at different pH values from 2.3 - 9.3. After 24 hr exposure to the buffer, 0.1 ml. of each mixture (equivalent to 8 mouse LD) was injected intravenously into mice. The culture material that was held at pH 2.3 was no longer lethal, but samples exposed to pH values from 3.6 - 9.3 retained their lethal activity.

(ii) Effect of heating. Preliminary experiments suggested that the lethal factor in cultures of type B strains of Cl. oedematiens is rather heat-labile. The lethal titres (table XXV) obtained after heating a culture at different temperatures confirm that the lethal factor is particularly susceptible to moderate heating.

(iii) Toxoiding with formalin. The lethal activity of a concentrated culture of a type B strain of Cl. oedematiens was lost after exposure to 0.064 per cent. of formaldehyde for 18 hr at 37° C; at room temperature (18° C), 42 hr exposure to 0.124 per cent. of formaldehyde was necessary to render the same concentrate non-lethal. Subsequent work showed that the non-lethal, formalin-treated concentrates were antigenic in rabbits, indicating that the lethal factor was toxoided during treatment with formalin.

Table XXV

The effect of heat on the soluble lethal factor in a culture of Cl. oedematiens (pH 6.8)

Strain of Cl. oedematiens	Growth Medium	*Lethal titre in mice	*Lethal titre after heating a sample of the culture for 30 min. at	
			47.5° C	50.5° C
R3/2 (type B)	CMB	64	16	< 2

*Test dose of 0.1 ml. injected

only. The CPE of different cultures, and neutralisation tests with antitoxic sera are shown in figs. 16 - 22.

The results described above suggest that the lethal and cytopathic activities of cultures of type A and B strains of Cl. oedematiens may be due to the same factor - the alpha antigen. In order to confirm this observation it is necessary to demonstrate that in suitable tests the two activities are neutralised by different antisera according to the anti-alpha content of the sera. In the present work a concentrated IB culture of strain GR1B was used as the test material. The LD50 dose of the concentrate in mice was a dilution of 1 in 1150 calculated by the Reed and Muench (1938) technique; by the same method, the CP50 dose was 1 in 1254 after 24 hr rolling of the monolayers, and 1 in 1320 after 48 hr rolling. Clearly, the CP50 dose after overnight rolling of the fibroblasts is slightly more sensitive than the mouse LD50 test read after 48 hr. On the basis of these results, a dilution of 1 in 12 of the concentrate containing approximately 100 LD50 or CP50 in each test system was used as the level of test in the subsequent neutralisation studies.

Dilutions of Cl. oedematiens type A, B and D antisera were prepared at differences of 20 per cent; equal volumes of concentrate and antitoxin dilutions were mixed, and after allowing a period of 30 min. for neutralisation a test dose of each mixture was inoculated into the two indicator systems. The results of the neutralisation tests are shown in table XXVI.

The type D antiserum failed to neutralise the lethal and cytopathic

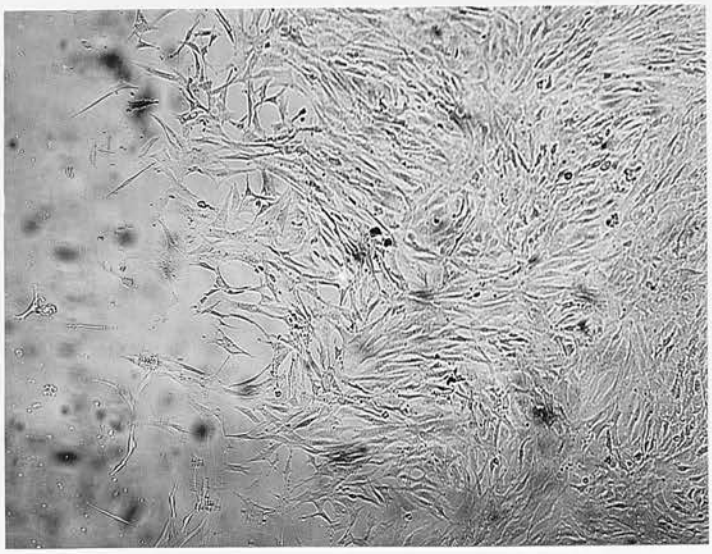


FIG. 16. - Control monolayer of chick-embryo fibroblasts. X 60.

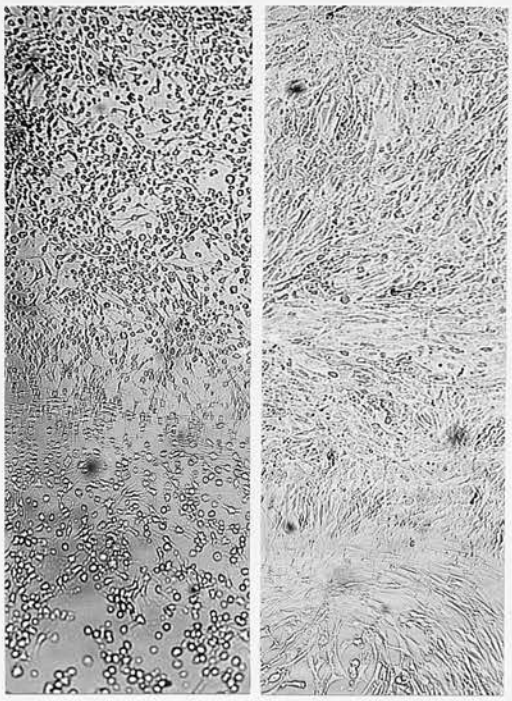


FIG. 17. - The cytopathic effect of a concentrated type B culture (left); neutralisation with a type B antiserum (right). X 60.

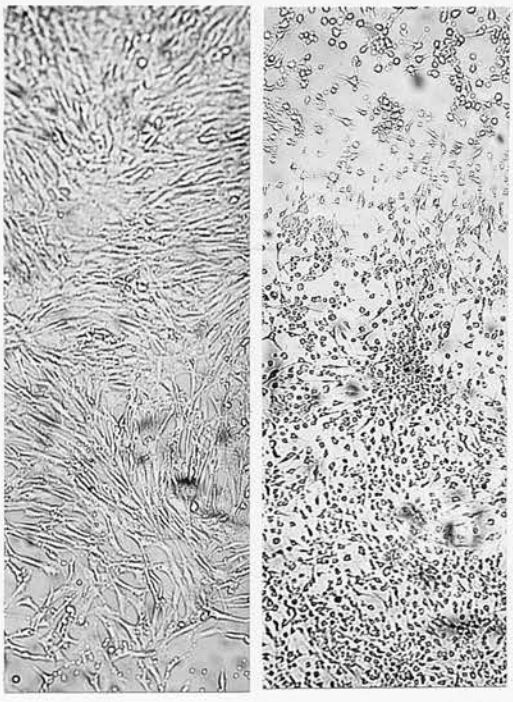


FIG. 18. - Neutralisation with a type A antiserum (left); failure of neutralisation with a type D antiserum (right). X 60.

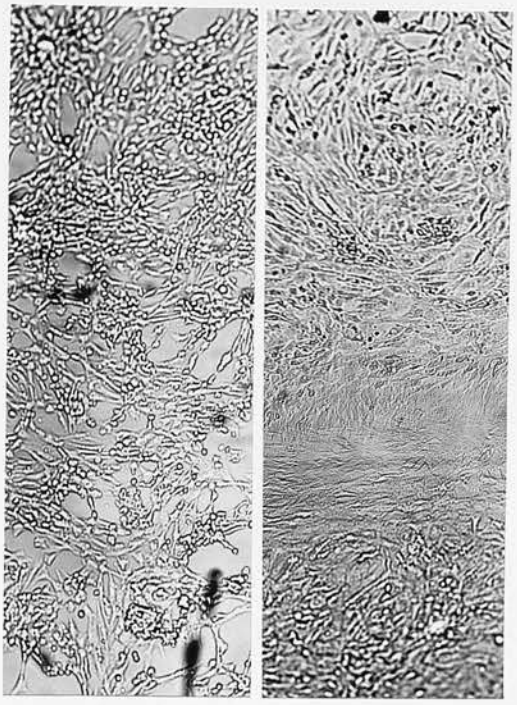


FIG. 19. - The cytopathic effect of a partially-purified type B concentrate (left); neutralisation with a type B antiserum (right). X 60.

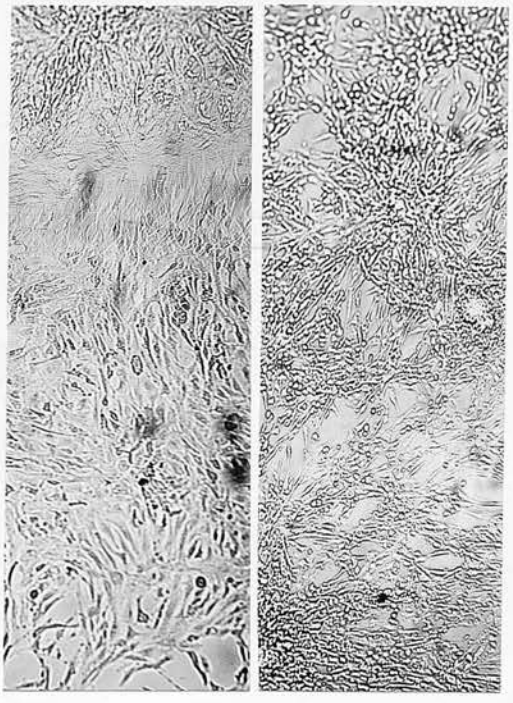


FIG. 20. - Neutralisation with a type A antiserum (left); failure of neutralisation with a type D antiserum (right). X 60.

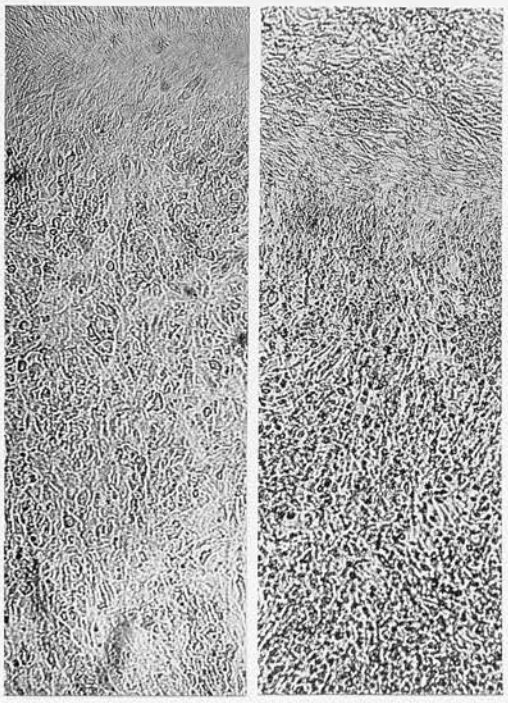


FIG. 21. - Control monolayer of chick-embryo fibroblasts (left); the cytopathic effect of a concentrated type D culture (right). X 60.

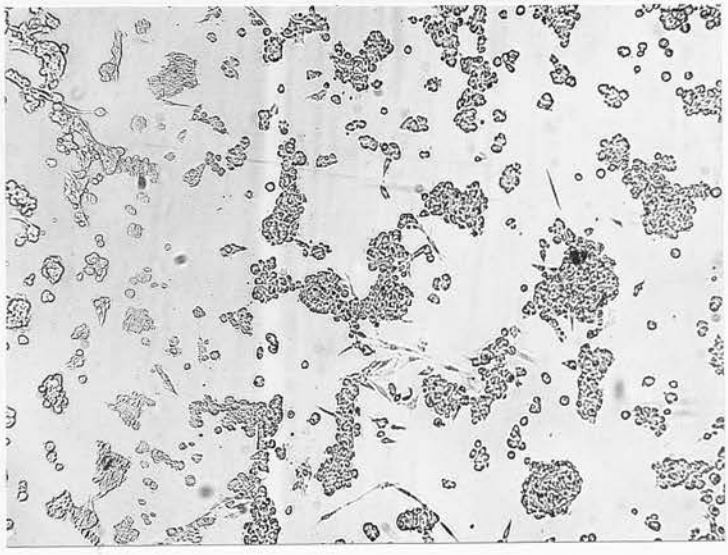


FIG. 22. - The cytopathic effect of a culture supernate of strain GR1A. X 60.

Table XXVI

Neutralisation of the lethal and cytopathic effects of Cl. oedematiens type B

Test system*	Neutralisation obtained with a test dose of a type B concentrate in the presence of type A antiserum diluted 1 in					
	60	73	89	109	132	156
Mouse lethality	+	+	+	-	-	-
	+	+	+	+	-	-
	+	+	+	+	-	-
CPE with chick fibroblasts	+	+	+	-	-	-
	+	+	+	-	-	-
	+	+	+	-	-	-
	+	+	+	+	-	-
	Neutralisation obtained with a test dose of a type B concentrate in the presence of type B antiserum diluted 1 in					
	240	294	357	435	503	625
Mouse lethality	+	+	+	-	-	-
	+	+	+	-	-	-
	+	+	+	-	-	-
CPE with chick fibroblasts	+	+	+	-	-	-
	+	+	+	-	-	-
	+	+	+	-	-	-
	+	+	+	-	-	-
	Neutralisation obtained with a test dose of a type B concentrate in the presence of type D antiserum diluted 1 in					
	10					
Mouse lethality	-					
	-					
	-					
CPE with chick fibroblasts	-					
	-					
	-					
	-					

+ = neutralisation; - = no neutralisation;

*3 mice and 4 tissue culture tubes were used for each dilution of antiserum.

effects of the type B concentrate. The type B antiserum clearly neutralised both effects at the same dilution of antiserum; the type A antiserum neutralised both effects but with slight discrepancies within 20 per cent. of the same dilution of antiserum. With the type A antiserum, the CPE was not neutralised in 3 of 4 tissue culture tubes by a dilution of the serum that suppressed the lethal effect in 2 of 3 mice. This may reflect the relative sensitivities of the two systems or it may be that other factors in the test dose were not suppressed by the heterologous antiserum. It seems reasonable to conclude that within the limits of experimental error, the results support the view that the lethal alpha antigen of Cl. oedematiens cultures produces the well-marked CPE that I have described above (see Discussion).

Experiments were also performed to further characterise the CPE of Cl. oedematiens. Cytopathic activity is detectable in cultures of types A and B of the organism; in order to obtain clear-cut positive results, particularly with cultures of type A strains, it may be necessary to use 0.1 ml. of the culture as the test dose. For routine tests with cultures of Cl. oedematiens the tissue culture medium should be renewed just before the test material is added; Eagle's maintenance medium was satisfactory in tests with chick fibroblasts during the present work.

The sensitivity of two other cell lines to the cytopathic agent was tested; it was found that chick fibroblasts, monkey-kidney cells and BHK cells are almost equally susceptible to the cytopathic factor

in cultures of types A and B strains of Cl. oedematiens.

Potent preparations of the cytopathic agent cause a recognisable effect in chick fibroblast cells within 2 hr rolling at 37° C, and 80 per cent. of the cells are affected within 4 hr. If the monolayers are exposed to the agent for 5 min. then washed thoroughly, a slight CPE is subsequently observed; exposure for 15 min. followed by washing causes a marked CPE. With less active preparations, the monolayer can be exposed to the test material for at least 70 min. without adsorption of the cytopathic agent. The CPE of potent material is neutralised by antiserum added up to 30 min. after exposure of the monolayer to the cytopathic agent. Thus, it appears that the cytopathic factor in potent material is rapidly adsorbed onto the tissue culture cells, but the adsorption can be reversed by the addition of a suitable antiserum.

The cytopathic factor in type D culture concentrates of Cl. oedematiens affects chick fibroblast cells; BHK cells do not appear to be susceptible. A correlation appears to exist between the lethal and cytopathic titres of type D concentrates, and the effects are neutralised by a homologous antiserum. Cultures of type D strains were neither lethal nor cytopathic in the present work. The CPE of the culture concentrates is difficult to quantitate and comparative experiments are not easy. It is possible that the potent beta activity of a type D concentrate of Cl. oedematiens may produce a CPE (see Discussion).

Other biological activities detectable in cultures of Cl. oedematiens

(i) Hyaluronidase. The hyaluronidase activity of culture supernates of Cl. oedematiens was tested with a modification of the method described by Oakley and Warrack (1951). The test employed bovine synovial fluid as the substrate and is a modification of the ACRA test (see Gadalla and Collee, 1968). Hyaluronidase activity was present in CMB and CMB/G cultures of type B strains (2 of 2 tested) and one type D strain of Cl. oedematiens that was tested; type A strains (2 of 2 tested) and strain GR1C gave negative results in the test. The level of hyaluronidase activity in the above cultures was not assayed, but the activity was clearly demonstrable.

(ii) Neuraminidase. The neuraminidase assay developed by Collee and Barr (1968) was used. The test detects free sialic acid that is liberated from mucoprotein-bound-complexes in a suitable substrate after incubation with test materials that contain neuraminidase. Neuraminidase activity was not detected in cultures of Cl. oedematiens during the present work.

(iii) Dermonecrotic activity. The dermonecrotic effect of cultures of Cl. oedematiens was not investigated critically during the present work. Dermonecrotic activity was demonstrated in a shaven guinea-pig skin, but in the early experiments the guinea-pig often died as a result of several injections of potent material. It was

observed that the injection of cagsal intradermally caused a marked oedematous reaction. There is a considerable variation in the diameter of the reaction zone that is produced by dermonecrotic material depending on the test animal, but a gradation occurs within the same animal. The results of these experiments suggested that the dermonecrotic test with a type B culture concentrate is considerably more sensitive than the LD50 dose or the CP50 effect of the same test material.

The fractionation of culture products of Cl. oedematiens

It is clear that the major soluble antigens of Cl. oedematiens have more than one biological activity, and that several antigens may possess the same biological activity. Identification of the different factors would be simpler if the antigens were readily purified, and the antigenic preparations would then be of great value in the production of relatively monospecific antisera. Modern fractionation techniques are applicable to the separation of the biological activities of culture products, and it is often possible to achieve some degree of purification during the preparation of the starting material. Clearly, it is necessary to have optimally active starting materials, and the following studies were performed to obtain suitably potent preparations for gel-filtration procedures.

Cultures of Cl. oedematiens possess haemolytic, LV and lethal

activities that are readily demonstrable. The lecithinase-C enzyme in culture products of the organism provides an explanation for some of the haemolytic and LV activities, but the nature of the lethal antigen has not yet been established. In the present study the haemolytic, LV and lethal activities of culture products were assayed to indicate the degree of purification that was obtained in each of the various procedures. The design of the fractionation techniques assumed that the soluble antigens are large protein-like molecules.

(i) Fractional precipitation. Fractional precipitation with salts is often the initial step in the purification of enzymes; ammonium sulphate is the salt of choice because it is extremely soluble in water, and it has no harmful effects on most enzymes. The results of initial fractionation experiments with ammonium sulphate-precipitated CMB cultures of Cl. oedematiens suggested that most of the biological activity was present in the fraction that precipitated at 20 - 60 per cent. ammonium sulphate saturation; more detailed precipitations were then performed. It was observed during these experiments that a precipitate often appears during the dialysis step to remove ammonium sulphate from the re-dissolved precipitated fraction; the dialysed solution can be clarified by filtration, but the precipitate contains almost as much biological activity as the solution.

The results of haemolytic and LV titrations with 500-ml. CMB cultures of type A strains of Cl. oedematiens suggested that individual

haemolytic and LV factors were present; the LV activity of these cultures increased between 1 and 5 days' incubation and the haemolytic activity decreased in the same period. Experiments performed with concentrated fractions prepared from CMB cultures of type A strains indicated that the biological activities may be separated in fractions that precipitate at different percentage saturations of ammonium sulphate (see table XXVII).

On several occasions, 500-ml. CMB cultures of type A strains contained no detectable lethal factor; in the cultures mentioned above, the overnight culture was lethal but the 5-day culture possessed no lethal activity. The lethal alpha antigen is the most significant component of Cl. oedematiens cultures, and although the separation of haemolytic from LV activity in type A cultures is of interest, I turned my attention to type B cultures which are more potently lethal than type A cultures.

Fractional precipitation of a CMB culture of a type B strain of Cl. oedematiens showed that lethal activity was detectable in fractions precipitated at 20, 40, 60 and 80 per cent. saturation with ammonium sulphate; haemolytic activity was also present in each fraction with maximum activity in the 40 - 60 per cent. fraction; and LV activity was detectable in only the 40 - 60 per cent. fraction. In order to estimate the recovery of biological activity after salt precipitation, a fractionation experiment was performed with strain GR1B grown in CMB and in IB. The titres of the concentrated fractions are given in table XXVIII, and the results are treated in table XXIX

Table XXVII

The ammonium sulphate fractionation of a type A CMB culture of Cl. oedematiens

Strain of Cl. oedematiens	Percentage saturation range of $(NH_4)_2SO_4$ precipitating fraction	HL titre* (human cells)	LV titre*
GR1A	0 - 40	80 : 160	160 : 320
	40 - 60	80 : 320	80 : 80
	60 - 80	80 : 160	<10 :<10

*first reading after 1 hr at 37° C;

second reading after cooling at 4° C overnight.

Table XXVIII

Ammonium sulphate fractionation of cultures of Cl. oedematiens strain GR1B

Culture medium	Percentage saturation range of $(NH_4)_2SO_4$ precipitating fraction	HL titre* (human cells)	LV titre*	¤ Lethality titre in mice
Cooked-meat broth	...	< 10 : 10	< 10 : < 10	40
	0 - 40	10 : 20	10 : 20	160
	40 - 60	10 : 320	40 : 80	640
	60 - 80	< 10 : < 10	< 10 : < 10	40
Infusion broth	...	< 10 : 10	< 10 : < 10	160
	0 - 40	10 : 40	< 10 : 10	> 80
	40 - 60	80 : 320	40 : 80	640
	60 - 80	20 : 160	20 : 20	80

*first reading after 1 hr incubation at 37° C;
second reading after cooling at 4° C overnight.

¤Test dose of 0.1 ml. injected.

Table XXIX

The degree of purification obtained during

ammonium sulphate fractionation of cultures of Cl. oedematiens strain GR1B

Indicator system	Culture medium	Percentage saturation range of $(NH_4)_2SO_4$ precipitating fraction	Volume (ml.)	Units per ml.	Total units	Protein mg. per ml.	Specific activity units per mg.	Yield per cent.	Purification
Haemolysis of human cells	Cooked-meat broth	...	445	20	8,900	2.6	7.6	100	1
		0 - 40	1.54	40	60	12.8	3.3	< 1	0.43
		40 - 60	5.1	640	3,264	30	21	37	2.8
		60 - 80	12.4	< 20	...	21.9	...	...	...
	Infusion broth	...	460	20	9,200	0.75	26.7	100	1
		0 - 40	0.44	80	35	6.9	11.6	< 1	0.43
		40 - 60	3.1	640	1,984	8.8	73.1	21.6	2.3
		60 - 80	7.3	320	2,336	8.8	36.6	25.4	1.4
LV reaction in egg-yolk emulsion	Cooked-meat broth	...	445	< 20	...	2.6	...	...	...
		0 - 40	1.54	40	60	12.8	3.3	...	...
		40 - 60	5.1	160	816	30	5.3	...	...
		60 - 80	12.4	< 20	...	21.9	...	...	...
	Infusion broth	...	460	< 20	...	0.75	...	...	...
		0 - 40	0.44	20	8.8	6.9	1.2	...	...
		40 - 60	3.1	160	496	8.8	18.3	...	...
		60 - 80	7.3	40	292	8.8	4.6	...	...
Lethal activity in mice	Cooked-meat broth	...	445	400	178,000	2.6	152	100	1
		0 - 40	1.54	1,600	2,464	12.8	123	1.4	< 1
		40 - 60	5.1	6,400	32,640	30	213	18.3	1.4
		60 - 80	12.4	400	4,960	21.9	18.3	2.8	< 1
	Infusion broth	...	460	1,600	736,000	0.75	2,133	100	1
		0 - 40	0.44	> 800	...	...	...	...	...
		40 - 60	3.1	6,400	19,840	8.8	731	2.7	< 1
		60 - 80	7.3	800	5,840	8.8	89	1	< 1

as an enzyme fractionation experiment (see Dixon and Webb, 1964, p. 32). The protein recovered in the three concentrated fractions of CMB or IB was 38 per cent. and 27 per cent. respectively; the yield of haemolysin was about 40 per cent; and about 20 per cent. of the lethal activity was recovered. The purification factors calculated on the basis of specific activity of the fractions are rather low.

At this point, preliminary experiments suggested that the biological activities of Cl. oedematiens were separable by gel-filtration. Thus, it seemed reasonable to concentrate the culture supernate in order to provide active starting materials, and then to fractionate the product on Sephadex columns. Polyethylene glycol (PEG) was used as the concentrating agent and the results of a typical concentration procedure are given in table XXX. Clearly, no purification is involved in this technique; the protein recovery is about 65 per cent., and 45 - 55 per cent. of the biological activity is retained. Subsequent work with type A and type D cultures of the organism suggested that the recovery of biological activity after concentrations with PEG may approach 100 per cent.

On the basis of the above results, the ammonium sulphate precipitation step does not appear to be justified with these cultures, and direct culture concentration followed by gel-filtration was used in the latter part of this work.

(ii) Gel-filtration. Gel-filtration studies were performed on columns of Sephadex gel during the present work. Initial experiments

Table XXX

The biological activities of a concentrated culture of Cl. oedematiens strain CR2B

Culture medium	Indicator system	Culture supernate (CS) or culture concentrate (conc.)	Volume (ml.)	Units per ml.	Total units	Protein mg. per ml.	Specific activity units per mg.	Yield per cent.	Purification
Infusion broth	Haemolysis of human cells	CS	427	32	13,664	2.5	13	100	1
		Conc.	40	160	6,400	17.3	9.3	47	< 1
	LV reaction in egg-yolk emulsion	CS	427	4	1,708	2.5	1.6	100	1
		Conc.	40	20	800	17.3	1.2	45	< 1
	Lethal activity in mice	CS	427	4,000	1,708,000	2.5	1,600	100	1
		Conc.	40	24,000	960,000	17.3	1,400	56	< 1

were carried out with a glass column (60 x 0.9 cm.) with a water-cooling jacket; the column was packed with Sephadex G-100. Phosphate buffer or PBS (pH 6.8, 0.02M with respect to phosphate) was used in most of the work; buffers such as tris-maleate buffer and Michaelis buffer gave UV absorption peaks on the protein recorder and their use in this part of the study was discontinued.

In the early experiments, concentrated ammonium sulphate-precipitated fractions prepared from cultures of Cl. oedematiens were used as the starting materials. The result of a typical column experiment with a concentrated fraction prepared from a CMB culture of a type A strain of the organism is given in fig. 23 (p.168a). The protein trace of the eluate from Sephadex columns in most of the experiments was similar; the fractions separated into an initial narrow peak of protein material followed by a second diffuse peak; the biological activities appeared predominantly before the second protein peak. In general, the lethal factor was eluted slightly faster than the haemolytic and LV components. A larger volume of the starting material was then applied to the column; the protein trace in this experiment formed a single, large diffuse peak and the biological activities were present in the same fractions of the eluate. It appears that the column was overloaded with material during this experiment.

The results of similar gel-filtration experiments with concentrated fractions prepared from type B and D strains of Cl. oedematiens are given in figs. 24 and 25 (p. 168b and c). The haemolytic and LV activities in both of the eluates are closely related; however,

FIG. 23. - The distribution of biological activities in fractions eluted from a column of Sephadex G-100 loaded with concentrated culture prepared from a type A strain of Cl. oedematiens.

= protein content;


= haemolytic activity;


= factor producing opalescence in egg-yolk emulsion.

* = Arbitrary estimate of level of activity in test material.

a

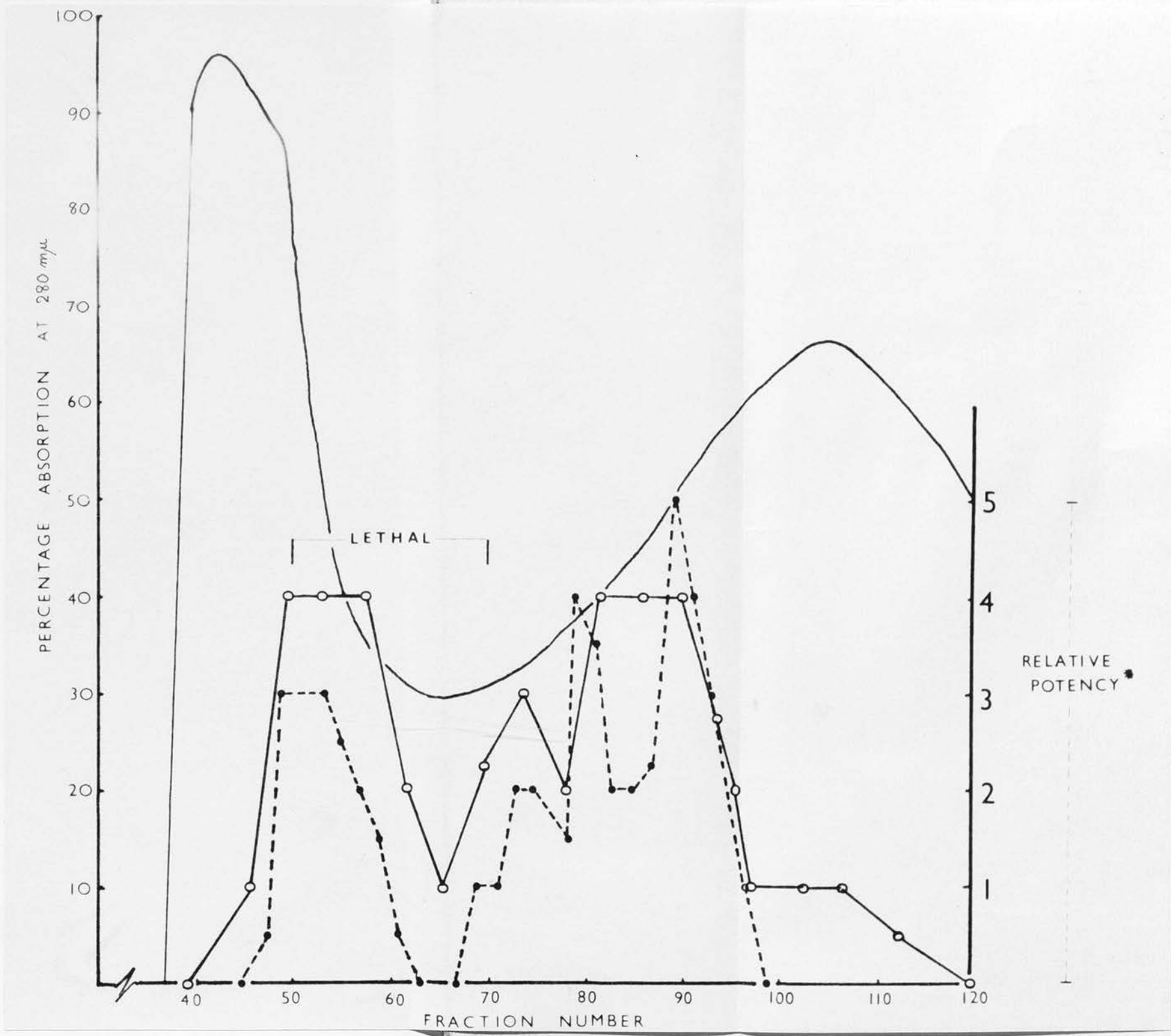
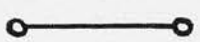

PERCENTAGE ABSORPTION AT 280 mμ
100
90
80
70
60
50
40
30
20
10
LETHAL
5
4
3
2
1
RELATIVE POTENCY
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

FIG. 24. - The distribution of biological activities in fractions eluted from a column of Sephadex G-100 loaded with concentrated culture prepared from a type B strain of Cl. oedematiens.

= protein content;


= haemolytic activity;


= factor producing opalescence in egg-yolk emulsion;


= cytopathic activity.

* = Arbitrary estimate of level of activity in test material.

b

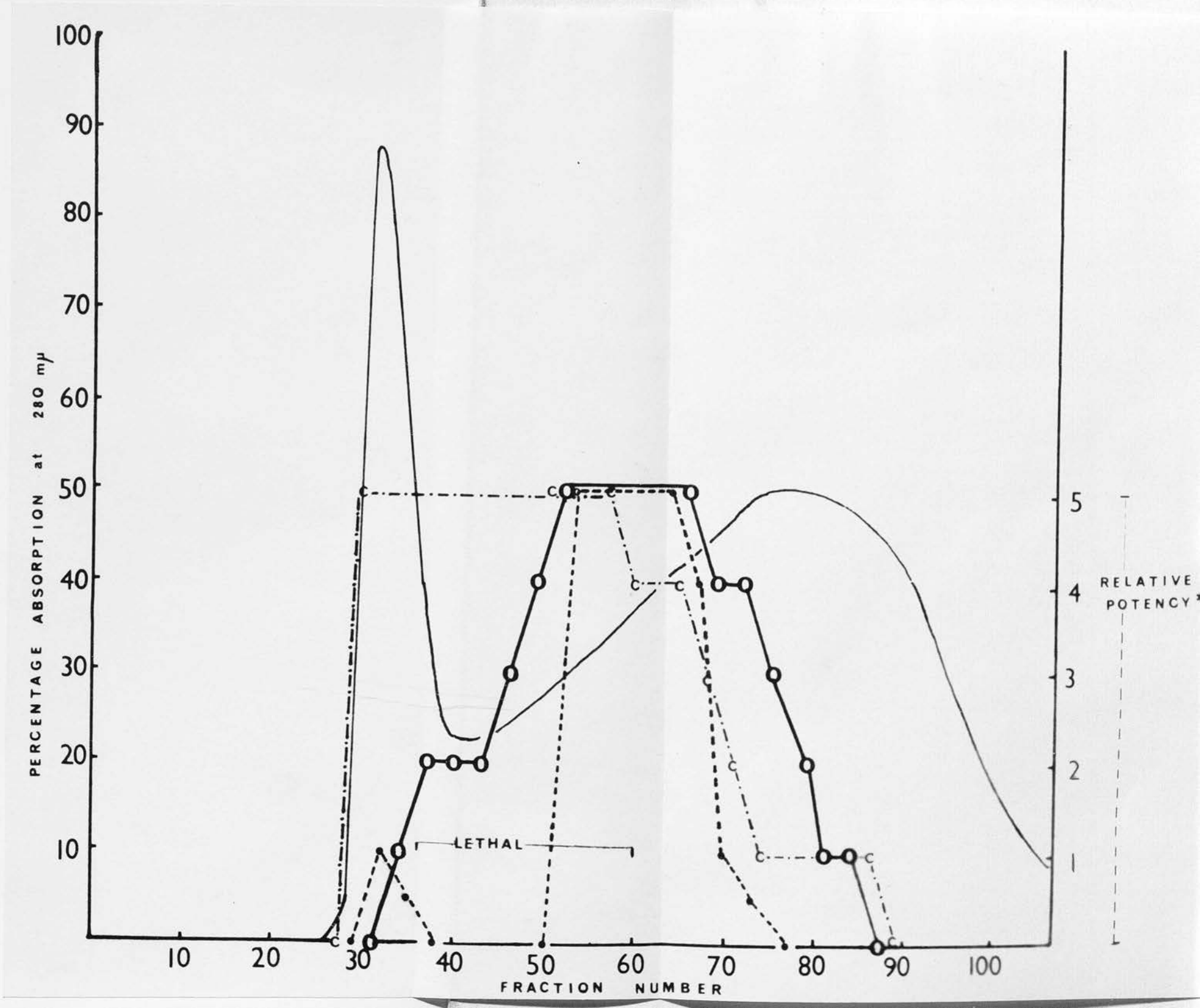
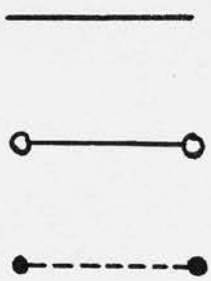

PERCENTAGE ABSORPTION at 280 mμ
RELATIVE POTENCY*
FRACTION NUMBER
LETHAL
100
90
80
70
60
50
40
30
20
10
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100

FIG. 25. - The distribution of biological activities in fractions eluted from a column of Sephadex G-100 loaded with concentrated culture prepared from a type D strain of Cl. oedematiens.



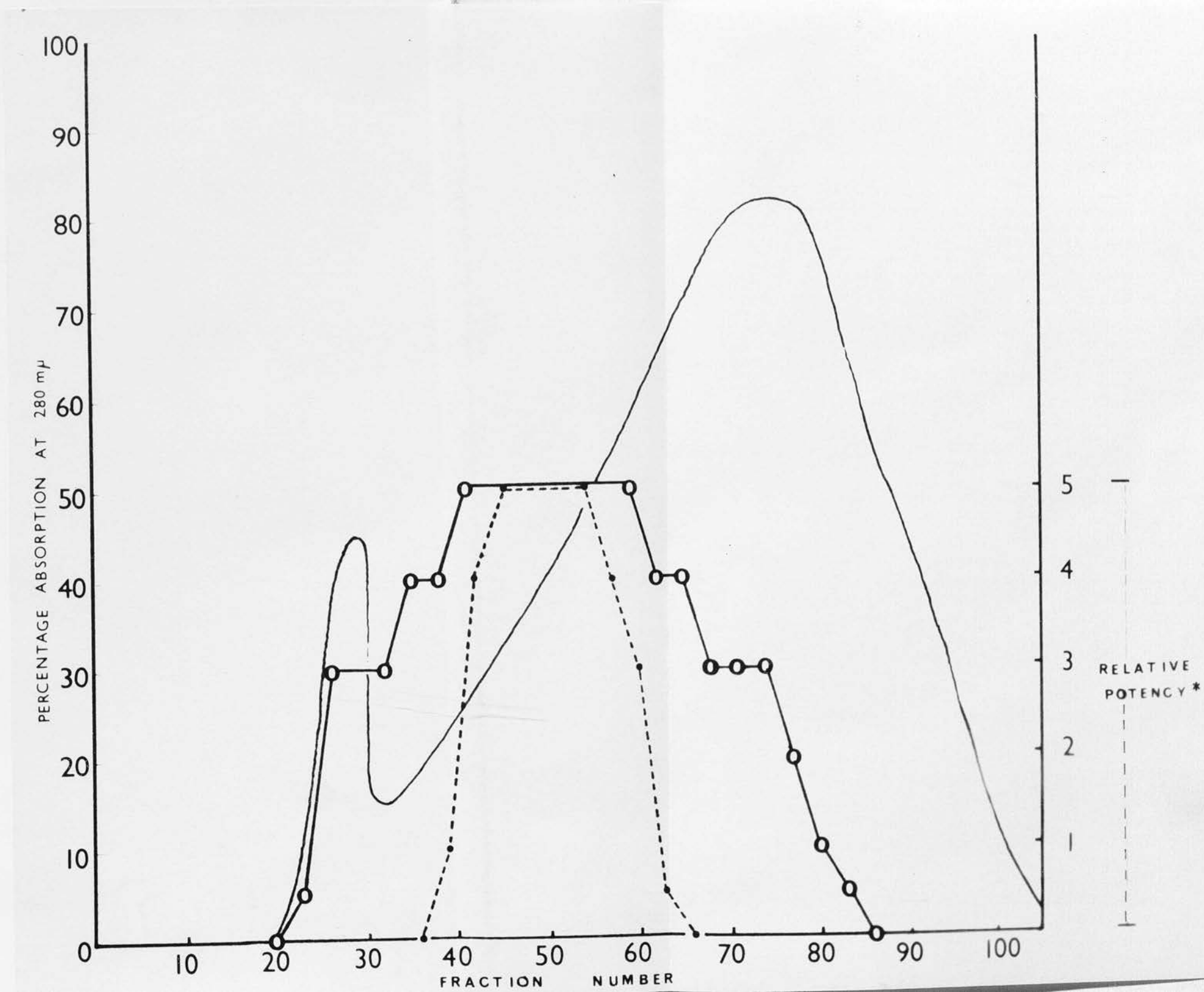
= protein content;

= haemolytic activity;

= factor producing opalescence in egg-yolk emulsion.

* = Arbitrary estimate of level of activity in test material.

C


100
90
80
70
60
50
40
30
20
10
0
PERCENTAGE ABSORPTION AT 280 mμ
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER
5
4
3
2
1
RELATIVE POTENCY*

there appears to be a slight separation of the cytopathic activity and the haemolytic activity in fig. 24.

As the separation of biological activities with Sephadex G-100 in the above column was not satisfactory, a similar column of Sephadex G-200 was prepared. The results obtained with this column were similar to those described above; there was a slight separation of the lethal and haemolytic activities; the recovery of biological activity was 45 per cent. of that applied and the purification factor was 1.5. Subsequent experiments with buffers at pH 5.0 and 8.0 did not enhance separation of the biological activities.

The results above suggested that Sephadex gel was capable of separating the soluble products of Cl. oedematiens, and it seemed that a longer column might give better resolution of the biological activities. In the following work a larger column (100 x 2.7 cm.) packed with Sephadex G-200 was employed; the column did not have a cooling jacket. Problems associated with settling of the gel in the column were encountered; in the early experiments the flow rate gradually decreased and the protein trace was greatly extended. A gravity-feed reservoir was used in the present work and the hydrostatic head (measured from the surface of the reservoir to the level of the outlet above the fraction collector) should not exceed 15 cm. if a satisfactory flow rate is to be maintained.

The separation of biological activities with the larger column of Sephadex G-200 was considerably better than in previous experiments. The results of a typical fractionation experiment are shown in fig. 26

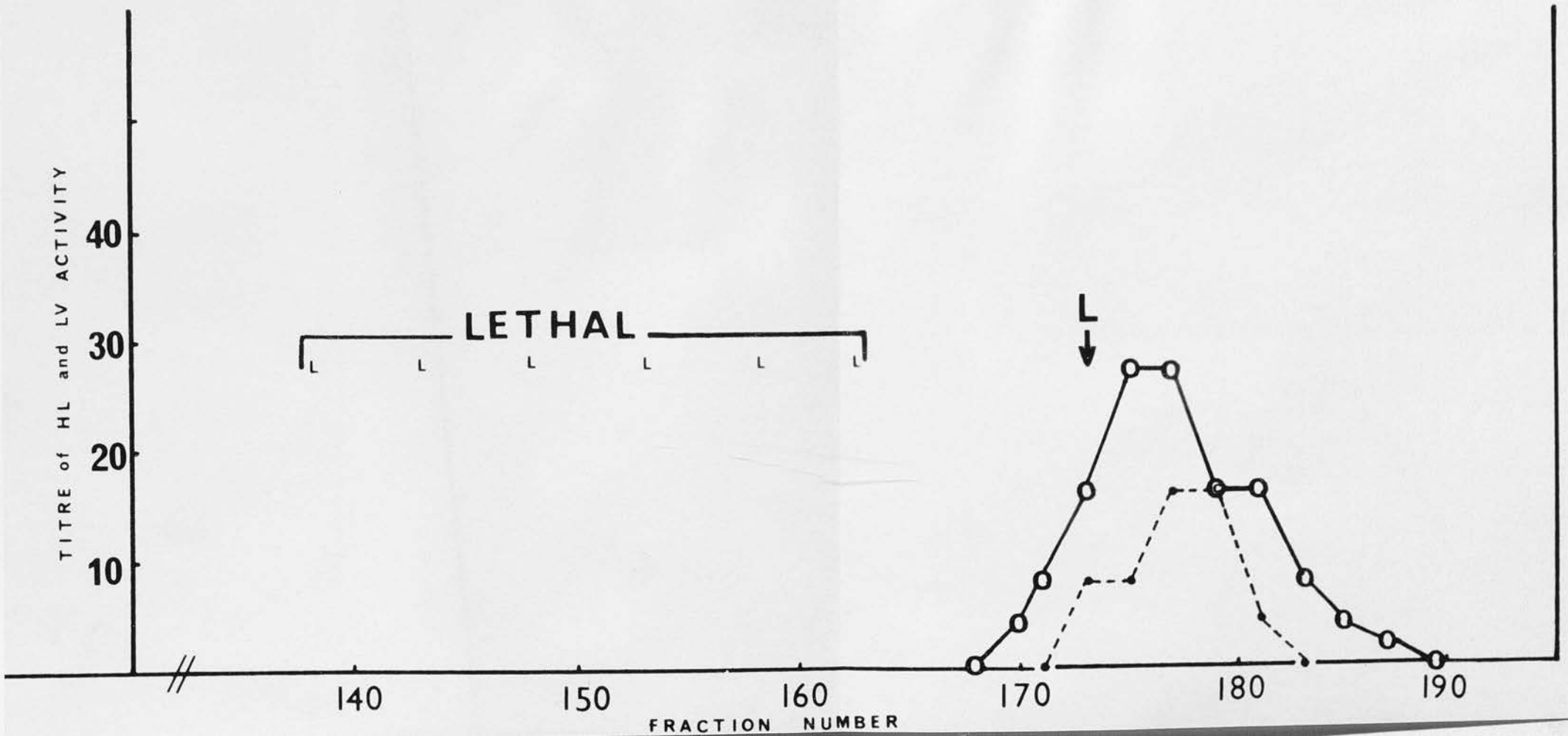
FIG. 26. - The distribution of biological activities in fractions eluted from a column of Sephadex G-200 loaded with concentrated culture prepared from a type B strain of Cl. oedematiens.

 = haemolytic activity;

 = factor producing opalescence in egg-yolk emulsion.

Note: (i) Lethal activity was detected in fraction 173 that presumably represents the 'tail' of the alpha antigen;

(ii) The titrations of haemolytic and LV activity were performed in perspex plates; inaccuracies associated with this technique (see text) may account for the apparent partial separation of the activities.


TITRE of HL and LV ACTIVITY
40
30
20
10
LETHAL
L
L
L
L
L
L
L
140
150
160
170
180
190
FRACTION NUMBER

(p. 169a). The protein traces in these experiments again show a double peak, but the traces are more elongated than those in figs. 23-25 (pp. 168a - c). The lethal activity appears just after the void volume and the initial protein peak; haemolytic and LV activities are eluted much later during the early part of the second protein peak, but there is a partial overlap of biological activities in the centre of fig. 26. Eluted fractions that contained lethal or haemolytic activity were pooled and reconcentrated. The recovery of haemolytic and LV activity in the reconcentrated fractions was 25 per cent. of the total applied to the column; 2-fold purification was effected. Only 6 per cent. of the lethal factor was recovered, and the degree of purification was less than 1 in terms of the specific activity. Appropriate neutralisation tests indicated that the alpha and the beta antigens were responsible for the respective biological activities.

It appears from these results that the lethal factor of Cl. oedematiens is more labile than the haemolytic factor. This observation may be related to a steadily decreasing flow rate during the experiment and the slow passage of soluble products through an unjacketed column. Subsequent results obtained with columns with more rapid flow rates showed that 34 per cent. of the lethal factor was recovered in one experiment; purification was almost 3-fold in terms of the specific activity.

The preparation of starting material that was potently lethal was achieved at the expense of haemolytic and LV activities, which are

usually rather weak in infusion broth cultures. Further dilution occurred during the fractionation procedure and on several occasions haemolytic activity without apparent LV activity was detected in the eluted fractions. Experiments with a concentrated type D culture of the organism indicated that the haemolytic and LV activities of this organism eluted in identical fractions; the haemolytic titre of the reconcentrated partially-purified fraction was 8-fold greater than the LV titre. It was calculated that the haemolytic activity detected in experiments with type B cultures occupies a similar position in the elution volume to that of the beta antigen of type D cultures. It was therefore concluded that the absence of LV activity in the above experiments reflects the relative sensitivities of the indicator systems.

On the basis of the above results it appears that the lethal activity in cultures of type B strains of Cl. oedematiens can be separated from the haemolytic and LV activities with a suitable column of Sephadex G-200. Small columns do not give satisfactory resolution of the biological activities. The yield of the biological activities is rather low and thus the degree of purification is comparatively poor; this may be related to the lability of the soluble products and the need for more efficient protective measures is indicated during the fractionation procedures.

The production of antisera

A preliminary attempt was made to compare the antigenicity of crude and partially-purified fractions of Cl. oedematiens. The antisera were prepared in rabbits (see Methods) and were tested for their protective capacity in mouse lethality neutralisation tests.

The biological activities of the two antigenic preparations are given in table XXXI; the anti-lethal antibody levels in the respective sera are shown in table XXXII.

It should be borne in mind that the results given in table XXXII were obtained in experiments that employed doubling dilutions of the test materials; the values merely represent an indication of the level of activity. Clearly, the neutralising power of these antisera is low compared with the experimental and commercial antisera; however, the levels were produced after only two injections of the antigen; the rabbits were not hyperimmunised. It is significant that the partially purified antigenic preparation stimulated a reasonably potent anti-lethal component, bearing in mind the low protein content and the low level of lethal activity in Fraction I.

Immunodiffusion studies

Ellner and Green (1963) used an agar-gel diffusion system in studies of the precipitating soluble antigens produced by a number of clostridia. The soluble antigens of the species that they examined

Table XXXI

The biological activities of the antigens used for the production of antisera

Antigen	Protein content (mg. per ml.)	Lethal activity (mouse LD per ml.)	Haemolytic activity (haemolytic units per ml.)	LV activity (LV units per ml.)
Fraction I	0.7	200	< 20	< 20
Fraction II (crude fraction)	17.3	24,000	160	20

Table XXXII

The antibody levels produced in the rabbit

Antigen	Antibody levels (anti-mouse LD per ml. of antiserum) present in the antisera on day		
	0 (control)	8	20
Fraction I	< 150	300 (2)*	3000 (20)*
Fraction II (crude fraction)	< 75	3000 (20)*	12000 (75)*

*The figure in brackets refers to the approximate level of anti-alpha units in each antiserum, calculated on the basis that 1 anti-alpha unit of Cl. oedematiens experimental antiserum EX1546 neutralises 160 mouse LD.

were very heterogeneous and reciprocal and non-reciprocal cross-reactions occurred. These workers speculated whether the cross-reactions that they observed with type A and B strains of Cl. oedematiens might be related to the alpha, beta or gamma soluble antigens described by Oakley et al. (1947). Gel-diffusion tests with culture products of Cl. oedematiens were performed during the present studies in order to determine the components that are involved in some of these reactions.

My early experiments were with ammonium sulphate-precipitated fractions derived from CMB cultures of type A strains of Cl. oedematiens; several precipitation lines were obtained in tests with homologous or heterologous antisera (figs. 27 and 28, pp. 175a and b). Further work showed that the fraction precipitated at 40 - 60 per cent. salt saturation gave the greatest number of lines in these tests, and the 0 - 40 per cent. fraction gave no visible lines. Tests with concentrated culture products obtained from strains of other types of the organism gave similar results (see figs. 29 and 30, pp. 175c and d). Partially-purified culture fractions prepared in gel-filtration experiments gave fewer precipitation bands than the parent material, and cross-reactions with heterologous antisera still occurred.

It is possible that certain constituents of culture media are antigenic and may be responsible for some of the cross-reactions described above; however, no precipitation bands were observed in tests with concentrated, uninoculated culture media. It seemed probable that most of the cross-reactions were caused by bacterial cell

Note. In the following figures, the reactants were prepared from cultures of Cl. oedematiens that were precipitated at different levels of saturation of ammonium sulphate. Thus, GR1A 0/60 is the product of strain GR1A precipitated by the addition of ammonium sulphate to 60 per cent. saturation.

a

Immunodiffusion experiments

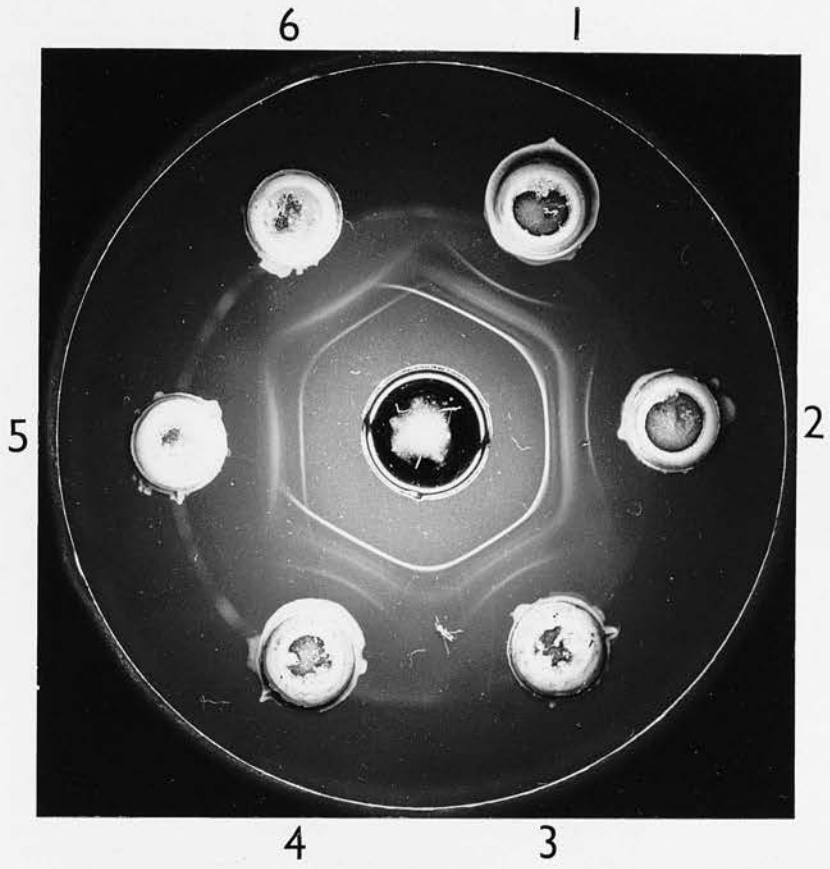


FIG. 27. - Centre well: Cl. oedematiens type A antiserum;

Outer wells: 1 and 2 = GR1A 0/60,
3 and 4 = GR2A 0/60,
5 and 6 = GR1A 60/100.

Immunodiffusion experiments

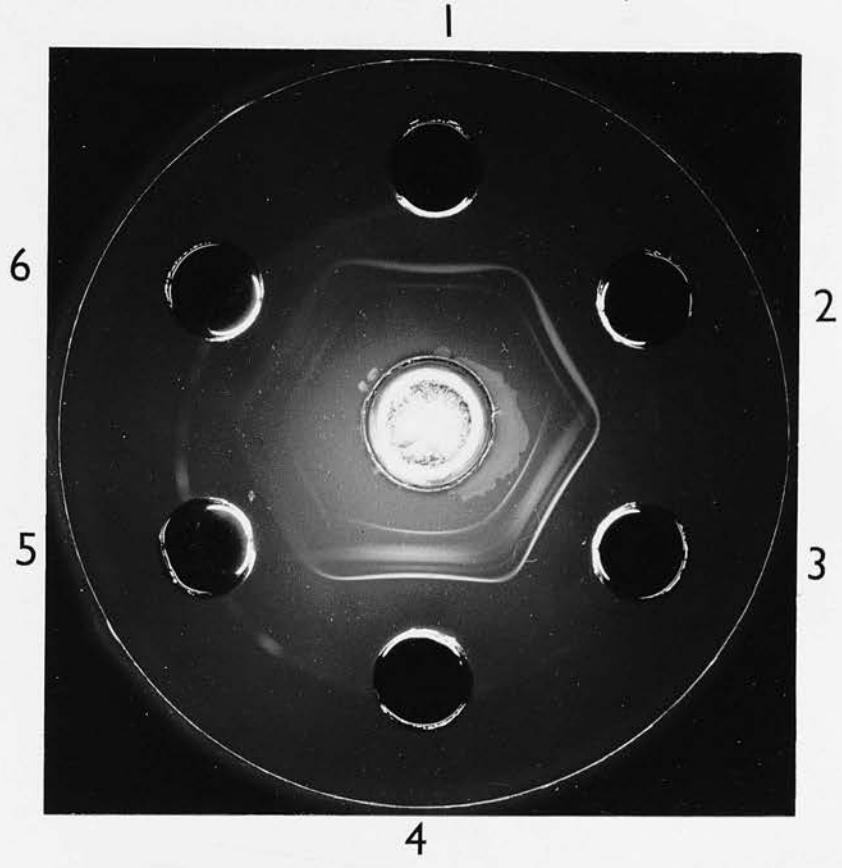


FIG. 28. - Centre well: GR1A 0/60;

Outer wells: 1 and 2 = Cl. oedematiens type B antiserum,

3 and 4 = Cl. oedematiens type A antiserum,

5 and 6 = Cl. oedematiens type D antiserum.

Immunodiffusion experiments

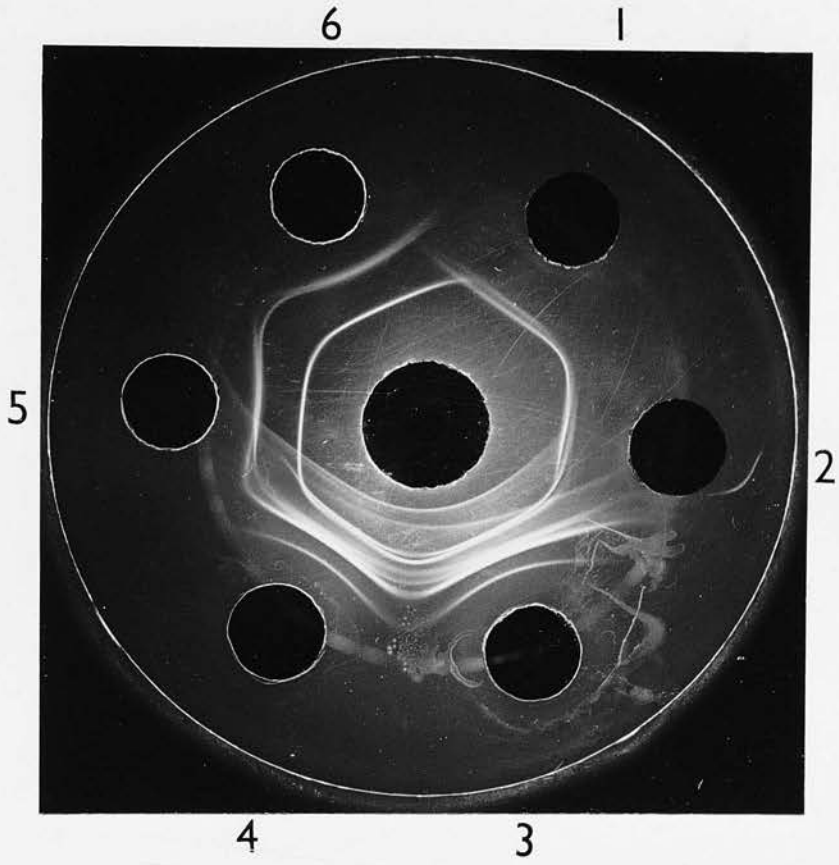


FIG. 29. - Centre well: Cl. oedematiens type B antiserum;

Outer wells: 1 and 2 = GR1D 0/100,
3 and 4 = GR2B 0/60,
5 and 6 = GR2A 0/60.

Immunodiffusion experiments

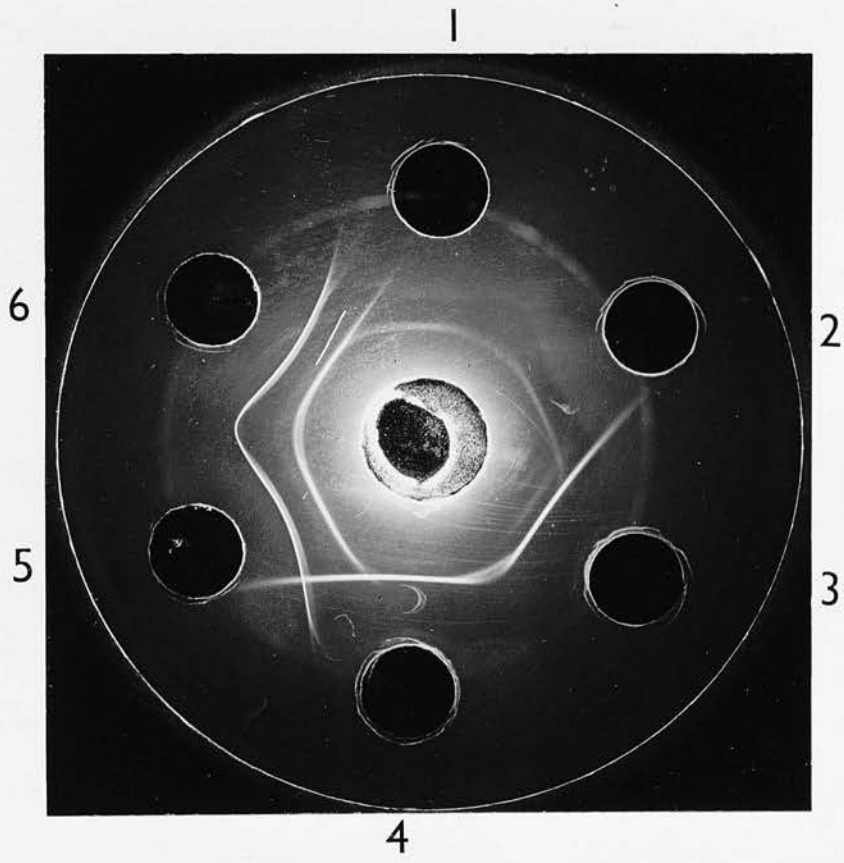


FIG. 30. - Centre well: Cl. oedematiens type D antiserum;

Outer wells: 1 and 2 = GR2A 0/60,
3 and 4 = GR1D 0/100,
5 and 6 = GR2B 0/60.

antigens that are present in each type of the organism. Twice-washed and resuspended bacterial cells obtained from an infusion broth culture of a type B strain of Cl. oedematiens gave at least five lines in tests with a homologous antiserum; it was found that most of the lines that develop with concentrated products prepared from type A and B strains of the organism are identical with lines that appear with the bacterial cells. The cell suspension also gave two lines with a type D serum and three lines with a type A serum that were identical with lines obtained in tests with the homologous (type B) serum. Washed cell suspensions obtained from CMB cultures usually give fewer lines in gel-diffusion tests than the concentrated product that is prepared from the same cultures. Previous results indicate that thrice-washed bacterial cells possess traces of haemolytic activity demonstrable only after ultrasonic disintegration of the cells; thus, it is unlikely that the precipitation lines that develop with the bacterial cell suspensions are caused by biologically active soluble products.

These results confirm the findings of Ellner and Green that several cross-reactions occur in tests of the precipitating antibodies of Cl. oedematiens; it appears that most of these cross-reactions are caused by antigens that readily become soluble and are associated with the bacterial cells. This observation is consistent with our previous knowledge of the serology of this group of organisms. The number of precipitation lines that develop in these tests depends to some extent on the culture medium, but it is clear that a type B

strain gives the greatest number of lines with homologous and with heterologous antisera. At least one type-specific line was obtained in each case with a type A and type D culture tested against the respective antiserum. A concentrated product derived from strain GR1C in CMB gave one or sometimes two lines in tests with heterologous antisera; the bacterial cell suspension obtained from this culture gave no precipitation lines in similar tests.

The writer then decided to use the immunoelectrophoretic procedure to detect the precipitating antigens of Cl. oedematiens, as this technique is more sensitive than double diffusion tests in agar plates. Preliminary experiments suggested that a phosphate buffer of low molarity (see Methods) was satisfactory in the electrophoretic system; runs were performed at a constant voltage of 250V for 90 - 120 min. It was found that a suspension of bacterial cells of strain GR1B grown in infusion broth gave eight precipitation lines with a commercial homologous antiserum; five lines migrated towards the anode and three moved towards the cathode. The cell suspension gave five lines in tests with a commercial type A antiserum and at least three of these were shared with the type B serum.

Note: The immunoelectrophoretic results suggest that some of the antigens associated with the bacterial cells of Cl. oedematiens might be separated on charged DEAE columns; and it would also be of interest to develop the precipitation lines after electrophoresis of the antigen with the fluorescent labelled globulin. Further immuno-

diffusion investigations may elucidate the relationship of the cellular antigens within the Cl. oedematiens group, and it is possible that some of the antigens may be related to the cellular antigens of other clostridial species; this information may be of value to the taxonomist. However, it is clear that considerable caution must be exercised in the provisional identification of the precipitation lines, and it seemed that this approach was unlikely to yield further significant information during the present study.

DISCUSSION

The reported occurrence of Clostridium oedematiens depends largely on the success of techniques for the isolation and identification of this demanding organism. The work of Oakley and his colleagues (1947, 1959) provided a serological basis for the recognition of four distinct types of Cl. oedematiens, but the knowledge has not been fully utilised in the field. This is almost certainly related to the difficulties of isolating and subculturing the organism, and the problems that were encountered early in the present study made it clear that more laboratory work with this organism is essential before further field studies can yield accurate data. There are numerous reports of the isolation of this demanding anaerobe in deep-agar cultures (Turner, 1930; Smith and Jasmin, 1956), and Williams (1962, 1964) commented on the frequent failures of isolation of type B and D strains on solid media. Although the deep-agar culture technique is now superseded in anaerobic bacteriology, the success that accompanies this procedure should not be too readily discounted.

The introduction of the fluorescent staining technique for Cl. oedematiens was followed by several descriptions of the presence of this organism in smears prepared from various organs in cattle, sheep and pigs; in only two of these reports was the organism isolated from the infected material. Corbould (1966) encountered considerable difficulty: "a few isolates" were obtained from 82 ovine livers that contained Cl. oedematiens demonstrable with the fluorescent technique. Bourne and Kerry (1965) isolated an organism from cases of sudden

death in pigs that was identified as Cl. oedematiens on the basis of the fluorescent stain; the strain was not typed and the authors then stated without further evidence that "the post-mortem findings do not suggest that it is type B." The fluorescent globulin is apparently species-specific, but the recent tendency to use the fluorescent procedure as the sole criterion for identification of Cl. oedematiens is not justified. Isolation and type identification of the organism are subsequently necessary in order to evaluate the specificity of the commercial product and to characterise the organism that is involved. Thus, the writer was obliged to accept the challenge of investigating the problems associated with the isolation, subculture and identification of this apparently fastidious anaerobe.

In spite of my reservations concerning the fluorescent technique, the procedure has an obvious application to studies of Cl. oedematiens and was investigated extensively during the present work. The technique gave good results, although certain technical problems merit further investigation. A report of my initial impression of the fluorescent procedure was sent to the Wellcome Laboratories; the report criticised the keeping quality of the stain and noted that a variable degree of fluorescence often occurred in the same smear. Batty (personal communication) agreed that the manufacturers relied too heavily on the bactericidal effect of fluorescein isothiocyanate and thiomersal; the thiomersal apparently gave rise to the granular precipitate that was observed in the stain. The sterilisation procedure for the product had already been changed and subsequent batches

of the stain gave no further trouble. Batty pointed out that the manufacturers prepare the antigen from "very short-growth cultures" in order to get a monospecific antiserum, and agreed that the degree of fluorescence seems to depend largely on the age of the culture that is used to prepare the smear.

The success of the procedure with strains of all types of the organism confirms the observations of Turner and Eales (1943) that species-specific somatic antigens are present throughout the group. The irregular staining of organisms taken from older cultures and from cultures in different growth media may reflect the absence of these common antigens or their inaccessibility to the labelled globulin. In spite of these criticisms, the writer believes that the fluorescent technique has an important place in studies of Cl. oedematiens.

Early in the present work, it became clear that type A strains of Cl. oedematiens grew consistently well on solid media incubated anaerobically, but that strains of the other types usually failed to grow; there seemed to be little point in attempting to isolate the organism from natural sources until more reliable methods were developed for culture on solid media.

At first, it seemed that the failures of growth associated with type B, C and D strains might be related to failures of the anaerobic environment; accordingly, the early part of this investigation involved a reappraisal of the anaerobic technique. Cl. oedematiens often grows as a spreading film on solid media, and the spreading effect

appears to be related to the humidity of the environment. In order to eliminate the moisture that is contributed to the jar environment by passing hydrogen gas through liquid in a wash-bottle before admission to the jar, some time was spent perfecting the use of simple manometry as an indication of catalyst activity. It was found that the rapid development of a secondary vacuum in the anaerobic jar is a satisfactory indication that the room-temperature catalyst is active, but an increasing negative pressure was observed in anaerobic jars over a period of 120 hr, suggesting that the catalytic reaction between hydrogen and oxygen may occur rather slowly. It should be borne in mind that a small volume of air is admitted to the jar each time the manometer is used and this may allow successive reactions leading to an increasing vacuum, nevertheless it seemed necessary to consider the rate of production of anaerobiosis in the BTL jar. The results of experiments performed with an indicator of anaerobiosis suggest that an anaerobic environment is produced in the BTL jar within 2 - 3 hr, however, this may not be a critical demonstration of the absence of oxygen at a level that may yet affect the viable particles. Parallel experiments showed that inconsistent surface growth of Cl. oedematiens occurs in jars from which 2/5 or 6/7 of the air is evacuated initially, and a prolonged series of tests indicated that the degree of evacuation does not regularly affect the initiation of surface growth. It was concluded that the irregular growth of these strains is not necessarily related to faults in the normal anaerobic procedure. Despite these observations, apparently superior results were often obtained in the

electrically-operated BTL jar and this may be related to the rapid development of a suitable microenvironment within this type of jar (q.v.).

The sensitivity of anaerobic bacteria to oxygen has led various workers to exclude air from the environment during the subculture of these organisms; Drasar (1967) claimed that he obtained better recovery of certain anaerobes by this method. Recent studies in this laboratory suggest that the subculture of Cl. oedematiens in an environment of carbon dioxide does not give more reliable recovery than subculture in the presence of oxygen, although the method designed by Drasar was considerably more exacting.

The controversy as to why anaerobic organisms fail to grow in the presence of oxygen is still unsettled. Gordon, Holman and McLeod (1953) reviewed an earlier suggestion (McLeod and Gordon, 1923) that the intolerance of anaerobes to oxygen is due to the formation of hydrogen peroxide. These authors observed that "the production of traces of hydrogen peroxide when reducing systems are oxidised with oxygen is well known," and they presented evidence that centrifuged deposits of anaerobic bacteria that were freed from traces of medium and metabolites give positive reactions for peroxide after oxygenation. They also suggested that proteolytic activity may increase the concentration of substances that are capable of masking the presence of hydrogen peroxide. Catalase is one enzyme that splits hydrogen peroxide and it is well known that many anaerobic bacteria are catalase-negative. Holman (1955) suggested that anaerobic bacteria grow

nearer to the surface of shake cultures if a preparation of catalase is added to the upper layer of the medium. Mateles and Zuber (1963) pointed out that the catalase used by Holman was impure and may have contained a number of reducing agents; these workers were unable to confirm Holman's observations with a purified preparation of crystalline catalase. Mechanisms in addition to catalase are now thought to exist for the breakdown of peroxides (see Whittenbury, 1964), and the difficulties associated with the design of critical experiments are reflected by our present superficial knowledge almost 50 years after the original suggestion that peroxide toxicity is involved.

Willis (1965, p. 83) attempts to relate the presence of oxygen to the problem of subculturing Cl. oedematiens; he states that the organism is so sensitive to free oxygen that it dies unless special precautions are taken during subculture. The present writer accepts that the formation of peroxides may inactivate vegetative cells, but considers that bacterial spores are likely to be resistant to oxygen; thus, an inoculum on solid medium should be viable if conditions are suitable for the germination and outgrowth of spores. The following experiments were designed to investigate whether the initiation of growth of Cl. oedematiens is related to the presence of spores in the inoculum. This theory was supported by the following observations: (i) type A strains of Cl. oedematiens grow readily on a variety of solid media and preliminary observations suggested that these strains sporulate more readily than strains of the other types; (ii) growth of type A and B strains was obtained on solid media after heating the

inocula; and (iii) heavier growth of type B strains was obtained on plates that were heated after inoculation. The latter observation indicates that heat activation of the spores occurs. It was also noted that a spreading film of growth on solid medium gives an impression of profuse growth, although it may be derived from only a few viable particles.

Experiments were then performed to relate the spore content of cultures to their viability; it was found that overnight cultures of type B strains of the organism invariably contain no visible spores; sporulation occurs with prolonged incubation, and viable counts performed on these cultures show a significant increase between 1 and 4 days. The ratio of the estimated spore count to the viable count was about 1 to 150 and this implies that a large number of spores fail to germinate. It is necessary to be cautious in interpreting estimates of the number of spores present in these cultures; for example, when dealing with bacterial populations of the order of 10^6, a spore estimate of "less than one per cent." may represent $0 - (9 \times 10^3)$ spores in the culture. The presence of spores thus appears to be related to the viability manifested in deep agar cultures and on spread-plate cultures, but it seems that other factors are also involved. Stained smears of cultures of type C and D strains confirmed that these strains do not sporulate readily after overnight incubation, and as a corollary to these observations, it is possible that the absence of spores may account for the non-viability of these strains on solid media.

There is ample evidence that some anaerobic bacteria require the

medium to be partially reduced before proliferation can occur (see Hewitt, 1950, p. 115). In the present work, all strains of Cl. oedematiens grew consistently well in tubes of cooked-meat broth medium, but failed to grow in tubes of nutrient broth or peptone water medium. Cooked-meat broth and nutrient broth are identical except that cooked-meat broth contains meat particles, and it seems that one or more factors present in meat particles allows the initiation of growth of this demanding organism. It is well known that meat particles lower the redox potential of a broth medium; experimental results in the present work with redox indicator dyes show reasonable agreement with the observations of Lepper and Martin (1930) who found that the Eh in cooked-meat broth approaches -200mV. However, the pitfalls associated with the estimation of redox potentials by means of indicator dyes were borne in mind. The addition of reducing agents such as glucose or sodium thioglycollate to tubes of nutrient broth does not allow the consistent growth of type B, C and D strains of Cl. oedematiens, and it may be significant that observations with the indicator dyes suggest that these reducing agents do not lower the Eh of nutrient agar shake-cultures to the level obtained in cooked-meat broth.

In conjunction with the above investigations, experiments have continued in an effort to obtain consistent growth of type B, C and D strains of Cl. oedematiens on solid media. It was found that the addition of iron filings to the surface of human blood agar plates gives more profuse, much more reliable and more rapid growth of these strains than is obtained on control plates. It appears that the

effect is specific for Cl. oedematiens and does not occur with Cl. tetani, another demanding anaerobe. Metallic iron is oxidised (i.e. loses two electrons) to the ferrous ion at a redox potential of -440mV, and it is possible that the availability of electrons at this low redox potential is critical for the outgrowth of viable particles. Heated ('chocolate') blood agar is the most satisfactory medium for growth of Cl. oedematiens in the absence of iron filings, and the relative success of this medium is presumably attributable to the release of one or more factors from the disrupted blood cells; these probably include iron and reducing agents. Although catalase may play a part in metabolic processes that involve available oxygen, it may be unnecessary to introduce theories concerning this enzyme into considerations of the initiation of growth of Cl. oedematiens. If we accept that the vegetative cells of this organism are readily inactivated on exposure to oxygen, the growth obtained on routinely used solid media in an anaerobic jar may primarily depend on the presence of spores and the consistent development of a suitable redox potential around the viable particles. This involves considerations of the spore content of the inoculum, the efficiency of the anaerobic procedure, and the local Eh of surface cultures.

The above observations are speculative. The present results merely indicate that the presence of spores appears to be associated with the viability of Cl. oedematiens, and that reliable growth of type B and D strains occurs in the presence of iron filings. Much more work is required to determine whether the surface growth of

Cl. oedematiens is derived from spores alone, and whether the outgrowth of viable particles of type B and D strains is related to a low redox potential. It would now be of interest to determine whether viable counts on solid media with iron filings approximate more closely to the spore content of cultures. A simple synthetic medium would be useful in future studies, and experiments with single cell or spore isolates transferred to liquid media that are poised at different levels of Eh may be of value. Whatever the explanation of the iron filings phenomenon, the effect can now be utilised to obtain relatively reliable surface growth of type B and D strains of Cl. oedematiens and this will facilitate the isolation of pure cultures.

It became clear during the present investigation that a straightforward description of a recommended method for the isolation of Cl. oedematiens is not recorded in the modern literature. As a guide for future workers, an isolation procedure (fig. 31) has been compiled on the basis of previous reports and the writer's own experience. The procedure was used successfully in the present studies, and it is now being evaluated in field trials (Bagadi, personal communication); the outcome of further studies is awaited with interest.

A number of cultural procedures are described in fig. 31 and the organism should be isolated by at least one of these. Differential heating is of considerable value in reducing the viability of contaminating organisms; the samples should be heated in sealed, thin-walled glass ampoules, and two different heating schedules are recommended as spores of type D strains may be inactivated at the higher

LIVER LESION:

I Remove aseptically with adjacent tissue.

II a) make impression smears, stain with Gram's method and the fluorescent globulin technique;

b) make an impression culture on selective blood agar plates*;

c) extract a pipette core of tissue into Brewer medium and into CMB; incubate anaerobically for 6 hr then subculture onto selective blood agar*.

III Macerate a portion of the lesion with CMB, then

a) make smears of the macerate (see II above);

b) subculture into CMB and onto selective blood agar plates*;

c) heat 0.5 ml. of the macerate in sealed glass ampoules for 15 min. at 65° C, and for 4 min. at 100° C; subculture from each ampoule into CMB and onto selective blood agar plates*.

IV *Incubate the plates anaerobically at 37° C with 10 per cent. of carbon dioxide added to the environment for at least 48 hr.

FIG. 31. - A scheme for the isolation of Cl. oedematiens from infected material.

temperature. The selective solid medium described on p. 75 is not specific for Cl. oedematiens, and growth of the organism is not guaranteed on this medium; it may be necessary to modify the medium in the light of our developing knowledge; for example, the inclusion of iron filings on the surface of the medium should now be considered.

The classification of isolates provisionally identified by the fluorescent staining technique is best achieved by analysing culture products of the organism for the presence of biological activities that are characteristic of the alpha, beta or gamma antigens. Strains may be encountered in the field, however, that fail to produce detectable amounts of the major soluble antigens; presumably, these strains will be identified as type C strains in the above system, but if other tests are available it might be possible to assess the relationship of these strains to poorly toxigenic strains of types A and B. At present, our knowledge of the characters of the Cl. oedematiens group is poorly defined, and a re-examination of the cultural and biochemical properties of the organism was indicated. The fermentation reactions of the group were selected as being most likely to yield meaningful results with the different biotypes.

Preliminary experiments indicated that Cl. oedematiens fails to grow consistently in most of the recognised fermentation media. After extensive trials with variations of media, cooked-meat broth was chosen as the basal medium for these tests. Cooked-meat broth contains a small amount of fermentable substrate and this presumably accounts for the fall of pH that occurs during incubation of test

strains in the control medium. The choice of a basal medium that contains inherent fermentable material can be criticised; however, suitable controls were included in each series of tests and the medium had the advantage of supporting reliable growth on every occasion.

Delayed fermentation reactions occurred in this medium and may be attributable to a diauxie effect; there may be preferential utilisation of fermentable material in the medium followed by a delay during which suitable enzyme systems are produced to degrade the added substrate. In addition, 'doubtful reactions' were obtained with some of the freshly-isolated type B strains in tests with glycerol and to a lesser extent with fructose; in these tests, the final pH of the medium is about 0.5 unit below that of the substrate-free control. Most of the tests were repeated and similar results were obtained after 10 days' incubation. It appears that the doubtful reactions are consistent with individual strains, and although it is conceivable that inhibitory factors are produced during degradation of the substrates, it seems more likely that these strains lack enzymes required for the complete utilisation of the carbohydrates.

Large volumes (100 ml.) of cooked-meat broth were used in the present fermentation reactions so that there was sufficient material for serial tests during prolonged incubation. Tests performed in 5 x $\frac{5}{8}$ in. tubes of the basal medium gave equally satisfactory results and are easier to handle; the pH of the culture supernates should be determined after 2 and 5 days' incubation of the test cultures.

Time did not permit a more complete characterisation of the fermentation reactions of Cl. oedematiens during this section of the work. Tests performed in Brewer medium during the early part of the present investigation gave unsatisfactory results, and the object of my experiments was to develop a reproducible method for testing the fermentation reactions of this group of organisms. A limited number of strains and substrates were intensively studied and the results suggest that cooked-meat broth is a suitable basal medium for these tests; it should now be possible to exploit the procedure that has been developed in a wider range of tests. Recent studies indicate that type A strains of Cl. oedematiens may give delayed fermentation reactions with mannose. This substrate was not tested in earlier experiments with these strains in cooked-meat broth, but it should be borne in mind that glucose and mannose are closely related aldohexoses.

Oakley et al. (1947) stated that 45 type A strains and 14 type B strains of Cl. oedematiens showed considerable variation in the fermentation of maltose and glycerol. These authors noted that irregular growth of type B strains occurred in the fermentation medium that they used. Screening tests with a large number of strains of this organism and a variety of substrates are now necessary in order to assess the consistency of the results in cooked-meat medium. The fermentation reactions of Cl. oedematiens do not give sufficiently rapid results to be of value in a routine system of typing, but the writer suggests that they are a useful adjunct to the present system

that is based on detection of the soluble antigens. In addition, a knowledge of the fermentable substrates may be of value in the design of enrichment media for this organism.

The present studies of the soluble products of Cl. oedematiens also exploited the reliable growth that was obtained in tubes of cooked-meat broth. Although yields of certain of the biologically active components of this organism are greater in infusion broth or horseflesh digest broth, cultures in cooked-meat broth reliably produce detectable amounts of the soluble antigens which are important in the typing procedure.

Two problems associated with the use of cooked-meat broth medium in a routine system of typing were identified in the present work. The first of these is that occasional negative LV reactions occur with type B strains in this medium; it is significant that Keppie (1944) and Jamieson (1949) reported similar findings. Prolonged incubation of the test mixtures at 37° C may cause apparently negative reactions to become positive, but this does not appear to be a generally recognised procedure.

Oakley et al. (1947) showed that the beta antigen of Cl. oedematiens is a haemolytic lecithinase, and the writer proceeded to examine the haemolytic activity of cooked-meat broth cultures with a view to incorporating this test in a routine system of typing. It was found that haemolysin tests with human cells give consistently positive results with cultures of type B strains of the organism that do not give a positive LV reaction. The haemolytic reaction appears

to be attributable to the beta antigen, and it seems that the HL test provides a more sensitive index of the presence of this product than does the LV test. The writer suggests that the HL neutralisation test with human cells should be employed with the LV neutralisation test as the first stage in the provisional typing of unknown strains of this organism. Red cells of horse and sheep can be considered as alternative indicators of haemolysis in this test system, but they are less sensitive than human cells and give a markedly 'hot-cold' reaction; they are not recommended if human cells are available.

Tests were also performed in the present studies to investigate the production of factors that are responsible for the haemolytic and LV reactions in a number of different media. These tests confirmed that with a type B strain, the LV factor is not detectable in a variety of different media, although a haemolytic reaction with human cells can be demonstrated after overnight incubation. In tests with strains of types A and D, it was found that in general, the haemolytic and LV activities are reduced if culture is performed in the presence of fermentable substrates; type A strains with maltose and type D strains with fructose are exceptions to this observation. The present tests suggest that variations in the production of haemolytic and LV factors are associated with the strain and with the medium; this information was valuable in the latter part of the work, when potent culture products were required for fractionation studies.

The second problem associated with the choice of cooked-meat medium in the present system of provisional typing was that in most of

the tests the haemolytic effect produced by cultures of type A strains against human cells was not neutralised by the experimental homologous antiserum. This observation is consistent with the results described by Hayward and Gray (1946). Neutralisation tests with commercial antiserum showed that the haemolytic activity of these cultures is neutralised by a suitable antiserum, thus, the HL neutralisation test is sufficient for the provisional identification of unknown strains. However, until further information regarding this factor is available a combination of the HL and LV neutralisation tests is recommended.

Further studies indicated that the 'non-neutralisable' haemolytic factor is associated with the growth of type A strains in the presence of meat particles. A similar but more potent effect is obtained after culture in nutrient broth with added egg-yolk emulsion.

The rapid and complete nature of the haemolytic reaction suggested that traces of lysolecithin might be present in cooked-meat broth cultures and in the egg-yolk reaction mixtures. It has not been possible to identify lysolecithin in these mixtures with the thin-layer chromatographic technique and further information is clearly necessary. The minor haemolytic components of this organism are poorly characterised and deserve further attention; although they do not appear to be significant in terms of pathogenicity they may complicate the interpretation of HL neutralisation tests.

The present work demonstrates that it is impossible to identify the minor biologically active components in culture products without well documented antisera. A common difficulty in neutralisation

tests is that trace amounts of minor components may appear to be underneutralised in an otherwise acceptable pattern of results. This may be avoided by (i) increasing the amount of neutralising antitoxin, or (ii) appropriate dilution of the test sample, but the writer endorses the statement of Oakley et al. (1947) that "The more information there is available about the serum values of the sera used, ... the more certainly can they be used for typing."

Fully documented antisera are not readily available; their preparation is an exacting and time-consuming exercise. A solution may now be provided by the use of thin-layer chromatographic techniques for the identification of different factors that affect egg-yolk emulsion. Although this procedure may not have an immediate application in a system of routine typing, its value in the characterisation of culture products is obvious. For example, in the present studies, thin-layer techniques suggest that the lipase activity of type A strains grown in cooked-meat broth plus glucose, and presumed to be associated with the epsilon antigen, is not responsible for the apparent underneutralisation of the LV reaction. The effect appears to occur in media with added fermentable substrates and may be related to the fall in pH that occurs in such media.

Lecithinase and lipase activities were identified in the present studies by thin-layer chromatographic analyses of reaction mixtures of egg-yolk emulsion with culture products of Cl. oedematiens. This simple technique confirms the careful work of MacFarlane (1948, 1950a), although of course the lecithinase enzyme was not characterised

in the present studies. The breakdown of cephalin was provisionally demonstrated in one of the reaction mixtures, and it is of interest to note that the lecithinase-C enzyme of Cl. welchii is now thought to be capable of splitting cephalin in the presence of lecithin (see Rossiter, 1967).

The writer was hopeful that the thin-layer technique might identify the haemolytic factor that occurs in reaction mixtures of egg-yolk emulsion with type A strains of the organism. At present, no biochemically recognised haemolytic factor other than lecithinase has been identified in these mixtures. Further studies will include attempts to detect trace amounts of lysolecithin, and it may be advisable to test the spot provisionally identified as diglycerides for haemolytic activity.

Although the lecithinase and lipase activities in cultures of type A and B strains of Cl. oedematiens are important in provisional typing systems, the alpha antigen is the pathogenic component of these cultures. This factor has been recognised as a soluble product for many years, but as yet, we have little knowledge of its nature or its mode of action. Elder and Miles (1957) showed that the alpha antigen is a permeability factor in vivo and that its action is rather prolonged.

The present results confirm that the lethal factor is rather heat-labile and is rapidly inactivated at pH 6.8 over a narrow temperature range; it appears to withstand exposure at room temperature to buffer solutions at pH values from 3.6 to 9.4, although the lethal

activity was not estimated quantitatively in these experiments. Culture in the presence of glucose markedly reduces the amount of the lethal factor produced. There is little evidence to suggest that the biological activities of cultures of Cl. oedematiens increase with prolonged incubation provided that adequate growth occurs initially.

The results of gel-filtration studies in the present work indicate that the alpha antigen can be readily separated from the haemolytic and LV activities in culture products of Cl. oedematiens. The recovery of biological activity was very variable during these fractionation studies, and it is probable that considerable toxoiding occurred. It is now possible to prepare a relatively pure sample of the lethal factor and this might be utilised in suitable experimental systems to determine the mode of action of this potent substance. Mice injected intravenously with preparations that contain the lethal factor usually die within 48 hr. but occasionally survive for 72 - 96 hr. The animals become sedated, and death occurs quietly with no signs of distress or of nervous involvement. The clinical signs are similar to those reported to occur in naturally infected sheep. The illness is more protracted in cases of gas gangrene in humans (MacLennan, 1962); type A strains of the organism are usually involved in this syndrome and it may be significant that these strains produce less of the lethal factor than type B strains in culture media.

The effects observed after the injection of preparations containing the alpha antigen contrast sharply with the haemolytic effect that occurs in mice injected with preparations from type D strains of the

organism. The extremities of these animals become dark and there is a bloody discharge from the natural orifices. At necropsy, there is a generalised internal haemolysis and the appearance is consistent with the administration of a highly potent haemolytic factor. In the present studies, injection of 0.1 ml. of the undiluted culture products of type D strains was not lethal to mice and experiments with concentrated culture products suggest that there is a minimum level of beta activity below which the test material is not lethal.

Several commercial organisations have recently introduced multi-component vaccines that offer protection against a range of clostridial diseases including Black disease. In one of these products, the Black disease component is toxoided and purified by ammonium sulphate precipitation, but the present results indicate that this is not an efficient method of purification. Such preparations almost certainly contain a large amount of extraneous antigenic material, and it is unlikely that the recipient's response to the protective antigens is maximal. In the present work, a partially purified lethal factor was prepared from a type B culture of Cl. oedematiens by gel-filtration and was then injected into a rabbit. Antibodies were produced in the rabbit that neutralised a normally toxic challenge of Cl. oedematiens in mice; thus, it seems that the purified fraction contained the alpha antigen. Highly purified antigens are worthy of consideration in multicomponent vaccine preparations, and the writer considers that the alpha antigen of Cl. oedematiens is the most likely constituent of the vaccine to yield satisfactory results

in this respect.

Culture products of type A strains of Cl. oedematiens frequently contained no detectable lethal factor after prolonged subculture of the strain in laboratory media. Type B strains of the organism remained potently lethal during the present work. Turner (1930) mentioned that he isolated a highly virulent but relatively atoxic strain of Cl. oedematiens after repeated passage of a type B strain through VF agar; the strain was not characterised. Turner also suggested that naturally virulent and poorly toxigenic strains were occasionally encountered in the field.

Smith, Claus and Matsuoka (1956) described variant strains of Cl. haemolyticum that they isolated from the livers of apparently healthy cattle; these strains produced weak haemolytic, LV and lethal activities in culture media; the haemolytic and LV activities were neutralised by "antitoxin to Cl. oedematiens type B" but not by "antitoxin to Cl. oedematiens type A"; the levels of antitoxic activity were not stated. In addition, these strains were late fermenters of maltose in Brewer medium, and produced indole in an undefined (but presumably Brewer) medium. The writer does not accept the claim of Smith et al. that these strains are variants of Cl. haemolyticum; the authors appear to use indole production to differentiate their strains from type B strains of Cl. oedematiens and this cannot be justified. Corbould (1966) stated that strains of Cl. oedematiens isolated from normal sheep livers and identified with the fluorescent technique appeared to be type B strains of low pathogenicity, but no

further information is given.

Strains of Cl. oedematiens isolated from infected sheep livers in the present work included two strains (R1 and R3/4) that were provisionally identified as type B strains on the basis of HL and LV neutralisation tests. Subsequent experiments to confirm the presence of the alpha antigen in culture products of these strains gave unexpected results. Intravenous injections of 0.1 ml. of cooked-meat broth culture supernates into mice were not lethal; the same amount of test material from strain R1 was not cytopathic in chick-embryo fibroblasts, and 0.1 ml. of R3/4 culture supernate gave a cytopathic effect only after 48 hr. Both of these strains fermented maltose. A more intensive investigation of these strains is now envisaged; the nature of the cytopathic effect observed with strain R3/4 suggests that small amounts of the alpha antigen may be produced by this strain. Three potently lethal type B isolates were obtained from the same lesion as strain R3/4; strain R1 was the only isolate from the first liver specimen and it is possible that a toxigenic type B isolate was missed. It would now be of interest to determine whether these apparently feebly toxigenic strains are pathogenic. It is possible that such strains represent only a part of the bacterial population of the lesion, and that their reported pathogenic effect is related to failures to isolate a potently toxigenic strain.

The cytopathic activity of culture products of Cl. oedematiens has been investigated and partially characterised during the present work. The alpha antigen of Cl. oedematiens appears to produce a well-defined cytopathic effect in chick-embryo fibroblast monolayers.

The tissue culture cells become rounded and highly refractile, and this may reflect the permeability effect observed *in vivo*. The cytopathic test is a slightly more sensitive indication of the alpha antigen than mouse lethality tests, and the effect is readily demonstrable in culture supernates of the organism.

The lethal and cytopathic factors in a culture concentrate derived from a type B strain of the organism were completely neutralised by the same dilution of a homologous antiserum; the two effects were neutralised to within 20 per cent. dilutions of a type A antiserum. The discrepancy noted with the latter antiserum might be explained on the basis that hyaluronidase in type B cultures has a synergistic effect with the alpha antigen; appropriate mixtures of type A and type D antisera should completely neutralise the cytopathic effect of type B cultures if this is so. Further studies with several concentrated products and different antisera are necessary to confirm beyond doubt that the alpha antigen is the cytopathic agent in these cultures of *Cl. oedematiens*; the present results strongly suggest that this assumption is correct.

The cytopathic effect of type D culture concentrates is distinguishable from the effect described above and appears to be related to the lethal factor of these strains. The writer considers that potent beta activity may be responsible for the cytopathic effect of type D strains of the organism, although the presence of hyaluronidase in these cultures should be borne in mind. The cytopathic activity of these strains was not further investigated in the present work as the

effect is not sufficiently well defined to be of value in a system of routine typing.

A system of typing based on the presence of the alpha, beta and gamma antigens is sufficient for the identification of unknown strains of Cl. oedematiens, and the writer recommends the following procedure. A pure culture of the strain in cooked-meat broth is tested in HL and LV neutralisation tests with type A and B antisera; the results of these tests differentiate type A strains from type B and D strains. Type C strains should give negative HL and LV tests. Further tests to differentiate type B strains from type D strains depend on the demonstration and neutralisation of effects that are characteristic of the alpha antigen; the production of this factor by type A strains should also be confirmed. Ideally, these tests should include mouse lethality tests, dermonecrotic tests and the cytopathic test with chick-embryo fibroblasts; the writer prefers the cytopathic test but in practice the choice is influenced by the facilities of the laboratory and the experience of the worker. The lethal and cytopathic effects of type B strains are neutralised by both type A and type B antisera, and the nature of the cytopathic effect indicates the presence of the alpha antigen. The lethal and cytopathic effects of type D strains are less potent than those of type B strains, and are unaffected by type A antisera; these effects are neutralised by homologous antisera, and may be neutralised by type B antisera that possess sufficient anti-beta activity. In addition, mice dying of a type D toxaemia show considerable signs of haemolysis. Further

information about the isolates may be provided by their pattern of fermentation reactions, and cooked-meat broth is the basal medium of choice in these tests.

Results obtained in the present work suggest that the problem of surface growth of Cl. oedematiens may be related to the presence of spores in the inoculum and to the development of a low redox potential in the microenvironment. Whatever the correct explanation, surface cultures of type B and D strains can now be obtained regularly on media with iron filings. The development of a defined synthetic medium has applications not only to studies of factors that influence the growth of this organism but also to investigations of the soluble antigens, and work is continuing along these lines. The soluble antigens can now be characterised more precisely, and a combination of these in-vitro studies with in-vivo investigations of the pathogenesis of diseases associated with this organism should greatly extend our understanding of Cl. oedematiens as a pathogen of man and animals. Procedures are thus available for the development of this outstanding model of microbial pathogenicity, and it is hoped that studies of some of these aspects may assist in the elucidation of related problems in other fields.

REFERENCES

AIKAT, B. K., and DIBLE, H. J. 1960. J. Path. Bact., 79, 227.

ALBISTON, H. E. 1927. Austral. J. Exp. Biol. Med. Sci., 4, 113.

ALWEN, J., and SMITH, D. G. 1967. J. Appl. Bact., 30, 389.

AUB, J. C., ZAMECNIK, P. C., and NATHANSON, I. T. 1947. J. Clin. Invest., 26, 404.

BARD, R. C., and McCLUNG, L. S. 1948. J. Bact., 56, 665.

BARNES, ELLA M., and INGRAM, M. 1956. J. Appl. Bact., 19, 117.

BATTY, IRENE, and WALKER, P. D. 1964. J. Path. Bact., 88, 327.

BATTY, IRENE, BUNTAIN, D., and WALKER, P. D. 1963. Vet. Rec., 75, 1339.

BATTY, IRENE, BUNTAIN, D., and WALKER, P. D. 1964. Ibid., 76, 1115.

BATTY, IRENE, KERRY, J. B., and WALKER, P. D. 1967. Ibid., 80, 32.

BERGEY, D. H., BREED, R. S. MURRAY, E. D. G., and HITCHEN, A. P. 1939. Bergey's manual of determinative bacteriology, 5th ed., Baltimore.

BERGEY, D. H., HARRISON, F. C., BREED, R. S., HAMMER, B. W., and HUNTOON, F. M. 1923. Bergey's manual of determinative bacteriology, 1st ed., Baltimore.

BOURNE, F. J., and KERRY, J. B. 1965. Vet. Rec., 77, 1463.

BREED, R. S., MURRAY, E. G. D., and SMITH, N. R. 1957. Bergey's manual of determinative bacteriology, 7th ed., London.

BREWER, J. H. 1940. J. Amer. Med. Assoc., 115, 598.

BROOKS, ELIZABETH M., STERNE, M., and WARRACK, G. HARRIET 1957. J. Path. Bact., 74, 185.

COLESHILL, G. G. 1968. B.Sc. Honours dissertation, Univ. Edinburgh.

COLLEE, J. G., and BARR, W. A. 1968. J. Path. Bact., in press.

CORBOULD, A. 1966. Vet. Rec., 79, 861.

CORBOULD, A., and MUNDAY, B. 1966. Ibid., 78, 218.

COTRAN, R. S. 1967. Lab. Invest., 17, 39.

CROOK, E. M. 1942. Brit. J. Exp. Path., 23, 37.

CRUICKSHANK, R. 1965. Medical microbiology, 11th ed., Edinburgh and London.

DIXON, M., and WEBB, E. C. 1964. Enzymes, 2nd ed., London.

DOCUMENTA GEIGY. 1962. Scientific tables, 6th ed., edited by K. Diem, Manchester.

DRASAR, B. S. 1967. J. Path. Bact., 94, 417.

ELDER, J. M., and MILES, A. A. 1957. Ibid., 74, 133.

ELLNER, P. D., and GREEN, S. S. 1963. J. Bact., 86, 1084.

EVANS, D. G.	1947.	J. Gen. Microbiol., 1, 378.
GADALLA, M. S. A.	1965.	Studies on the haemagglutinin and diffusible products of Clostridium septicum. Ph.D. Thesis, Univ. Edinburgh.
GADALLA, M. S. A., and COLLEE, J. G.	1968.	J. Path. Bact., in press.
GORDON, J., HOLMAN, R. A., and McLEOD, J. W.	1953.	Ibid., 66, 527.
GRANT, N. H., and ALBURN, H. E.	1959.	Arch. Biochem. Biophys., 82, 245.
HART, C. B.	1965.	Vet. Rec., 77, 229.
HAYWARD, NANCY J.	1941.	Brit. Med. J., 1, 811.
HAYWARD, NANCY J., and GRAY, J. A. B.	1946.	J. Path. Bact., 58, 11.
HAYWARD, NANCY J., and MILES, A. A.	1943.	Lancet, II, 116.
HELLER, C. L.	1954.	J. Appl. Bact., 17, 202.
HEWITT, L. F.	1950.	In Oxidation-reduction potentials in bacteriology and biochemistry, 6th ed., Edinburgh.
HOBBS, BETTY C., SMITH, M. E., OAKLEY, C. L., WARRACK, G. HARRIET, and CRUICKSHANK, J. C.	1953.	J. Hyg., Lond., 51, 75.
HOLMAN, R. A.	1955.	J. Path. Bact., 70, 195

HOLMAN, R. A.	1957.	Ibid., 73, 91.
JAMIESON, S.	1949.	Ibid., 61, 389.
JAMIESON, S., THOMPSON, J. J., and BROTHERSTON, J. G.	1948.	Vet. Rec., 60, 11.
JASMIN, A. M.	1947.	Amer. J. Vet. Res., 8, 289.
KEPPIE, J.	1944.	A study of the antigens and toxins of Cl. oedematiens and Cl. gigas by in-vitro and in-vivo methods. Ph.D. Thesis, Univ. Cambridge.
KNIGHT, B. C. J. G., and FILDES, P.	1930.	Biochem. J., 24, 1496.
KRANEVELD, F. C.	1934.	12th International Veterinary Congress, New York, p. 187.
KRANEVELD, F. C., and DJAENOEDIN, R.	1933.	Ned-ind. Bl. Diergeneesk., 45, 80.
KRAYER, O., AUB, J. C., NATHANSON, I. T., and ZAMECNIK, P. C.	1947.	J. Clin. Invest., 26, 411.
LEPPER, ELIZABETH, and MARTIN, C. J.	1929.	Brit. J. Exp. Path., 10, 327.
LEPPER, ELIZABETH, and MARTIN, C. J.	1930.	Ibid., 11, 137.
McCLEAN, D., ROGERS, H. J., and WILLIAMS, B. W.	1943.	Lancet, I, 355.
McCLUNG, L. S., and TOABE, R.	1947.	J. Bact., 53, 139.

McCLUNG, L. S., HEIDENREICH, P., and TOABE, R. 1946. Ibid., 50, 715.

MACFARLANE, MARJORIE G. 1942. Biochem. J., 36, Proc. iii.

MACFARLANE, MARJORIE G. 1948. Ibid., 42, 590.

MACFARLANE, MARJORIE G. 1950a. Ibid., 47, 267.

MACFARLANE, MARJORIE G. 1950b. Ibid., 47, 270.

MACFARLANE, MARJORIE G. 1955. Ibid., 61, 308.

MACFARLANE, R. G., OAKLEY, C. L., and ANDERSON, C. G. 1941. J. Path. Bact., 52, 99.

MACLENNAN, J. D. 1962. Bact. Rev., 26, 177.

McLEOD, J. W., and GORDON, J. 1923. J. Path. Bact., 26, 326.

MACPHERSON, I., and STOKER, M. 1962. Virology, 16, 147.

MARSHALL, S. C. 1959. N.Z. Vet. J., 7, 115.

MATELES, R. I., and ZUBER, B. L. 1963. Antonie van Leeuwenhoek, 29, 249.

MILES, A. A., and MILES, E. M. 1952. J. Physiol., 118, 228.

MILES, A. A., and MISRA, S. S. 1938. J. Hyg., Lond., 38, 732.

NAGLER, F. P. O. 1944. Nature, London, 153, 1536.

NAGLER, F. P. O. 1945. Austral. J. Exp. Biol. Med. Sci., 23, 59.

NAKAGAWARA, G., and NISHIDA, S.	1963.	Med. and Biol. (Tokyo), 67, 17.
NARAYAN, K. G.	1966.	Zbl. Bakt. (1. Abt. Orig.), 201, 57.
NISHIDA, S., and IMAIZUMI, M.	1966.	J. Bact., 91, 477.
NISHIDA, S., and NAKAGAWARA, G.	1964.	Ibid., 88, 1636.
NISHIDA, S., and NAKAGAWARA, G.	1965.	Ibid., 89, 993.
NOVY, F. G.	1894.	Z. Hyg. Infekt Kr., 17, 209.
OAKLEY, C. L., and WARRACK, G. HARRIET	1951.	J. Path. Bact., 63, 45.
OAKLEY, C. L., and WARRACK, G. HARRIET	1959.	Ibid., 78, 543.
OAKLEY, C. L., WARRACK, G. HARRIET, and CLARKE, PATRICIA H.	1947.	J. Gen. Microbiol., 1, 91.
PENSO, G., and VICARI, G.	1957.	In Proceedings of the 3rd International Meeting of Biological Standardisation.
PHILLIPS, A. W., and BATTY, IRENE	1963.	Nature, London, 198, 1200.
RECORDS, E., and VAWTER, L. R.	1945.	Univ. of Nevada Agric. exp. Sta., Technical Bulletin, no. 173.
REED, R. W.	1942.	J. Bact., 44, 425.

REED, L. V., and MUENCH, H. 1938. Amer. J. Hyg., 27, 493.

REED, G. B., and ORR, J. H. 1941. War Med., Chicago, 1, 493.

REED, G. B., and ORR, J. H. 1943. J. Bact., 45, 309.

ROBERTS, T. A., and INGRAM, M. 1965. J. Appl. Bact., 28, 125.

ROBERTSON, MURIEL 1915. J. Path. Bact., 20, 327.

ROSSITER, R. J. 1967. In Lipids and Lipidoses, ed. by G. Schettler, Berlin, p. 112.

SCOTT, J. P., TURNER, A. W., and VAWTER, L. R. 1934. 12th International Veterinary Congress, New York, p. 168.

SCRIMGEOUR, G., KEANE, P. M., and ALDER, V. G. 1967. J. Path. Bact., 93, 688.

SMITH, L. DS. 1953. J. Bact., 65, 222.

SMITH, L. DS. 1955. Introduction to the Pathogenic Anaerobes, Chicago.

SMITH, L. DS., and JASMIN, A. M. 1956. J. Amer. Vet. Med. Assoc., 129, 68.

SMITH, L. DS., CLAUS, K. D., and MATSUOKA, T. 1956. J. Bact., 72, 809.

SOLTYS, M. A., and JENNINGS, A. R. 1950. Vet. Rec., 62, 5.

TURNER, A. W. 1930. Bull. Coun. sci. industr. Res., Australia, no. 46.

TURNER, A. W., and DAVESNE, J.	1927.	C.R. Soc. Biol., Paris, 96, 921.
TURNER, A. W., and EALES, CATHARINE E.	1943.	Austral. J. Exp. Biol. Med. Sci., 21, 79.
VAN HEYNINGEN, W. E.	1950.	Bacterial Toxins, Oxford.
VAWTER, L. R., and RECORDS, E.	1925-26.	J. Amer. Vet. Med. Ass., 68, 494.
VAWTER, L. R., and RECORDS, E.	1931.	J. Infect. Dis., 48, 581.
VETO, I., BACKHAUSZ, R., and HORVATH, I.	1957.	In Proceedings of the 3rd International Meeting of Biological Standardisation, p. 33.
WALBUM, L. E., and REYMANN, G. C.	1937.	J. Path. Bact., 44, 379.
WEINBERG, M., and SÉGUIN, P.	1915.	C. R. Soc. Biol., Paris, 78, 507.
WETZLER, T. F., MARSHALL, J. D., and CARDELLA, M. A.	1956.	Amer. J. Clin. Path., 26,
WHITTENBURY, R.	1964.	J.Gen. Microbiol., 35, 13.
WILLIAMS, B. M.	1962.	Vet. Rec., 74, 1536.
WILLIAMS, B. M.	1964.	Ibid., 76, 591.
WILLIS, A. T.	1960.	J. Path. Bact., 80, 379.
WILLIS, A. T.	1964.	Anaerobic bacteriology in clinical medicine, 2nd ed., London.
WILLIS, A. T., and HOBBS, G.	1958.	J. Path. Bact., 75, 299.

WILLIS, A. T., and HOBBS, G. 1959. Ibid., 77, 511.

WILSON, G. S., and MILES, A. A. 1964. In Topley and Wilson's Principles of Bacteriology and Immunity, 5th ed., London.

ZEISSLER, J., and RASSFELD, L. 1929. Arch. wiss. prakt. Tierheilk., 59, 419.

ZEMSKOV, E. M. 1964. Fedn. Proc. (Transl. Supp.), 23, 85.

APPENDICES

Appendix 1

Strains of Cl. oedematiens obtained from Mr J. R. Hepple.

(i) Strains of NCTC origin:

GR1A	NCTC 538 Batch I
GR3A	NCTC 6737
GR4A	NCTC 6735
GR1C	NCTC 9747 (OBB 4)
GR1D	NCTC 8350
GR2D	NCTC 8145

(ii) Strains from Aberystwyth Veterinary Investigation Centre:

	Glaxo collection number
GR2A	S277/64 (B)
GR2B	S281/64
GR8B	S277/64 (A)
*GR9B	S277/64 (C)
GR10B	S284/64
GR11B	S286/64
GR12B	S291/64
GR13B	S292/64

*Results in the present studies confirm Hepple's view (personal communication) that this is a type D strain.

(iii) Strains from Glaxo collection:

GR1B	474E
GR3B	475E
GR4B	479E
*GR5B	480E
GR7B	513E

*Results in the present studies indicate that this is a type A strain.

Appendix 2

Suggested procedure for operating the BTL anaerobic jar:

1. Connect inlet H (closed) to pump and inlet V (open) to gauge.
2. Start pump and slowly open inlet H.
3. Evacuate to -660 mm. Hg (25 in. Hg).
4. Close inlet H; disconnect pump line; stop pump.
5. Connect a bladder of carbon dioxide to inlet H and run in until gauge registers - 600 mm. Hg. (Omit this step if CO_2 is not required.)
6. Close inlet H; change to hydrogen bladder; run in H_2 until gauge shows zero.
7. Close both inlets; leave jar on bench.

AFTER 10 MINUTES:

8. Either admit more H_2 via Woulff bottle, OR PREFERABLY check with manometer that a secondary vacuum of at least 10 mm. Hg has been produced. Admit more H_2.
9. Close inlets. Disconnect hydrogen bladder. Check gas cylinders are closed.
10. Incubate jar.

To avoid failures, the following useful checks may be made:

The needle inlet valves require periodic cleaning and greasing with high-vacuum grease.

The gasket should not be greased but should be kept clean and dry.

The catalyst should not be in contact with water. Keep new catalysts in a dry place.

Store unused jars in a warm dry place.

Wash out a jar occasionally; rinse with spirit; dry open. Do not include the lid in the washing process but keep it particularly clean. Take care to dry out the side-arm and renew the rubber connection on the side-arm when necessary.

Appendix 3

Preparation of Trypsinised Embryonic "Carcass" (10-13-day-old chick embryos):

1. Sterilise shell over air sac with alcohol.
2. Remove shell over air sac.
3. Peel off shell membrane.
4. Make a cruciform cut in CA medium.
5. Cut amniotic membrane.
6. Hook embryo round neck and transfer to Petri dish.
7. Decapitate - remove stomach, crop and intestines - cut off legs.
8. Chop up remaining tissue with scissors and scalpels.
9. Wash twice in Hank's balanced salt solution (BSS) (50 ml. each time).
10. Add 50 ml. 0.25 per cent. trypsin (Difco 1 : 250) in Hank's BSS forewarmed to 37° C, pH 7.2 - 7.8.
11. Place on magnetic stirrer in "hot room" (37° C) or place in 37° C water-bath. Leave for 20 - 30 min. Shake from time to time if placed in water bath.
12. Using a wide bore pipette, suck up and down for 5 min.
13. Leave for 1 min. to allow larger pieces of tissue to settle.
14. Transfer supernatant to tubes or universal containers.
15. Spin gently for 5 min.

16. Resuspend in 3 ml. growth medium.

17. Count cells in haemocytometer.

18. Dilute to 1 x 10^6 cells/ml.

19. Transfer 1 ml. to tubes.

Growth Medium

Eagle's medium	-	90 ml.
Calf serum	-	10 ml.
4.4% $NaHCO_3$	-	2.5 ml.
Penicillin	-	100 units
Streptomycin	-	100 µg.

Maintenance Medium

Eagle's medium	-	98 ml.
Calf serum	-	2 ml.
4.4% $NaHCO_3$	-	4 ml.
Penicillin	-	100 units
Streptomycin	-	100 µg.