A BIOCHEMICAL STUDY OF THE SOLUBLE ANTIGEN AND HAEMOLYSIN OF CLOSTRIDIUM CHAUVOEI.

by

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CHAPTER I.

INTRODUCTION.

The Aim of the investigation

Clostridium chauvoei, referred to as Cl. feseri and Bazillus rauschbrand in the American and German literature respectively, is one of the organisms involved in gas gangrene. They belong to a well-defined group of bacteria, namely, the genus Clostridium , which are characterized by growth under conditions of more or less anaerobiosis. Most of the anaerobic bacilli are saprophytic bacteria and have been shown to be normal inhabitants of the intestinal tracts of animals. Clostridium chauvoei is responsible for symptomatic anthrax, referred to as "blackleg" or "sponssiekte", an infectious disease occurring mainly among sheep, cattle and goats. This widely distributed infectious disease is usually acquired through the agency of fecally contaminated soil in which the bacillus is present in the form of spores which may retain their viability for several years.

In South Africa, one of the main wool-producing countries, this blackleg disease chiefly occurs in sheep. Even the smallest wound or abrasion, such as one caused by careless shearing, can allow the entry of infected soil. Wound infection is nearly always set up by a group composed of several distinct anaerobic organisms, making identification of the fatal bacillus more complicated.

At the Onderstepoort Veterinary Laboratories a good preventive vaccine is prepared, which gives a high immunity against blackleg.

Though the first successful active immunization against bacilli responsible for blackleg dates from as early as 1880 (Arloing, Cornevin and Thomas), practically

nothing /.....

nothing is known about the working of vaccines. We started the investigation of this vaccine to obtain information on both the chemical nature of the immunizing component and its action. This information is of importance in a study of the production of vaccine and its immunizing capacity.

General outline of the anaerobes associated with gas gangrene.

The bacteria that cause gas gangrene are gram-positive rods and they form heat-resistant spores. These organisms are further characterized by their growth under anaerobic conditions. In 1863 Pasteur laid the foundation of all the work on the anaerobes by his discovery of Cl. butyricum, a non-pathogenic anaerobe. The anaerobes show a tendency to grow in mixed colonies. To obtain these bacilli in pure cultures frequently repeated isolation was necessary. The difficulty of obtaining pure cultures and of maintaining them in a pure state not only delayed the investigation of the anaerobes, but also makes the early descriptions of anaerobic bacilli unreliable.

No wonder that an extensive study of the anaerobic organisms responsible for gas gangrenes started during World War I after the development of the modern anaerobic methods, which made surface growth feasible. In medical practice gas gangrene is generally attributed to the presence of Cl. perfringens. But although this bacillus was found in about 70-80 per cent of the cases of gas gangrene during the two World Wars, it practically never occurred in pure culture (Stock, 1947).

In veterinary practice we find that besides Cl. perfringens Cl. septicum, Cl. chauvoei and Cl. novyi

also /.....

also contribute to gas gangrene to a considerable extent.

As first suggested by von Hibler in 1908, the anaerobes responsible for gas gangrene can be divided into two general groups :

a) The Saccharolytic group, with a greater avidity for carbohydrates than,

b) The Proteolytic group. The anaerobes of this latter group can be distinguished by their ability to liquefy coagulated horse serum. Organisms of the saccharolytic group fail to liquefy this medium even after prolonged incubation.

The saccharolytic group includes the most toxic anaerobes, such as :

Cl. perfringens
Cl. septicum
Cl. chauvoei
Cl. novyi and
Cl. fallax.

To the proteolytic group belong organisms like :

Cl. histolycum and
Cl. sporogenes

The organisms of the proteolytic group are not pathogenic in themselves but they complicate gangrenes by their intense proteolytic action and are usually responsible for the foul odour of wounds. Thus in 1918 Weinberg and Séguin isolated Cl. sporogenes in 27 per cent of their gangrene cases.

Description of the Cl. chauvoei species.

Cl. chauvoei is responsible for the infectious disease symptomatic anthrax. The bacillus is about 4-6µ long and 0.4-0.6µ wide. It usually occurs singly and never forms long chains. In young cultures (about 18 hours)

Cl. chauvoei /.....

Cl. chauvoei is gram-positive; in older cultures, however, gram-negative individuals are also found. In the vegetative form the bacillus is mobile.

The organism is strictly anaerobic and was discovered by Bollinger in 1875 and by Feser (1876). In 1887 Arloing, Cornevin and Thomas isolated Cl. chauvoei and in 1889 Kitasato was the first to cultivate it on solid medium and he obtained the bacillus in pure culture. Though Cl. chauvoei can be grown on ordinary meat media, the addition of glucose makes the media more favourable. Two very favourable media must be mentioned :

a) a liver-broth with glucose and
b) a von Hibler medium, consisting of liver-meat-broth with glucose and pieces of brain added.

In all these media there is an active gas formation. Cl. chauvoei, as we saw, belongs to the saccharolytic group of anaerobes and ferments carbohydrates like glucose, maltose, lactose and saccharose quite easily, with the production of gas and acid.

Salicin, however, is not fermented by Cl. chauvoei and for this reason it is important in post-mortem investigations. Though Cl. chauvoei has only slight proteolytic activity, it can liquefy gelatine.

Pathogenic action of Cl. chauvoei.

Cl. chauvoei bacilli are pathogenic for sheep, cattle, goats, guinea-pigs and mice. Horses are slightly susceptible (according to Wagener (1925) they are immune), and dogs, cats, rabbits and birds are immune. The symptomatic anthrax disease has never been observed in man, indicating that man also is immune.

Infection /.....

Infection, natural or experimental, depends to a large extent upon the degree of virulence of the bacillus, a very variable factor in the Cl. chauvoei species. If 0.1 - 0.5 ml supernatant of a von Hibler infusion culture is inoculated into the thigh of a guinea-pig, a soft, puffy swelling appears at the point of entrance within 24 hours after inoculation. Pressure or slight touching of this swelling causes an emphysematous crackling and the emphysema spreads rapidly often reaching the abdomen and chest within a day. The course of the disease is acute, the fever high and death may result within 3-4 days after inoculation. The swollen area is found to be infiltrated with a thick blood-stained exudate and often bursts open. Subcutaneous tissue and muscles are edematous and when touched the gas formed will make a crackling sound. The internal organs show haemorrhagic areas and degenerative symptoms. Immediately after death few bacilli are found in the blood and internal organs. They are demonstrable in enormous numbers in the edema fluid surrounding the centre of inoculation. The spores are formed after death of the inoculated animal but are not as a rule present during life. The course of the disease is practically identical, whether the infection was spontaneous or caused by experimental inoculation.

Identification of Cl. chauvoei after death of the animals.

On post-mortem examination an extensive blood-stained edema of a deep colour is seen, and a considerable amount of gas is developed in the tissue round the muscles concerned. Those appearances, however, are also seen in the case of a Cl. septicum infection. These two organisms, Cl. chauvoei and Cl. septicum, are indeed closely related

and are /.....

and are very similar morphologically. Robertson (1929) distinguishes between Cl. chauvoei and Cl. septicum by the fact that the former ferments saccharose and not salicin, whereas Cl. septicum ferments salicin and not saccharose.

In 1900 Leclainche and Vallée describe another point of distinction which is found in the smears of the peritoneal surface of the liver. Long snake-like filaments are demonstrable in smears from the liver of animals which died of Cl. septicum infection. Such forms are lacking entirely in the case of Cl. chauvoei. The two organisms, Cl. septicum and Cl. chauvoei, can also be distinguished serologically (see Chapter VII).

The toxins of Cl. chauvoei.

The literature which has appeared on the metabolites of Cl. chauvoei is comprehensive, but shows many discrepancies.

According to the investigations of Duenschmann (1894) and of Leclainche and Vallée (1900), Cl. chauvoei produces an exotoxin, very active against guinea-pigs and only slightly active against rabbits. Leclainche and Vallée demonstrated that this exotoxin is heat stable. Kelser in 1918 states that even 1.0 - 1.5 ml of a 12 days old culture filtrate cultivated after Leclainche and Vallée was lethal to guinea-pigs. Further evidence for the presence of a toxin in culture filtrates of Cl. chauvoei is found in the work of Nicolle, Césari and Raphaël (1915), Okuda (1922) and Berg (1923). The former authors state that the toxin is destroyed by heating at 55°C for 30 min. and that the toxin is lethal after intravenous injection and also, but in a much lesser degree, after subcutaneous

injection /.....

injection. They were the first to describe the specifity of a haemolysin which is active for the erythrocytes of guinea-pigs, rabbits and sheep. Eisenberg in 1907 already reported the haemolysin of Cl. chauvoei as partially oxygen-labile, being easily activated by reduction after many months of storage. Kojima (1923) and Basset (1925) produced filtrates of Cl. chauvoei cultures of low toxicity, when injected intravenously into guinea-pigs. Both Kojima and Basset found that the toxin is heat-labile. Kojima distinguished a haemolysin and a lethal factor; both are destroyed by heating in a waterbath at 52°C for 30 min., and both can be dialysed but only with difficulty. The haemolysin can be precipitated by a half saturated solution of ammonium sulphate, while the lethal factor remains to some extent in the supernatant fluid. Pepsin and trypsin destroy the action of the two toxins of Cl. chauvoei.

Scott (1925, 1926 and 1928) and Viljoen and Scheuber (1926) were unable to demonstrate a toxin in Cl. chauvoei filtrates. Scott (1925), however, demonstrated an endo-toxin. Zeller (1925) used filtrates from 3 weeks old cultures which only produced swellings in sheep after subcutaneous injection of 25 - 50 ml. Production of a toxin was further denied by Haslam and Lumb (1919), McEwen (1926) and O'Brien (1929).

In 1934 Kerrin, followed by Karube (1934) and Uenaka in 1938, again demonstrated toxic and haemolytic activities in filtrates of Cl. chauvoei. Kerrin, who found strong haemolytic activities in 48 hours old culture filtrates came to the conclusion that the toxin and haemolysin are closely related. Both are destroyed by heating at 52°C for 5-10 min., both are precipitated by a two third saturation of ammonium sulphate and both can be

stored /.....

stored fairly well. Absorption of filtrates by red blood cells in addition to removing the lysin also removed the lethal factor. Kerrin claimed that the haemolysin and toxin are different substances because their behaviour in formaline solution differs. The lethal factor is destroyed by 0.5 per cent formaline at 37°C, whereas the haemolytic activity is still present even after 3 weeks. The toxin of Cl. chauvoei acted strongly for mice and guinea-pigs; the minimal lethal dose (intravenous injection) varied from 0.025 to 0.5 ml for mice and guinea-pigs respectively. Rabbits were not killed by 3 ml.

Mason (1936) demonstrated both a toxin and a haemolysin in the filtrates of Cl. chauvoei cultures. Attempts to concentrate the toxin were only partially successful. He found that concentration was possible by ammonium sulphate or sodium sulphate precipitation. The toxicities of the dried powders obtained by ammonium sulphate precipitation, 30 g and 60 g per 100 ml respectively, were approximately the same. Increase in the necrotizing effect of the toxin, when intradermally injected into guinea-pigs, was obtained by Mason when he mixed the concentrated filtrate with adrenaline.

An interesting report was published by Uenaka in 1938. He found that the toxin of Cl. chauvoei adhered well to the bacilli, up to 24 hours. The bound toxin was far more thermo-stable than the toxin present in the culture filtrate. Uenaka presumed that the toxin is formed by the spores.

In 1948, Guillaumie and Kréguer reported on the existence of both a toxin and a haemolysin. They confirmed the results of Kerrin with regard to the action of formol upon the components. They found the haemolytic activity

to be /.....

to be stronger in saline than in a saline-buffer mixture of pH 7.4. Heating of the two toxins, according to Guillaumie and Kréguer, gave confusing results. These authors found that the lethal toxin was destroyed by heating at 70°C for 5 minutes, but also that this toxin could be regenerated at 100°C, as was the case for the haemolysin also. Only after prolonged heating at 100°C (for at least one hour) did the components lose their activities. Adrenalin gave no increase of the lethal activity, as reported by Mason.

Moussa (1958) described not only a necrotoxin and a haemolysin, the latter consisting of an oxygen-stable and an oxygen-labile part, but also a hyaluronidase and a deoxyribonuclease. Both the oxygen-labile and the oxygen-stable haemolysin were inactivated by oxidation with hydrogen peroxide and iodine. Whereas the oxygen-labile haemolysin could be easily reactivated by hydrogen sulphide gas or sodium-thioglycolate, the oxygen-stable component was irreversibly inactivated by oxidation. Unlike the instant lysis produced by the oxygen-labile haemolysin, an induction period preceded haemolysis of sheep erythrocytes brought into contact with the oxygen-stable compound. The oxygen-stable haemolysin and the necrotic toxin were found to lose at least half their activity within 48 hours of storage. Parallelism appeared between the oxygen-stable haemolysin and the necrotic toxin in titers and inactivations by anti-sera.

In a very recent report (1962), Jayaraman, Lal and Dhanda describe the appearance of a toxin in culture filtrates. They tested this lethal toxin in mice, and describe conditions for high yields. According to the authors the toxin is destroyed by heating at 52°C for

5 minutes /.....

5 minutes. Good antitoxic sera in rabbits could be obtained.

Because of the low toxin yield and because a number of strains repeatedly failed to show any trace at all, Cl. chauvoei was often considered to be a transitional organism between the toxigenic and the non-toxigenic clostridia. (McCoy and McClung, 1938, and Moussa 1958). Indeed, non-toxic strains are more generally encountered in the Cl. chauvoei species than in other common anaerobes (Haslam and Lumb, 1919, Allen and Bosworth, 1924, and Moussa, 1958).

Phagocytosis and Aggressive activity.

Goss and Scott (1919), published an article on the possible determination of aggressin in culture filtrates. They inoculated guinea-pigs with a well-known standard dose of protective rabbit-antiserum. After 1 hour they challenged the guined-pigs with a dose of a suspension of washed bacteria. This inoculation was sub-lethal after the immunization of the guinea-pigs with the antiserum but by adding culture filtrate to the suspension of washed cells, this sublethal dose became lethal. The minimum amount of culture filtrate necessary for obtaining lethal activity is a fairly good measure for the immunizing power of the culture filtrate.

Scott demonstrated in 1925, that filtered muscle juice of an animal which died of blackleg and culture filtrate of Cl. chauvoei possesses equal qualities of aggressivity when injected into animals with sublethal doses of culture. Viljoen and Scheuber (1926) reported that a culture filtrate of Cl. chauvoei was able to activate spores; this was confirmed by Mason in 1936.

In 1939 /.....

In 1939 DeMoulin published the results of phagocytic studies in blackleg. He made histological sections from tissues artificially infected with Cl. chauvoei and found that phagocytosis is the most important bodily defence-mechanism against blackleg infection. Immunization with a filtrate or formalized whole culture vaccine increased the degree of phagocytosis. It was further demonstrated that the addition of filtrate to a small dose of bacilli reduced phagocytosis in the infected tissues considerably.

Live (1940) made the same observations as DeMoulin but he experimented in vitro. However, whereas DeMoulin attributes the decreased phagocytosis to the action of the aggressin upon leucocytes, according to the theory of Bail (1904) Live demonstrated another action of the aggressin.

By allowing the aggressin (muscle juice, culture filtrate or sonic bacillus extract) to act upon serum and upon leucocytes, it was shown that the inhibition of phagocytosis was due to fixation of opsonin, (a factor in serum involved in phagocytic reactions) and not to a direct reaction upon the leucocytes.

Smith and Martin (1948) say that the term aggressin was used to designate a substance possessed by bacteria which had the power to overcome body defences and to prevent their phagocytosis by leucocytes. This term has been discarded in view of the development of knowledge concerning the blocking action of haptenes and the recognition that some bacteria produce toxins attacking leucocytes. The latter may be the case with the Cl. chauvoei species, for it is already a well-known fact with the Cl. septicum species. Meisel (1961) described a method of determining a

deoxyribonuclease /.....

deoxyribonuclease in Cl. septicum culture filtrates that has the power to destroy rabbit leucocytes.

Agglutination.

The agglutination reaction was studied in 1911 by Markoff. A specific agglutination reaction was found for both Cl. chauvoei and Cl. septicum. Specific agglutination was further demonstrated by Wulff (1912), Gaehtgens (1918), Scott (1925), Gins and Hussein (1927), Rottgardt (1929), and Uenaka (1938), among others.

Cross-agglutination, however, was reported by Grosso (1913), Wolters and Dehmel (1928) and Weinberg and Mihailescu (1929).

Fürth (1916), followed by Conradi and Bieling, also in 1916, reported that their strains showed two "antigenic phases" when grown on different media. On meat media, and other media that promoted sporulation, Cl. chauvoei and Cl. septicum strains shared a "common phase" which was lost when the same strains were grown on carbohydrate media. Kreuzer (1939) reported on the presence of a common thermo-stable antigen between the two organisms.

The presence of a common S-antigen, being a spore antigen, is shown by Moussa in 1959. This antigen may explain the results of Fürth and Kreuzer.

Moussa (1959) examined 37 strains with most of the cultural characters of Cl. septicum and 37 strains with those of Cl. chauvoei. He concluded that all but two Cl. chauvoei strains possess a single O-antigen and that there was no cross-agglutination with Cl. septicum O-antisera. Further, Cl. chauvoei possesses two H-antigens which are also distinct from those of Cl. septicum, as stated by Moussa.

C) /.....

A serological comparison between ovine and bovine strains.

Some authors report that blackleg infection in cattle occurs spontaneously, whereas in sheep it usually occurs as a direct and obvious result of wounding. (Viljoen and Scheuber (1926); Marsh, welch and Jungherr (1929); McEwen and Roberts (1932)). This fact gave rise to an investigation into the nature of the Cl. chauvoei bacilli isolated from sheep and cattle which died of blackleg.

Zeissler (1924) carried out an extensive study of the agglutination reaction of two different organisms isolated from cattle and sheep, referred to as a bovine strain and an ovine strain respectively. He found no serological difference but did not attempt to distinguish them by cross adsorption of agglutinins (the serological factor involved in agglutination).

Miessner and Meyn (1926) demonstrated distinct serological reactions of both the ovine and bovine strains. They found that the blood of a cow or a sheep immunized with a bovine or ovine strain, inhibited growth for the homologous bacilli. So for instance, the growth of a bovine strain on blood-agar was very strongly inhibited when the blood was derived from a cow immunized with a bovine strain. The growth was only slightly inhibited when the blood of a sheep immunized with an ovine strain was used. Miessner and Meyn also found a strong homologous protection in mice immunized with one of the cultures, and only a weak protection against the other strain.

Zeller (1927), Jungherr (1928) and Bosworth (1930) found it impossible to distinguish between the ovine and bovine strains and they state that the two organisms are identical morphologically, as well as culturally and serologically.

In /.....

In 1931 Roberts made a careful study of eleven strains of Cl. chauvoei isolated from sheep which died of gas gangrene and of a bovine strain. A preliminary investigation had shown that bovine and ovine rabbit antisera agglutinate homologous suspensions to a high titer and the heterologous suspensions only to a low one. The results of cross-agglutination tests point to a community of antigens but as shown by absorption tests neither the ovine nor the bovine bacillus suspension is capable of removing from the heterologous serum its agglutinins for its homologous antigens. It therefore appears that the two strains possess both a common and a specific antigen. After heating saline suspensions of a bovine and an ovine strain at 100°C for 2 hours, antisera in rabbits were prepared. The sera each caused agglutination and cross-agglutination of ovine and bovine suspensions, from unheated bacilli, to an equal but very low titer. Attempts to cause agglutination of the heated suspensions failed with all the sera. An alcohol treatment had the same effect as heating on agglutination. The common antigen of the ovine and bovine strains is therefore a heat-stable antigen referred to as "O" or somatic antigen.

The other antigen, which is thermolabile and destroyed by alcohol, is referred to as "H" antigen or flagellar antigen (see Robertson and Felix 1930).

The results obtained by Roberts were confirmed by Henderson in 1932.

Precipitation reaction.

Hecht (1913) reports on specific precipitation reactions between horse-immune sera and heated organ extracts. The organs were isolated from animals which

died /.....

died of blackleg. A cloudy ring is formed at the junction of the serum with the extract. The specificity of this reaction is strongly criticised by Ramazotti (1915) because the Cl. chauvoei cultures in blackleg cases are very seldom pure. As is shown by the publications of Miessner and Lange (1914), Gaehtgens (1918), Wolters and Dehmel (1928), and Robinson (1929), a specific precipitation was possible. Gaehtgens described a precipitation reaction with autolysates from 4 to 5 days old. The specificity of the precipitation reaction, however, is very suspicious, when one considers the reports of Koegel (1920), Gerlach (1921), Todorovitsch (1923), Wagener (1925) and Rottgardt (1929). It was found by Koegel that even normal sera of the ruminants may give precipitation reactions with Cl. chauvoei filtrates. Lindley (1955) claims a good precipitation reaction between sheep or cow immune-sera and bacterial extracts. The precipitin titer (precipitin is a serum factor involved in the precipitation reaction) is found to be an indication of the level of immunity produced in the animals.

The stimulation of immunity.

Active immunity in animals can be stimulated very successfully by injection of one of the following materials:

1) Spores or preparations of the sporing culture.

More generally the dried, or dried and heated, muscles from an infected animal are very potent as was first shown by Kitt in 1887.

2) Aggressin .

Aggressin is the juice from infected muscle, pressed out and filtered, it can also be used in dried form. These methods were developed by Roux in 1888.

3) /.....

3) Culture filtrate.

This method was first demonstrated in 1888, also by Roux, who referred to the culture filtrate as "artificial aggressin". The method had been widely used as the immunity produced proved to be satisfactory. Since the material was free of living organisms it lent itself to precise control. To destroy possible lethal toxins most producers added formaline, which did not lessen the antigen potential. Further writers on this subject are : Schöbl (1910 and 1912); Leclainche and Vallée (1913 and 1923); Nitta (1918); Eichorn (1918); Gräub and Zschokke (1920); Viljoen and Scheuber (1926) and many others.

4) Whole cultures.

A formalized whole culture is nowadays most widely used for the production of immunity. Satisfactory results were obtained by Leclainche and Vallée (1925), McEwen (1926) and Karmann (1927). McEwen made a comparative study of different methods for the production of immunity and found the anaculture to be the best vaccine (see also Leclainche and Vallée, 1925).

A method of preparing a very potent vaccine is described by Sterne, Thorold and Scheuber (1953). The vaccine is prepared in cellophane cases which are immersed in broth. The bacteria grow inside the case in physiological saline and get their growth nutrients by dialysis from the broth. The work reported indicates that "dialysated" toxoids of Cl. chauvoei were far more potent antigens than toxoid prepared in the conventional ways.

Passive immunity can also be produced in susceptible animals. The antiserum can be prepared by the injection of a series of increasing doses of culture

filtrate /.....

filtrate in horses. Sheep serum was also used by Viljoen and Scheuber (1926). In experimental work this method is often used but antisera have not been employed much in the treatment of blackleg, although there is every reason to assume that if given in adequate doses sufficiently early it would be effective.

From the comprehensive literature emerges the fact that almost nothing is known about the chemical properties of the immunizing component. Although very important for the production of highly potent vaccines, the localization and the nature of the antigen are never studied intensively. In chapter three some chemical and physical reactions and their influences on the antigenic activity are discussed. The localization of the antigen has also been briefly studied. We examined the presence of various toxins which could be of interest for a possible correlation with the antigen.

After this preliminary study and a discussion of the results, the method of the isolation of the soluble antigen is found in chapter four. The isolation of the haemolysin is dealt with in the procedure of the antigen isolation, but is also shortly discussed in this chapter. In chapter five some criteria for purity are reviewed. Some chemical and physico-chemical characters of the antigen and haemolysin are also given in this chapter.

Biological properties of the antigen and haemolysin are found in chapter six, where also some experiments with the haemolysin are described. The seventh and last chapter deals with the serological and immunological work that was carried out in the course of this investigation.

Chapter II /.....

CHAPTER II.

MATERIALS AND METHODS.

I. MATERIALS.

1) Strain.

Unless otherwise stated the work was carried out with Cl. chauvoei strain 64. This strain was isolated from a muscle of a heifer which died of blackleg in Transvaal (South Africa) in 1929. The strain is highly pathogenic for sheep and guinea-pigs, its pathogenic action being maintained by regular passing through guinea-pigs, and has been and is still being used for the routine production of blackleg vaccine (anaculture) at Onderstepoort Laboratories.

2) Cultures.

A meat broth, to which were added 0.5 per cent glucose and 1 per cent horse serum, was used to cultivate Cl. chauvoei when small volumes of a pathogenic culture were needed. In practice these cultures were only used for inoculation purposes. We found that they were fairly constant in their pathogenic action, the minimal lethal dose for guinea-pigs varied a little, but was mostly around 0.1 ml when injected intra-muscularly. Because only small volumes of culture were needed the medium was generally freshly prepared before use. It was also found that a rapid deterioration appeared during storage, so that the culture did not grow well. After inoculation the media were placed in a McIntosh jar from which the air was removed by the application of a vacuum and replaced by hydrogen which was freed from traces of oxygen by using a glowing platinum wire. (McIntosh and Fildes, 1916).

As /.....

As stated these cultures were not used as a source of the antigen but could serve for the isolation of bacterial cells.

When young bacilli were wanted, liver broth to which 0.5 per cent glucose was added, was also used. Good growth was obtained in the McIntosh jar, provided the medium was not more than a few days old. Unfortunately the yield of bacteria varied considerably, as did the pathogenic action of these cultures.

For the isolation of the antigen, and the haemolysin, we used both a 48-hour-old culture of Cl. chauvoei in buffered liver-meat broth to which 0.5 per cent glucose was added, and a culture from a cellophane case immersed in the same medium. The 48-hour-old liver-meat broth culture, the basical substance for the preparation of vaccines at Onderstepoort, was prepared in 20 litre quantities from which the samples were drawn. The media were boiled for 1 to 2 hours prior to the inoculation, in order to remove the dissolved air. The inoculate was a constant volume of a von Hibler culture, a culture on liver-meat broth to which were added 0.5 per cent glucose and minced horse brain, and the media were placed in an incubator at 37°C without further anaerobic precautions being taken. We also experimented with cellophane case cultures because of the high immunizing potency as cited in the literature. The cellophane case, containing about 500 ml of air-free, sterile saline was immersed as far as possible beneath the surface in 20 litres of liver-meat broth. The saline was inoculated as before. Growth was retarded because it depends on diffusion of nutrients through the cellophane membrane. For this reason we used the cultures after 5 days of growth.

3) /.....

3) Sera.

Much of the immunochemical work has been done with a hyper-immune horse serum. This serum was prepared by Dr. Mason of the Medical Research Institute in Johannesburg by injection of a series of Cl. chauvoei culture filtrates. The pepsin digestion technique of Pope (1939) was applied to refine the serum, which appeared to be specific as no passive immunization against any Clostridium except Cl. chauvoei was obtained. The serum had been used for analytical purposes only, over a long period. Other sera used in the course of this work were rabbit sera recovered from hyper-immunized rabbits.

4) Other biological materials.

Most of the experimental work on the haemolysin was carried out with sheep erythrocytes. A small quantity of blood was obtained from sheep and Alsever's solution was used to prevent clotting.

The sheep blood mixed with Alsever's solution was allowed to stand for 24 hours in a stoppered measuring cylinder at 4°C. The erythrocytes settled under gravity, and the supernatant, formed after 24 hours, was removed by suction. The erythrocytes were resuspended in an equal volume of Alsever's solution and were stored at 4°C for not more than two weeks.

For several assays we needed rabbit leucocytes which were obtained from exudative fluids by the method of Mudd-Lucké-McCutcheon and Strumia (1929). They were obtained by injecting 150 - 200 ml of sterile 0.9 per cent saline intra-abdominally on the evening before the experiment and again in the morning. Three hours after the second injection about 100 ml of the fluid was recovered

with a /.....

with a stout needle from the peritoneal cavity (near the mouth of the needle side openings had been drilled to avoid blocking). The fluid was collected in a flask containing 30 ml of a solution of 0.7 per cent saline plus 1.1 per cent sodium citrate. The suspension was centrifuged for 2 minutes at 2000 r.p.m., the leucocytes washed with 0.9 per cent saline and centrifuged again. The leucocytes from one rabbit were finally suspended in 5 ml of 0.9 per cent saline.

II. BIOLOGICAL METHODS.

1) The assay of antigen.

We were obliged to examine samples in vivo for their antigenic potency, because no other satisfactory method was available. Guinea-pigs and sheep had proved to be reliable test-animals so we decided to use guinea-pigs in our experiments. The assay of the antigenic activity was carried out in the following manner :
ten guinea-pigs were inoculated subcutaneously with 1 ml of the samples three times at intervals of two weeks. Two weeks after the third and last inoculation the guinea-pigs were challenged with 0.5 ml of a pathogenic culture of strain 64. At the same time three groups, each of three control guinea-pigs, were challenged with 0.5 - 0.3 and 0.1 ml respectively of the same culture. As stated before the minimal lethal dose (referred to as m.l.d.) of this culture proved to be fairly constant, usually being approximately 0.1 ml. Thus we challenged the immunized guinea-pigs with about 5 m.l.d., which value could vary between 2 and 8. We were not interested in an m.l.d. lower than 0.1 ml, so the control series was set up with 0.1 ml as the lowest point. It was found over a period of

several /.....

several years that the lowest m.l.d. ever found was 0.06 ml and this explains that the challenge dose could go up to 8 m.l.d. On the other hand we only accepted results if the control animals that were challenged with 0.3 ml of culture succumbed within 5 days.

The immunizing capacity is recorded as the percentage of the total number of animals used in an experiment that were still alive 5 days after the challenge. The arbitrary time of 5 days was chosen because the animals which were still alive at that time would, with possible exceptions, survive.

2) Determination of the haemolytic activity and the rate of haemolysis.

To determine the haemolytic activity, 2 ml of the above erythrocyte suspension were washed in saline and the red cells resuspended in 50 ml of saline. To 1 ml haemolysin solution in saline was added 0.5 ml of the latter suspension. The mixture was placed at 37°C and left, being shaken occasionally. The results of the experiments are given in the lowest amount of haemolysin that gave an absolutely clear solution after 4 hours.

The rate of haemolysis was measured according to the method of Bernheimer (1944). In this case 2 ml of the above erythrocyte suspension were suspended in 750 ml of buffered saline, after being washed. After establishment of the temperature, equal volumes of this erythrocyte suspension were mixed with a solution of the haemolysin in buffered saline (0,453 per cent (w/v) sodium chloride in M/15 phosphate buffer, a buffer of pH 6 being generally used). The reaction mixtures were left at the temperature employed for the haemolysis and samples of 2 ml volume were taken from the mixtures at regular intervals and

centrifuged /.....

centrifuged immediately. The supernatant fluids were removed from the unlysed cells and the optical density measured in a Zeiss spectrophotometer at 410 mμ wavelength. The degree of haemolysis is expressed as a percentage of complete haemolysis (which was obtained by freezing and thawing).

3) Anti-phagocytic activity estimation.

The anti-phagocytic test was carried out according to the method of Live (1940), who found that culture filtrates of Cl. chauvoei organisms could prevent phagocytosis of the bacilli, in vitro experiments.

A mixture of 0.2 ml of normal rabbit serum, 0.2 ml of a leucocyte suspension, obtained from exudative fluid, 0.2 ml of a washed bacterial cell suspension and the sample was shaken at 37°C for 10 minutes. At the end of the 10 minute period the tubes were transferred to an ice-bath in order to stop phagocytosis. Smears were made of the mixture and, after drying, they were fixed in methanol-formaline, dried again and stained according to Giemsa's method. The degree of phagocytosis was estimated by observing 100 leucocytes and counting the number which had ingested bacilli.

4) Estimation of the deoxyribonuclease activity.

This estimation was carried out in two different ways, using the methods of Kurnick and of Warrack and his co-workers. The method of Kurnick (1953) was followed after minor modifications. The reaction was carried out as follows :

10 mg sodium-deoxyribonucleate was dissolved in 10 ml of 0.01 M phosphate buffer pH 7.5 containing also 0.01 M

magnesium /.....

magnesium sulphate. This solution was incubated at 37°C before 2 ml of the sample were added. Every 10 minutes a 1 ml sample of the reaction mixture was pipetted in 2 ml ice-cold 4% perchloric acid. After chilling in an ice-bath for 1 hour the precipitate was removed by centrifuging at 6 000 r.p.m. for 1 hour and the optical density of the supernatant was measured at 260 mμ in 1 cm quartz cuvettes. The optical density at this wavelength must increase, for the concentration of soluble fragments, formed by the action of the deoxyribonuclease increases.

The method of Warrack, Bidwell and Oakley (1951) was also used (see Meisel (1961)). Rabbit leucocytes were used to detect the action of the deoxyribonuclease. A smear of rabbit exudative leucocytes on an object glass was fixed with methanol for 15 minutes and placed vertically in a test-tube with the solution to be tested. Care was exercised not to have more than one half of the slide immersed in the liquid. The tube was then placed in an incubator at 37°C. After 24 hours at this temperature the slide was withdrawn from the tube, rinsed with water and stained according to Giemsa's method. The percentage of the leucocytes that had been destroyed or had decomposed nuclei is a measure of the activity.

5) Determination of the hyaluronidase activity.

This determination was most easily carried out by the method of Oakley and Warrack (1951). Both horse- and ox-synovial fluids, obtained directly after the animals had been slaughtered, were used after dilution with 0.05 M borate buffer pH 8 to which 0.5 per cent sodium chloride was added. The test relies upon the fact that mixtures of synovial fluid and aqueous congo red will,

under /.....

under suitable conditions, form a compact blue "blob" when a drop of the mixture is allowed to fall into acid alcohol. Reduction of the amount of hyaluronic acid in the mixture leads to changes in the blob formation; at sufficient dilution the drop of synovial fluid and congo red spreads freely into the acid alcohol.

To each tube of a series of synovial fluid dilutions in 0.5 ml volumes, 1.5 ml buffer was added. The mixture was incubated in a waterbath of 37°C for 1 hour. After rapid cooling of the tubes in a melting ice-bath, 0.4 ml of a 0.5 per cent aqueous congo red solution was added. The solutions were mixed and allowed to stand 5 minutes before a suitable quantity was removed with a Pasteur pipette (internal diameter 1 mm). One drop was allowed to fall from a height of 1 cm into an acid alcohol solution in a petri dish. The acid alcohol (1 per cent concentrated hydro-chloric acid in 70 per cent alcohol) reached a depth of 5 mm in the dish. To test the hyaluronidase activity of a sample approximately 8 times the smallest amount of synovial fluid giving a good cohesive blob was used. The test was carried out as described but instead of adding 1.5 ml buffer we now added 1.5 ml of the filtrate diluted with buffer.

III. SEROLOGICAL METHODS.

1) The gel diffusion technique.

Precipitin reactions were studied with the aid of the gel diffusion technique.

The reactions were carried out in small tubes with an inner diameter of 4 mm. Equal volumes of undiluted serum and 1 per cent (w/v) agar were mixed and a layer of 1 cm was allowed to gelatinate in the tube. We then added a 0.5 per cent (w/v) "neutral" agar to a layer also of 1 cm.

After /.....

After gelatinating, the agar column was covered with 0.5 ml of an antigenic solution, containing 3 mg material per ml. The reaction was mostly carried out in physiological saline, but also buffered saline was used, employing acetate and Tris (tris-(hydroxy-methyl)- amino-methane) - sulphuric acid buffers.

In the latter cases the sera were dialysed against the buffer before use. To prevent mould and bacterial growth 0.1 per cent (w/v) merthiolate (Eli Lilly and Company, Indianapolis, U.S.A.) was added to the agar and antigen solution.

2) The fluorescent antibody technique.

The immune sera (5 ml) were precipitated with equal volumes of a saturated ammonium sulphate solution at 2°C. The precipitate was collected by centrifuging and redissolved in physiological saline to a total volume of 5 ml. The precipitation was repeated three times and the material finally dissolved in 5 ml of saline and dialysed against 10 litres of a physiological saline solution for at least 16 hours at 2°C. The protein concentration was determined by the aid of a refractometer and adjusted to 1 per cent by the addition of saline. The pH of the solution was then established at 9 by adding 0.5 M carbonate buffer to a final concentration of 0.05 M. Conjugation of the proteins with fluorescein isothiocyanate (obtained from the British Drug House, England) was carried out at 2°C for 24 hours using 0.05 mg of the fluorescein per mg of protein. The unreacted fluorescein was removed from the fluorescent protein conjugates by gel filtration on a 30 x 2.5 cm column of Sephadex G-75.

The column /.....

The column, prepared as described below, was washed with buffered saline (0.15 M sodium chloride in 0.01 M phosphate buffer of pH 7.2). The fluorescent antibody solution was dialysed against water for about 4-5 hours, and freeze-dried. Before use, a sample of the material was again dissolved in buffered saline and fractionated on a Sephadex column. The final solution was used within several hours after preparation. A smear of the culture to be examined on a thin microscope slide was fixed by heat or by the use of a ether-ethanol mixture (1 : 1). The smear was then covered with a small volume of the fluorescent antibody solution and the conjugation was carried out for 45 minutes at room temperature.

Before being observed under an ultraviolet microscope, the slides were carefully rinsed in buffer of pH 7.2 and, after drying, covered with a few drops of buffered glycerol (of pH 7.5). This pH was employed to enhance the fluorescence.

IV. PHYSICO-CHEMICAL METHODS.

1) Chromatography.

a) D.E.A.E.-cellulose.

Diethylaminoethyl-cellulose, an anionexchanger, was used for preparatory work as well as for purity tests.

Two different commercial products were used, namely,

cellulose N.N diethylaminoethyl ether from Eastman Organic Chemicals - Rochester, New York and

Whatman powder D.E. 50 manufactured by W. and R. Balston Ltd., London.

These types differed mainly in particle size of the resin. The Whatman powder, having a coarser particle

size /.....

size, gave a greater rate of flow of the effluent than the Eastman product when treated in the same way, as described by Peterson and Sober (1956). Before use the resin was twice washed with 0.5 N sodium hydroxide on a glass sintered filter and again with distilled water until the filtrate was neutral. The resin was then twice washed in the buffer which was used as the first solvent for the elution of the column.

Columns were prepared from slurries of the resin by allowing the material to settle in glass tubes fitted with coarse sintered glass bottoms. The flow conditions during the packing of the column were induced by gravity after the fluid column had been lengthened by the connection, at the bottom of the column, of about 1 metre of rubber tube, filled with water. This connection was supplied in order to enhance the rate of flow during the packing and also to obtain a homogeneous column. (When packed under gravity alone, the material sometimes tended to form layers, the resin being bound together in belts which seemed to be piled on each other without being attached). Then air-pressure of 50-60 cm mercury was applied to compact the resin. Prepared under these conditions, the column did not run dry under gravity flow. After each use the anion-exchanger was treated twice with 0.5 N sodium hydroxide to remove the residual protein and then washed with water until alkali free.

b) Sephadex.

Dextran powders manufactured by Pharmacia (Uppsala, Sweden) are sold under the name of Sephadex. These dextran powders form gels consisting of hydrophilic chains that are cross-linked, the polar character being due to the high content of hydroxyl groups. For a

description /.....

description of the chemical and physical properties of the gels, we refer to the dissertation of Flodin (1962).

We used the types G-75, G-100 and G-200 respectively, the powders being dissolved in the volumes of fluid as prescribed by the Pharmacia Laboratories. After the suspensions had stood for 24 hours at room temperature, the gels were formed and columns packed by allowing the gel to settle in glass tubes fitted with coarse sintered glass bottoms which had been covered with filter paper to avoid blocking. The rate of flow during packing was induced by gravity alone.

c) Calcium phosphate gels.

Very good results were obtained by use of columns of calcium phosphate, the hydroxyapatite, prepared according to Tiselius, Hjertén and Levin (1956). Because our antigen purification relied, to a great extent, upon the properties of this material, a brief description of this preparation of the gels is given here.

Two litre quantities each of 0.5 M aqueous solutions of calcium chloride and di-sodium phosphate were allowed to run at an equal rate of flow (about 10 ml per minute) from two separatory funnels into a glass beaker under stirring. After the precipitate had settled, the supernatant fluid was removed by suction and the precipitate washed 4 times with about four litres distilled water. Water was again added to a volume of four litres and 100 ml of a 40 per cent sodium hydroxide solution (freshly prepared) was added. The suspension was boiled under stirring for one hour and the precipitate was again washed four times with four litres of water. After the last washing four litres of 0.01 M sodium phosphate buffer pH 6.8 were added. The solution was heated to about 95°C,

care /.....

care being taken to prevent boiling at this stage. The gel was then boiled twice in 0.01 M buffer for 5 and 15 minutes respectively, after which it was twice boiled in 0.001 M sodium phosphate buffer of pH 6.8 for 15 minutes. After each boiling the gel was allowed to settle and the supernatant was removed by suction. The hydroxyapatite thus obtained was stored in 0.001 M buffer of pH 6.8 at 4°C.

Columns were packed under gravity flow in tubes with sintered glass bottoms which had been covered with filter paper to avoid stoppage.

d) γ-alumina-gel.

This gel was only used in a few experiments. The preparation was carried out according to Willstätter and Kraut (1923). A hot solution of 50 g aluminium sulphate ($Al_2(SO_4)_3 .18H_2O$) in 100 ml water was poured quickly into 650 ml of an aqueous solution containing 30g ammonium sulphate and 42 ml of 20 per cent ammonia. The solution was stirred for 15 minutes at a temperature of 60 - 70°C. The suspension formed was diluted to four litres and the alumina allowed to settle. The supernatant was removed and the precipitate washed several times with four litre quantities of water. At the fourth washing, 8 ml of 20 per cent ammonia was added to remove traces of aluminium ions. After more washings with water the supernatant no longer became clear and only two washings were done after this. The alumina was stored in the coldroom at 4°C for 3 months prior to use, in order to obtain a high yield of γ-alumina.

Columns were packed in the same way as calcium phosphate columns. Before packing, however, phosphate buffer of pH 6.8 was added to a final concentration of 0.01 M.

e) /.....

e) Operation of the columns.

The column surfaces were covered with a filter paper to prevent disturbance by the loading procedure. After equilibration of the columns with the initial buffer, which was allowed to flow through them for at least 16 hours, the columns were loaded. The samples were allowed to drain in the columns and washed in with a few ml of the initial solvent. When loaded a layer of a few mm of the first effluent was brought upon the column and a vessel containing it was connected to the top of the column.

To adjust the rate of flow of the solvent through the column the height of the vessel was adjusted. When gradient elution was employed, two similar vessels were connected by means of a bridge, a U-tube with the legs immersed down to the bottoms of both vessels.

The volume of fluids in the two vessels was kept equal to provide a hydrostatic equilibrium. Simple linear gradients thus obtained proved to be suitable in our experiments. The outlet of the vessel containing the buffer eluent with the lowest ionic strength was connected to the top of the column and this fluid, its ionic strength continuously being increased, was mixed by a magnetic stirrer.

The effluent was collected in fractions by means of a fraction collector (a Central Aimer fraction collector, from Aimer Products Ltd., London).

2) Electrophoresis.

a) Starch gel electrophoresis.

The starch-gel electrophoresis as developed by Smithies (1955) seemed to have the advantage of greater

resolubility /.....

resolubility than the paper electrophoresis.

We used the "Starch-Hydrolysed" as manufactured by Connaught Medical Research Laboratories, Toronto, Canada. The gels were prepared according to the recommendations of the manufacturer. The samples were introduced into the gel on a piece of filter-paper which was previously soaked in a buffer solution of the sample. The filter-paper used for this purpose, Whatman paper 3 MM, was cut accurately to the cross-section of the gel, and was placed in a transverse slit made in the gel. The electrical contact was supplied to the ends of the gel with filter-paper wads soaked in a suitable buffer solution which dip into vessels containing the same solution. This solution had the same pH of the buffer in which the gel was prepared but was 12 times concentrated. Agar bridges saturated with potassium chloride were used to connect these vessels to the electrode chambers. Current was passed for 6 hours at a low temperature. About 10 volts per cm gel were applied. The gel was then removed from the tray and sliced along its length in a horizontal plane with the aid of a very thin metal wire. The sliced planes were stained with a saturated amido black solution in a methanol-acetic acid-water mixture (50:10:40 volumes) for five minutes under swirling of the stain solution. The excess dye was washed out of the gel by repeated addition of the same mixture.

We used two different buffers to extablish the purity of our fractions, namely, a borate buffer of pH 8.4 (recommended by Smithies for serum separations) and a phosphate buffer of pH 6 of the same molarity as the borate buffer. This pH change could be applied without difficulty because the stability range of the starch-gels

lies /.....

lies between pH 3 and 9, according to Smithies who obtained good and reproducible results at pH values in this range. The protein sample was dissolved to a 3 per cent solution in the same diluted buffer, which was also used for the preparation of the gels.

b) Column electrophoresis.

The procedure described by Gedin and Porath (1957) was followed, using a vertical column of 2 x 135 cm filled with Munktell's cellulose powder. The electrophoresis was carried out for 45 hours applying a current of 22½ mA. The column was loaded with 5 ml of the refined horse serum (about 500 mg of protein) which was previously dialysed against 2 litres of the buffer for 18 hours at 2°C. During the experiment the column was cooled with water at 4°C in an outer jacket. The buffer used in the experiments was a phosphate-borate buffer of pH 8.4 and an ionic strength of 0.05 (2.34 g $NaH_2PO_4.2H_2O$ and 4.29g $Na_2B_4O_7.10H_2O$ per litre). After electrophoresis the column was eluted with the same buffer and the optical density of the effluent, collected in 3 ml fractions, was measured in a Zeiss spectrophotometer at a wavelength of 280 mμ using quartz cuvettes with a width of 0.5 cm.

c) Moving boundary electrophoresis.

The moving boundary electrophoresis of the antigen and haemolysin was carried out at 4°C in a Hilger-Watts instrument. For the assembly and operation of the apparatus we refer to the publication of Tiselius (1937).

The movement of the boundary under the influence of an electrical field was recorded photographically during the course of the experiments. The mobility under unit electrical field, u, was calculated by the expression :

u /.....

$u = \frac{x}{Ft}$ where, x is the distance moved by the boundary in t seconds, and F is the field strength.

The electrical field, F, may be computed by the expression

$F = \frac{i}{Ax}$ where i is the current in amperes, A is the area of cross section of the cell and x is the specific conductivity of the protein solution.

Hence from a combination of these two formulae

$$u = \frac{xAx}{it}$$

The position of the boundary was taken at the peak maxima on the photographic recordings. The correct conductivity to be used in the calculation of the mobility would be that of the solution at the position of the boundary. In practice, however, we equilibrated the protein solution by dialysis for 18 hours at a low temperature against 2 litres of buffer, whose conductivity was measured after the dialysis. This value was used in the evaluation of the mobilities. An elementary type of cell in conjunction with a Cambridge conductivity bridge was used for the measurements.

3) Ultracentrifugal methods.

a) The establishment of purity in the ultracentrifuge.

This test was carried out in a Spinco model E ultracentrifuge. The experiments were made at 56 100 r.p.m. using the synthetic boundary cell. The photographs were taken at 8 minute intervals. Approximately 1 per cent protein solutions were used. The sedimentation constants were calculated from the equation :

$$S = \frac{dx/dt}{w^2x}$$

where : w = the angular velocity of the rotor and

x = the displacement of the centroid of the boundary from the axis of rotation.

By /.....

By taking photographs at equal intervals of time it was possible to examine the progress of the peak down the cell. The equation may be written :

$$S = \frac{dx/x}{w^2 dt}$$

and hence we find for any displacement of the boundary from x_1 to x_2

$$S = \frac{\ln x2/x1}{w^2(t_2-t_1)}$$

These sedimentation constants were corrected to 20°C and also to the viscosity and density of the pure solvent (water) at this temperature by the expression (Svedberg, 1940) :

$$S^o_{20} = S \times \frac{n}{n^o_{20}} \frac{(1 - \bar{v}^o_{20} \rho^o_{20})}{(1 - \bar{v} \rho)}$$

where : S^o_{20} is the sedimentation constant corresponding to n^o_{20} and ρ^o_{20}, the viscosity and density respectively of pure water at 20°C and $\bar{v}^o_{20}$ the partial specific volume of the protein under these conditions. The other symbols refer to the experimental conditions of temperature and solvent viscosity.

b) Molecular weight determinations in the ultracentrifuge.

Molecular weights of macromolecules can be obtained from the well-known equation :

$$M = \frac{S}{D} \frac{RT}{(1-\bar{v}\rho)}$$

where R is the gas constant, T the absolute temperature $\bar{v}$ the partial specific volume of the macromolecules, ρ the density of the buffer solvent at temperature T S the sedimentation coefficient and D the diffusion coefficient. (S and D refer to the macromolecules).

The /.....

The sedimentation coefficient is readily evaluated from ultracentrifuge measurements at high speed 56 100 r.p.m.) and must be combined with the independently determined diffusion coefficient for computing the molecular weight. For precise molecular weight estimates the partial specific volume $\bar{v}$ must be determined; this factor, however, has the value of approximately 0.74 for most proteins at 20°C.

Another method of computing the molecular weight of macromolecules in the centrifuge is the sedimentation equilibrium method. When the ultracentrifuge is operated at a low speed, a situation is obtained after a prolonged centrifuge time, in which there is no net mass transport across a given surface at a distance x from the axis of rotation. At this stage the material migrating across the surface in the centrifugal direction is exactly balanced by the transport centripetally, due to diffusion. Or :

$$cSw^2x = D\frac{dc}{dx}$$

where c is the concentration of solute, w the angular velocity, x the distance surface to axis of rotation and dc/dx the concentration gradient in the region of the surface.

When this equation is combined with that for the molecular weight the formula becomes :

$$M = \frac{2\,RT}{(1-\bar{v}\rho)w^2}\quad\frac{d\ \ln\ c}{dx^2}$$

It is obvious that measurement of the concentration distribution at sedimentation equilibrium gives the molecular weight of the sedimenting molecules directly.

To /.....

To reach the sedimentation equilibrium, however, a long centrifuge time is involved. Archibald (1947) has shown that the above equilibrium equation applies also to the region near the meniscus and the bottom of the cell at any time during the approach to equilibrium. As Archibald pointed out the cell is closed and for this reason the transport of solute across the meniscus and the bottom of the cell must be zero. Though the Archibald method does not deal with a real equilibruim, because there is no mass transport at all across the surfaces under observation, the method provides a rapid and accurate determination of molecular weights. A knowledge of the concentration distribution in the region of the meniscus and the bottom of the cell can be used to evaluate the molecular weight.

For the calculation of the molecular weight from these measurements the formula of Ehrenberg (1957) was employed :

$$\frac{S}{D} = \frac{y_m}{w^2 x_m K(A_s - A)}$$

where y_m is the concentration gradient at the meniscus.
x_m is the distance from the meniscus to axis of rotation.
K is the inverse of the enlargement in the x direction
A is the area between the curve, baseline and meniscus and
A_s is the area between the curve and baseline of a corresponding exposure using a synthetic boundary.

We used the technique as described by Joubert and Haylett (1962). The area A_s was determined in the same experiment as A by running a solution of the material simultaneously in the synthetic boundary cell and a wedge window cell in the ultracentrifuge. The areas were estimated from enlargements of the ultracentrifugal patterns traced on to graph paper. A relation similar to the above is

valid /.....

valid at the bottom of the cell but because of the piling up of the material in this region the curves are usually steep there. Thus the y co-ordinate of the intercept is difficult to determine, making the results unreliable.

c) <u>The estimation of the diffusion coefficient in the ultracentrifuge</u>.

The diffusion coefficient, D, can be estimated from the peaks produced by the synthetic boundary cell during the approach to equilibrium experiments, using the equation :

$$\frac{dc}{dx} = \frac{C_o}{2\sqrt{\pi Dt}} e^{-x^2/4Dt}$$

where : dc/dx is the concentration gradient
C_o is the initial concentration of solute
x is the distance from the original boundary
t is the time at which the exposure was taken.

To evaluate the diffusion coefficient, the curves were enlarged on graph paper and analysed by :

1) the statistical method, first applied by Lamm (1937) and
2) the height-area method (see Neurath 1942).

The above equation can be modified by making use of the proportional relation between increments of concentration and refractive index, a linear function of the type $n - n_o = kc$
where n and n_o are the refractive indices of the solution and solvent respectively.

In the height-area method we examined the refractive index gradient at the position of the original boundary, where x equals zero and the curve reaches its maximum height.

It follows from the above equation that at the position x = 0 the formula can be written :

$$\left(\frac{dn}{dx}\right)_{x=o} = H_m = \frac{n - n_o}{2\sqrt{\pi Dt}}$$

and /.....

and therefore :

$$D = \frac{(n - n_o)^2}{4\pi t\ (H_m)^2} = \frac{A^2}{4\pi t\ (H_m)^2}$$

where A is the area of the curve and Hm is the maximum ordinate.

To estimate the diffusion coefficient according to the statistical method we treated the curves as ideal displacement distribution curves. The base-line of the curve is divided into evenly spaced units (e.g. 0.2 cm) numbered outward from an arbitrarily chosen origin near the centre of the base-line. If Xi is the respective distance on the base-line and Yi the corresponding ordinate then we can write :

$$u_1 = \frac{\Sigma X_i Y_i}{\Sigma Y_i} \quad \text{and} \quad u_2 = \frac{\Sigma X_i^2 Y_i}{\Sigma Y_i}$$

where u_1 and u_2 are respectively the first and second moment about this arbitrarily chosen ordinate.

The second moment of the curve about the centroidal ordinate, that ordinate for which the first moment equals zero, is

$$u_2^o = u_2 - (u_1)^2$$

where u_2^o is the true second moment of the curve.

The diffusion coefficient can readily be evaluated from this true second moment by the relation :

$$D = \frac{u_2^o}{2t}$$

In both methods we had to correct the calculated diffusion coefficients for the photographic magnification. The values of $A^2/4\pi H_m^2$ and of u_2^o were plotted against the time, t, for every series of photographs taken in one experiment. The diffusion coefficients were computed from the slope of the regression lines, which were determined

by /.....

by the method of least squares, and corrected to 20°C and the viscosity of pure solvent (water) by the formula

$$D^{o}_{20} = D \frac{T}{293} \frac{ns}{n_{H_2O}}$$

4) The variable solvent solubility test.

The test was carried out according to Smithies (1954). The protein sample to be tested was dissolved in 0.1 M phosphate buffer of pH 7.4 to a final concentration of 1.25 per cent. This solution was centrifuged at 40 000 r.p.m. before use, to ensure its clarity.

To ensure a constant pH the ammonium sulphate was also dissolved in this buffer until saturation at 20°C. This saturated ammonium sulphate solution was diluted with the same buffer at 20°C, using a burette for accurate dilution. We made up 32 different dilutions and added 2 ml of each to 0.2 ml of the antigen and haemolysin solution. After 40 hours at 25°C the precipitates were removed by centrifuging at 6 000 r.p.m. and the optical densities of the solutions were measured at 275 mμ.

V. CHEMICAL METHODS.

1) Determination of the nitrogen content.

The method of McKenzie and Wallace (1954) was followed. This method relied upon the Kjeldahl digestion technique using mercuric sulphate as a catalyst. To raise the boiling-point of the digestion mixture, potassium sulphate was added. The ammonia was distilled into a 2 per cent boric acid solution containing 1 per cent of a mixed indicator which consisted of 2 parts of a 0.2 per cent methyl red and 1 part of a 0.2 per cent methylene blue solution in 95 per cent alcohol. The boric acid solution was directly titrated with a 0.01 N potassium bi-iodate ($KH(IO_3)_2$) solution. This solution was prepared by

dissolving /....

dissolving 389.9 mg of the salt (p.a.) in 100 ml distilled water. This solution is acid and reacts with the ammonia in the borate solution where 1 ml is equivalent to 0.01 mM NH_3 or 0.1401 mg nitrogen.

2) Determination of carbohydrates.

We employed the methods of Devor (1950) using sulphonated α-naphthol, as well as the method of Surgenor (1949) using orcinol for the colourimetric determination of carbohydrates.

The α-naphthol was dissolved in concentrated sulphuric acid to a 0.4 per cent (w/v) solution and left in the dark at 20°C for 8 hours prior to use. To a volume of 2 ml of a test solution 5 ml of the sulphonated α-naphthol was added, the mixture heated at 100°C for 10 minutes and, after chilling it in an ice-water bath, the colour densities were measured (within one hour of the beginning of the experiment) at 490 mμ and 570 mμ for hexoses and at 490 mμ and 550 mμ for pentoses.

The orcinol method was used for hexoses only and the colour was developed by heating a mixture of 1 ml test solution with 5 ml of a 0.1 per cent (w/v) orcinol solution in 67 per cent sulphuric acid at 80°C for 12 minutes. In this case the colour densities were measured at 540 mμ.

3) The amino acid analysis.

a) Paper chromatography.

The materials were hydrolysed with constant boiling hydrochloric acid in sealed glass tubes, using about 0.5 ml acid per mg of protein. The hydrolysis was carried out for 16 hours at 105°C. After removal of the acid by evaporation in a vacuum the residue was dissolved in water and again evaporated. Approximately

150 /.....

150 µ of the hydrolysed material, after being dissolved in 10 per cent isopropyl alcohol (about 0.5 ml per mg of the original protein), was brought upon Whatman paper No. 1. Two-dimensional chromatography was employed with the solvents :

1) phenol-water (80 per cent w/v phenol in water) and
2) butanol-acetic acid-water (4:1:5).

The first solvent was allowed to run for 24 hours, and the paper was dried before the chromatography was continued in the second direction, where it was carried out for 18 hours. The experiment took place at 25 - 30°C. The amino acid spots were examined after staining with ninhydrin. The paper was sprayed with a fresh solution of 2 g ninhydrin (Eastman Organic chemicals) in a mixture of 200 ml ethanol, 8 ml collidine and 60 ml acetic acid. The colours were developed at 80°C for 5-10 minutes.

b) Column chromatography.

We used the procedure of Moore, Spackman and Stein (1958) with the ion-exchanger Amberlite CG-120 type II (obtained from Fisher Scientific Co., New York, U.S.A.).

After several washings in hydrochloric acid and sodium hydroxide the resin was fractionated by the hydraulic separation method of Hamilton (1958). The resin was finally washed with boiled citrate buffer of pH 4.25 and 150 x 0.9 cm columns were packed in 6 layers with a suspension of the resin in the same buffer. For a description of the buffers and the packing of the column we refer to the literature. Good separations were obtained when the temperature was maintained at 50°C and when the rate of flow remained constant (12 ml per hour). To maintain the temperature at 50°C, water from a thermostat was

pumped /.....

pumped through a jacket around the column. The rate of flow was adjusted by the use of nitrogen pressure, regulated by a manostat (manufactured by Edwards High Vacuum Ltd., Crawley, England).

Small columns, 15 x 0.9 cm, were prepared to estimate the basic amino acids. The columns were loaded with 1 - 1.5 mg of the hydrolysed proteins and the effluent was collected in 2 ml volumes. The fractions were then stained according to the method of Rosen (1957).

To every fraction, 1 ml of concentrated acetate buffer of pH 5.3 containing 0.002 M sodium cyanide (Na CN) and 1 ml of a 3 per cent (w/v) ninhydrin solution in methyl cellosolve were respectively added.

The mixtures were heated in a bath of 100°C for 15 minutes, then removed, after which 5 ml of an isopropyl alcohol-water mixture (1:1) was added and the solutions shaken. After cooling to room temperature the colour density was measured at 570 mμ in a spectrophotometer.

The concentrations of the amino acids were estimated with the aid of a leucine standard curve, plotted with the method of least squares, and corrected for the percentage of colour yield as given by Rosen. Ammonia was estimated by the use of an ammonia standard curve because of the low colour yield of this component.

4) Determination of tryptophane.

Because tryptophane was destroyed during the hydrolysis we adopted the following method from the South African Wool Research Institute. To 5 mg of the material were added 0.4 ml of a 1.5 per cent (w/v) dimethyl-amino-benzaldehyde solution in 1 N sulphuric acid, and 2 ml of 22.8 N sulphuric acid. The material was dissolved by

shaking /.....

shaking and left for 8 days at 20°C in the dark. Then 0.02 ml of a 0.007 per cent sodium nitrite solution, freshly prepared, was added and the colour developed at 20°C for 45 minutes. When a precipitate was formed in the solution, it was removed with a sintered glass filter. The colour density was measured at 570 mμ.

5) Determination of phosphate.

The phosphate determination was carried out according to Scheffer, Ulrich and Benzler (1960). Approximately 3 mg of the material was digested with concentrated sulphuric acid (0.5 ml) and perchloric acid (0.1 ml) in Kjeldahl flasks of 10 ml volume. During the digestion the material became dark but after some time this colour disappeared and the digestion was continued until all the perchloric acid had evaporated. The residue was diluted with 6 ml distilled water after which 1 ml of a 1.44 per cent (w/v) ammonium molybdate solution was added. The flasks were then heated in a waterbath at 70°C for 5 minutes before 0.5 ml of a 1 per cent (w/v) ascorbic acid solution was added.

The latter solution was used within one hour of being prepared. The mixtures were heated at 70°C for a further 20 minutes, after which the volumes were corrected to 10 ml by the use of volumetric flasks. The colour density of the mixture was measured at 670 mμ. The method is highly sensitive and traces of phosphate could be determined.

To determine inorganic phosphate, the material was dissolved in 1 ml sulphuric acid (200 ml concentrate acid diluted to 1 litre) and the reagents were added directly.

VI /.....

VI. OTHER TECHNIQUES AND METHODS.

1) Determination of protein concentrations.

The optical density was taken as a measure of protein concentration and was determined in a Zeiss spectrophotometer at a wavelength of 275 or 280 mμ. In the ultraviolet region, quartz cuvettes of 1 cm or 0.5 cm width were used.

For the estimation of protein concentrations of 0.5 - 1.5 per cent, as used in molecular weight determinations in the ultracentrifuge, we had a refractometer at our disposal.

2) Dialysis of protein solutions.

The protein solutions were dialysed in 23/32 cellophane tubes obtained from the Visking Company (division of Union Carbide, Chicago, U.S.A.). This cellophane membrane did not allow insulin with a molecular weight of about 6 000 to pass. After heating the cellophane in boiling water for about 10 minutes it became even less permeable, a method we applied for the dialysis of the haemolysin which we suspected was passing the membrane in considerable amounts at prolonged dialysis. The cellophane case was rotated in a long cylindrical vessel, through which a continuous flow of distilled water ran at a rate of about 10 ml per minute (by using a capillary connected just above the bottom of the vessel). The dialysis was carried out for at least 18 hours in a coldroom of 2°C.

3) Freeze-drying of protein solutions.

Because the isolated proteins were labile in aqueous solutions, we had to store the proteins in another way. We decided upon the most widely-used method of freeze-drying, evaporation of the solution, in the frozen state, in high vacuum. The principle of the method

relies /.....

relies upon the fact that the solution stays in the frozen state when the evaporation occurs quickly enough.

Solutions up to about 100 ml volumes were frozen around the wall of a 500 ml cylindrical flask by rotating the latter in an acetone-dry-ice bath of -80°C. The flask was transferred immediately to the vacuum apparatus and then evacuated to a pressure of about 10^{-3} - 10^{-2} mm. The evaporated water was condensed in a big vessel, which was immersed in a dry-ice-acetone bath of -80°C, before it could reach the pumping system. Under these conditions the protein solution remained in the frozen state until complete dehydration, which occurred after 6 - 8 hours. To allow the last traces of water to evaporate the freeze-dried material was left overnight in the apparatus under continuous vacuum.

4) The preparation of bacterial extracts by ultrasonic vibration.

For the preparation of ultrasonic extracts a Sonblaster ultrasonic generator (manufactured by Narda Ultrasonic Corp., Westbury, New York) was used. The treatment was performed for 1 to 2 hours with a frequency of 10 000 Hz and a capacity of 500 Watts.

5) The estimation of the energy of activation.

According to the theory of Arrhenius (1889), the energy of activation of a reaction can be evaluated from the formula :

$$k = Ae^{-u/RT}$$

where k is the rate of reaction

A is the constant in connection with the collision and probability factors, which both determine the rate of reaction between different molecules,

u is the energy of activation

T /.....

T is the temperature in absolute units

R is the gas constant in cal/^{o}C.

On differentiation of the logarithmic form of this equation with respect to 1/T (assuming that A remains constant) we obtain :

$$\frac{d \ln k}{d(1/T)} = -\frac{u}{R}$$

where u/R is given by the slope of the straight line of the plot of ln k against 1/T.

Chapter III /.....

CHAPTER III.

A PRELIMINARY STUDY OF THE CULTURES OF CL. CHAUVOEI STRAIN 64.

Robertson (1929) states that the lethal toxin of Cl. chauvoei is an important immunizing antigen. Although it has never been proved directly, since the toxin has never been isolated, it is certainly true that the toxin is an antigen. This can be shown by the high anti-toxin titers of various sera claimed by different workers. These sera also proved to have protective power against cultures in susceptible animals.

A correlation between the lethal toxin and a haemolysin is reported by various authors (Kerrin 1934, Guillaumie and Kréguer 1948 and Moussa 1958).

Because of the immunizing power of the lethal toxin, according to Robertson, and because of the correlation between the lethal toxin and the haemolysin as predicted and partly proved by a group of different authors, we were also interested in the latter component. Our main interest was of course the immunizing antigen.

1) The localization of the immunizing antigen.

It may be stated here that all the authors who have studied the process of immunization of susceptible animals against infection with virulent cultures are agreed upon the general antigenic and immunizing capacity of the bacteria-free, non-toxic filtrates of Cl. chauvoei, and upon their specific nature.

It was also found that anaculture vaccines were more potent vaccines than the culture filtrates.

This /.....

This indicates the presence of an immunizing antigen bound to the bacterial cell. This antigen may be related to that in the culture filtrate. Many experiments in the localization study were carried out with a five-day-old cellophane case culture. We used this culture for two reasons, namely :

a) the bacterial cells could be obtained free from impurities present in the liver-meat-broth, and

b) bacterial products are relatively pure in this culture when compared with the liver-meat-broth cultures, which contain many other substances.

We compared the immunizing capacities of the sedimented materials from the cellophane case and liver-meat broth cultures obtained by centrifuging at 3 000 r.p.m. for 30 minutes. The supernatant fluids were removed and the residues washed and resuspended in 0.85 per cent saline. Formaline was added to a final concentration of 0.6 per cent and the suspensions were incubated at 37°C for 48 hours. The volumes were corrected with sterile saline to the initial culture volumes. The supernatant fluids were sterilized by filtering through a Seitz filter and were also tested for their immunizing capacities.

Before the test was carried out the sterility of the suspensions and the filtrates was investigated by inoculating liver-glucose broth with the samples. The immunizing capacity was then determined by using two different quantities of the samples, e.g. 1 ml and 0.6 ml, for the injections.

Table /.....

Table III-1.

The immunizing capacity of culture filtrate and bacterial cells.

	5-day-old cellophane case culture.		48-hour-old culture	
	bacterial cells	filtrate	bacterial cells	filtrate
1 ml	100%	100%	100%	90%
0.6 ml	100%	80%	100%	70%

All the control animals died within five days.

The suspensions and the Seitz filtrates proved to be sterile as no growth was obtained.

It was anticipated that the lower immunizing potencies of the Seitz filtrates were the results of absorption of antigen on the filter. We were, however, obliged to remove the germs to obtain information about the soluble antigen. In another experiment we sterilized the cultures in the ultracentrifuge at 40 000 r.p.m. for 1 hour. The supernatant, which was sterile as no growth was detected in liver-glucose broth, gave the same results as those recorded in Table III-1.

After obtaining encouraging results, shown in Table III-1, we decided to investigate the antigen or antigens, present in the bacterial cells because the immunizing potency of this antigen seems to be much higher.

The cells were again recovered from a five-day-old cellophane case culture by centrifuging at 3 000 r.p.m. for 30 minutes. After two washings in 0.85 per cent saline the cells were resuspended in saline and the suspension divided into two equal volumes. One part was boiled for two hours and the other was centrifuged and the bacterial material dried by acetone washing. The acetone-dried bacteria were stored at 25°C for 1 month, and resuspended in saline. The bacterial

suspension /.....

suspension volumes were corrected with 0.85 per cent saline to the corresponding culture volumes from which the bacteria originated. The immunizing capacities of the cells, recorded after the treatments described above are shown in Table III-2.

Table III-2.

The immunizing capacity of boiled and acetone-dried bacterial cells.

	capacity.
Boiled bacteria	20%
Acetone-dried bacteria stored for 1 month	100%

All the control animals died within five days except one, which may have been naturally immune; for it was challenged with 0.3 ml of culture. The suspension of acetone-dried bacterial cells had to be sterilized because viable spores were still present, as was detected after the inoculation of guinea-pigs, which all succumbed. We sterilized with 0.6% formaline in an incubator of 37°C for 48 hours.

The results shown in Table III-2 indicate also the presence of an immunizing antigen bound to the bacterial cell, although this antigen is not heat-stable as reported in the literature.

2) The immunizing antigen in bacterial cell extracts.

The following experiments were carried out to obtain information on the localization of the immunizing antigen bound to the cell.

The bacterial cells recovered from a five-day-old cellophane case culture were washed three times in

0.85 per cent /.....

0.85 per cent saline and resuspended in about 1/10 of the culture volume of 0.85 per cent saline. This suspension was divided into two equal volumes. To one part, lysozyme was added to a concentration of 0.3 per cent, and the bacteria in the second part were treated by a combination of autolysis and freezing in cold acetone-dry-ice mixtures.

Although lysozyme is supposed to attack the cell walls of most gram-positive bacteria, it had almost no effect on the Cl. chauvoei cells when treated for 48 hours at 37°C. When a sample of the suspension was centrifuged at 6 000 r.p.m. for one hour the supernatant of the lysozyme treated cells had only a very low optical density at 260 mμ and 280 mμ. The process of autolysis, one week at 30°C, followed by freezing and thawing three times in acetone-dry-ice mixtures of -80°C and in a waterbath of 30°C respectively, followed again by autolysis for one week yielded a greenish coloured, slightly viscous solution after centrifuging at 6 000 r.p.m. for one hour. (This solution had an extremely high absorption at 260 mμ but slightly less at 280 mμ).

The supernatant of the autolysate was divided into two parts, one of which was sterilized by filtration through a Seitz filter. These extracts, the filtered and not-filtered autolysates, were examined for their immunizing capacities; also after heating in a water-bath at 80°C for 30 minutes. The cell residues after lysozyme treatment and autolysis were both sterilized by 0.6 per cent formaline and incubating at 37°C for 48 hours.

Table III-3 /.....

Table III-3.

Immunizing capacity of cell extracts and cell residues.

		capacity, %
Bacterial cells after autolysis		100
Bacterial cells treated with lysozyme		100
Cell extract not sterilized	(a)	100
Cell extract not sterilized but heated at 80°C	(a)	70
Cell extract sterilized	(a)	100
Cell extract sterilized and heated at 80°C	(a)	30
Cell residue after prolonged autolysis		0

(a) only cell extracts from the autolysate were used, because of the very weak reaction of the lysozyme.

All the control animals challenged with 0.5 and 0.3 ml culture succumbed, whereas two (of the three) animals challenged with 0.1 ml survived. The m.l.d. seems to be higher than 0.1 ml.

Unsterilized cell extract tested before heating, gave a weak growth in liver-glucose broth, consisting of spore bearing rods which gave blackleg in guinea-pigs.

As can be seen from Table III-3, absolutely sterile bacterial cell-extracts possess a heat-labile immunizing antigen.

The bacterial residue after autolysis and freezing still contains an immunizing antigen. This suspension was stored at 30°C for one month and frozen in acetone-dry-ice mixtures of -80°C twice a week. The suspension was then centrifuged at 40 000 r.p.m. for 30 minutes and the supernatant discarded. Absolutely no immunizing activity was found in the cell residue (see Table III-3). The challenge dose was, however, higher in this case. The guinea-pigs challenged with 0.1 ml succumbed within 5 days.

The results indicate the presence of an immunizing antigen inside the cell which is soluble after disintegration

of the/.....

of the cells. Perhaps it is present in the plasma but it also may be adsorbed to cell-particles from which it is released during the reaction. We decided to investigate this antigen further.

3) The study of the immunizing antigen in the bacterial cell.

We tried to isolate the antigen from the bacterial cell in order to obtain information about its nature. The phenol extraction method of Westphal, Lüderitz and Bister (1952) was chosen. This method was modified by Davies (1956) for the isolation of somatic antigens. We followed the warm 45 per cent phenol extraction method for cold phenol extraction so far had only been applied in the case of gram-negative bacteria.

The bacterial cells were recovered from a 5-day-old cellophane case culture, washed in saline and dried by acetone washing. To remove the readily soluble protein before proceeding to the extraction of other compounds the acetone-dried cells were suspended in 2.5 per cent saline at 20°C (about 12 g dried material per 150 ml) and stirred for 24 hours. To prevent mould and bacterial growth, 1 per cent toluene was added. The suspension was centrifuged at 3 000 r.p.m. and the supernatant, after sterilization through a Seitz filter, was tested for its immunizing activity.

The sedimented cells, resuspended in saline, were re-extracted twice for periods of 24 hours. Both extracts were also tested. The cell residue remaining after the saline extraction was suspended in water and warmed to 50°C, and an equal volume of 90 per cent aqueous phenol at 50°C was added. The mixture was stirred for 45 minutes

and cooled /.....

and cooled to 0°C, whereupon a separation of water and phenol phases occurred, this separation being hastened by centrifuging. The clear aqueous phase was removed and sterilized by filtration through a Seitz filter. The addition of 3 vol. of ethanol at -10°C to the solution precipitated most of the material it contained. After reprecipitation with ethanol, the material was redissolved in water at 0°C. Cold 1 N hydrochloric acid was added to pH 2 and the nucleic acids were precipitated. The material in the supernatant consisted mostly of lipo-polysaccharides as was proved by the low nitrogen content of freeze-dried material.

Table III-4.

Immunizing capacity of several fractions isolated from bacterial cells.

		Capacity.
First saline extract		60%
Second and third saline extracts		0%
Bacterial cells after 3 saline extractions	(a)	100%
Nucleic acid precipitate		0%
Lipo-polysaccharide solution	(b)	0%

(a) The bacterial cells were sterilized by 0.6 per cent formaline and incubated at 37°C for 48 hours prior to the experiment.

(b) Nitrogen content of the freeze-dried material was 1.4 per cent.

All the control guinea-pigs inoculated with 0.5 ml, 0.3 ml and 0.1 ml succumbed within five days.

As recorded in Table III-4, the results were fully negative. It seems that the immunizing antigen was destroyed by the phenol extraction. We already lowered the temperature from 60°C, as employed by Davies, to 50°C, but still no active soluble antigen was found. One fact

emerges /.....

emerges from this experiment : the nucleic acids and the lipo-polysaccharide extract possess no immunizing activity.

Because of the bad results as far as the phenol extraction was concerned we decided to investigate some properties of the immunizing potency of the Cl. chauvoei cultures.

4) Some properties of the whole Cl. chauvoei culture-antigen.

Until now very little was known about the chemical and physical properties of the antigen or antigens present in the whole culture. A series of simple tests was set up with the anacultures in order to investigate the stability of the antigen(s). The work, as reported here, had been carried out with a 5-day-old cellophane case culture and also with the 48-hour-old liver-meat-glucose broth culture.

The results were approximately the same.

A one-litre sample of both cultures was divided into several 50 ml portions. The portions from each culture were treated in the same way, and tested immediately after sterilization with 0.6 per cent formaline at 37°C for 48 hours.

Table III-5.

The influences of physical and chemical reactions on the immunizing capacity of whole cultures.

	Capacity of cellophane case culture, %	Capacity of 48-hour-old culture, %
Boiling for 2 hours	30	20
Heated at 80°C for 1 hour at pH 4	20	10
At 25°C for 24 hours at pH 2	30	30
At 25°C for 24 hours at pH 9	100	100
At 37°C for 18 hours with 0.5% pepsin at pH 3	20	10
At 37°C for 18 hours with 0.5% trypsin at pH 6	50	40
At 37°C for 18 hours with 1% amylase at pH 7	100	100

To establish the pH of the culture N HCl and N NaOH were used and the pH measured with a glass electrode.

All the control animals challenged with 0.5 ml culture succumbed, and so did 2 out of 3 and 1 out of 3 challenged with 0.3 ml and 0.1 ml culture respectively.

The resistance of the immunizing potency against storage was also investigated in both cultures. The ana-culture was kept at 30°C for 30 days whereupon a dense precipitate was formed in the 48-hour-old culture and hardly any in the cellophane case culture. The precipitate was removed by centrifuging at 6 000 r.p.m. and had to be sterilized by the addition of 0.6 per cent formaline and incubating at 37°C for 48 hours. The clear supernatant was filtered through a Seitz filter. The immunizing capacity was tested immediately.

Table III-6.

The influence of storage upon the immunizing capacity of culture filtrate and bacterial cells.

	Capacity after storage at 30°C for 30 days.	
	Filtrate	Precipitate
48-hour-old culture	70%	100%
5-day-old culture	80%	100%

All the control animals succumbed within five days.

As can be seen from Table III-6 the antigen did not deteriorate much during storage; only the antigen present in the filtrate seems to lose a bit of its potency.

5) The immunizing antigen in the filtrates.

For the study of the soluble antigen, cellophane case cultures were used, as we expected a higher antigen content in their filtrates than in the filtrates of the

liver-meat /.....

liver-meat broth cultures. To investigate the nature of the antigen, trichloro-acetic acid precipitation and the chloroform-protein gel formation were applied.

a) To 250 ml of the filtrate an equal volume of 1 N trichloro-acetic acid was added slowly and under vigorous stirring at 2°C. After standing for 15 minutes at a low temperature, the precipitate was removed by centrifuging at 6 000 r.p.m. for 15 minutes. The precipitate was again suspended in 35 ml 0.1 M phosphate buffer of pH 7 (in which it did not dissolve) and tested for its antigen content. The supernatant was also examined for activity after dialysis and concentrating to 35 ml by freeze-drying.

b) The culture filtrate was also treated according to the method of Sevag, Lackman and Smolens (1938). When a mixture of carbohydrates, nucleic acids and proteins is shaken with chloroform, only the proteins and chloroform seem to form a loose molecular combination. This molecular combination, of both chloroform and proteins, forms a gel-like structure and this gel can readily be separated by centrifugation (Sevag, 1934).

A mixture of 250 ml of the culture filtrate, 65 ml of chloroform and 25 ml of amylalcohol, to avoid foaming, was shaken for 30 minutes at 20°C. After centrifuging at 2 000 r.p.m. for 15 minutes the mixture divided into two layers. The lower (chloroform) layer was completely gel-like and was separated from the upper layer, which was then repeatedly shaken with fresh chloroform. After the fourth shaking no more protein precipitated in the chloroform and the separation was stopped. To the chloroform-protein gel 96 per cent alcohol was added; this removed the

chloroform /.....

chloroform and precipitated the protein at the same time. The precipitated protein was recovered by centrifuging at 3 000 r.p.m. for 30 minutes and dissolved in 35 ml 0.1 M phosphate buffer pH 7. The water phase after the chloroform extraction was concentrated to 35 ml by evaporation in a vacuum. The two samples were tested for immunizing activity.

c) In the former experiments we employed dialysis but we realized that nothing was known of the behaviour of the antigen with dialysis. We investigated this after concentrating the culture filtrate and dialysed against an equal volume of 0.1 M phosphate buffer of pH 6.

We used buffer to lower the osmotic effect of the culture filtrate; pH 6 was chosen because this was also the pH of the filtrate. The concentration of the culture filtrate was achieved by freeze-drying and redissolving in half the volume of distilled water. After 18, 36, 72 and 120 hours of dialysis respectively, 35 ml samples of the inner and outer fluid of the dialysis were taken. These samples were all tested on the immunizing capacity.

Table III-7.

The influence of chemical and physical reactions on the immunizing capacity of culture filtrate.

	Capacity, %
Trichloro-acetic acid precipitate	50
Trichloro-acetic acid supernatant	0
Protein extracted with chloroform	40
Filtrate after chloroform extraction	10
Inner fluid after 18 hours dialysis	100
Outer fluid after 18 hours dialysis	0
Inner fluid after 36 hours dialysis	100
Outer fluid after 36 hours dialysis	0
Inner fluid after 72 hours dialysis	100
Outer fluid after 72 hours dialysis	0

Inner /.....

Table III-7 Contd.

	Capacity, %
Inner fluid after 120 hours dialysis	100
Outer fluid after 120 hours dialysis	0
Precipitate formed at pH 2	10
Supernatant after precipitation at pH 2	10
Filtrate after alkaline treatment	100
Boiling for 20 minutes	0

All the control animals died within five days.

d) The filtrate was also acidified with N hydrochloric acid to pH 2 whereupon a precipitate was formed. After standing at 20°C for 24 hours, alkaline was employed to bring the pH back to 6. The precipitate did not redissolve and was removed by centrifuging at 6 000 r.p.m. The clear supernatant was examined for immunizing activity and so was the precipitate after being suspended in 35 ml 0.1 M phosphate buffer of pH 7 in which it did not redissolve.

e) When the pH of the filtrate was established at 9 with the aid of 1 N sodium hydroxide, and after standing at 20°C for 24 hours the pH being again reduced to 6, no loss of its immunizing capacity could be detected.

Boiling of the filtrate for 20 minutes completely destroyed the antigen; no precipitate was formed although the filtrate became slightly opaque. The results are shown in Table III-7.

From Table III-7 it can be seen that the antigen has a protein character and that it cannot pass a cellophane membrane.

6) The haemolytic activity of Cl. chauvoei strain 64.

It was quite easily shown that filtrates of 48-hour-old cultures of Cl. chauvoei in liver-meat broth with 0.5 per cent glucose contain haemolytic activity to sheep erythrocytes /.....

erythrocytes. Moussa (1958) described the appearance of an oxygen-labile and an oxygen-stable haemolysin, which are distinguished by :

a) the ability to withstand storage; the oxygen-labile haemolysin retains its activity after months of storage in a reducing medium, whereas the oxygen-stable haemolysin loses activity within five days.

b) The oxidation-reduction reactions; unlike the oxygen-labile haemolysin, whose activity can be restored after oxidation, the oxygen-stable haemolysin is irreversibly inactivated after oxidation with chemical reagents;

c) the induction period which precedes haemolysis of sheep erythrocytes when brought into contact with the oxygen-stable haemolysin.

We could not properly distinguish between an oxygen-labile and -stable haemolysin. The haemolytic activity in our filtrates was very low but withstood storage at 10°C for 2 weeks, even without a reducing agent being added. Oxidation by 0.5 per cent hydrogen peroxide inactivated the haemolysin irreversibly. An induction period preceded the reaction and lasted about 5 - 10 minutes when high haemolysin concentrations were used. Heating the filtrate at 80°C for 10 minutes diminished the activity considerably.

Table III-8

The haemolytic activity of the culture filtrate of Cl. chauvoei, strain 64.

	Volume of haemolytic filtrate in ml									
	1-	.9-	.8-	.7-	.6-	.5-	.4-	.3-	.2-	.1
Filtrate	+	+	+	+	+	+	-	-	-	-
Filtrate heated at 80°C	-	-	-	-	-	-	-	-	-	-
Filtrate oxidized with H_2O_2	+	-	-	-	-	-	-	-	-	-
Oxidized filtrate reduced with cysteine	+?	-	-	-	-	-	-	-	-	-
Storage	+	+	+	+?	-	-	-	-	-	-

Storage /.....

Table III-8 Continued.

	Volume of haemolytic filtrate in ml 1-.9-.8-.7-.6-.5-.4-.3-.2-.1
Storage with $NaHSO_3$	+ + + + - - - - - -
Cell extract	+ + +? - - - - - - -
Cell extract heated at 80°C	+? - - - - - - - - -

The volumes of the filtrate were corrected to 1 ml with 0.85 per cent saline. Filtrates of 48-hour-old cultures on liver-meat broth were used.

We assumed that only the oxygen-stable haemolysin was present in our filtrates and that this haemolysin withstood storage fairly well.

A weak haemolytic activity was also found inside the bacterial cell. Bacterial cells were recovered from liver-glucose broth cultures, 18 - 22 hours old, resuspended in 1/20 of the culture volume of 0.85 per cent saline after three washings, and disintegrated by ultrasonic vibration. The suspension was then filtered through a Seitz filter and the filtrate showed to possess a weak haemolytic activity. The latter activity was also destroyed by heating at 80°C for 10 minutes (see Table III-8).

7) Other substances in filtrates of strain 64 cultures.

Moussa (1958) described the presence of deoxyribonuclease and hyaluronidase activities in culture filtrates apart from the haemolysins. He sometimes noted a necrotic activity also, whose presence had very often been reported by various authors, before. We examined all the toxins mentioned, in our culture filtrates.

a) /.....

a) The lethal toxin :

None of the cultures of Cl. chauvoei strain 64 showed any lethal toxin. We studied the filtrates of a 48-hour-old liver-meat-glucose broth, and of cellophane case cultures after 24, 48, 72, 96 and 120 hours of growth. We tested for activity in mice and guinea-pigs by intravenous injections. The animals were not examined for any necrotic effect as was done by Mason in 1936. Precipitation of the toxin by ammonium sulphate followed by dialysis and freeze-drying, as reported by different workers (Mason, 1936, a.o.) had no effect.

When the bacterial cells of liver-glucose broth cultures were centrifuged and resuspended in 1/20 of the culture volume of distilled water and disintegrated by autolysis (10 days at 10°C), ultrasonic treatment or alternate freezing and thawing, the filtrates contained no lethal toxin.

Knowing that the strain used is highly pathogenic for guinea-pigs and sheep, we must assume, however, that the lethal toxin produced by this strain (if any) is very labile because no activity could be detected. Though the lethal activity of Cl. chauvoei cultures can be explained by secondary effects on the inner organs in the animal, the course of the disease certainly indicates the presence of toxins, for death occurs sometimes within 24 hours and in this case practically no bacilli are detectable in the organs.

b) Deoxyribonuclease activity.

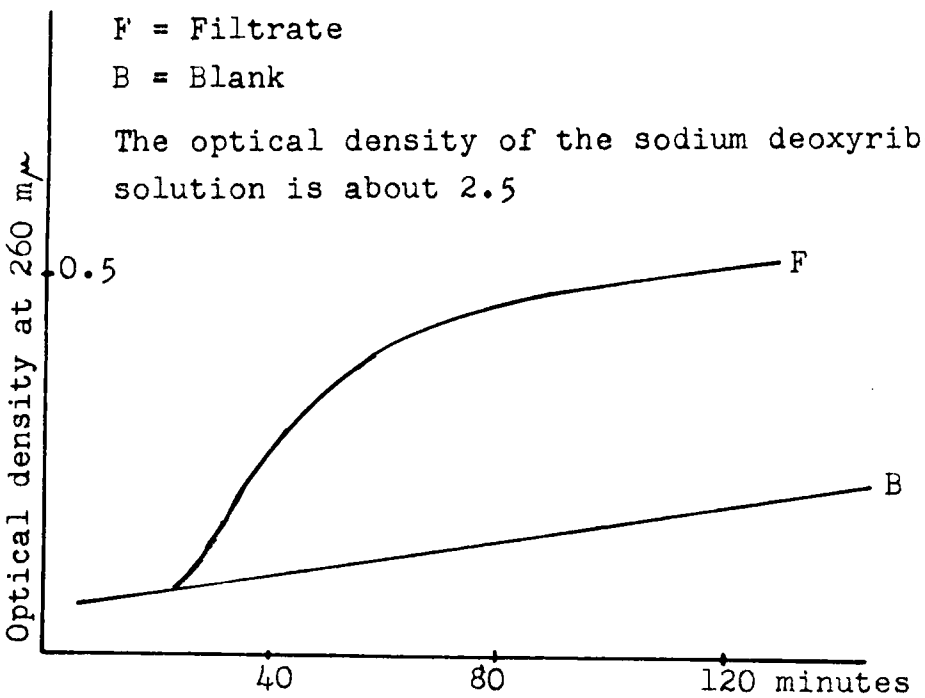
We detected a weak deoxyribonuclease activity in filtrates of liver-meat broth cultures when the method of Kurnick (1953) was employed (see Graph III-1).

We failed /.....

We failed, however, to detect any reaction with rabbit leucocytes, according to the method of Warrack, Bidwell and Oakley as modified by Meisel (1961).

Graph III-1.

Deoxyribonuclease activity of 48-hour-old filtrates.



c) Hyaluronidase activity.

A weak activity was found in liver-meat broth filtrates. This activity was destroyed by boiling for two minutes.

Table III-10.

Hyaluronidase activity of 48-hour-old culture filtrates.

	Ox-synovial fluid	Horse-synovial fluid
Filtrate	0.2 ml	0.3 ml
After 2 min. boiling	---	---

0.2 ml /.....

0.2 ml filtrate prevented ox-synovial fluid blobbing whereas 0.3 ml was needed to prevent horse-synovial fluid from blobbing.

We observed that the filtrates possess hyaluronidase activity which is destroyed by boiling for only two minutes.

Discussion.

The results in this chapter clearly indicate that an immunizing antigen is present in the culture filtrates, as well as in the bacterial cell. This was to be expected, since the anaculture vaccines possess a higher immunizing capacity than the culture filtrates (McEwen, 1926 and Leclainche and Vallée, 1925).

We found only a small difference in the potencies of the cellophane case cultures and the vaccines prepared in the conventional way. This seems to be in contrast with the findings given in the publication of Sterne-Thorold and Scheuber (1953). These authors claimed a far more potent vaccine when Cl. chauvoei was cultured in cellophane cases. After a comparative study over a long period, it was found at Onderstepoort Laboratories that, with the particular strain (strain 64), the capacity of the cellophane case cultures rose only 30 per cent above that of the conventional vaccines. The cellophane cases had a volume of about 500 ml and were immersed in 20 litres of medium, which was liver-meat broth with 0.5 per cent glucose. The medium could be restored and used over again but it seemed to be unpractical and uneconomical to produce vaccines in this manner on a large scale. We mention this here because it is one of the reasons which made us decide to use the liver-meat broth cultures after 48 hours of growth to isolate the antigen.

The soluble /.....

The soluble antigen found in the culture filtrates as well as in the plasma of the bacterial cells appeared to be a protein-like component which was not dialysable. Evidence for this hypothesis is found in the action of pepsin, trypsin, acid and in the chloroform extraction. The influence of pepsin digestion on the immunizing antigen was very strong but it is not certain if this is due to the action of pepsin itself or whether it must be attributed to the acidity of the medium. It was well known at Onderstepoort Laboratories that a low pH could destroy the immunizing potency of anaculture vaccines, prepared by the use of Cl. chauvoei strain 64, and this was confirmed by our results. A pH below 3 can lead to complete loss of immunizing activity.

The action of trypsin on the antigen is less powerful but also indicates that the antigen consists, at least partly, of protein material.

The phenol extraction method from Westphal, Lüderitz and Bister, as modified by Davies, was often used for the extraction of somatic or "O" antigens from various bacteria. Most of those somatic antigens proved to be lipo-polysaccharide complexes which are thermo-stable. The cold phenol extract method is recommended by Westphal, Lüderitz and Bister in the case of gram-negative bacteria, but is not often used. We decided to do no more fractionations on the bacterial cells because the soluble antigen was not a lipo-polysaccharide complex but a protein-like substance.

The influence of high temperatures on the immunizing antigen had been studied by various authors, without their reaching unanimity. Henderson in 1932 reported the successful immunization of guinea-pigs with

bacterial /.....

bacterial suspensions heated at 100°C for two hours. We found that boiling the anacultures, or bacterial cell suspensions, for 2 hours diminished the immunizing capacity to 20 - 30 per cent. This result, however, is in agreement with that of Roberts (1933), who noted that an amount of boiled "O" antigen corresponding to 143 ml of culture did not give 100 per cent protection against about 1 m.l.d. The objection against the work of Roberts and Henderson is that they did not challenge with Cl. chauvoei germs but with spore suspensions activated by calcium chloride, whereas the immunization was done by germs. Mason and Scheuber (1936) found that a dense washed and boiled bacterial suspension gave immunity in sheep when challenged with anaculture. When, however, the same boiled suspension was diluted to the original volume, a considerable loss was noticed. A dose of culture which gave protection against 20 m.l.d. was, after boiling in a dense suspension and diluting to the original volume, only able to protect against less than 1 m.l.d.

Our results with culture filtrates showed that boiling for 20 minutes led to a complete loss of the immunizing activity. Though the filtrate became opaque, no precipitate was formed as reported by Viljoen and Scheuber (1926). These authors found that the immunizing antigen in formalized filtrates withstood heating at 80°C for 30 minutes but that the antigen was destroyed and a precipitate formed when a higher temperature was employed. We therefore assume that heating at 80°C for 30 minutes is the maximum the antigen in our culture filtrates can endure.

The effect of temperature can possibly be correlated with the influence of the temperature on the

lethal /.....

lethal toxin which was also strongly deteriorated by heat (Mason, 1936). We were never able to detect this lethal toxin in filtrates of strain 64 as did Mason, who used the same strain in 1936. Mason, however, stressed that, unless it could be concentrated, no useful work could be carried out with the toxin.

A haemolysin was also found in our work but it was of a very low titer as already claimed by Mason. Only a few properties of the haemolysin were investigated, as we considered the material to be impure and results at this stage could be confusing. Mason could not show that the haemolysin and the lethal toxin were the same component. We found that the haemolytic titer could vary considerably whereas the immunizing potency of the culture filtrate was fairly constant. This seems to indicate that the antigen and the haemolysin are different components, unless the haemolysin is responsible for part of the immunizing activity and that it keeps its immunizing activity after the haemolytic character has disappeared.

Other substances found to be present in culture filtrates are a deoxyribonuclease and a hyaluronidase, both having low titers, however. The deoxyribonuclease activity was so low that we doubt if it is worth while investigating further.

Most researchers are agreed upon the presence of two immunizing antigens in Cl. chauvoei cultures, namely, a heat-labile one present in the filtrates and related to the lethal toxin, and a heat-stable one, mostly bound to the cell. Moussa in 1959 attributes the discrepancies in the results obtained by heating mainly to the presence of spores. Our results with sterilized and non-sterilized bacterial cell content indeed point in this direction

(see /.....

(see Table III-3 on page 53). We also know that spore suspensions are capable of immunizing susceptible animals against toxic cultures and we therefore assume that the heat-stable antigen is nothing else but the spore.

Chapter IV /.....

CHAPTER IV.

THE ISOLATION AND PURIFICATION OF THE ANTIGEN.

1) Raw materials.

As we saw in the previous chapter, immunizing potency is present in the filtrates of 48-hour-old liver-meat-glucose broth cultures as well as in the filtrates of 5-day-old cellophane case cultures. We also found that an immunizing antigen adhered to the bacterial cells; this antigen may differ from the one in the filtrates.

We tried to isolate the immunizing antigens from :

a) liver-meat broth culture filtrates,

b) cellophane case culture filtrates,

c) bacterial cell extracts.

The filtrates of a cellophane case culture and of a liver-meat broth culture gave, after dialysis and freeze-drying respectively, 3.2 and 14 g of good soluble materials (soluble to about 2 per cent solutions in water).

The bacterial cells, obtained from a cellophane case culture were resuspended in 1/10 of the original culture volume of saline and the extracts were prepared by :

a) autolysis; the cell suspension was kept at 10°C for 10 days;

b) freezing and thawing alternately five times;

c) ultrasonic vibration.

The cell residues were removed by ultracentrifuging at 40 000 r.p.m. for 30 minutes. Greenish, viscous supernatants were obtained with high optical densities at 260 and 280 mμ.

These extracts yielded white but almost insoluble materials after dialysis and freeze-drying. Suspensions

of /.....

of these materials in saline showed low antigen contents when they were compared with the extracts (see Table IV-1).

Table IV-1.

The immunizing capacities of bacterial cell extracts.

Cell extract prepared by	Capacity	
	Extract	Dried material
autolysis	100%	40%
freezing-thawing	100%	30%
ultrasonic treatment	100%	40%

All the control guinea-pigs, except one which was challenged with 0.1 ml culture, died within five days.

Because of the insolubility of the cell extract after dialysis and freeze-drying which we also feared during the concentrating of the antigen, we decided to use the filtrates. For reasons of economy we preferred the liver-meat broth filtrates to those from the cellophane cases.

2) The concentration of the immunizing antigen.

We tried several methods, which relied upon adsorption and precipitation of proteins, to reach our aim which was the quantitative removal of the immunizing antigen from culture filtrates. Precipitation with methanol and with ammonium sulphate and also the adsorption on aluminium hydroxide were successively investigated.

a) Methanol precipitation.

The method of Roth and Pillemer (1953) was adopted. These authors used the methanol precipitation to isolate the lecithinase from Cl. perfringens type A culture filtrates. After cooling the filtrates to a temperature

near /.....

near freezing point, the pH was adjusted to 4.6 with 1 N acetic acid. Sometimes a highly insoluble precipitate formed; this was removed by centrifuging and could be discarded as no immunizing capacity was found after suspending it in 0.85 per cent saline. The antigen content of the acid supernatant showed no decrease when compared with that of the filtrate (see Table IV-2).

The clear supernatant, after centrifuging at 6 000 r.p.m. for 30 minutes, was precipitated with methanol at a low temperature. At first methanol was added to a concentration of 5 per cent, the methanol having been previously cooled in a dry-ice-acetone bath of -80°C. The mixture was then itself cooled to about -10°C, after which the methanol concentration was increased to 35 per cent by adding the methanol slowly from a separatory funnel. The temperature was maintained at -10°C. The precipitate formed was allowed to settle for 1 week and was centrifuged at 10 000 r.p.m. for 20 minutes. The supernatant was evaporated in a vacuum until most of the methanol had been removed.

The precipitate, a dark brown mass, was suspended in 1/10 of the filtrate volume of 0.1 M phosphate buffer of pH 6 (the same pH as the original culture). Only a little of the precipitate dissolved and the insoluble material was centrifuged.

Table IV-2 /.....

Table IV-2.

The immunizing capacity of several filtrate fractions.

	Capacity
Filtrate	100%
Acid supernatant	100%
Acid precipitate	10%
Methanol precipitate soluble	100%
Methanol precipitate insoluble	10%
Methanol supernatant	90%

The control animals, except one, challenged with 0.3 ml and two challenged with 0.1 ml, died within five days.

The insoluble part of the methanol precipitate and also the pH 4.6 precipitate from 1 litre filtrate were suspended in 100 ml 0.85 per cent saline. The supernatants and the soluble part of the methanol precipitate were corrected to 250 ml volumes before the test was carried out; this means that the concentration of these latter test samples is four times the concentration of the filtrate.

A fractionation by methanol precipitation was also carried out. The results at a pH of 4.6 were not successful as shown in Table IV-3.

Table IV-3.

The methanol fractionation of filtrates.

Methanol added ml	Total volume ml	Percentage Methanol	Capacity (%)
100	1100	9.1	20
250	1250	20	30
450	1450	31	70
700	1700	41.2	100
1000	2000	50	70
1500	2500	60	40
3000	4000	75	20
Supernatant			10

The /.....

The control guinea-pigs challenged with 0.3 or 0.1 ml culture all died within five days except one of each group. The precipitates were allowed to settle at -10°C for 1 hour. After centrifuging more methanol was added to the supernatants while the temperature was kept at -10°C. The precipitated materials were dissolved in 1/10 of the culture volume of saline. After the last precipitation the supernatant was evaporated in a vacuum to a volume of 1/10 of the original culture.

b) Ammonium sulphate precipitation.

As we saw in the previous chapter, the results of the experiments done with the soluble antigen from the culture filtrate indicate that the antigen consists of a protein-like substance, perhaps bound to another compound. It was therefore likely that the antigen could be precipitated by ammonium sulphate. Indeed dense flocculates formed when the culture filtrates were saturated with ammonium sulphate at 2°C. The materials were allowed to settle at 2°C for 8 hours before being centrifuged. The clear supernatants were dialysed and freeze-dried, after which brown, hygroscopic powders were obtained. These powders, however, possessed no immunizing activities at all. The precipitates were also dialysed at 2°C and, because they did not completely dissolve, the dialysed solutions were centrifuged before being freeze-dried. Slightly brown powders were obtained which readily dissolved in water (to a 4 per cent solution). The yield was 6 g material per litre of filtrate.

In Table IV-4 the antigenic potency of the ammonium sulphate precipitated material is given.

Table IV-4/.....

Table IV-4.

The immunizing capacity of ammonium sulphate precipitate and supernatant.

Sample in 30 ml solutions	Filtrate equivalent per guinea-pig, ml	Capacity %
400 mg precipitate	6.5	100
200 mg precipitate	3.3	100
100 mg precipitate	1.7	80
50 mg precipitate	0.8	50
About 400 mg freeze-dried supernatant	---	0
insoluble precipitate	---	0

All the control guinea-pigs except two out of three challenged with 0.1 ml died within five days.

The filtrate equivalent of the precipitate is calculated to a yield of 6 g precipitate from 1 litre filtrate.

The results compare well with the finding in the previous chapter, where it was found that three injections of 0.6 ml of a same culture filtrate gave a 70 per cent protection.

c) Adsorption by aluminium hydroxide.

In 1936 Mason and Scheuber demonstrated a higher potency of the blackleg vaccines after the addition of an alum solution. This can be attributed to a slow absorption of the antigen from the hydroxide by the body, providing a constant dose of antigen over a long period; this results in an increased immunity. (Glenny, Pope, Waddington and Wallace, 1926 and Glenny, Buttle and Stevens, 1931).

It was anticipated that the antigen could be removed from the filtrates by using the adsorption. A concentrated potassium alum solution ($KAl(SO_4)_2.12H_2O$)

was added /.....

was added to the filtrates to a final concentration of 1 per cent, and the pH was re-adjusted to 5 with 1 N alkali. The precipitate was stirred for 30 minutes and centrifuged. The hydroxide was extracted about five times by stirring with 0.5 M phosphate buffer of pH 7, after which the suspension was centrifuged. The extracts were collected, dialysed and freeze-dried and yielded a water-soluble material, soluble to about 4 per cent.

The immunizing potency of this material is shown in Table IV-5.

Table IV-5.

The immunizing capacity of alumina adsorbed material and of the supernatant.

Sample in 30 ml solutions	Capacity, %
400 mg adsorbed material	100
200 mg adsorbed material	80
100 mg adsorbed material	50
50 mg adsorbed material	10
Supernatant (3 times concentrated)	10

All the control guinea-pigs except one out of three challenged with 0.1 ml culture died within five days.

The materials obtained from the 48-hour-old culture filtrates, isolated according to the above given methods, were also studied by D.E.A.E.-cellulose chromatography. The effluent patterns of approximately the same quantities of the materials are shown using the same column sizes and gradients. See Diagrams IV-1, IV-2 and IV-3.

The precipitation of the antigenic material with ammonium sulphate seemed to have the following advantages over the two other methods :

a) an almost quantitative yield was obtained

b) the material was readily soluble.

The methanol /.....

Diagram IV-1

Chromatography of 600 mg methanol precipitate of culture filtrate.

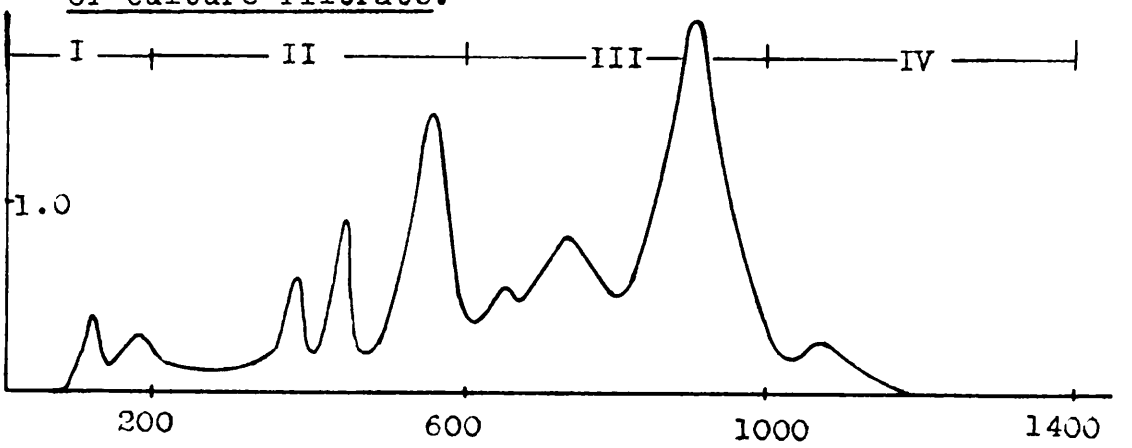


Diagram IV-2

Chromatography of 400 mg ammonium sulphate precipitate of culture filtrate.

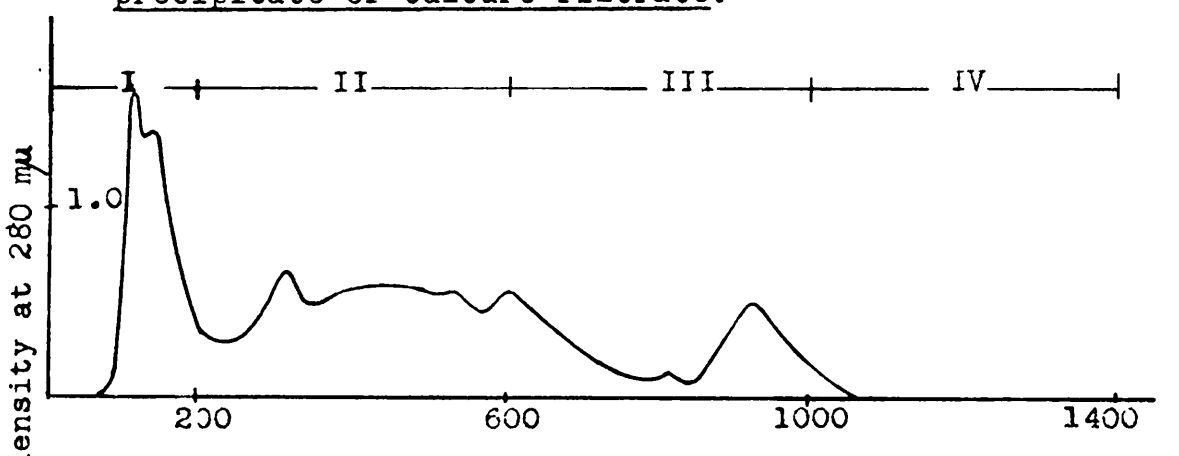
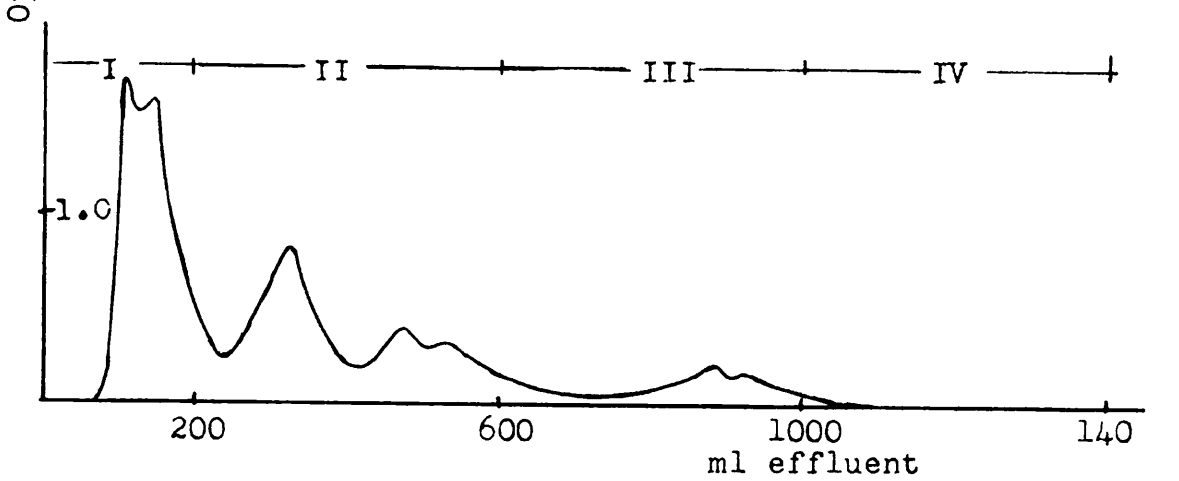


Diagram IV-3

Chromatography of 400 mg aluminium hydroxide adsorbed material of culture filtrate.



Columns : D.E.A.E.-cellulose; 40 x 2.5 cm
Rate of flow : 50 ml per hour
Temperature : 2°C

I : 0.05 M buffer pH 6 (phosphate)
II : gradient 0.05 M buffer ⟶ 0.1 M buffer + 0.2 M NaCl
III : gradient 0.1 M buffer + 0.2 M NaCl ⟶ 0.1 M buffer + 0.5 M NaCl
IV : gradient 0.1 M buffer + 0.5 M NaCl ⟶ 0.1 M buffer + 1 M NaCl

The methanol procedure seemed partly to denature the antigen, for the yield of antigenic material, precipitated by 50 per cent methanol, is lower and this precipitate is only partly soluble. By adsorption on aluminium hydroxide the antigen could be quantitatively removed from the filtrate but this method was complicated by the strong adsorption, which made it difficult to obtain the material.

3) Fractionation of the crude material, precipitated by saturation of the filtrates with ammonium sulphate.

a) Fractionation by precipitation methods.

Fractionation of proteins can sometimes be achieved easily by the employment of precipitation. In these cases several factors influencing the solubility of the components must be strictly controlled. A good procedure can usually be found when the temperature and the pH of the solution are established and kept constant.

we used ammonium sulphate, methanol and polyethylene glycol as precipitating agents. The crude material was dissolved in 0.01 M phosphate or acetate buffer with pH values of 6 and 5 respectively. Using 1 per cent solutions (3g per 300 ml) the results shown in the Tables IV-6, IV-7 and IV-8 were obtained. The fractions were studied for their immunizing capacities after dissolving in saline (100 mg per 30 ml).

Table IV-6 /.....

Table IV-6.

Fractionation by ammonium sulphate precipitation of a 1 per cent solution of crude material at pH 6 and 20°C.

Per cent (w/v) ammonium sulphate	Amount of precipitate mg	Yield of the material %	Immunizing capacity %
10	210	7	0
20	360	12	70
30	425	14	90
40	650	22	100
50	625	21	80
60	205	7	40
supernatant	--	--	0

All the control guinea-pigs succumbed.

Table IV-7.

Fractionation of a 1 per cent solution of crude material by methanol precipitation at pH 5 and -10°C

Per cent of methanol	Amount of precipitate mg	Yield of the material %	Immunizing capacity %
10	90	3	20
20	740	25	30
30	380	13	60
40	95	3	20
60	270	9	10
supernatant	--	--	30

All the control guinea-pigs succumbed.

Table IV-8.

Fractionation of a 1 per cent solution of crude material by polyethylene glycol precipitation at pH 6 and 20°C.

	Amount of precipitate	Yield of material	Immunizing capacity
Precipitate	515 mg	17%	0%
supernatant	--	--	60%

All the control guinea-pigs died.

Only at 8 per cent (w/v) polyethylene glycol was a precipitate formed. The average molecular weight of

the /.....

the polyethylene glycol was 6 000.

Fractionation of the crude material by precipitation methods seemed unpractical because of the tendency of the antigen to precipitate in a wide range. Methanol and polyethylene glycol gave denaturation of the antigen, although good results of the latter agent have been claimed by Polson in case of Cl. botulinum type C toxins.

The only conclusion is that a 10 per cent ammonium sulphate concentration does not precipitate the antigen, for we often found dense flocculates in the filtrates at this low concentration.

b) Fractionation by chromatographic methods.

Chromatography on D.E.A.E.-cellulose.

In Diagram IV-2 we saw that a fractionation by chromatography on this resin could be achieved. We were, however, interested in a preparatory fractionation and for this purpose 30 x 5 cm columns were used. The columns were loaded with 5 g material dissolved in 100 ml phosphate buffer of pH 6. The results are shown in Diagram IV-4.

The fractions were dialysed and freeze-dried after which quantities of 100 and 50 mg were dissolved in 30 ml saline and tested for their immunizing potencies.

Chromatography on calcium phosphate columns.

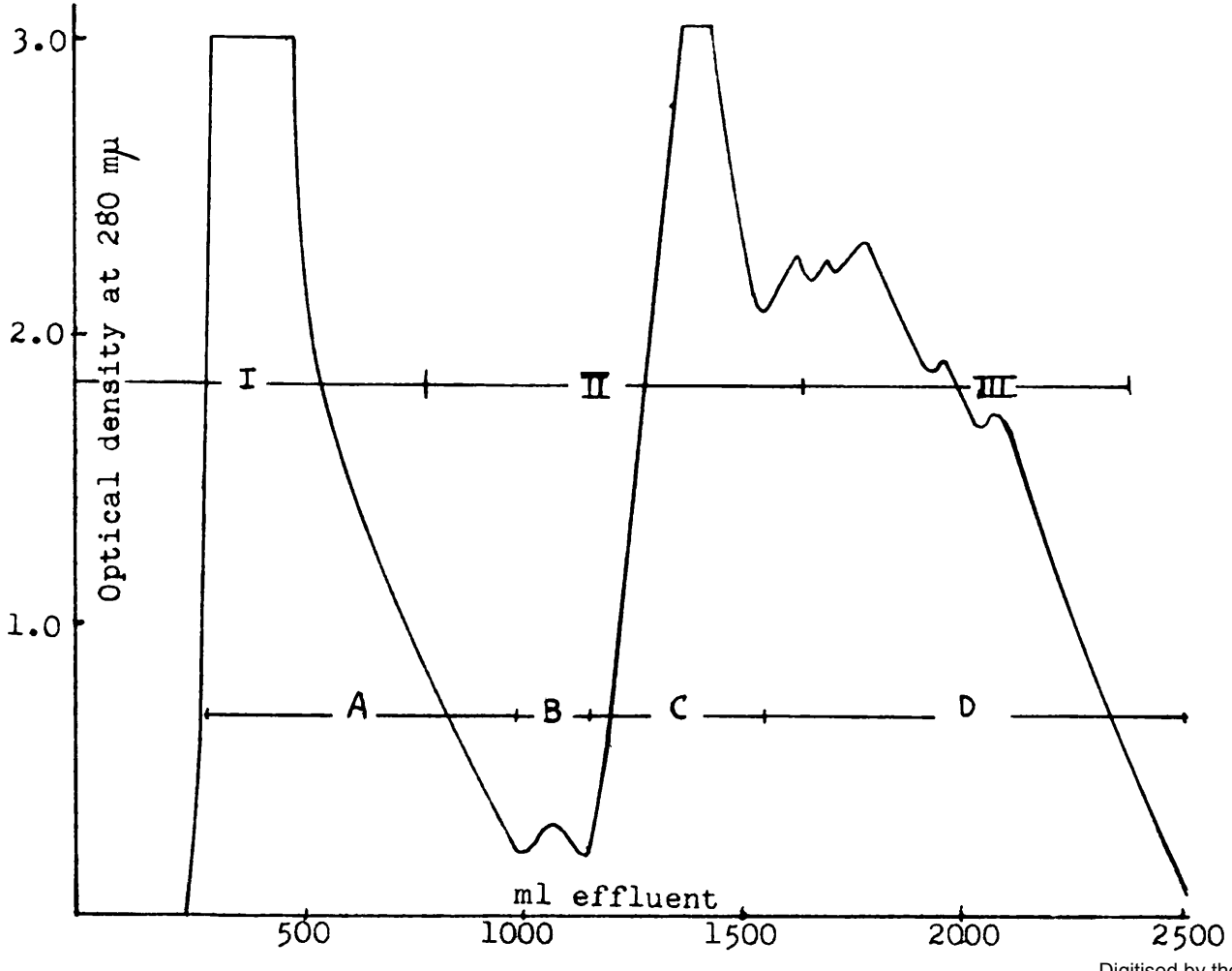
The results are shown in Diagram IV-5. The effluent was divided into two parts, dialysed and freeze-dried. The materials were dissolved in saline, 75 mg per 30 ml, and the antigenic potency estimated.

From the Diagrams IV-4 and IV-5 it can be seen that the antigen adsorbs strongly on the D.E.A.E.-cellulose and the calcium phosphate.

c)/.....

Diagram IV-4

Chromatography of 5g ammonium sulphate precipitate



Column : D.E.A.E. - cellulose; 30 x 5 cm
Rate of Flow : 120 ml per hour
Temperature : 2°C

I : 0.05 M buffer pH 6 (phosphate)
II : gradient 0.05 M buffer ⟶ 0.1 M Buffer + 0.2 M NaCl
III : gradient 0.1 M buffer + 0.2 M NaCl ⟶ 0.1 M buffer + 0.5 M NaCl

	Immunizing capacity of 50 mg	100 mg
A	0%	0%
B	0%	-
C	0%	40%
D	80%	100%

All the control animals died.

Diagram IV-5

Chromatography of 250 mg ammonium sulphate precipitate on a calcium phosphate column.

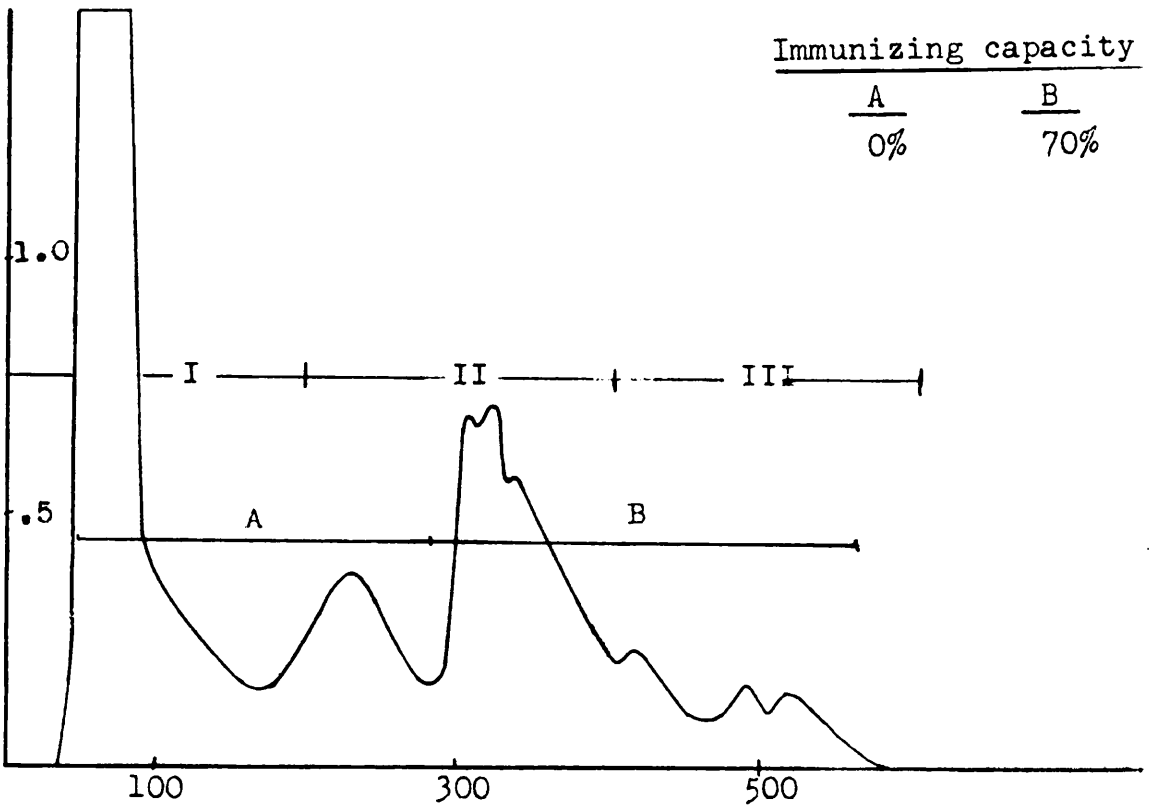
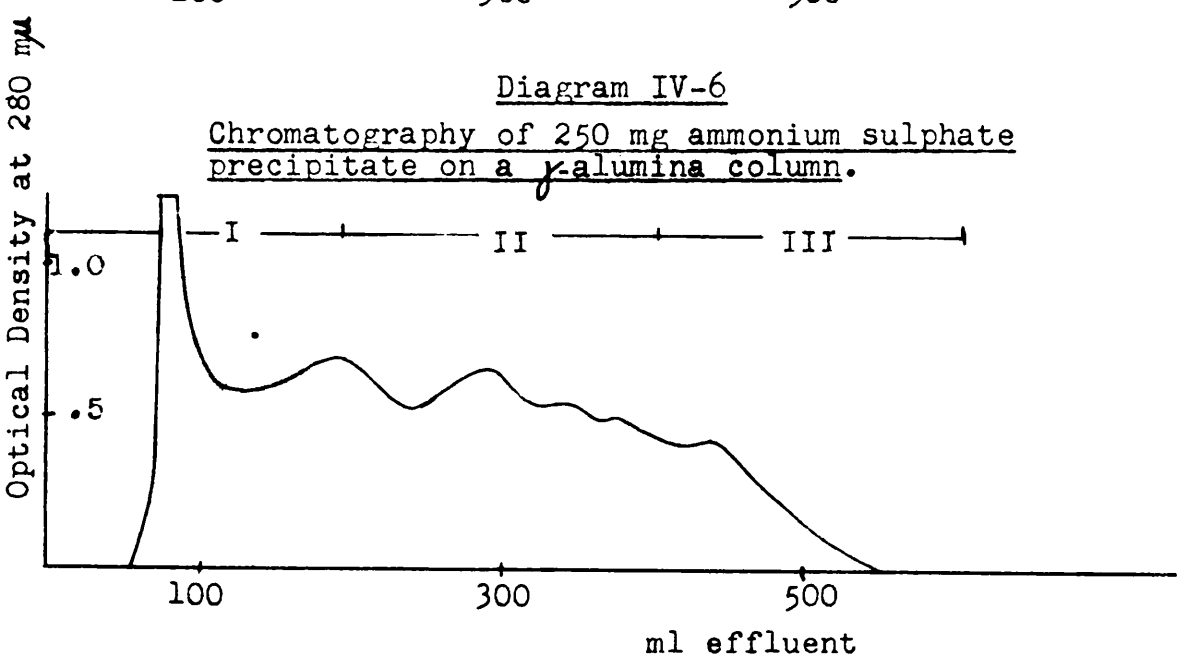


Diagram IV-6

Chromatography of 250 mg ammonium sulphate precipitate on a γ-alumina column.



Columns : 10 x 2.5 cm
Rate of flow : 40 ml per hour
Temperature : 2°C

I : gradient 0.005 M buffer ⟶ 0.05 M buffer
II : gradient 0.05 M buffer ⟶ 0.2 M buffer
III : gradient 0.2 M buffer ⟶ 0.6 M buffer

Buffer : phosphate buffer pH 6.8

Other chromatographic methods.

We also investigated the fractionation on Sephadex and γ alumina columns. The results, however, were not encouraging, as can be seen from the Diagrams IV-6, IV-7 and IV-8.

c) Fractionation by adsorption methods.

Adsorption by D.E.A.E.-cellulose.

The crude material was dissolved in 0.05 M phosphate buffer of pH 6 to a 1 per cent solution. A thick slurry of washed resin was added and the suspension stirred for 1 hour at a low temperature. (We used 3 g of dry Whatman powder per g crude material). The resin was filtered off, on a Büchner funnel, and washed with several 100 ml volumes of 0.05 M buffer. A column was packed and eluted with phosphate buffers with increasing ionic strengths. The effluents were collected, after the optical densities became constant again, and dialysed and freeze-dried. We tested 75 mg of the various materials obtained for their immunizing capacities after dissolving in 30 ml saline. The results are given in Table IV-9.

Table IV-9.

The immunizing capacity of various fractions obtained by elution of the D.E.A.E. adsorbed compounds.

Fractions eluted by :	Capacity, %
0.05 M buffer	20
0.08 M buffer	10
0.1 M buffer	20
0.1 M buffer + 0.05 M NaCl	60
0.1 M buffer + 0.1 M NaCl	100
0.1 M buffer + 0.2 M NaCl	100
0.1 M buffer + 0.3 M NaCl	60
0.1 M buffer + 0.4 M NaCl	0
0.1 M buffer + 0.5 M NaCl	0
0.1 M buffer + 1 M NaCl	0

All /.....

Diagram IV-7

Chromatography of 500 mg ammonium sulphate precipitate on Sephadex G-100

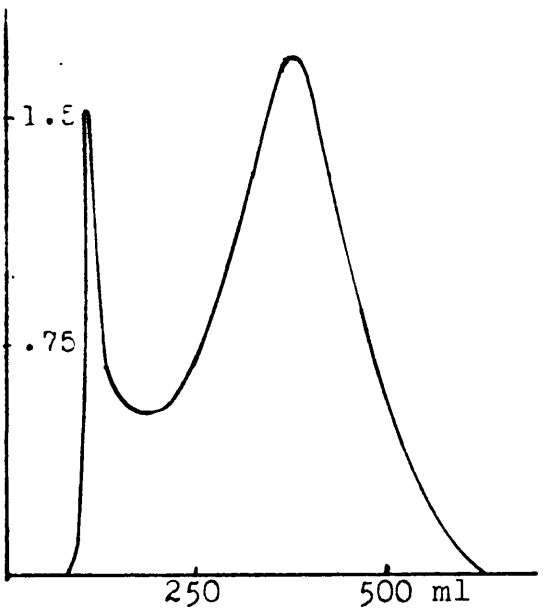
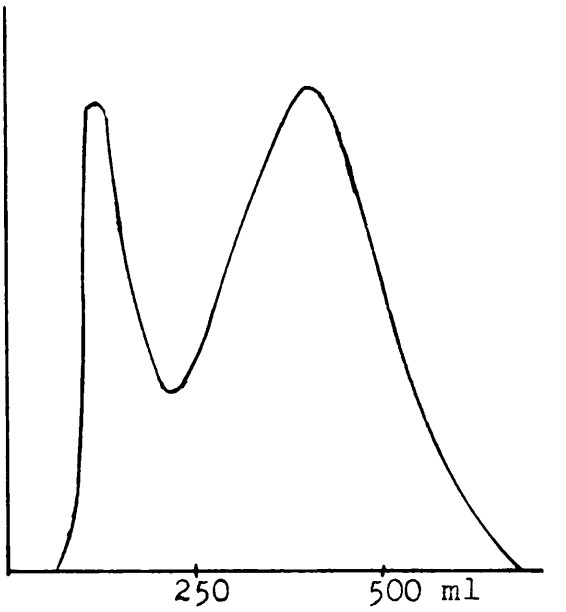


Diagram IV-8

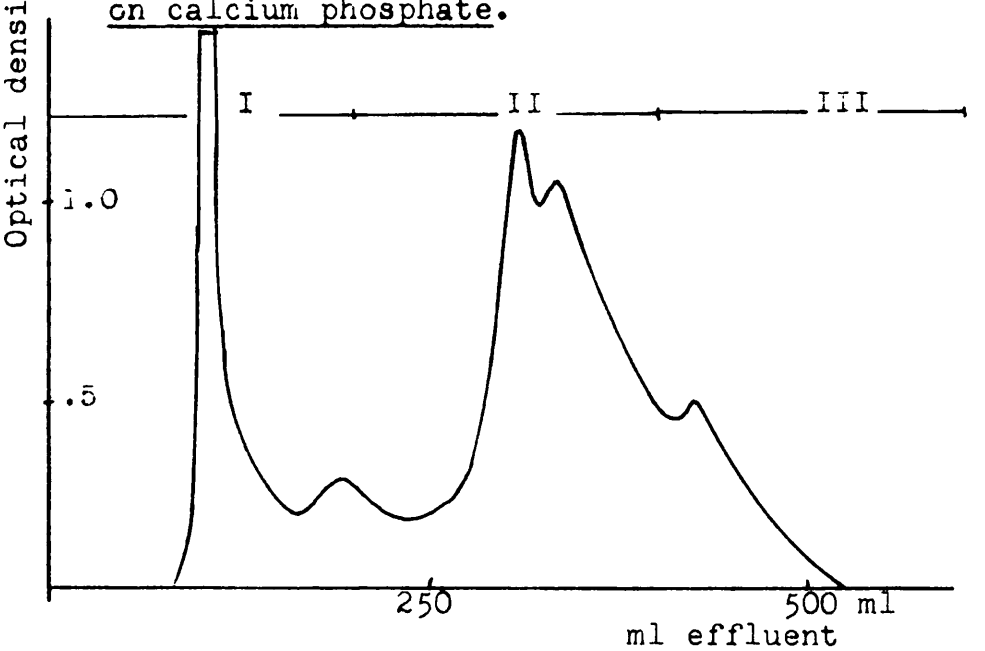
Chromatography of 500 mg ammonium sulphate precipitate on Sephadex G-200



Column sizes : 40 x 5 cm
Temperature : 2°C
Effluent : 0.05 M phosphate buffer of pH 6
Rate of flow : 25 ml per hour

Diagram IV-9

Chromatography of 250 mg D.E.A.E. adsorbed material on calcium phosphate.



Column size : 10 x 2.5 cm
Rate of flow : 40 ml per hour
Temperature : 2°C

I : gradient 0.005 M buffer ⟶ 0.05 M buffer
II : gradient 0.05 M buffer ⟶ 0.2 M buffer
III : gradient 0.2 M buffer ⟶ 0.6 M buffer

Buffer : phosphate buffer pH 6.8

All the control guinea-pigs died within five days.

We observed, in Table IV-9, that a purification was achieved. The yield of dialysed and freeze-dried material from the four fractions containing immunizing activity was about 300 mg per g of material precipitated by ammonium sulphate.

We studied this material by chromatography on a calcium phosphate column. The resulting diagram (IV-9) was compared with the effluent pattern obtained by the chromatography of the crude material on calcium phosphate (see Diagram IV-5). An increase in the area of the peak containing the antigen was observed.

Adsorption by calcium phosphate.

We found that an enormous amount of gel had to be used to achieve complete removal of the antigen from one per cent solutions of the crude material in 0.01 M potassium phosphate buffer of pH 6.8. This is a disadvantage of the calcium phosphate gel and makes the method unpractical. According to the literature (Zittle, 1953 and Sörbo, 1961), maximum adsorption on the calcium phosphate gels occurs at the iso-electric point of the compounds. However, the gels are only stable in a narrow pH range (5.6 - 7.8) as cited by Sörbo (1961). Because the adsorption on D.E.A.E.-cellulose indicate a low iso-electric point of our antigen the calcium phosphate method was not further investigated.

Adsorption on stannous-stannic oxide gels.

We tried this method because of the good results claimed by Messing and Ness (1961).

To a solution of 1 g of crude ammonium sulphate precipitate in 100 ml 0.85 per cent saline was added a

suspension/.....

suspension of 300 mg stannous chloride ($SnCl_2.2H_2O$) in 10 ml of water at a low temperature. The pH was then adjusted to 6.5 by the addition of 1 M ammonia and checked with a glass-electrode.

The mixture was stirred for an additional 30 minutes and the precipitate was centrifuged at 3 000 r.p.m. for 30 minutes. The clear supernatant fluid was dialysed and freeze-dried and gave 785 mg dry powder, still antigenic. The antigen was not noticeably purified by this method as can easily be seen from the high yield of non-adsorbing material.

The fractionation method using D.E.A.E.-cellulose seemed the most favourable, because it has the advantage of a rapid separation, fractionation being carried out in 10 - 12 hours. The use of 3 g Whatman D.E.A.E.-cellulose powder per g of the crude material gave quantitative adsorption. This is in agreement with the values given by Peterson and Sober (1956) for the adsorbing capacity of D.E.A.E.-cellulose.

4) The further purification of the D.E.A.E. adsorbed material.

After the fractionation on D.E.A.E.-cellulose, which is itself an anion-exchanger, the employment of other anion-exchangers seemed superfluous.

The investigation of the fractionation on cation-exchangers (for instance, carboxymethyl-cellulose) is also unnecessary, for the strong adsorption on D.E.A.E.-cellulose indicates a strong negative charge of the antigen at pH 6, the antigen being an acid protein with a low iso-electrical point.

We decided to investigate other chromatographic methods which do not rely upon the electrical charge of

the /.....

the proteins like calcium phosphate-, Sephadex-, and alumina columns. An indication of the possibility of a good purification on calcium phosphate columns had already been obtained (see Diagram IV-9). We tried various column sizes, several buffer gradients and amounts of material that could be separated at the same time and were able to find a method which gave excellent results.

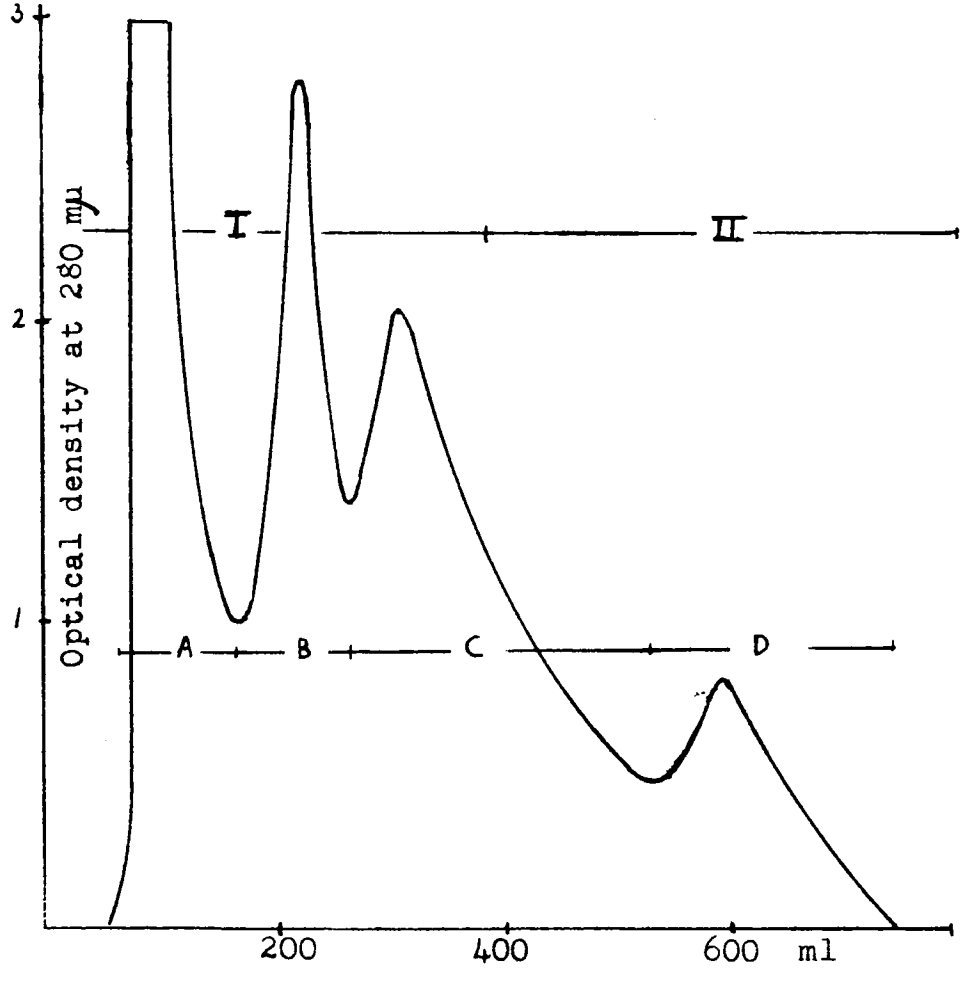
750 mg Of the D.E.A.E.- cellulose adsorbed material was dissolved in 20 ml of 0.01 M potassium phosphate buffer of pH 6.8 and brought upon a calcium phosphate column of 12 cm length and 5 cm diameter at 2°C. After the solution had been drained in the gel, the glass wall of the column was cleaned by two washings with 5 ml 0.01 M buffer each. Then 5 ml of the same buffer was layered upon the column and the fractionation started with a linear gradient of 200 ml 0.01 M buffer to 200 ml 0.1 M buffer. A second linear gradient was applied for which we used 200 ml 0.1 M buffer to 200 ml 0.6 M buffer. The complete elution was carried out at a constant pH of 6.8 and took about 24 hours. The fractions were collected in 5 ml volumes and gave the effluent shown in Diagram IV-10.

After some experience the method proved fully reproducible. The four components were dialysed and freeze-dried prior to an examination of their immunizing potency. We dissolved respectively 100, 50 and 25 mg of the dry material obtained from the peaks in 30 ml 0.85 per cent saline and tested these solutions in the usual way.

We also tried columns of Sephadex G-100 and G-200 but the results were rather disappointing. This was also the case when the hydroxyapatite was replaced by alumina and the /.....

Diagram IV-10

Chromatography of 750 mg prepurified material on calcium phosphate.



Column : 12 x 5 cm
Rate of flow : 40 ml per hour
Temperature : 2°C

I : gradient 0.01 M buffer ⟶ 0.1 M buffer
II : gradient 0.1 M buffer ⟶ 0.6M buffer

Buffer : phosphate buffer pH 6.8

	Immunizing capacity of		
	25mg	50mg	100mg
A	0%	0%	0%
B	0%	0%	0%
C	10%	40%	40%
D	100%	100%	100%

and the chromatography was carried out in the same way. None of these methods gave more than two distinct peaks in the effluent pattern.

5) The complete isolation and purification procedure for the soluble antigen from cultures of Cl. chauvoei, strain 64.

The 48-hour-old culture was centrifuged at 3 000 r.p.m. for 1 hour and the supernatant decanted and cooled in an alcohol bath of -10°C to a temperature of about 2°C. We then slowly added pulverized ammonium sulphate (100 gm per litre) under vigorous stirring. The formed precipitate was allowed to settle for 2 hours at 2°C and centrifuged at 3 000 r.p.m. for one hour. The supernatant was decanted and the ammonium sulphate concentration increased to 55 per cent (w/v) by the addition of solid salt under stirring at a low temperature. We recovered the material after allowing the precipitate to stand for five hours at 2°C by centrifuging at 3 000 r.p.m. for 30 minutes, and suspended it in approximately 1/5 of the culture volume of distilled water. The suspension was dialysed for 18 hours against distilled water and the dialysate was cleared from the remaining precipitate by centrifuging at 3 000 r.p.m. for 30 minutes. The almost clear supernatant was corrected to about half of the culture volume by the addition of distilled water, the volume having already been increased by dialysis.

The pH of the solution was established at 6 by adding 1 M phosphate buffer to a final concentration of 0.05 M. Per litre of the original culture, we added a slurry of 18 g D.E.A.E.-cellulose powder, washed with 0.5 M sodium hydroxide and water prior to use, in 0.05 M phosphate buffer. The suspension was stirred for 45

minutes /.....

minutes at a low temperature and the resin, which adsorbed the antigen, was allowed to settle. Most of the supernatant was removed by decanting and a column of 5 cm diameter was packed with the resin, applying 60 cm mercury pressure to compact the cellulose. The column was directly eluted with 0.1 M phosphate buffer of pH 6, with a rate of flow of 120 - 150 ml per hour; until after the peak had emerged the effluent again had a low and constant optical density at 280 mμ. The elution was then continued with 0.1 M buffer to which 0.3 M sodium chloride was added. As soon as the peak had emerged and the optical density of the effluent at 280 mμ became constant, the elution was stopped. Care was exercised because with prolonged elution with this effluent nucleic acids might come off the column. For this reason we used a fraction collector to control the effluent at this stage. The solution was dialysed and freeze-dried and in our case about 1.6 g material was obtained from 1 litre of culture.

This material was further purified in 750 mg quantities on calcium phosphate columns as described before and gave a yield of about 160 mg of pure antigen per litre of culture.

6) <u>The purity of the successive antigen fractions produced during the isolation</u>.

A comparative study of the immunizing capacity and the nitrogen content of the successive antigen fractions was made in order to obtain information about the degree of purity. We examined the following materials :

A) the crude ammonium sulphate precipitate,

B) the material adsorbed by D.E.A.E.- cellulose,

C) the purified antigen obtained by calcium phosphate chromatography.

The /.....

The results of this comparative study are shown in Table IV-10.

Table IV-10.

The immunizing activities of the antigenic materials in relation to their yield and nitrogen content.

Material	Yield per litre culture	%N	Minimal protecting dose for 10 guinea-pigs.
Ammonium sulphate precipitate	about 6 g	10.1	150 mg
D.E.A.E. adsorbed material	about 1.6 g	14.6	50 mg
Purified antigen	about 0.16 g	15.1	20 mg

The minimal protecting dose was determined by dissolving increasing quantities of each material in 30 ml of 0.85 per cent saline. The lowest quantity that gave 100 per cent protection in guinea-pigs was taken as minimal protecting dose. The figures given are obtained by one test.

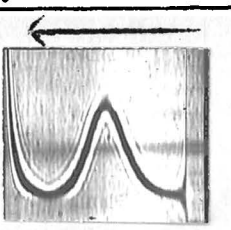
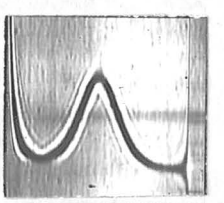
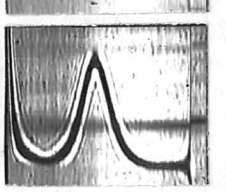
These materials were also examined in the analytical untracentrifuge and by starch-gel electrophoresis.

The starch-gel electrophoresis was carried out at two different pH values, namely at pH 6 and 8.4. We found that the ammonium sulphate precipitate gave broad smears but the other materials revealed only one band. The width of these bands decreased with increasing purity. As shown in Talbe IV-11 the ultracentrifuge patterns of the materials reveal one peak.

Table IV-11 /.....

Table IV-11.

The sedimentation of the successive antigenic materials in a centrifugal field, using phosphate buffers of pH 6.

Material	S^{o}_{20}	Photograph taken after 72 minutes.
By ammonium sulphate precipitated material	1.24	
By D.E.A.E.-cellulose adsorbed material	1.54	
Purified antigen	2.03	

7) The haemolytic activities of the successive antigenic fractions obtained during the isolation.

All the antigenic materials contained haemolytic activity as can be seen from Table IV-12.

Table IV-12.

The haemolytic activity of the antigenic materials.

Material	Haemolytic activity	Filtrate equivalent
Filtrate	0.5 ml	---
Precipitated by ammonium sulphate	4 mg	0.6 ml
Adsorbed on D.E.A.E.-cellulose	1.3 mg	0.8 ml
Purified antigen	1.3 mg	8.5 ml

The purified antigen contained very little haemolytic activity, which seemed to indicate that the antigen and haemolysin are distinct substances. We therefore studied the calcium phosphate chromatography in connection with the haemolysin purification.

The results /.....

The results of this study are shown in Table IV-13.

Table IV-13.

The haemolytic activity of the four fractions obtained by the chromatography of the D.E.A.E. adsorbed material (Diagram IV-10).

	Haemolytic activity
Fraction A	--
Fraction B	6 mg
Fraction C	0.4 mg
Fraction D	1.3 mg

It was observed that fraction C has the strongest haemolytic activity. The weak activity present in peak B can be attributed to the overlapping of this peak with the strong haemolysin. The same explanation for the haemolytic activity in peak D does not hold, for it was found that,after repeated chromatography of this material on a calcium phosphate column, the activity remained. This seems to indicate a relation between antigen and haemolysin, which, however, are not identical, for they gave distinct peaks when calcium phosphate chromatography was applied.

8) Other biological activities of the successive antigenic fractions obtained during the isolation.

Lethal activity was never found in sterilized solutions of any of the antigenic materials. Quantities of 100 and 200 mg of the crude material precipitated by ammonium sulphate, did not possess lethal activity when injected in mice and guinea-pigs respectively. (100 mg is equivalent to 16 ml of culture filtrate).

The hyaluronidase activity was also precipitated from the cultures by the use of ammonium sulphate, but

this /.....

this activity was not adsorbed by D.E.A.E.-cellulose.

We repeatedly failed to detect any deoxyribonoclease activity when sodium-deoxyribonucleate was used as substrate. The optical density of the solution at 260 mμ was constant, after precipitation of the nucleate with perchloric acid at regular intervals of time.

Anti-phagocytic activity was not detected. When quantities up to 50 mg of the material, precipitated by ammonium sulphate, were mixed with serum prior to the phagocytic test, we found that the per centage of leucocytes that contained bacilli amounted to about 50.

9) The antigen bound to the bacterial cell.

The purification method (as described above) gave no satisfactory results when applied to bacterial cell extracts. The ammonium sulphate precipitate proved to be practically insoluble. The soluble part of this precipitate, however, was tested for immunizing capacity after centrifuging at 40 000 r.p.m. for 1 hour and it was found that 100 mg of this material gave only a 70 per cent protection. This potency compared reasonably with that of the ammonium sulphate precipitate of the culture filtrate.

No more tests were made on the cell extracts, the latter being only used for a few immunochemical experiments.

Chapter V /.....

CHAPTER V.

A CHEMICAL AND PHYSICO-CHEMICAL STUDY OF THE SOLUBLE ANTIGEN AND HAEMOLYSIN.

As we saw in the previous chapter, the antigen and haemolysin, isolated according to the given method, revealed no impurities in the ultracentrifuge and the starch-gel electrophoresis. The latter technique was employed at two different pH values but only one distinct component was obtained. The materials proved to be homogeneous as far as the main characters of proteins, namely the electrical charge and the molecular weight, are concerned. However, we thought it necessary to establish purity also by the use of other techniques, before a chemical analysis was carried out.

First of all we studied the dried materials by chromatography on D.E.A.E.-cellulose and calcium phosphate, (the hydroxyapatite) columns. We employed long linear gradients in both methods and the optical density of the effluents was measured at two different wavelengths, namely at 280 mμ and 260 mμ respectively. This was done in order to detect nucleic acid impurities. Cation-exchange chromatography seemed superfluous because we knew already that the materials were strongly negatively charged in the pH range where they proved stable. Because of the specific adsorption of the components on calcium phosphate we preferred this gel, for such a specificity was not found on Sephadex, or any other adsorbent.

We also applied the variable solvent solubility test to establish purity (see Falcomer and Taylor, 1946).

This /.....

This test relies upon the precipitating effect of salts upon proteins, the pH and temperature being kept constant during the experiment. A simple method was developed in 1952 by Derrien, Steyn-Parvé, Cotte and Laurent who showed that ammonium sulphate could be used to precipitate proteins in a variable solvent solubility test. The concentration of the proteins remaining in solution could be readily determined by measuring the optical density after filtration. Smithies (1954) employed this method after minor alterations and found that quantities of about 25 mg dried material were sufficient for the test.

The mobilities of the antigen and haemolysin during free boundary electrophoresis were computed and compared with each other. This technique was used once again to prove purity of the two components. As pointed out by Smithies (1955) the theoretical advantages of starch-gel electrophoresis, being a zone-electrophoresis technique, are :

1) freedom from boundary anomalies,
2) quantitative separation of the components, and
3) the adaptability of the technique to small quantities.

The separation of the components during starch-gel electrophoresis is very effective because the mobilities of the proteins are not only determined by their electrical charges but also by their molecular sizes. Proteins with identical charges have a similar mobility in the free electrophoresis, whereas the gel-structure retards movement of the large molecules. The main advantage of the free boundary electrophoresis, however, is that the different components are visible at any stage of the experiment, no staining technique is necessary as in the case of the starch-gel electrophoresis. In the latter method several

dyes /.....

dyes have to be employed to reveal impurities of a nature other than proteins; this is a complication of this technique. The mobilities were calculated at two different pH values to obtain a picture of the electrical charges of the two components.

The molecular weights of the antigen and haemolysin were estimated in the ultracentrifuge with the Archibald approach to equilibrium method. At the same time the diffusion was studied in the ultracentrifuge and the coefficients were computed by the methods of height-area and moments. As pointed out by Herdan (1953) the agreement between the values calculated by these methods provides also a test of homogeneity. By combining this diffusion coefficient with the sedimentation constant, determined with a second ultracentrifuge experiment, the molecular weights were also evaluated.

A chemical analysis was carried out to compare the antigen and haemolysin. The amino acids were assayed with the use of paper chromatography whereupon a quantitative estimation followed with the column chromatographic method of Moore, Spackman and Stein (1958). Both methods were applied after hydrolysis of the materials with constant boiling hydrochloric acid. Tryptophane however, is destroyed during the hydrolysis and was estimated directly in the protein by the aid of Ehrlich's reagent. Further analyses were made for phosphate and carbohydrate contents.

RESULTS /.....

RESULTS.

1) Chromatography.

The two materials obtained according to the procedure described in the previous chapter will be called the antigen and haemolysin respectively in order to distinguish them. When the antigen and haemolysin were examined by calcium phosphate chromatography, it was observed that both still contained some impurities. It was impossible to obtain an antigen which was absolutely free from other compounds. The haemolysin, however, proved homogeneous after repeated calcium phosphate chromatography (see the Diagrams V-1, V-2, V-3 and V-4). It seemed to us that the antigen decomposed whereupon a little haemolysin was also formed. This can explain the weak haemolytic activity of the antigen. Though the haemolysin showed no distinct components when calcium phosphate chromatography was employed, the peak was always asymmetric. The effluent was therefore divided into four parts and the materials obtained after dialysis and freeze-drying were examined for thier haemolytic activity (see Diagram V-4).

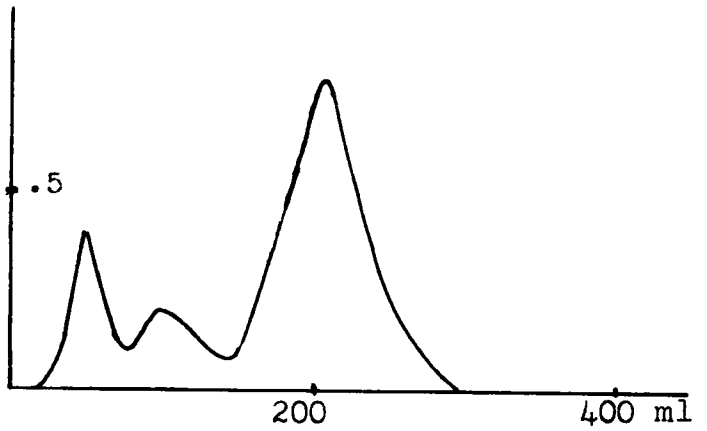
When tested for homogeneity on a D.E.A.E.-cellulose column at 2°C, it was found that the antigen was homogeneous, giving a symmetric effluent peak. The haemolysin on the other hand at first appeared to be homogeneous but when "long" gradients, (very slow increases in the salt concentration of the effluent), were used, more components were observed in the effluent pattern (see the Diagrams V-5, V-6, V-7 and V-8).

It was found that the latter peak of Diagram V-7 contained approximately four times more haemolytic

activity/.....

Diagram V-1

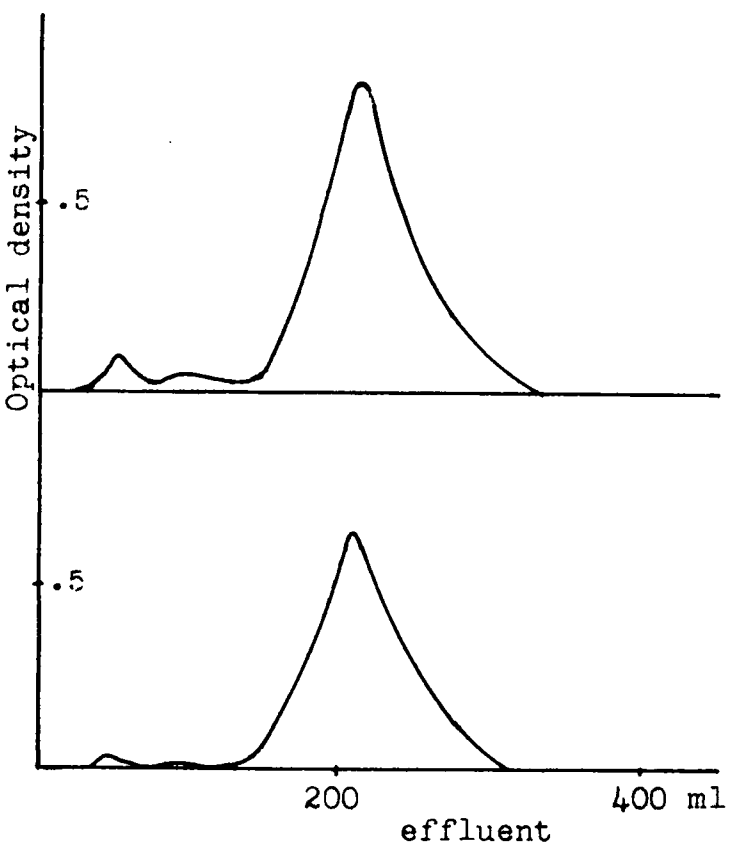
Chromatography of 100 mg antigen on calcium phosphate.



Optical density measured at 280 mμ

Diagram V-2

Chromatography of 100 mg material obtained from the large peak in Diagram V-1 on calcium phosphate.



Optical density measured at 280 mμ

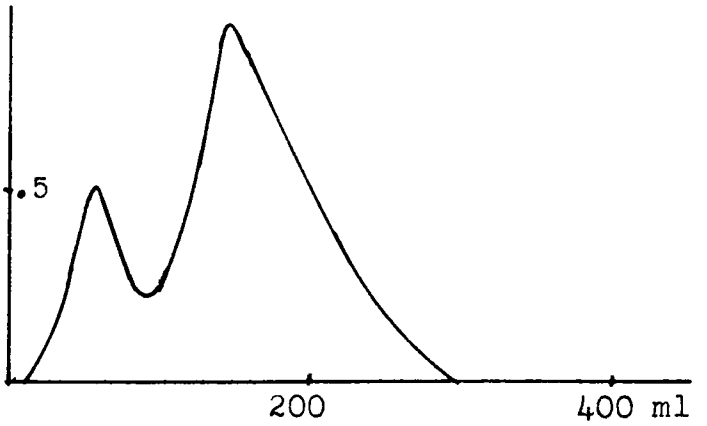
Optical density measured at 260 mμ

Column size : 12 x 2 cm
Rate of flow : 20 ml per hour
Temperature : 2°C
Gradient : 0.02 M buffer ⟶ 0.5 M buffer

Buffer : phosphate buffer of pH 6.8

Diagram V-3

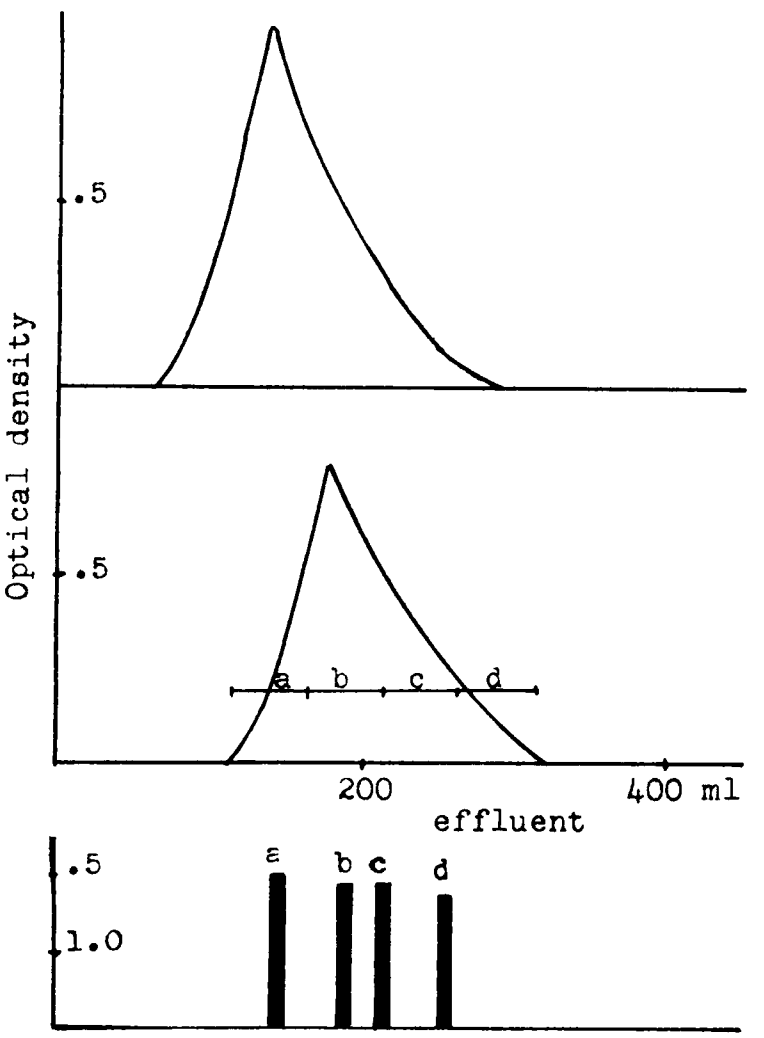
Chromatography of 100 mg haemolysin on calcium phosphate.



Optical density measured at 280 mμ

Diagram V-4

Chromatography of 100 mg material obtained from the large peak in Diagram V-3 on calcium phosphate.



Optical density measured at 280 mμ

Optical density measured at 260 mμ

Haemolytic activity of 4 fractions in mg material

Column size : 15 x 2 cm
Rate of flow : 20 ml per hour
Temperature : 2°C
Gradient : 0.02 M buffer ⟶ 0.5 M buffer

Buffer : Phosphate buffer of pH 6.8

Diagram V-5

Chromatography of 100 mg antigen on D.E.A.E.-cellulose

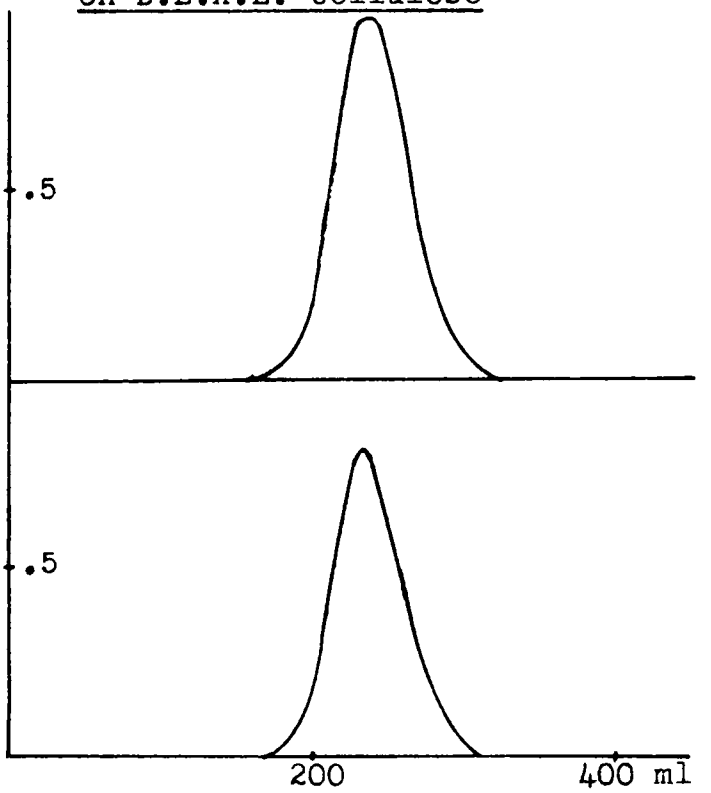
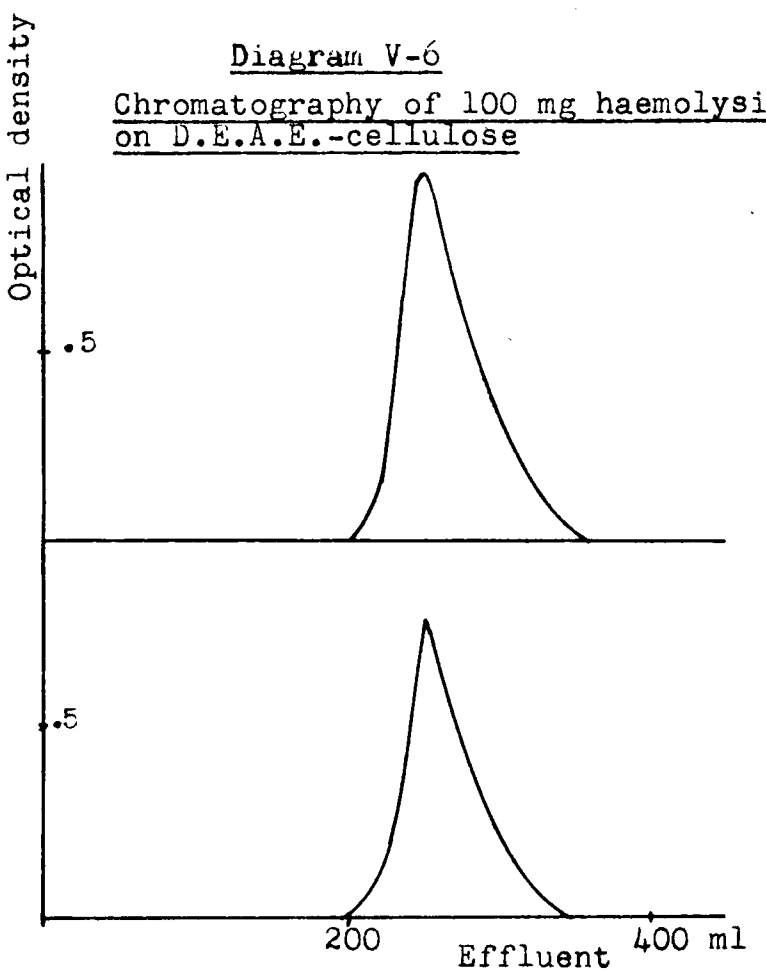


Diagram V-6

Chromatography of 100 mg haemolysin on D.E.A.E.-cellulose



Column size : 28 x 2 cm
Rate of flow : 30 ml per hour
Temperature : 2°C
Gradient : 0.1 M buffer ⟶ 0.1 M buffer + 1 M NaCl

Buffer : phosphate buffer pH 6.

Diagram V-7

Chromatography of 100 mg haemolysin on D.E.A.E.-cellulose, using a long gradient.

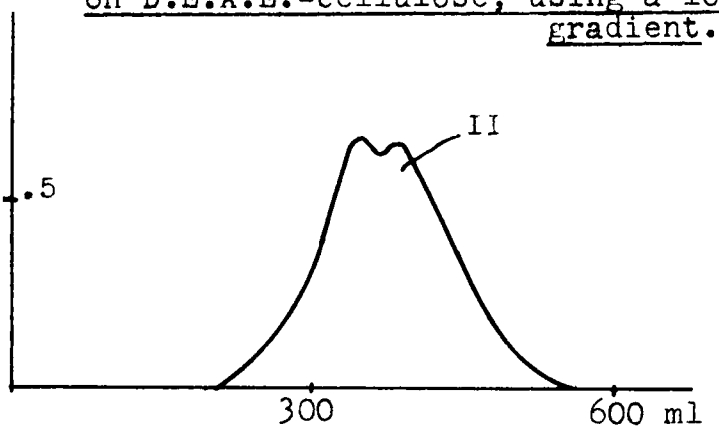
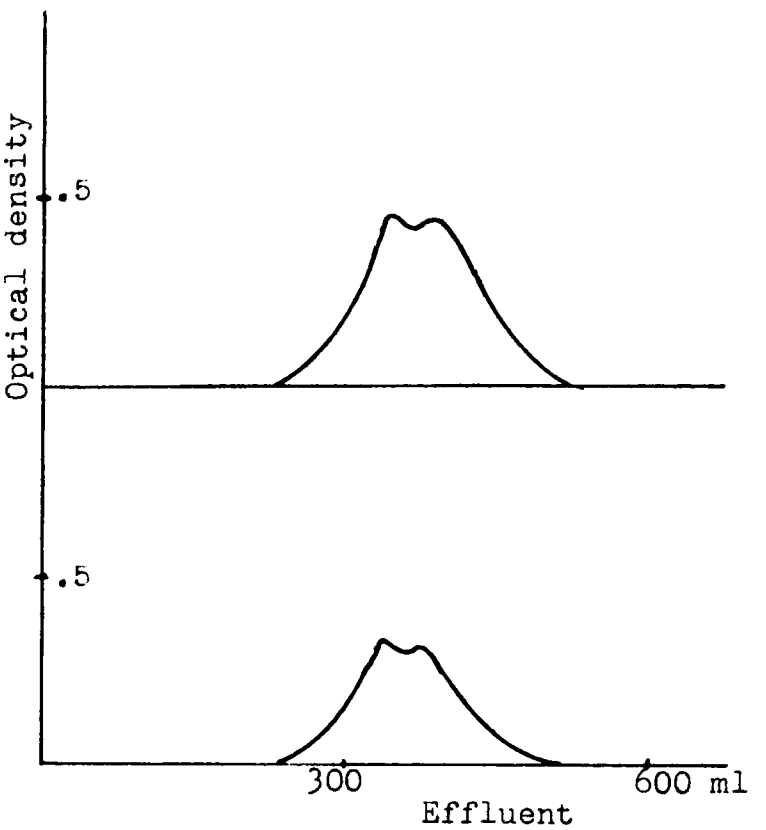


Diagram V-8

Chromatography of 80 mg material from peak II of Diagram V-7 on D.E.A.E.-cellulose, using a long gradient.



Column size : 28 x 2 cm
Rate of flow : 30 ml per hour
Temperature : 2°C
Gradient : 0.1 M buffer ⟶ 0.1 M buffer + 0.5 M NaCl
Buffer : phosphate buffer pH 6.

activity than the first peak, when the test was carried out as described on page 22. This latter peak contained the same activity per mg dried material as the component brought upon the column. It was therefore expected that the fractionation procedure on D.E.A.E.-cellulose would cause a heavy loss of haemolytic activity. As the whole procedure only consists of dissolving the protein in phosphate buffer of pH 6, fractionating this solution on D.E.A.E.-cellulose, dialysing and freeze-drying, we investigated the influence of these treatments on the activity.

We dissolved the haemolysin to a 1 per cent solution in 0.1 M phosphate buffer of pH 6 containing 0.5 M sodium chloride and left it for five days at 10°C. After dialysis and freeze-drying the material was tested again for haemolytic activity.

Table V-1.

The haemolytic activity after storage in phosphate buffer of pH 6, dialysis and freeze-drying.

	Activity.
Original material	0.4 mg
After freeze-drying once	0.4 mg
After freeze-drying twice	0.4 mg
After freeze-drying three times.	0.4 mg

The activity was recorded as the minimum amount of material that gave an absolutely clear solution.

It can be seen from Table V-1 that no loss of haemolytic activity was found even when the material was repeatedly freeze-dried and the samples were taken from the dry powders between the successive freeze-dryings. For this reason we assumed that the D.E.A.E.-cellulose itself caused the inactivation and that this inactivation

reaction /.....

reaction was coupled to a loss of charge, the reason of the appearance of the first peak in the effluent pattern. The dialysed and freeze-dried material from the active component after the D.E.A.E.-cellulose fractionation was re-chromatographed on a D.E.A.E.-cellulose column. The effluent pattern, as shown in Diagram V-4, again showed two distinct components, which were tested for their haemolytic activity. Again a loss of activity was found, for the first peak was very weakly active and the activity of the latter peak per mg dried product was the same as that of the material brought upon the column.

To see if the inactivation resulted in molecular alterations we studied the sedimentation in the ultra-centrifuge and the amino acid contents of the following materials :

1) the haemolysin before the D.E.A.E.-cellulose chromatography was carried out,
2) the material obtained after dialysis and freeze-drying of the second peak after the first D.E.A.E.-cellulose chromatography, and
3) the materials obtained after dialysis and freeze-drying of both peaks after a second D.E.A.E.-cellulose chromatography of haemolytic material.

The sedimentation coefficients, determined in 0.05 M phosphate buffer of pH 6.8, were calculated and showed no significant differences, especially where, as will be seen, the coefficient shows a concentration effect.

Table V-2 /.....

Table V-2.

The sedimentation constants in relation to the haemolytic activity of 1 per cent solutions in phosphate buffer of pH 6.8 and I 0.1.

	S^{o}_{20}	Activity.
Haemolysin	1.05	0.55 mg
Haemolysin after D.E.A.E chromatography	1.02	0.35 mg
Haemolysin after second D.E.A.E chromatography	1.13	0.50 mg
First peak after second D.E.A.E. chromatography	1.07	3 mg

The amino acid contents will be given below.

The use of D.E.A.E.-cellulose for any further purification was avoided for :

1) the antigen proved to be homogeneous on this resin and
2) the haemolysin, or better the haemolytic activity, was partly destroyed.

To ascertain antigenic and haemolytic products with reproducible properties, we decided to repeat the calcium phosphate chromatography after the isolation as described in the previous chapter. All the following experiments were carried out after twice repeated chromatography of the materials on calcium phosphate columns.

2) The absorption spectra of the antigen and haemolysin.

From the chromatographic patterns of the antigen and haemolysin it can be seen that there is only a slight difference in absorption of ultraviolet light of the wavelengths 280 and 260 mμ respectively. We made ultraviolet absorption spectra of the materials dissolved in phosphate buffer of pH 6.8 (ionic strength 0.1) and in 0.1 N sodium hydroxide. The materials were dissolved to a concentration of 1 mg per ml and measured in cuvettes

with a /.....

with a width of 1 cm in a Zeiss spectrophotometer (see Diagrams V-9 and V-10).

The absorption spectra in phosphate buffer of pH 6.8 show maximum values at 276 mμ and minimum at 254 mμ. They resemble the spectra found in many proteins except for a low ratio of the absorption values at 280 and 260 mμ. We found O.D. 280/O.D. 260 ≈ 1.1 whereas most proteins give a value of about 1.3-1.7 for this optical density ration. For this reason phosphate determinations were carried out to detect nucleic acids.

In 0.1 N sodium hydroxide an increased absorption as well as the disappearance of the maxima and minima in the absorption spectra was noticed. It was assumed that only very little ionizable tyrosine is present.

3) The variable solvent solubility test.

In Diagrams V-11 and V-12 the optical densities are plotted against the percentages of saturation of the ammonium sulphate solutions (at 20°C in 0.1 M phosphate buffer of pH 7.4, when it was assumed that no volume alterations found place by diluting).

As can be seen the haemolysin was not homogeneous but the antigen revealed only one component, although the chromatography on calcium phosphate columns indicated the presence of more compounds in very low concentrations. We assumed that the discontinuity in the salting-out curve for the haemolysin resulted from heterogeneity and not from the situation where a single protein in solution is in equilibrium with different solid phases, according to the salt concentration. This latter system was observed with lactoglobulins as explained by Ogston and Tombs (1956). These authors stated that the

discontinuity /.....

Diagram V-9

Absorption spectra of antigen.

Antigen dissolved in:
I) Phosphate buffer pH 6.8 I 0.1
II) in 0.1 N NaOH

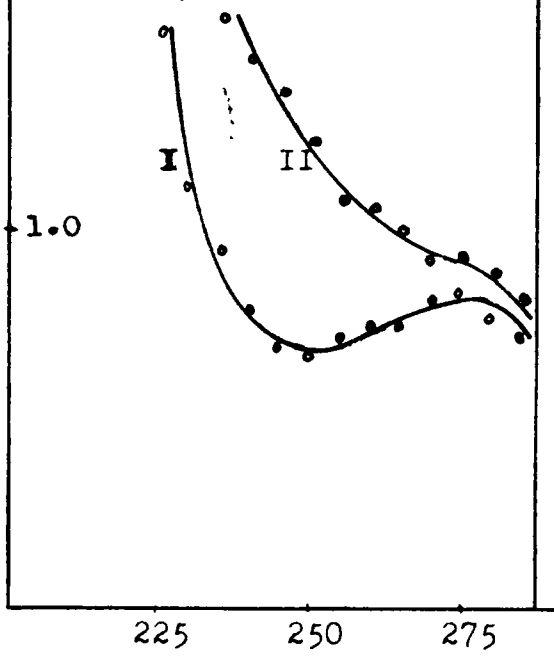
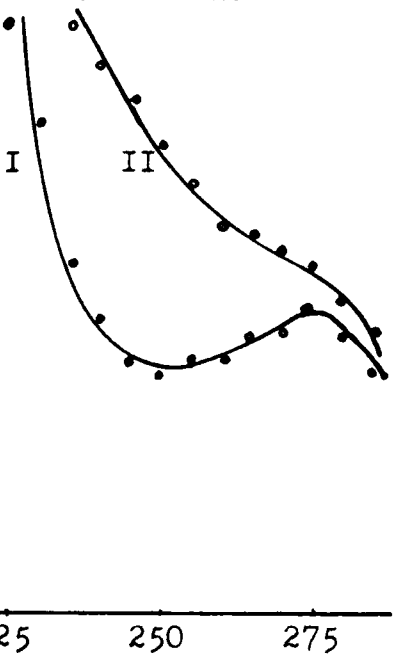


Diagram V-10

Absorption spectra of haemolysin.

Haemolysin dissolved in:
I) In phosphate buffer pH 6.8 I 0.1
II) In 0.1 N NaOH



Wave length in mμ

Optical density

Diagram V-11

Variable solvent solubility curve of the antigen at pH 7.4

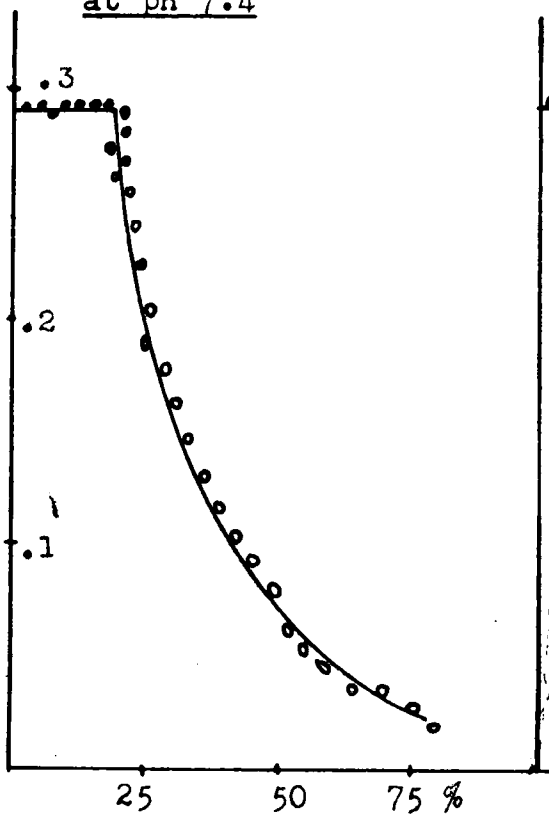
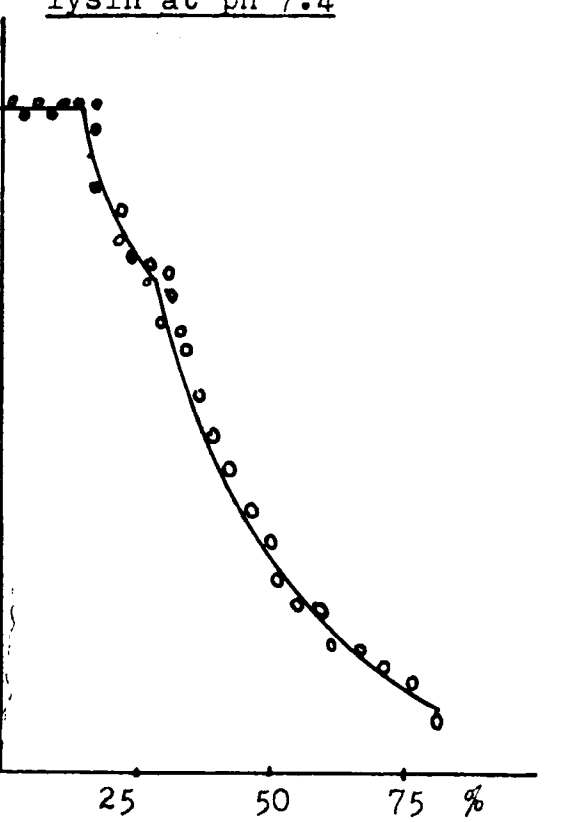


Diagram V-12

Variable solvent solubility curve of the haemolysin at pH 7.4



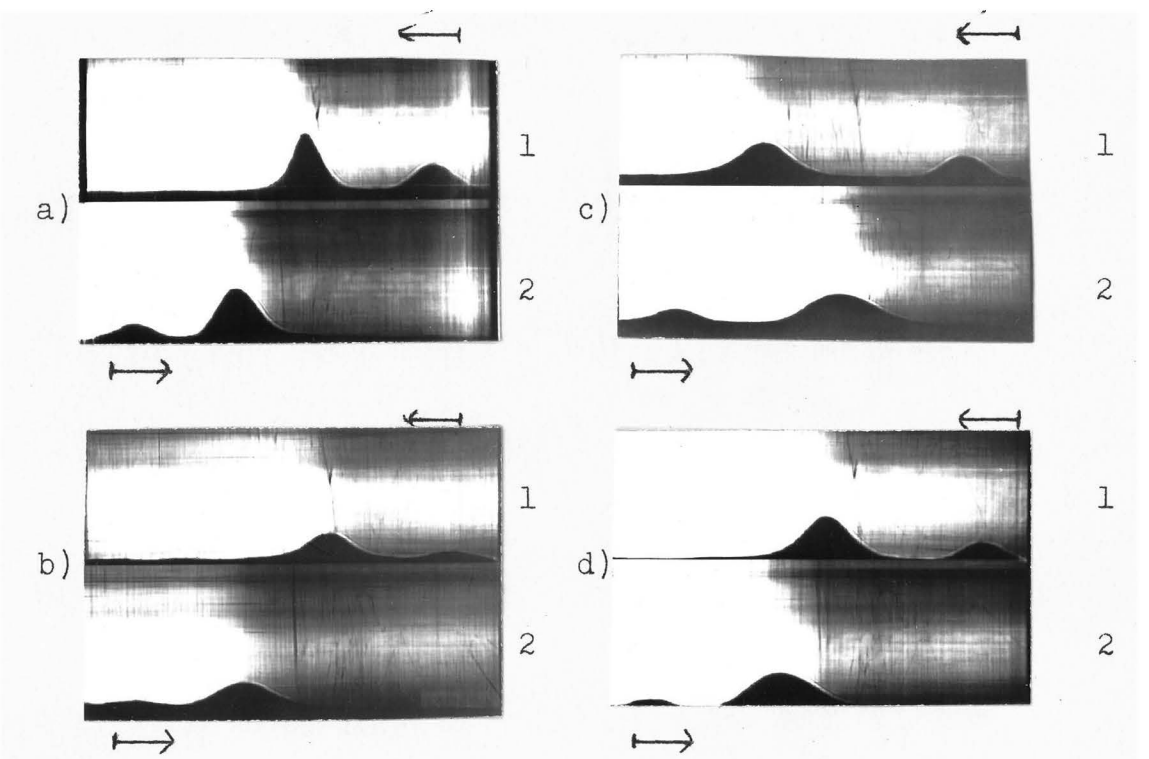
Per cent ammonium sulphate

discontinuity of a salting-out curve due to more solid phases of a single component can easily be detected by lowering the initial concentration of the solute, where the discontinuity must disappear. In our case this seemed superfluous, because the initial concentration was already very low and we feared inaccuracy in measuring the optical density when we had to lower this concentration.

4) Moving boundary electrophoresis.

Both the antigen and haemolysin were studied in the electrophoresis. We used a barbiturate buffer of pH 8.6 and also a sodium phosphate buffer of pH 6.2 both with an ionic strength of 0.1.

In Figure 1 the electrophoretic patterns are given after $3\frac{1}{2}$ hours of electrophoresis.



1) ⟵ ascending.

2) ⟶ descending.

Figure 1 /.....

Figure 1. Electrophoretic patterns of antigen and haemolysin in different buffers of I=0.1 after about 3½ hours of electrophoresis.

a) antigen in barbiturate buffer pH 8.6

b) antigen in phosphate buffer pH 6.2

c) haemolysin in barbiturate buffer pH 8.6

d) haemolysin in phosphate buffer pH 6.2

No impurities were revealed and the resultant peaks were symmetrical. The mobilities of the components are tabulated in Table V-3.

Table V-3.

Electrophoretic mobilities of the antigen and haemolysin at 4°C in different buffers of ionic strength 0.1.

Material	Buffer	pH	Mobility x 10^{-5} cm^2/sec./V. Ascending	Descending
Antigen	barbiturate	8.6	-6.7	-6.3
Antigen	phosphate	6.2	-4.7	-4.4
Haemolysin	barbiturate	8.6	-7.3	-6.2
Haemolysin	phosphate	6.2	-5.5	-4.4

It is noticeable that no significant difference in the mobilities of the antigen and haemolysin were found.

5) Estimation of the molecular weight of the antigen.

The molecular weight estimates of the antigen according to the Archibald technique were carried out at 14 290 r.p.m.

The specific volume being used was taken at 0.74. In Table V-4 the physico-chemical constants of the antigen are recorded.

Table V-4 /.....

Table V-4.

The molecular weight, sedimentation and diffusion constants of the antigen in phosphate-borate buffer of I 0.2 and pH 8.4 at different concentrations.

Concentration. mg/ml	Δn	S^o_{20}	D x 10^{-7} cm^2/sec. Height area	Moments	M.w. by the Archibald method
15	0.0017	2.22	4.54	4.34	53.300
11	0.0012	2.24	4.41	4.49	53.500
8	0.0009	2.25	4.37	4.51	54.100

Δn is the increment of the refractive index by the solute.

There is a definite agreement in the values of the diffusion coefficients calculated by the methods of height-area and moments. The average value of the diffusion coefficient is 4.44. When this value is combined with the sedimentation constant, which after extrapolation to zero concentration is 2.28 S, a molecular weight of 48.400 is calculated. This latter molecular weight is somewhat lower than that evaluated by the Archibald method.

The Archibald method gives the weight-average molecular weight of the solute or :

$$M = \frac{\sum c_i M_i}{\sum c_i}$$

It is therefore possible that larger molecules (e.g. aggregates) have a considerable influence on this molecular weight. On the other hand as pointed out by Ehrenberg (1957) the value of the area of the curve between the meniscus and base-line increases during the experiment. When more components are present, they would all contribute to the area increase. The contribution of the larger molecules to this area increase is very large in the beginning (for their higher S/D ratio), but becomes relatively smaller during the experiment and

hence /.....

hence the molecular weight computed according to the Archibald method decreases. We found, however, a fairly constant molecular weight during the course of an experiment as can be seen from the example given in Table V-5.

Table V-5.

The molecular weight of the antigen, computed by the Archibald method during the course of an experiment.

Δn = 0.0013 phosphate-borate buffer pH 8.4 I = 0.2.

Time in minutes	Molecular weight.
0	54.000
8	52.100
16	53.300
24	52.900
32	52.000
40	54.800
48	55.200
56	53.400
64	54.400
72	52.600
80	53.200
88	55.300
96	52.800
104	53.600
112	53.100

For this reason the discrepancy in the values of the molecular weight is to be sought in the value of the diffusion coefficient. The method of estimating the diffusion coefficient in the ultracentrifuge is rather inaccurate because :

1) the temperature in the ultracentrifuge is not sufficiently constant, (the ultracentrifuge not being equipped with a temperature regulator);
2) small inhomogeneities of the solution will have a significant influence on the apparent diffusion coefficient.

We also investigated the stability of the antigen at different pH values in the ultracentrifuge. The sedimentation constant was determined in approximately 1 per

cent /.....

cent solutions using buffers of ionic strength 0.1.

Table V-6.

The sedimentation constants of the antigen in buffered solutions with I 0.1.

Buffer	pH	S^{o}_{20}
Acetate (a)	4.1	2.08
Phosphate	6.8	2.11
Borate	8.4	2.22

a) The antigen did not completely dissolve in this buffer.

In Figure 2 photographic recordings are shown of the sedimentation of the antigen taken after 72 minutes of centrifugation.

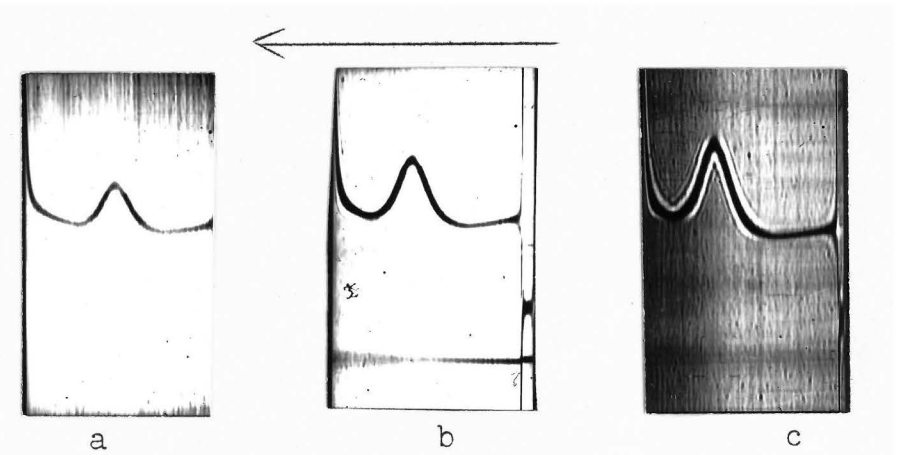


Figure 2. The sedimentation pattern of the antigen taken after 72 minutes centrifuging at 56 100 r.p.m.

a) the sedimentation in acetate buffer of pH 4.1

b) the sedimentation in phosphate buffer of pH 6.8

c) the sedimentation in borate buffer of pH 8.4

No further investigations were carried out as no significant difference was found.

We only once studied the antigen with the Archibald technique in phosphate buffer of pH 6.8, using an approximately 1 per cent solution. The same results were found as those recorded in Table V-4. In this case the molecular weight according to the Archibald method

was /.....

was 53 100 and by combining the sedimentation and diffusion co-efficients we found a molecular weight of 45 800.

6) Estimation of the Molecular weight of the haemolysin.

We also studied the haemolysin in the ultra-centrifuge, although it was realised that this component was not pure, as found with the solubility test.

Preliminary investigations in the ultracentrifuge, in phosphate buffer of pH 6.8 and ionic strength 0.1 indicated a molecular weight of about 11 000 and a sedimentation constant of about 1.1 S. It was evident that the constant showed a pronounced concentration effect, and when extrapolated to zero concentration a value of approximately 1.25 was estimated. Because this material showed a very asymmetrical diffusion peak in the ultra-centrifuge, run at 23 150 r.p.m., we feared contamination of low molecular weight components. The experiments were repeated with the same preparation that was also used in the constant solubility test, as described before. In this case we used a similar phosphate-borate buffer as in the study of the antigen. In Table V-7 the constants are recorded with the different concentrations of the solute substance as measured by the increment of the refractive index. We used a centrifuge speed of 16 200 r.p.m.

Table V-7.

The molecular weight of the haemolysin and the sedimentation and diffusion constants, in phosphate-borate buffer of pH 8.4 and I 0.2 at different concentrations.

Δn	S^{o}_{20}	D x 10^{-7} cm^2/sec. Height area	Moments	M.W. by the Archibald method.
0.0026	1.32	5.44	5.47	24.600
0.0019	1.40	5.61	5.64	27.300
0.0013	1.48	5.53	5.51	28.800
0.0008	1.56	--	--	---

The /.....

The diffusion coefficients computed by the methods of height-area and moments are in agreement. When the average value of D is combined with the sedimentation constant of 1.64, extrapolated to zero concentration, a molecular weight of 24 800 is found. This molecular weight is lower than that evaluated by the Archibald method. The latter reaches a value of 33 000 when extrapolated to zero concentration. This difference, however, can readily be explained by the presence of larger molecules as already indicated by the solubility test. It was also observed that the molecular weight computed by the Archibald method decreased during the first 30 minutes of an experiment. In Figure 3 photographic sedimentation patterns are shown of the haemolysin in phosphate-borate and phosphate buffer after 72 minutes of centrifuging at 56 100 r.p.m.

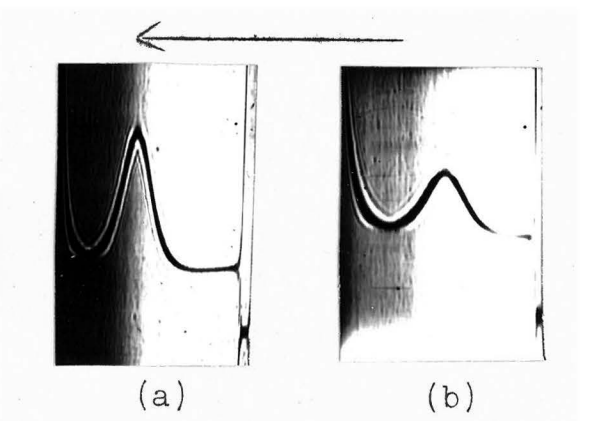


Figure 3. The sedimentation patterns of the haemolysin in phosphate-borate buffer of pH 8.4 and I=0.2 (a) and in phosphate buffer of pH 6.8 and I = 0.1 (b).

It is noticeable that although the haemolysin is not pure, symmetrical peaks were obtained in the ultracentrifuge.

It was found that the sedimentation constant of the haemolysin was much higher than was expected from the preliminary experiments. The buffer, however, was of a

composition /.....

composition other than that used in the preliminary experiments. For this reason we studied the sedimentation also in phosphate buffer of pH 6.8 and ionic strength 0.1. The results are shown in Table V-8.

Table V-8.

The sedimentation constant of the haemolysin in phosphate buffer of pH 6.8 and I 0.1 at different concentrations.

$\triangle n$	S^{o}_{20}
0.0022	1.15
0.0018	1.30
0.0012	1.40
0.0008	1.46

When extrapolated to zero concentration a sedimentation constant of 1.55 was obtained. This value compares very well with that evaluated in phosphate-borate buffer of pH 8.4, but is higher than that obtained by earlier experiments.

It is evident, however, from the ultracentrifugal experiments that the antigen and haemolysin are distinct components with different molecular weights.

7) The amino acid contents of the antigen and haemolysin.

By the employment of paper chromatography it was found that the antigen and haemolysin contained the same amino acids, when hydrolysed in a sealed glass tube at 105°C for 16 hours. When this hydrolysis technique was used no humin forming was detected. The colour intensities of the spots, after staining with ninhydrin revealed that the concentrations of the amino acids in both components were approximately equal (see Table V-9).

Table V-9 /.....

Table V-9.

The amino acids present in the antigen and haemolysin with the colour intensities of their spots.

Amino acid	Antigen	Haemolysin
Aspartic acid	very strong	very strong
Threonine	weak	weak
Serine	weak	weak
Glutamic acid	very strong	very strong
Proline	very weak	very weak
Glycine	weak	weak
Alanine	strong	strong
Valine	strong	strong
Methionine	?	?
Isoleucine	weak	weak
Leucine	strong	strong
Tyrosine	very weak	very weak
Phenylalanine	weak	weak
Lysine	strong	strong
Histidine	very weak	very weak
Arginine	weak	weak
Tryptophane	positive	positive

Tryptophane was detected with Ehrich's reagent.

It is noticeable that no cysteine or cystine were detected. We were not completely sure in the case of methionine, as this amino acid is difficult to distinguish from valine. Because the amino acid contents were very similar for both components we decided to apply the column chromatographic method to investigate any difference.

It was found that by hydrolysis in constant boiling hydrochloric acid, under reflux, humin formation appeared after about 24 hours. The ninhydrin reaction, however, carried out on samples taken after various times of hydrolysis, showed a maximum colour intensity after 36 hours. For this reason the antigen was hydrolysed for 18, 36 and 54 hours respectively in constant boiling hydrochloric acid under reflux. The amount of acid taken was about 5 000 times the weight of the protein. We also hydrolysed for 16 hours in a sealed tube at 105°C.

The amino acid /.....

The amino acid content of the haemolysin was also determined after 36 hours of hydrolysis in hydrochloric acid (the time at which maximum hydrolysis also occurred) and after 16 hours in a sealed tube at 105°C. To detect if inactivation of the haemolysin on D.E.A.E.-cellulose was accompanied with an alteration in the amino acid contents, we determined the amino acids in 36-hour-hydrolysates of haemolysin fractions after a first and a second chromatography on D.E.A.E.-cellulose columns. These haemolysin samples had various activities. The results are shown in Table V-10, where we recorded the average values of duplicate experiments.

Table V-10.

The amino acid composition of the antigen and haemolysin in mg per 100 mg of hydrolysed material.

	Antigen after hours of hydrolysis			Haemolysin with activities given in mg			After hydrolysis in sealed tubes.	
	18	36	54	0.35	0.55	3.0	Antigen	Haemolysin
Aspartic acid	6.9	6.9	6.9	7.0	7.1	7.1	7.0	7.1
Threonine	2.8	2.9	2.9	2.9	3.0	3.1	2.9	3.0
Serine	3.7	3.8	3.5	3.5	3.6	3.2	3.6	3.4
Glutamic acid	20.4	20.9	19.9	20.4	20.1	20.0	20.8	20.3
Glycine	2.3	2.5	2.5	2.5	2.5	2.8	2.5	2.7
Alanine	5.5	5.7	5.2	5.1	5.0	4.8	5.4	5.0
Valine	5.0	4.9	5.2	5.1	5.1	5.3	5.1	5.2
Isoleucine	3.6	3.2	3.5	3.7	3.6	3.2	3.4	3.6
Leucine	7.2	7.4	7.4	7.3	7.3	7.1	7.2	7.1
Tyrosine	0.6	0.7	0.7	0.8	0.7	0.8	0.8	1.0
Phenylalanine	1.5	1.6	1.5	1.6	1.6	1.4	1.6	1.5
Lysine	8.0	8.1	7.8	8.0	8.2	7.9	7.8	8.1
Histidine	0.9	0.7	0.6	0.9	0.8	0.8	0.7	0.9
Arginine	3.3	3.2	3.1	3.2	3.1	2.9	3.3	3.1
Ammonia	3.0	3.0	3.4	3.0	2.8	3.1	3.3	3.0

The given /.....

Tryptophane is present in the proteins but could not be determined quantitatively because of very high blank values. The proteins dissolved in sulphuric acid show high densities at 570 mμ.

It can be seen that :

1) no proline is present, which we found by paper chromatography;
2) only about 70 - 75 per cent yield of amino acids is obtained;
3) much ammonia is found;
4) no difference in the amino acid contents of the antigen and haemolysin was detected; and
5) the amino acid composition of the haemolysin did not change with the activity.

The total nitrogen yield only amounts to about 12 per cent which is also lower than the content of the materials (about 15.2 per cent). We therefore assume that the humin formed during the hydrolysis consists of protein-like material.

When computed in mMol per g material the ammonia, glutamic and aspartic acids amount respectively to approximately 1.9, 1.4 and 0.5 mMol. It may be possible that these two acids are present as glutamine and asparagine.

8) Nitrogen, phosphate and carbohydrate analysis.

Though the nitrogen contents and the high yields of amino acids of the antigen and haemolysin indicated that these components consisted of protein material, we still carried out a phosphate and carbohydrate assay. The results are shown in Table V-11.

Table V-11.

Analysis of the antigen and haemolysin for nitrogen, phosphate and carbohydrates.

	Nitrogen	Phosphate	Carbohydrates	
			orcinol	sulphonated ∝ naphthol
Antigen	15.2%	trace	negative	negative
Haemolysin	15.4%	0.2% m	negative	positive

The haemolysin gave a very weak reaction with

the /.....

the sulphonated ∝naphthol. When compared with a standard ribose solution the colour density of the haemolysin with the sulphonated ∝naphthol indicated 0.3 per cent carbohydrate in this material.

Care had to be exercised with the phosphate determination for it seemed very difficult to free the material from inorganic phosphate by dialysis, that is, phosphate determined without the material being digested. After prolonged dialysis for 48 hours in running distilled water, only traces of phosphate were detected when digestion was applied. It seems possible that especially the haemolysin was contaminated with nucleic acids but this contamination was very slight.

Discussion.

The results of the chemical investigation, described in this chapter show a strong correlation between the antigen and haemolysin. The identical amino acid contents and electrophoretic mobilities of both biologically active materials are indeed remarkable. That the materials are yet distinct components is beyond any doubt for ultracentrifuge experiments showed different molecular weights. In the experiments described it was found that the antigen had a molecular weight of about twice that of the haemolysin. However, it had been demonstrated that the haemolysin was not homogeneous since the constant solubility test showed a discontinuity. We mentioned already that previous results with the haemolysin in the ultracentrifuge indicated a much lower molecular weight. A possible explanation of the discrepancy must be sought in the contamination of high molecular compounds in the latter experiments.

We /.....

We found already (see Chapter III) that the haemolytic activity could be attributed to an oxygen-stable haemolysin. The absence of cystine or cysteine seems to confirm this, as reversible oxidation is quite often due to these amino acids. The weak haemolytic activity of the antigen which we first attributed to contamination with haemolysin can now also be explained in another way. The antigen seems to decompose, very slowly but spontaneously, forming haemolysin also, as we saw by calcium phosphate chromatography. The identical amino acid contents of the antigen and haemolysin denote that this may very well be the case. We presume, however, that the antigen was originally a metabolite with another biological activity, as it seems unnatural that Cl. chauvoei should produce a large molecule (the antigen) when only a small molecule (the haemolysin) is needed.

Chapter VI /.....

CHAPTER VI.

A BIOCHEMICAL INVESTIGATION OF THE ANTIGEN AND HAEMOLYSIN.

In this chapter we will describe some experiments carried out with the antigen and haemolysin. It was found during the isolation procedure that a reasonably active immunity was obtained when the antigen was inoculated in guinea-pigs prior to a challenge with a lethal dose of virulent culture. The biological function of the antigen, however, was not clear; so far only a weak haemolytic activity has been detected.

The antigen was related to the haemolysin but was distinct from the latter : physically, by its higher molecular weight, and biologically, by its immunizing capacity. This biological distinction was further investigated for it could be anticipated that the haemolysin also might be capable of immunizing guinea-pigs.

As found in the literature, several authors presumed that the haemolysin and the lethal toxin of Cl. chauvoei were related, if not identical. (Kerrin, 1934; Guillaumie and Kręguer, 1948; and Moussa, 1958). For this reason we carried out an extensive number of experiments aiming at the detection of any lethal activity in filtrates as well as in the successive antigenic fractions at each stage of the isolation procedure. As no lethal activity was found we assumed that the lethal toxin, if any, produced by Cl. chauvoei, was very labile but could maintain its antigenic potency. MacFarlane (1955) in a lecture presented to the Society for General Microbiology, stressed the importance of lecithinases in gas

gangrene /.....

gangrene toxins. Special reference was given to Cl. perfringens a-toxin (the main lethal toxin of this species, which is a lecithinase) and to Cl. haemolyticum toxin. (See MacFarlane, Oakley and Anderson (1941), MacFarlane and Knight (1941); Jasmin (1947)). Lecithinases are also demonstrated in toxins of Cl. novyi (Crook, 1942 and Hayward 1943) and Cl. Bifermentans (Hayward, 1943). Since we found that the antigen also had a weak haemolytic effect, which can possibly be attributed to an enzymatic action upon the phospholipin of the erythrocyte membranes, we decided to test for lecithinase activity. Because the action of lecithinases is more pronounced upon lecithin than upon phospholipin (MacFarlane, 1950) we employed egg lecithin. Until now only Crook (1942) has reported on the lecithinase activity of Cl. chauvoei filtrates, an activity that was detected by the reaction of Nagler (1939).

The haemolytic activity was also investigated. It was realized that the method which we employed for testing haemolytic activity depended upon a linear relation between the concentration of haemolysin and the rate of haemolysis. Such a linear relation would also suggest that the reaction could be enzymatic in character. Bernheimer (1944 and 1946) demonstrated that the haemolytic reaction of Cl. septicum filtrates was indeed enzymatic. The biochemical action of the latter toxin, however, is unknown because Bernheimer only studied the kinetics of the lysis. In his second publication Bernheimer (1946) clearly showed that the haemolysis induced by Cl. septicum filtrates is remarkable for an induction period during which the cells swell and become translucent through the action of the lysin; lysis is the result of this action

and can /.....

and can be prevented by the addition of sucrose.

It seemed interesting to repeat some experiments with the Cl. chauvoei haemolysin in order to obtain information, not only about this haemolysin, but also about the relation between the two species, Cl. chauvoei and Cl. septicum. This relation was often predicted (see Prévot and Taffanel (1942), Guillaumie, Kréguer, Geoffroy and Ventre (1961) and Moussa (1959).).

RESULTS.

1) The immunizing capacities of the antigen and haemolysin.

We once again investigated the antigenic potency of the antigen when active immunization was employed. We knew that adsorption of the antigen on aluminium hydroxide enhanced the activity considerably. Therefore a comparative study was carried out, in which the immunizing potencies of the antigen and haemolysin in solution as well as adsorbed on aluminium hydroxide were determined. The results are shown in Table VI-1.

Table VI-1.

The immunizing activities of the antigen and haemolysin dissolved in phosphate buffer of pH 6.8 and co-precipitated on aluminium hydroxide (1 per cent alum added).

	Activity.
Antigen in solution	15 mg
Antigen on aluminium hydroxide	7.5 mg
Haemolysin in solution	over 100 mg
Haemolysin on aluminium hydroxide	over 100 mg

All the control animals died.

The activity is recorded as the lowest total amount of freshly prepared dry material that gave full protection /.....

protection to 10 guinea-pigs.

It can be noticed from Table VI-1 that the haemolysin is completely inactive. The antigen on the other hand produced a reasonably active immunity, from which the specificity was investigated. So far the antigen was only used to immunize guinea-pigs against its producer, namely strain 64. The following experiment revealed that not only a protection against strain 64 was obtained but also against several other Cl. chauvoei strains. We inoculated 20 guinea-pigs three times subcutaneously, each time with 20 mg antigen (1 mg per guinea-pig). Two weeks after the third and last inoculation we challenged the guinea-pigs in groups of four, using the following strains :

Cl. chauvoei,

strain 64
strain Lourenço marques
strain K 796
strain 2153

Cl. septicum, there was only one strain available at Onderstepoort Laboratories.

The challenge dose was 0.5 ml for each Cl. chauvoei culture and 0.2 ml for the Cl. septicum culture. The results are shown in Table VI-2.

Table VI-2.

The specificity of the soluble antigen.

	Immunity.
Cl. septicum	4†
Cl. chauvoei :	
Strain 64	1† 3 L
" Lourenço Marques	1† 3 L
" K 796	4 L
" 2153	4 L

† indicates the number of animals which died and L the number still alive five days after the challenge. The m.l.d. of each culture was tested and amounted to about 0.1 ml with the exceptions of the Lourenço Marques strain and the Cl. septicum strain, which had an m.l.d. of 0.2 ml and 0.075 ml respectively.

Though we cannot yet generalize, it still seems that the antigen produced by strain 64, and isolated according to the method described in Chapter IV, gives a reasonable protection against the whole Cl. chauvoei species.

THE HAEMOLYTIC REACTION.

1) Rate of haemolysis as a function of concentration of the haemolysin.

The haemolysin test of various fractions obtained during the isolation procedure depended upon a linear relation between the concentration of the haemolysin present in the sample and the rate of lysis.

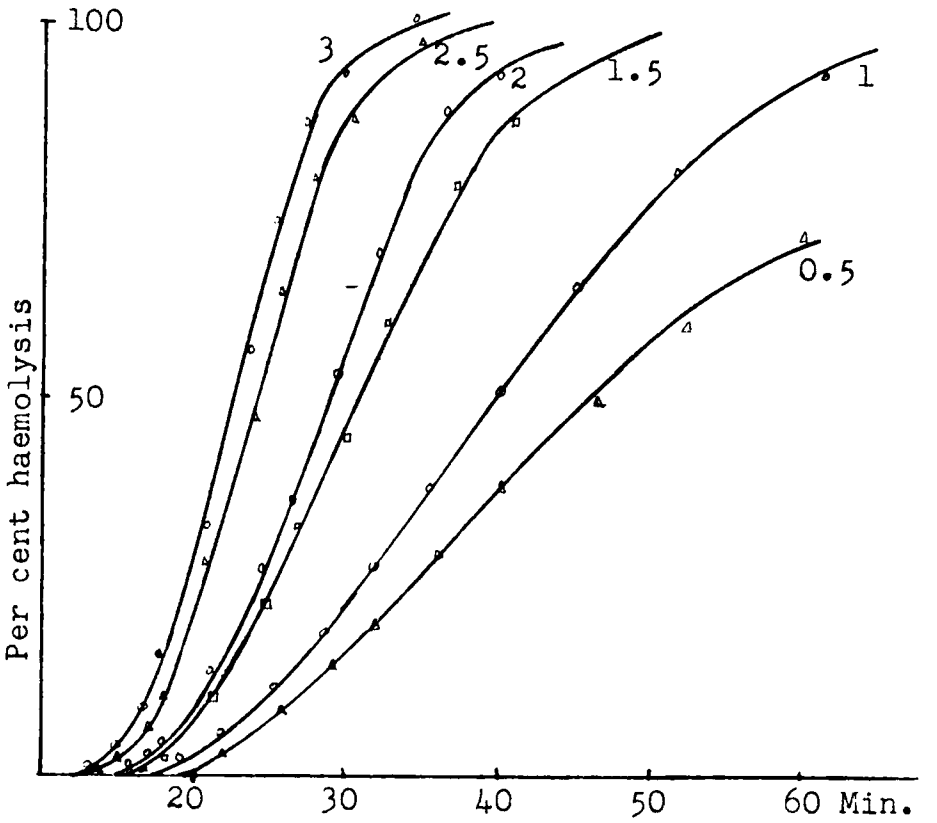
We had already carried out preliminary experiments to investigate if a linear relation existed. Thus we found a long induction period preceding haemolysis, and also that the rate of haemolysis increased with increasing haemolysin concentration.

A further study was made of the reaction in buffered saline (phosphate buffer of pH 6) at a temperature of 37°C. We mixed 20 ml of a sheep erythrocyte suspension with 20 ml of haemolysin solutions containing various concentrations, namely 3 - 2.5 - 2 - 1.5 - 1 and 0.5 mg dried material per ml solution. The results are shown in Graph VI-1 and Graph VI-2.

Graph VI-1 /.....

GRAPH VI-1.

The course of the haemolytic reaction for different haemolysin concentrations.

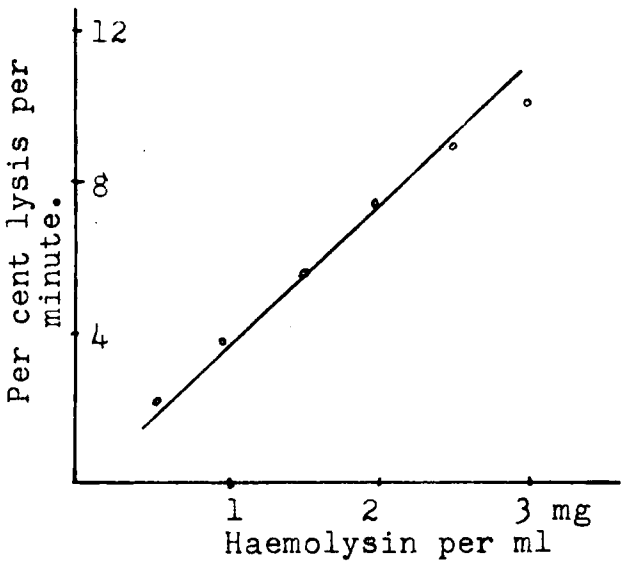


The rates of haemolysis were estimated from the slopes of the linear parts of each curve. When these values are plotted against the haemolysin concentration, as shown in Graph VI-2, a straight line is obtained.

Graph VI-2 /.....

GRAPH VI-2.

Rate of haemolysis as a function of haemolysin concentration at 37°C.



2. Haemolysis of erythrocytes from different species.

The haemolysis of erythrocytes prepared from the blood of different animals was studied and the rates compared at three different concentrations of haemolysin. The erythrocyte suspensions were prepared from the blood of :

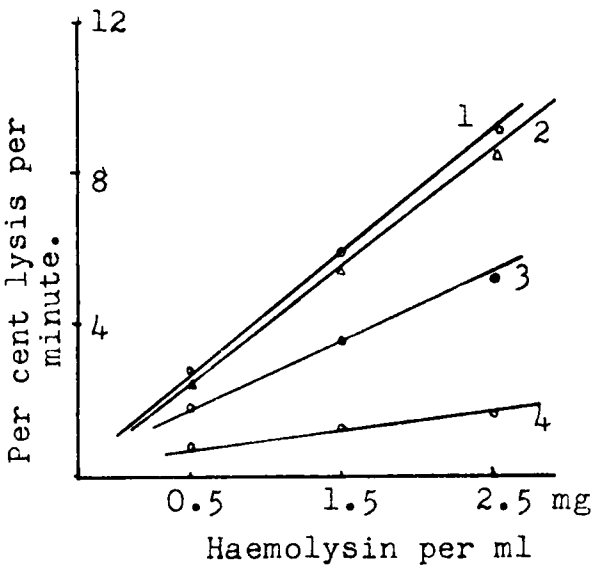
1) a normal sheep;
2) a sheep that just survived a blackleg infection, the dark coloured blood being derived from the edemous area;
3) a guinea-pig;
4) a cow;
5) a rabbit;
6) a horse.

Equal volumes of the suspensions and of the haemolysin solutions, with concentrations of respectively 2.5 - 1.5 and 0.5 mg haemolysin per ml buffered saline of pH 6, were mixed and the reaction was carried out at 37°C. The results were plotted in Graph VI-3.

GRAPH VI-3 /.....

GRAPH VI-3.

The linear functions of the rate of haemolysis and the concentration of haemolysin for different Erythrocytes at 37°C.



1) normal sheep cells
2) infected sheep cells
3) guinea-pig cells
4) cow cells

The functions for horse and rabbit erythrocytes are not shown because they are too low. In fact haemolysis of these erythrocytes equals zero when concentrations of 0.5 and 1.5 mg haemolysin are used.

It can be noticed from Graph VI-3 that the red blood cells of the susceptible animals show a higher rate of lysis than those of the less susceptible animals.

3) Rate of haemolysis in vitro as a function of pH.

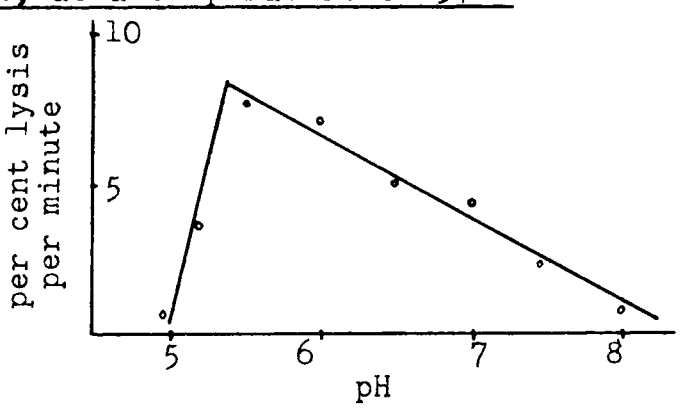
Preliminary experiments, varying the pH from 9 to 5, indicated an optimum rate of haemolysis at a pH value of about 5.5 - 5.6 (approximately the value of physiological saline solution as used). This result was in agreement with those of Guillaumie and Kréguer (1948) and Moussa (1958), who also found the haemolytic reaction more rapid in saline than in buffers of pH 7. During our preliminary experiments, however, we used different types

of /.....

of physiological buffers which were computed from the buffers given by Miller and Golder (1950). It might be anticipated that various ions can influence the lysis of red cells. We decided to repeat the experiment using only phosphate buffers as described by Bernheimer (1944). Thus we employed 0.015 M phosphate buffers (prepared from sodium salts and acid), which were made physiologically by the addition of 0.85 per cent (w/v) sodium chloride. The reaction was carried out at 37°C using a haemolysin solution with a concentration of 1.5 mg dried material per ml of the corresponding buffered saline. The results are shown in Table VI-4.

TABLE VI-4.

The rate of haemolysis of sheep cells as a function of pH, at a temperature of 37°C.



Once again a pH optimum was found at a value of approximately 5.5. Spontaneous haemolysis occurred at pH values below 5. An almost linear increase in the rate of haemolysis with decreasing pH was observed until the pH value of 6 was reached. A steep decrease in the rate of haemolysis was found on the acid side of the pH optimum. It was observed, however, that precipitates were formed at these pH values and we assumed that these

precipitates /.....

precipitates consisted of the haemolysin itself. We could confirm this by precipitating the antigen as well as the haemolysin in acetate buffers of pH 4.8 and 5.3. When culture filtrates were employed no precipitates were formed, only a little material precipitated at a pH of 4.6.

Bernheimer, who studied the pH effect upon the haemolysis of human red blood cells induced by Cl. septicum filtrates, could not detect an optimum pH value in the range from 5 to 9. It seems that the haemolysins of Cl. chauvoei and Cl. septicum behave in the same way with regard to the hydrogen ion concentration.

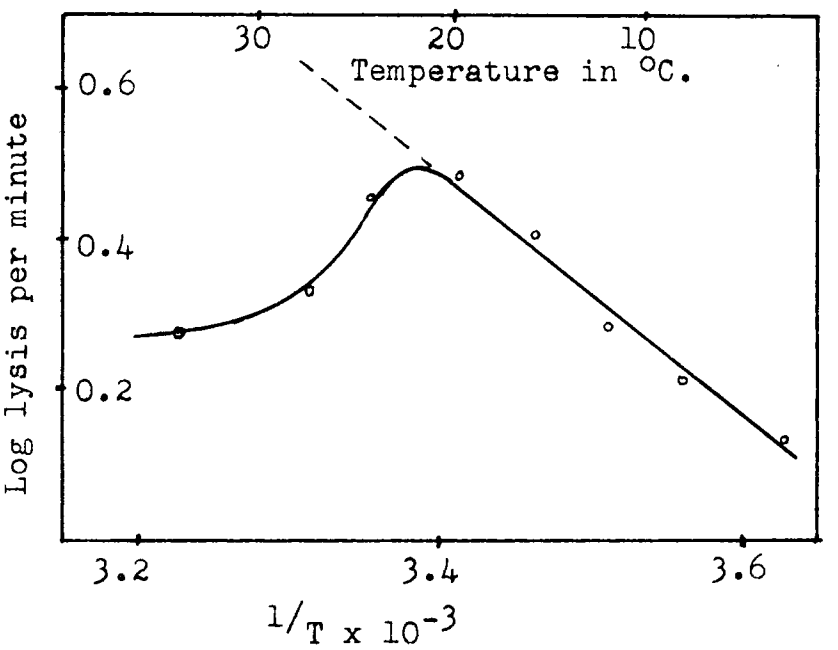
4) The rate of haemolysis as a function of temperature.

Equal volumes of a sheep erythrocyte suspension and a haemolysin solution (1.0 mg per ml), both in buffered saline of pH 6, were mixed after temperature equilibration. The rates of haemolysis were determined at temperatures of 2½ - 7½ - 11 - 15½ - 19½ - 24½ - 29 and 37°C. The results are shown in Graph VI-5, where we plotted the reciprocal of the absolute temperature against the logarithm of the rate of haemolysis at that temperature (Arrhenius plot).

GRAPH VI-5 /.....

GRAPH VI-5.

The rate of haemolysis, induced by a haemolysin solution with a concentration of 1 mg per ml, as a function of temperature.



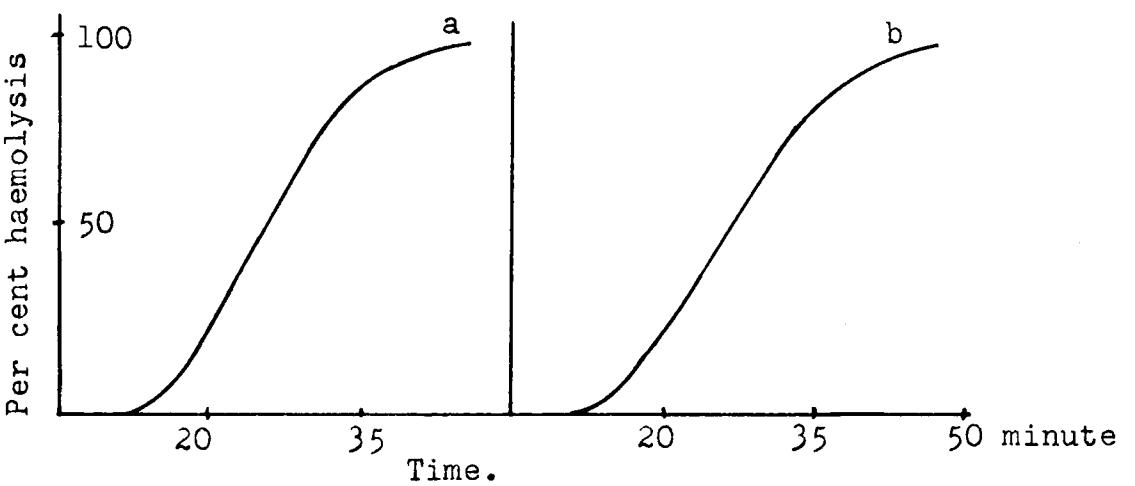
We see that a linear function is obtained below 20°C, which is also approximately the temperature of optimum haemolysis. A similar curve was obtained by Bernheimer (1944) for the temperature function of Cl. septicum haemolysin. Bernheimer also demonstrated a deviation from a linear function above 20°C, when using an Arrhenius plot. He found that the maximum rate of haemolysis of human erythrocytes occurred at 37°C. We, however, detected a maximum at about 20°C and a slow decrease in the rate of haemolysis above this temperature. Bernheimer (1944) attributed the deviation from the linear function above 20°C to the irreversible inactivation of the haemolysin at higher temperatures. This, however, was not the case for the Cl. chauvoei haemolysin, because the rate of haemolysis at 20°C was not altered when the haemolysin solution (1 mg per ml of buffered saline at a pH of 6) was incubated at 37°C for 5 hours and re-established

at /.....

at 20°C, prior to the experiment (see Graph VI-6).

GRAPH VI-6.

The course of haemolysis at 20°C for a haemolysin solution incubated at 37°C (b) as compared with that for a normal solution (a).

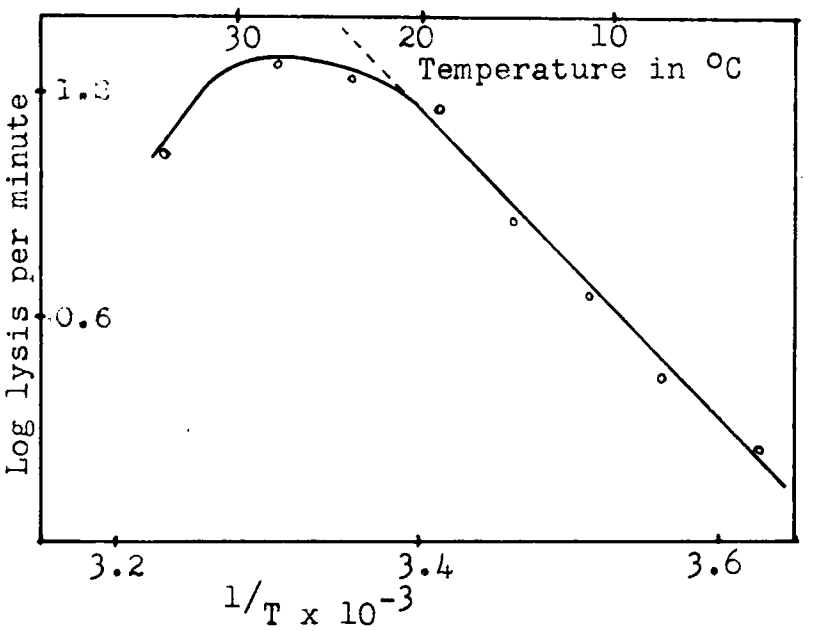


The energy of activation, computed from the slope of the linear function between 2½ and 20°C from Graph VI-5, is 7 400 mole $^{-1}$. This u value is considerably lower than that found by Bernheimer (1944) for the Cl. septicum haemolysin (u = 12 700). The Q10 value between 10 and 20°C amounts to about 1.5 which is also lower than that found by Bernheimer (Q10 is about 2). Though the energies of activation differ for the two lysins, they are both very low as can be seen from a table given by Bernheimer (1946). We repeated the experiment with a higher haemolysin concentration, as we could not explain the low u value. Using a concentration of 3 mg haemolysin per ml of buffered saline (pH 6) we found quite a different temperature function, as shown in Graph VI-7 (Arrhenius plot).

GRAPH VI-7 /.....

GRAPH VI-7.

The rate of haemolysis, induced by a haemolysin solution containing 3 mg per ml, as a function of temperature.



Again a linear function could be observed until a temperature of 20°C was reached. Above this temperature we found a deviation from the linear function, but then an increase in the rate of haemolysis was observed up to a temperature above 29°C. When the energy of activation was evaluated from Graph VI-7, a value of 18 700 was obtained whereas the Q10 between 10 and 20°C amounted to 2.8.

5) The influence of sucrose on the rate of haemolysis.

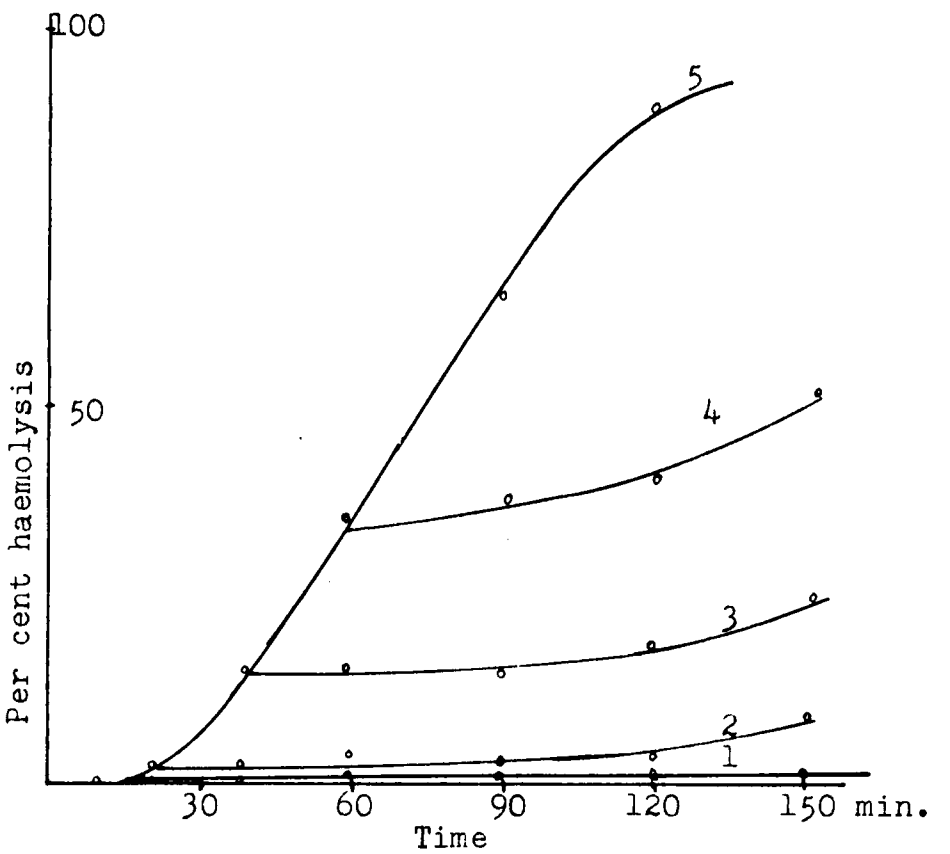
Bernheimer (1946) demonstrated that the presence of 0.15 M sucrose decreased the haemolysis induced by Cl. septicum toxins remarkably. As shown in Graph VI-8, this was also the case for the haemolysis induced by Cl. chauvoei haemolysin.

Equal /.....

Equal volumes of a sheep erythrocyte suspension and a haemolysin solution (containing 0.5 mg per ml) both in buffered saline of pH 6, were mixed after temperature equilibration. The reaction was carried out at 37°C and a solution of 3 M sucrose was added to a final concentration of 0.15 M after 0 - 20 - 40 and 60 minutes respectively.

GRAPH VI-8.

The effect of 0.15 M sucrose on the haemolysis induced by Cl. chauvoei haemolysin.



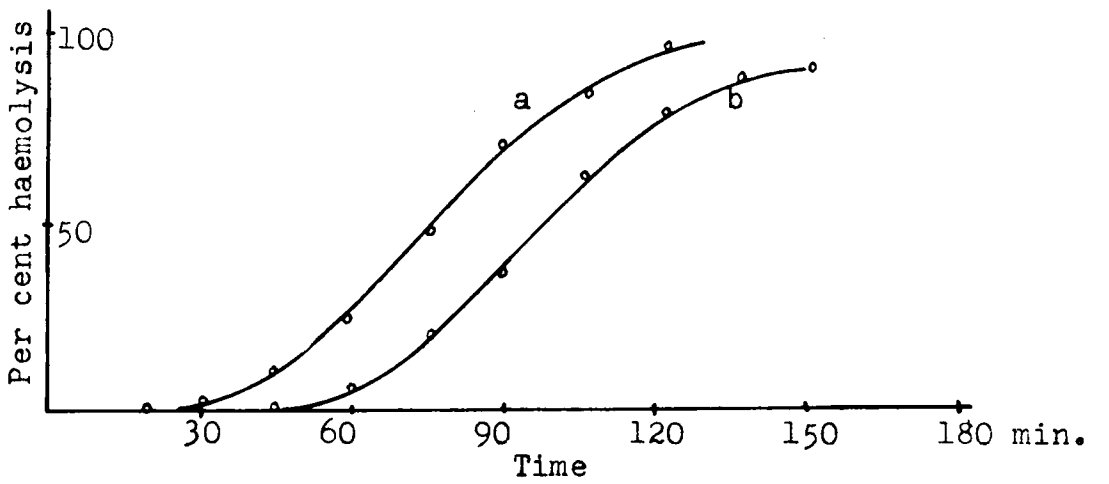
1) Sucrose added directly in the beginning.
2) Sucrose added after 20 minutes.
3) Sucrose added after 40 minutes.
4) Sucrose added after 60 minutes.
5) Normal curve.

The lysis-inhibiting effect of sucrose was temporary and lasted only a few hours (in the range of 1 to 4 /.....

1 to 4 hours). When the sucrose was added directly at the beginning of a haemolysis experiment, then the unlysed cells showed haemolysis with an almost unaltered rate when they were removed from the mixture by centrifugation after 45 minutes and resuspended in buffered saline (see Graph VI-9).

GRAPH VI-9.

The rate of haemolysis of sheep erythrocytes removed from a sucrose-containing reaction mixture 45 minutes after the beginning of the reaction.



a) normal curve.
b) red blood cells removed from the sucrose containing medium.

It may well be that the haemolysin alters the structure of the erythrocyte membranes, whereupon lysis follows. The lysis itself can be prevented by the addition of sucrose, according to the scheme of haemolysis given by Bernheimer (1946) for the Cl. septicum haemolytic filtrates.

6) The effect of horse anti-serum on the haemolysis.

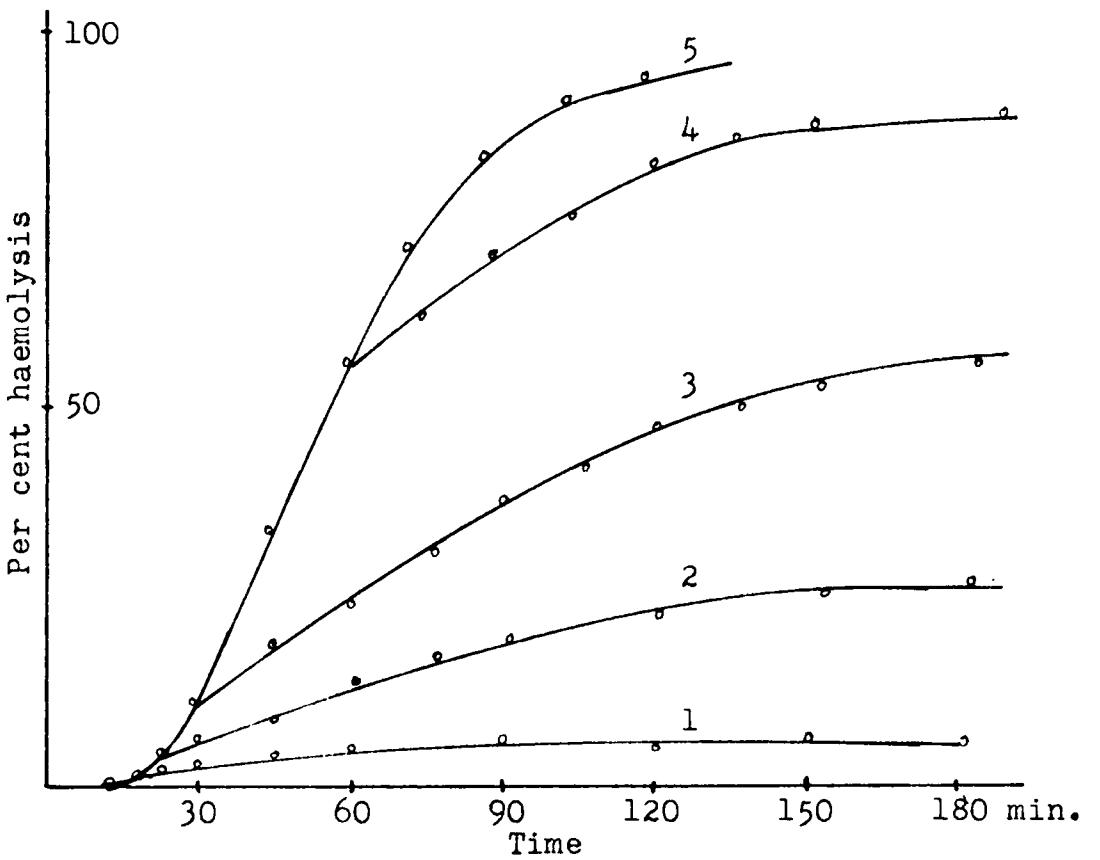
As in the case of Cl. septicum haemolysis, the available Cl. chauvoei horse anti-serum prevented the first

stage /.....

stage (the alteration of erythrocyte membranes by the lysin) of the haemolysis. The reaction was carried out at 37°C, using sheep erythrocytes and a haemolysin solution with a concentration of 0.5 mg per ml in buffered (pH 6) saline. After respectively 0 - 10 - 20 - 30 and 60 minutes we added 1 per cent horse serum to the reaction mixtures. The results are shown in Graph VI-10.

GRAPH VI-10.

The effect of horse anti-serum on the haemolysis induced by Cl. chauvoei haemolysin.



1) Serum added 10 minutes after the beginning of the lysis.
2) " " 20 " " " " " " "
3) " " 30 " " " " " " "
4) " " 60 " " " " " " "
5) Normal curve.

Notice the complete inhibition of haemolysis when the anti-serum was added at the beginning of the reaction.

8) /.....

The test of lecithinase activity.

Lecithin was obtained after the evaporation of an ether extract of an egg yolk. The material was suspended in 200 ml saline containing also 0.01 M calcium chloride. One ml of this emulsion was added to 2 ml of the antigen or haemolysin solution in 0.1 M tris-sulphuric acid buffer of pH 7.2. The mixtures were incubated at 37°C and the reaction was stopped by the addition of 2 ml 10 per cent (w/v) trichloro-acetic acid. The amount of acid-soluble phosphate was determined after prolonged centrifugation (1 hour) at 6 000 r.p.m. and filtration. The reaction was negative, no increase in the soluble phosphate being detected.

Hydrolysis of the phospholipin of sheep erythrocytes.

To detect any action of the antigen or haemolysin upon the phospholipin in the erythrocytes the haemolysis was carried out at 37°C. At suitable time intervals 1 ml of the mixture was pipetted into 1 ml of a 10 per cent (w/v) trichloro-acetic acid solution and chilled in an ice-bath. The precipitate was removed by centrifuging at 6 000 r.p.m. for 1 hour and filtration. The phosphate contents of the clear solutions were determined. No increase in the soluble phosphate was detected.

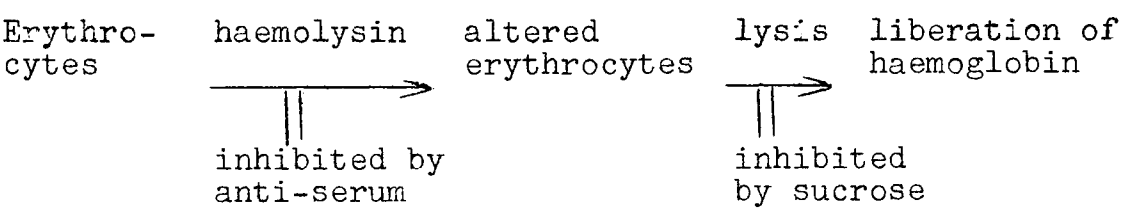
DISCUSSION.

It was found (see Chapter IV) that the antigen also possessed haemolytic activity. In Chapter V it was shown that the antigen spontaneously split into at least two fragments. One of these fragments showed the same adsorption on calcium phosphate as the haemolysin. It was assumed that this could explain the small haemolytic

activity /.....

activity of the antigen. In this chapter we investigated the reverse, namely, whether the haemolysin possessed antigenic properties. As this was not the case we must exclude the assumption that the antigen consists of haemolytic fragments only. However it remains to be seen if the haemolysin will not give immunizing activity when mixed with one or both biological inactive materials obtained by calcium phosphate chromatography (see Diagram IV-10). Our results so far, employing saline solutions, were completely negative. No other influences, however, have been investigated, as for instance the pH and the ionic strength of the medium.

The haemolysin of Cl. chauvoei shows a remarkable resemblance to that of Cl. septicum; in regard to the influences of pH and sucrose they seem identical. Our results also clearly indicate two stages in the haemolytic reaction as proposed by Bernheimer (1946) for the haemolysis induced by Cl. septicum filtrates. The simplified scheme of the haemolysis is as follows :



The action of the haemolysin on the erythrocytes is still obscure, in fact it has not been determined if the reaction is enzymatic, though it has some characters that indicate this possibility. The action of the haemolysin on the erythrocytes is not due to lecithinase D activity. Bernheimer, in his first publication (1944), mentioned the possibility of lecithinase activity in Cl. septicum filtrates but he never demonstrated it. It therefore seems that these gas gangrene species do not

produce /.....

produce the so-called lecithinase D, the lethal factor of another gas gangrene organism, namely Cl. perfringens.

As can be seen from the above scheme a heterogeneous reaction is presumed. This may explain the variation in the value of the activation energy when different concentrations of haemolysin are employed. Since the u value changes with the relative concentrations of enzyme and substrate we assume that the rate of the total reaction is determined by an adsorption (or desorption) process, e.g. the adsorption of the lysin on the erythrocyte surface. A further indication of the existence of an adsorption process is found in the deviation from a linear function above 20°C in the Arrhenius plots.

When the rate of reaction is controlled by an adsorption process the u value will also change with the temperature (Sizer 1943).

Of course the departure from the theoretical Arrhenius curve can also be due to an inactivation of the haemolysin at temperatures above 20°C. The inactivation reaction must be reversible as we clearly demonstrated. The haemolysin is not destroyed at 37°C as stated by Bernheimer (1944) in case of the Cl. septicum haemolysin.

It seems unlikely that the rate of the lytic reaction is determined by a diffusion process, because in this case a u value of approximately 3 000 is to be expected (see Sizer 1943).

As stated above we are not sure if the first stage of the haemolysis must be attributed to an enzymatic action. Evidence for an enzymatic character, however, is found in the low energy of activation, even at the higher haemolysin concentration; because the great efficiency of catalysis by enzymes results always in a decrease in

energy /.....

energy of activation (see Bray and White, 1957).

The influence of horse anti-serum and of sucrose upon the reaction was only temporary. The effect of sucrose can probably be explained by comparing it with that of glucose upon the permeability to cations (see Parpart and Hoffman, 1954). Lytic agents often increase the permeability to cations which results in an alteration of the Donnan equilibrium, so that more salts penetrate the cells which swell until they burst. Glucose, and probably sucrose too, will prevent the loss of potassium ions out of the cells and thus stabilizes the Donnan equilibrium in the altered erythrocytes.

The horse anti-serum on the other hand may prevent the alteration of the cell membranes. It is thought that this results from a soluble complex formation between a serum component, probably an antibody, and the haemolysin. A competitive inhibiting effect was excluded after it was found that normal sera did not show this haemolysis-preventing effect (see Chapter VII).

Chapter VII /.....

CHAPTER VII.

SOME SEROLOGICAL EXPERIMENTS IN RELATION TO THE STUDY OF THE SOLUBLE ANTIGEN AND HAEMOLYSIN OF CL. CHAUVOEI.

In this chapter some serological experiments, which we carried out during the course of our studies, will be discussed. A horse serum, prepared from a hyper-immunized horse (see Chapter II), was available for our experiments. We found that 0.1 ml of this serum could neutralise 8 m.l.d. of virulent culture of Cl. chauvoei, when titrated in guinea-pigs. It was also found during the testing of the protective capacity that a solution of crude ammonium sulphate precipitate of the culture filtrate (exhaustively dialysed against water) could lower this neutralizing effect. Though the decrease in the protective capacity was considerable, it did not result in a complete loss. No precipitation could be detected when the horse serum was mixed with the ammonium sulphate precipitate.

We decided to repeat the experiments with rabbit sera, prepared from rabbits immunized with various materials. Thus we endeavoured to prepare rabbit sera with high titers and for this purpose we hyper-immunized rabbits with a) antigenic materials, b) washed living cells and c) cell extract. The neutralizing capacity and the specificity of these sera were investigated.

Several authors have agreed upon the importance of agglutination in the defence-mechanism of susceptible animals against Cl. chauvoei infection. We found that a study of the agglutination was hindered by the auto-agglutination of the organisms. To avoid this difficulty we employed the fluorescent antibody technique, using

fluorescein /.....

fluorescein isothiocyanate. For a description of the technique we refer to Coons, Creech, Jones, and Berliner (1942), and for the use of the isothiocyanate to Marshall, Eveland and Smith (1958).

The precipitation reaction was also investigated, especially the gel diffusion technique in agar gels, according to the method of Oakley and Fulthorpe (1953).

The horse anti-serum was fractionated by the use of column-electrophoresis (Gedin and Porath, 1957) and it was clearly demonstrated that the culture-neutralizing antibody was electrophoretically identical to γ globulins. The activity of this purified antibody was further investigated.

The haemolysis-inhibiting effect of the sera was also tested because of the existing relationship between the antigen and haemolysin.

RESULTS.

1) The neutralizing effects of the immune sera.

Rabbits were inoculated 10 times intravenously (twice a week in the ear veins), with the following materials and quantities :

a) 1 ml of a dense suspension of living cells. The germs of 30 ml of a liver-glucose broth culture were washed in saline (three times) and suspended in 1 ml;

b) 2 ml of cell extracts prepared by ultrasonic treatment of the same suspensions for one hour; the extracts were centrifuged at 40 000 r.p.m. for one hour prior to the inoculation;

c) 20 mg of freeze-dried antigenic material, dissolved in 2 ml saline. This material was obtained by

D.E.A.E./.....

D.E.A.E.-cellulose chromatography, using gradient elution. The rabbit was injected with 200 mg of the material of peak D shown in Diagram IV-4;

d) pure antigen, injected in 10 mg portions after dissolving in 1 ml of saline. Sera were prepared from this rabbit after inoculation of 40, 70, 100 and 130 mg respectively of dry material.

The rabbits were allowed two weeks' rest before being bled. The sera were compared with regard to their capacities to protect guinea-pigs against a virulent culture. The results are shown in Table VII-1.

Table VII-1.

The neutralizing effect of different sera upon virulent culture.

Serum	Number of m.l.d. neutralized by 0.1 ml serum	0.1 ml serum mixed with 10 mg antigen
Horse	8	2
Rabbit inoculated with :		
living germs	15	15
cell extracts	1.5	1
Antigen from D.E.A.E	1	0.3
40 mg pure antigen	-	0
70 mg pure antigen	1	0
100 mg pure antigen	1.5	0.3
130 mg pure antigen	1.5	-

The experiment was carried out as follows : 0.3 ml of the sera was diluted with 0.3 ml of physiological saline before being mixed with varying volumes of virulent culture. The mixtures were left for one hour at room temperature and then used to inoculate guinea-pigs. The m.l.d. of the culture was tested by titration

in /.....

in guinea-pigs. The reaction was observed five days after the challenging of the guinea-pigs. It is quite clear that the rabbit serum, prepared from a rabbit hyper-immunized with living germs, is preferable even to the refined horse serum. The neutralizing effects of the other rabbit sera showed poor results when compared with the former serum. All the sera derived from animals immunized with antigenic solutions showed a remarkable decrease in protective capability after mixing with the antigen. The unaltered effect of the immune serum prepared by the inoculation of living organisms indicates another antigen, which is not soluble and might be responsible for the well-known agglutination.

All the sera proved specific for the Cl. chauvoei species as no neutralizing effect was observed against the Cl. septicum strain.

2) The agglutination reaction.

Preliminary experiments revealed that the sera of sheep immunized with formalized anaculture contained very low agglutination titers (1:20 - 1:40), when tested on a microscope slide. The titers of the horse serum and the rabbit sera, with one exception, were also very low, indeed so low that doubt arose if agglutination took place at all. A very high titer, about 1:5000, was obtained with the serum prepared from the rabbit which was inoculated with living germs. This serum also gave very good results when the fluorescent antibody technique was applied, whereas the results with the other sera were negative.

Care had to be taken with the fixation of the culture on the microscope slides. The best results were

obtained /.....

obtained when the organisms were fixed by heat; decrease in the conjugation of the fluorescent antibodies with the germs was observed when ether-ethanol mixtures were used.

It was found that the fluorescent antibody technique was species specific. No reaction was detected with the Cl. septicum strain, but all the Cl. chauvoei strains tested (Lourenço Marques, 64, K-796 and 2153) gave positive reactions.

3) The precipitin reaction.

When the serum of a sheep immunized with formalized anaculture was mixed with a solution of the antigenic component (pre-purified by D.E.A.E.-cellulose chromatography) in physiological saline, a precipitate was formed. We observed, however, several unusual properties which let us doubt about the nature of the reaction, namely :

1) a long induction period (about 12 - 16 hours) preceded the precipitation,
2) the precipitate was only formed above 30°C, and nothing was detected at 4°C, and
3) the precipitate redissolved at a low temperature (4°C).

Because of these complications we changed to the gel diffusion technique; it was readily detected that here also precipitin bands were formed at higher temperatures only. All the immune sera showed one precipitin band with the antigen, with the exception of the serum obtained from the rabbit which was immunized with the pure antigen itself. In the latter case two precipitin bands were always formed, whose presence was not influenced

by any /.....

by any of the following :

a) the temperature, between 20 and 40°C

b) the pH of the medium, between 5.5 and 8.5

c) the saline concentration, between 0.2 and 1.5 per cent, or by

d) the concentration of the antigen used in the test (1-5 mg per ml).

When the haemolysin was used, practically all the sera formed precipitin bands, except the horse serum and the rabbit serum, obtained after the inoculation with pure antigen. These sera completely failed to form precipitin bands with the haemolysin. It was also observed that in the case of the haemolysin a much longer induction period preceded the appearance of the precipitin bands.

When the antigen and haemolysin were replaced by cell extracts obvious precipitin bands were formed when sera from rabbits immunized with washed germs or cell extracts were used. The results are summarized in Table VII-2.

Table VII-2.

The precipitin reactions of various sera in agar gel at 37°C, using pure antigen, haemolysin and cell extract.

Serum	Precipitin reaction with : antigen	haemolysin	cell-extract
Horse	1 band	---	---
Rabbit inoculated with :			
living germs	1 band	2 bands	2 bands
cell extract	1 band	2 bands	2 bands
D.E.A.E. purified material	1 band	2 bands	---
40 mg pure antigen	2 bands	---	---
70 mg pure antigen	2 bands	---	---
100 mg pure antigen	2 bands	---	---
130 mg pure antigen	2 bands	---	---

The bands /.....

The bands formed by the antigen and rabbit sera obtained by the inoculation with viable germs and cell extract were very weak.

4) Haemolysis inhibition by various sera.

During the gel precipitation experiments we saw that several sera showed precipitin bands with the purified haemolysin whereas others failed to do so. But we still decided to study the haemolysis-inhibiting effect of the prepared sera. The test was carried out by mixing a series of dilutions of the sera with equal volumes of a haemolysin solution, containing 3 mg per ml of physiological saline. The mixtures were incubated at 37°C for 30 minutes prior to the experiment. To 1 ml of the mixtures was added 0.5 ml of a sheep erythrocyte suspension, prepared as described in Chapter II.

Because, as we saw already in the previous chapter, the inhibition of haemolysis is only temporary, we observed the reaction 2 hours after complete haemolysis of a blank test. The latter test was set up by mixing the haemolysin solution with physiological saline, and complete haemolysis occurred in about 30 minutes. The results are shown in Table VII-3.

Table VII-3.

The inhibition of haemolysis by various sera.

Serum	Titer
Horse	128
Rabbit inoculated with :	
viable germs	0
cell extract	0
D.E.A.E. purified material	16
40 mg pure antigen	8
70 mg pure antigen	16
100 mg pure antigen	64
130 mg pure antigen	128

It is /.....

It is remarkable that no haemolysis inhibition was detected in sera which gave positive results with the haemolysin in the gel diffusion tests, as did the sera obtained from rabbits immunized with living bacteria and cell extract. On the other hand the sera which produced evident precipitin reactions with the antigen also showed a haemolysis-inhibiting effect. The rabbit serum prepared by the inoculation of pure antigen showed a remarkable effect and still no precipitin bands were obtained with pure haemolysin. The absence of an inhibiting factor in the sera prepared by the immunization with living organisms and cell extract indicated that the inhibition does not depend upon competitive enzyme inhibition by one or another of the factors present in normal sera. This could be confirmed by the use of normal horse and rabbit sera, which both lacked any inhibitive effect.

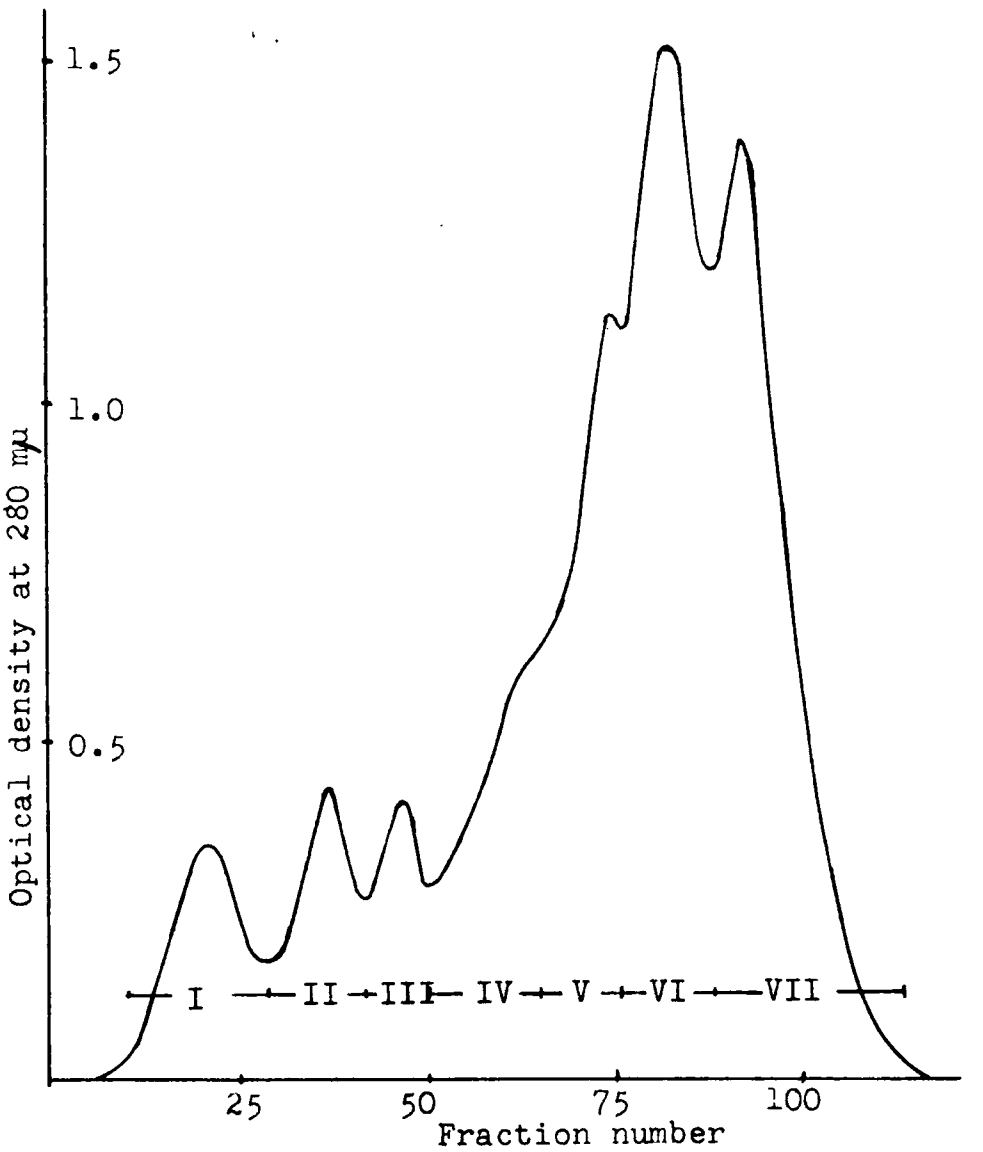
5) The fractionation of horse serum.

By means of column electrophoresis a fractionation was obtained, as shown in Diagram VII-1.

Diagram VII-1 /.....

Diagram VII-1.

The fractionation of 5 ml refined horse serum by column electrophoresis. Current 22½ mA.; time 45 hours.



By mixing the freeze-dried materials, obtained from the peaks of Diagram VII-1, with varying volumes of virulent culture, and titrating these mixtures in guinea-pigs, after standing at room-temperature for 1 hour, it was quite easily detected that the last peak contained most of the protective antibodies. This component also contained the strongest anti-haemolytic activity; about 0.5 mg of the material was sufficient to neutralise 3 mg of the haemolysin. The results are summarized in Table VII-4.

Table VII-4 /.....

Table VII-4.

The protective capacities and anti-haemolytic activities of the fractions obtained by column electrophoresis of the horse serum.

Fraction	Anti-haemolytic Activity	Protective Capacity.
I	more than 16 mg	0
II	more than 16 mg	0
III	more than 16 mg	0
IV	8 mg	0.5
V	2 mg	1.0
VI	2 mg	2.0
VII	0.5 mg	4.5

The anti-haemolytic activity is recorded as the amount of material that neutralized 3 mg of the pure haemolysin until 2 hours after complete haemolysis of the blank. The protective activity is recorded as the number of m.l.d. being neutralized by the amount of material obtained from 0.1 ml of the serum.

Besides its anti-haemolytic effect no other reaction with the last fraction of the electrophoretic fractionation could be detected.

Discussion.

The antigen proved its ability to produce sera which can be used for passive immunization. When these sera were mixed with the antigen, a loss of this property could be observed, but this loss was never complete. We assumed that the reaction between the antigen and antibody resulted in the formation of a reversible complex, during the so-called "complexing stage", and that this complex grew only very slowly, forming an aggregate which precipitates. This growing period is called the "aggregating stage" (see Crowle, 1961). This might also be the

reason /.....

reason for the long induction period preceding the formation of precipitin bands during the gel diffusion experiments. This aggregating stage might also be prevented by low temperatures.

It was clearly demonstrated that the soluble antigen is not the only immunizing factor, for agglutination also plays an important role, as can be seen from the high protective capacity of the rabbit serum prepared by the immunization with living bacteria. The agglutinative antigen, however, can easily be inactivated by the use of an ether-ethanol mixture for the fixation of the culture in the fluorescent antibody experiments. It had already been said by Roberts, 1931, that no agglutination was obtained after treatment of washed germs with alcohol. When we recall the preliminary investigation as described in Chapter II, the agglutination antigen is not only inactivated by organic solvents but also by acid and proteases. The latter inactivations were explained by presuming a protein-like character of the antigen. The inactivation by the ether-alcohol mixture might therefore be attributed to denaturation.

Batty and Walker (1963) published an article on the differentiation of Cl. septicum and Cl. chauvoei by the use of fluorescent labelled antibodies. The same procedure was followed, but they used boiled bacterial suspensions for the preparation of the anti-serum. According to these authors good results were obtained after fixation with acetone.

The relation between the antigen and haemolysin is again emphasized by the results of the serological investigation that we carried out. This relation is most clearly demonstrated by the haemolysis-inhibiting effect

of the /.....

of the rabbit serum prepared by the inoculation of pure antigen. We also tried to demonstrate the reverse, namely a decrease in the protective capacity of the latter serum by mixing with pure haemolysin. The results so far are not satisfactory and nothing conclusive can be said at this stage. Strong evidence for the relation between antigen and haemolysin is also found in the haemolysis-inhibiting effect of the material from the last fraction from the column electrophoresis (see Diagram VII-1). This material contained two activities, namely a haemolysis-inhibiting- and a virulent culture neutralizing activity.

We demonstrated the existence of a precipitin reaction in agar gels between the antigen and various sera. Because the antigen showed a reaction with all the immune sera, it might be presumed that the antigen possesses an enzyme activity for a certain serum component which results in precipitation. This explanation of the forming of precipitin bands seemed not to be the case because :

1) the precipitin bands were always sharp and remained distinct;
2) no precipitation was observed when normal horse and rabbit serum were used.

The haemolysin also formed precipitin bands with several sera. There seemed to be no relationship between precipitin formation and haemolysis-inhibitive activity of the sera.

The fact that two precipitin bands were observed when the antigen was allowed to react with its own antiserum prepared in rabbits may indicate the presence of two components, but this is not absolutely necessary, as cited by Crowle (1960 and 1961).

No /.....

No precipitin bands were obtained when the antigen was allowed to react with the material from the last fraction of the column electrophoresis of the horse serum. The reason may be that other serum components are needed for the precipitin-forming, or that the antibody lost its ability to form precipitates during the "aggregating stage".

When cell extracts were used, the precipitin reaction with the serum obtained from a rabbit immunized with pure antigen revealed no bands.

The reverse, however, namely the gel diffusion test carried out with the antigen and the sera prepared from rabbits immunized with cell extract and germ suspension showed positive results.

This is why we cannot exclude the presence of the antigen, in the cell plasma, or attached to the cell wall.

FINAL /.....

FINAL CONSIDERATION.

In reviewing the study of the antigen and haemolysin of Cl. chauvoei we shall accentuate two relationships which we clearly demonstrated, namely :

a) that between the soluble antigen and haemolysin of the Cl. chauvoei species, and

b) that between the haemolysins produced by Cl. chauvoei and Cl. septicum.

In connection with the first relationship it was found from a literature study that an affinity was presumed between the lethal toxin and haemolysin of the Cl. chauvoei toxins. Indeed several authors carried out experiments which indicate clearly that the lethal toxin and haemolysin are related, if not identical. (Kerrin, 1934, Guillaumie and Kréguer 1948, and Moussa 1958).

This comparison between the lethal toxin and haemolysin was also demonstrated in culture filtrates of the Cl. septicum species (Moussa 1958).

We therefore anticipated that the soluble antigen that we isolated could be identified with the lethal toxin. Unfortunately we were not able to confirm this identification because no lethal activity was found. We detected, however, a very weak haemolytic activity in solutions of the purified antigen in saline. This was explained by the discovery that the antigen decomposed spontaneously with the formation of a small quantity of haemolysin. We expected this reaction to be reversible but we failed to prove this. In spite of this failure evidence exists, serologically as well as chemically,

that /.....

that the haemolysin molecule is part of the larger antigen molecule.

The affinity between the haemolysins of Cl. chauvoei and Cl. septicum was, with the possible exception of the temperature function, striking. During the course of our investigation the common character of the haemolysins was strongly emphasized by Guillaumie, Kréguer, Geoffroy and Ventre (1961). These authors found that the serum prepared from a horse, which was hyper-immunized with Cl. septicum toxins, also inhibited haemolysis induced by the Cl. chauvoei haemolysin.

From our serological study it can be assumed that the antigen of Cl. chauvoei is not related to the lethal toxin of Cl. septicum, for the rabbit serum prepared by the immunization with pure antigen showed activity for the Cl. chauvoei species only. Though this is only a mere indication that the isolated antigen might differ from the α toxin of Cl. septicum, it is our conclusion (see also the findings of Moussa 1958, for the lethal toxins).

Nothing conclusive can yet be said about the affinity of the species Cl. chauvoei and Cl. septicum. With the aid of the literature and our own results, however, we formulated the following hypotheses :

1) the haemolysins are intermediary metabolites in the α toxin sythesis, or products formed by the decomposition (spontaneous or enzymatic) of the latter components; they are very closely related.
2) Therefore the lethal factors of the toxins of Cl. chauvoei and Cl. septicum are identical in their actions and properties, but they will differ serologically.

With /.....

With regard to the above-mentioned affinities it seems possible to consider the two distinct organisms as types (A and B) of one single Clostridium species.

This was already suggested by Moussa (1959), who made a careful study of the agglutination phenomena of both Cl. chauvoei and Cl. septicum.

When we survey the work reported in this thesis we notice a serious shortcoming, namely, the failure to give a satisfactory account of exactly what the antigen is, or what happens when this particular Cl. chauvoei organism invades a host.

It is, however, expected that an extensive study of the haemolytic reaction might cast some light upon the substrate specificity and upon the action, not only of the haemolysin itself but also of the lethal toxin and/or antigen with which the haemolysin has many properties in common.

Whenever a lethal filtrate of a Cl. chauvoei culture is obtained, it can be demonstrated that the isolated antigen is, as we expected, nothing else but the lethal component.

SUMMARY /.....

S U M M A R Y.

The aim of the study described in this thesis was to obtain information about the chemical nature of the immunizing antigen of the vaccines against blackleg disease and the mechanism of the immunization.

A literature study revealed the existence of much controversy about the reactions caused by Cl. chauvoei germs and toxins. Though the results of serological experiments carried out by various investigators led to the accepted theories about the relationships between homologous lethal toxins and haemolysins, and between the haemolysis and other reactions of the two different species Cl. chauvoei and Cl. septicum, a comparative chemical study of the toxins had not yet been carried out.

A preliminary investigation showed that the soluble immunizing antigen from the Cl. chauvoei cultures had the character of proteins. This was revealed by the loss of activity when pepsin and trypsin were allowed to act upon the antigenic solution, and further by the influence of acid and heat upon the antigenic potency. No lethal activity was found in the filtrates of Cl. chauvoei (strain 64) cultures; instead we detected a haemolysin and a hyaluronidase which were also both heat-labile.

The isolation procedures for the antigen and haemolysin relied on precipitation with ammonium sulphate and on chromatographic methods. By means of calcium

phosphate /.....

phosphate (hydroxyapatite) chromatography the two biological active components could be distinguished. No separation was obtained with D.E.A.E.-cellulose chromatography, used for pre-purification. This can be considered as proof of the identical electrical charge. The latter was readily confirmed by starch gel electrophoresis of mixtures of the two components, namely the D.E.A.E.-cellulose pre-purified material, which revealed only one band.

The purity tests demonstrated the spontaneous, but slow, decomposition of the antigen, with the formation of haemolysin and other - but inactive - materials. It is obviously difficult to purify the haemolysin, because after a repeated calcium phosphate chromatography impurities were still detected with a constant solubility test.

A comparative chemical and physico-chemical analyses of the antigen and haemolysin were carried out and a remarkable identity was found, emphasized by :

a) the amino acid contents, and

b) the electrophoretic mobilities at two different pH values.

The molecular weights estimated by the Archibald approach to the equilibrium method differed widely, namely, 53 500 and about 30 000 for the antigen and haemolysin respectively. Previous experiments, also carried out with a heterogeneous haemolysin preparation, indicated a molecular weight of about 11 000 and we therefore presume that the real value is between the two given.

The haemolytic reaction had been studied and a noticeable agreement with the haemolysis induced by the Cl. septicum toxins was detected, namely :

a)/.....

a) an induction period preceded the reaction,

b) the rate of haemolysis increased with decreasing pH,

c) sucrose and horse anti-serum showed an inhibitive effect, and

d) the value of the energy of activation was low.

The energy of activation appeared to be a function of the haemolysin concentration, which was explained by an adsorption process being the pacemaker of the reaction. Haemolysis was considered to consist of two stages which could be inhibited by anti-serum and sucrose respectively, according to the suggestion made for the lytic reaction induced by Cl. septicum culture filtrates. The first stage was presumed to be enzymic in character, the enzyme, however, not being the lecithinase D, produced by other gas gangrene organisms (Cl. perfringens and Cl. haemolyticum).

The serological investigation, emphasized once again the affinity between the antigen and haemolysin, because :

a) immune serum prepared from a rabbit with pure antigen contained a haemolysis-inhibitive effect,

b) the fraction, obtained by column electrophoresis of the horse anti-serum, which contained the protective anti-bodies against virulent culture, was also remarkable for its haemolysis-preventing activity.

It was clearly demonstrated with the fluorescent antibody method that, besides the soluble antigen which we isolated, an agglutinative antigen also plays an important role in the defence-mechanism of susceptible animals. Because of its instability the latter antigen was also presumed to be a protein-like material.

A precipitin /.....

A precipitin reaction was obtained in 0.5 per cent (w/v) agar gels, but the reaction was complicated by several factors, such as the temperature and the long period before the reaction became visible (compared with a normal precipitating system). The haemolysis and the bacterial cell extract failed to give evident precipitin bands with the rabbit serum prepared after the immunization with pure antigen. On the other hand the antigen gave a precipitin band with the sera from rabbits immunized with the cell extract and living germs respectively.

Because of this and because of the weak haemolytic activity detected in the cell extracts, it is still presumed that the antigen is also present in the bacterial cells.

It was suggested that :

1) the antigen could be identified with the lethal toxin of Cl. chauvoei,
2) the lethal toxins of the Cl. chauvoei and Cl. septicum organisms are related, and therefore,
3) the two apparently different species can be considered as two types of one single species.

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