THE ANTIGENICITY OF *MYCOPLASMA MYCOIDES* WITH PARTICULAR REFERENCE TO THE POLYSACCHARIDE ANTIGENS

by

ROBERT NIGEL GOURLAY

SUMMARY

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF VETERINARY MEDICINE AND SURGERY
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East African Veterinary Research Organization,
Muguga, Kenya

SUMMARY

Serum, plasma, lysed blood cells, urine and pleural fluid or lymph (inflammatory exudate from subcutaneous inoculation) were obtained from cattle naturally and artificially infected with Mycoplasma mycoides. These fluids were examined for antigens of M. mycoides by means of the agar gel double diffusion precipitin test and the quantitative agar gel precipitin test. They were also examined for antibodies against M. mycoides by means of the complement fixation and slide agglutination tests, and for viable M. mycoides by growth in broth cultures. Hyperimmune sera for use in these tests were prepared in sheep and cattle by the intravenous injection of washed organisms that had been grown in broth medium. From the natural cases of CBPP viable organisms were obtained from the pleural fluid only, while in the experimental cases the organisms were present in the serum, plasma and lymph. Antibodies against M. mycoides were demonstrated in sera and plasma samples of all cases. Specific precipitating antigens were demonstrated in all the fluids, urine possessing at least 5 serologically distinct antigens, lymph and pleural fluid at least 6 and serum and plasma at least 6 and sometimes 7 antigens. The 5 in urine were common to all fluids, while the extra 1 in lymph and pleural fluid was also present in serum and plasma. In addition to these so-called major antigens, minor ones, at least 6 in number, were also demonstrated, but these were apparently primarily associated with the organisms. The major precipitating antigens were predominantly extracellular with only small amounts present in the organisms. These major antigens were also elaborated by the organisms when grown in artificial culture medium, and those produced by fully virulent organisms were apparently identical to those produced by avirulent organisms.

Fractionation of pooled urine from the artificially infected cattle by precipitation with varying volumes of cold iso-propyl alcohol and deproteinization with a chloroform-butanol

mixture was undertaken. A total of 6 serologically distinct precipitating antigens were demonstrated in the AGT and separation of these antigens was possible to a limited extent by varying the volumes of alcohol used.

Fraction C/1/2/3, the fraction which contained all the antigens, was shown to contain approximately 5.6 per cent. Kjeldahl N, 0.5 per cent. P., 42.4 per cent. carbohydrate (estimated as galactose) and 11.9 per cent. hexosamine. One or possibly 2 of the precipitin bands was shown to contain lipid, but there was no indication of nucleic acid. By the use of paper chromatography, galactose was demonstrated and probably sorbose and arabinose, together with some amino acids. These antigens were resistant to a temperature of 94°C. for 60 minutes and to the action of trypsin. Separation of the individual antigens was not obtained by either ultracentrifugation or electrophoresis. The antigenic fraction fixed complement in the presence of hyperimmune sheep sera, and fraction C/1/2/3 absorbed 87.5 and 96.9 per cent. of the agglutinating antibodies and 93.75 and 87.5 per cent. of the complement fixing antibodies from hyperimmune sheep x and sheep 6 sera respectively. This fraction was pyrogenic in rabbits and relatively non toxic to cattle, rabbits and mice, but proved to be lethal to fowl embryos. The antigens were haptens in cattle, rabbits and mice, but precipitating antibodies were produced in rabbits when the antigens were combined with "shiga conjugated protein". Fraction C/1/2/3 possessed an aggressive action when inoculated together with viable M. mycoides in immune animals and appeared to enhance the virulence of the organisms in susceptible cattle.

It is suggested that these antigens play a part in assisting the growth of M. mycoides in the host tissues but are not in themselves significantly harmful.

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GENERAL INTRODUCTION

Prophylactic inoculation against contagious bovine pleuropneumonia, the disease caused by Mycoplasma mycoides, has been practised for over a century. The earliest inoculations consisted of subcutaneous inoculation of infected fluids from acute cases of the disease into the tip of the tail. Since then numerous vaccines, prepared empirically by means which have proved satisfactory in other diseases, have been used. These include dead whole organisms killed by various means, live attenuated strains, usually attenuated by repeated passage in artificial culture but also by passage in embryonated eggs, and natural strains of low virulence. Unfortunately none of these vaccines has proved entirely satisfactory as, generally speaking, dead vaccines have not produced sufficient protection and live vaccines, if sufficiently immunogenic, have been liable to produce severe vaccination reactions frequently leading to death, and there have also been reliable reports of living vaccines producing clinical cases of pleuropneumonia.

According to Turner (1959), recovery from the natural disease is usually followed by immunity. He quotes no experimental evidence to substantiate this statement, although it has become generally accepted that this is true, and it appears to be based on reports published many years ago. For example, Yvart (1851, quoted by Curasson, 1936) stated that immunity following natural infection lasted 3 or even 4 years, and Bouley (1854, quoted by Curasson, 1936) reported that recovery from the disease conferred immunity even on those cattle that had not shown any evidence of clinical illness at their first contact with the disease. Curasson (1936) stated that one could demonstrate, at least to subcutaneous challenge, a lengthy, if not strong, resistance in animals which had been previously infected.

There is no doubt that recovery from artificial subcutaneous infection does produce immunity to subsequent subcutaneous inoculations and also to the natural disease.

This observation gave rise to the practice used extensively by Willems (1852) of inoculating virulent fluids from acute cases of the disease into the tip of the tail, and also gave rise presumably to the methods of prophylactic inoculation practised by native tribes in Africa, which entailed the subcutaneous inoculation of infected material over the frontal bones. The relative safety of these methods probably depended on the self-limitation of the infection through local venous and lymphatic thrombosis (Turner, 1959). The disadvantage of Willems method of immunization was the high incidence of severe vaccination reactions which were variable, unpredictable and frequently fatal. Although under certain conditions it proved an effective method of reducing losses from the disease, it did not effect eradication (Hutyra, Marek and Manninger, 1949). Nevertheless, vaccination by this method was used extensively in Europe, in South Africa until the policy of eradication by slaughter was adopted, and in Australia until 1931, when pathological fluids were replaced by broth cultures of suitable strains of the organism.

Evidence of the passive transfer of immunity is very scanty and there appears to be no information on this since the experiments carried out at the end of the last century. Nocard, Roux and Dujardin-Beaumetz (1899, quoted by Nocard and Leclainche, 1903) reported that the serum of a cow that had been inoculated subcutaneously and intraperitoneally with increasing doses of pure culture of M. mycoides, giving a total of 5 litres, acquired immunizing properties. A dose of 40 ml. produced an immunity to subcutaneous challenge lasting up to 10 days, and a dose of 100-200 ml., repeated at intervals of 24 hours if necessary, cured or inhibited the further development of an artificially produced inoculation swelling. It appears, therefore, that passive transfer of immunity is possible and hence immunity is at least partly humoral.

Further evidence to support this was published in 1954, when Edward and Fitzgerald demonstrated the presence of

neutralizing antibodies in the sera of hyperimmunized rabbits which specifically inhibited the growth of PPLO's and Mycoplasma mycoides, and which did not require the presence of complement. The neutralizing antibodies appeared to be independent of the agglutinating antibodies. Earlier, Priestley (1952) had shown that sera of cattle which had recovered from CBPP were capable of killing M. mycoides. The effect was rapid and was dependent on the presence of complement. This action of antibody thus differed from the inhibition of growth and appears to be similar to the bactericidal and bacteriolytic effects of antibody acting in the presence of complement on certain bacteria. Provost and Villemot (1961) showed that blood from immune cattle strongly phagocytosed M. mycoides whereas blood from susceptible cattle did not.

While antibodies do appear to play a part in immunity to M. mycoides, the demonstration of agglutinating and complement fixing antibodies in the serum of an animal does not necessarily indicate that the animal is immune, at least to subcutaneous challenge, as there are a number of instances of cattle possessing strongly positive agglutinating and complement fixing titres which have not withstood challenge by this route (Gourlay, to be published).

There seems to be no doubt that inactivated vaccines prepared from killed organisms do produce some degree of immunity provided an adequate dose is given (Turner, 1959; Anon., 1960b). The large dose required seems to be the limiting factor in the use of these vaccines, and thus living vaccines have proved relatively more successful. These have usually been 'wild' strains treated or attenuated in various ways and inoculated subcutaneously usually into the tip of the tail. Many of the methods of production used in the past, for example the use of glycerine, lanoline and saponin, proved unsatisfactory as either the vaccines were dangerous and produced excessive vaccination reactions or the immunity produced was inadequate. The same

disadvantages apply to the more successful vaccines but to a less extent. These vaccines include 3 main groups, namely, broth culture attenuated, broth culture attenuated with the addition of adjuvants and fowl embryo attenuated vaccines. With these vaccines it appears that true attenuation occurs on repeated sub-culture but unfortunately together with the loss of virulence is a corresponding loss of immunizing power. This is recognised by the Expert Panel on CBPP (Anon, 1960b) who state: "with most strains of M. mycoides immunizing power is correlated with virulence", and they go on to say: "It is probable that as attenuation increases a greater number of organisms must be inoculated in order to ensure establishment and multiplication but, with some strains at least, continued transfer in media may lead eventually to loss of immunizing power, however large a dose is administered".

The findings of Priestley and Dafaalla (1957), that the addition of agar as an "adjuvant" to a living vaccine reduced greatly the minimum number of organisms necessary to produce immunity, were confirmed by Turner (1960). The agar probably does not act as a true adjuvant but rather creates an environment favourable for multiplication.

The length of immunity produced by recovery from natural infection, recovery from subcutaneous inoculation with virulent materials and treatment with different vaccines is unknown and varying lengths are claimed by different workers. Provost, Villemot and Queval (1959) and Knight (1960) claim that with fowl embryo attenuated vaccines the length of immunity is proportional to the number of organisms inoculated.

In order to overcome the problem of loss of immunogenicity on repeated subculture, Provost, Villemot and Queval (1959) inoculated a moderately virulent fowl embryo attenuated vaccine into the muzzle of cattle. Although a necrotic nodule formed at the site of inoculation, there was

apparently no further extension of the infection and a good degree of immunity was produced. Orue and Memery (1960) extended this method further and inoculated fully virulent lymph into the muzzle of cattle with relative innocuity. The disadvantage of this method is that the site of injection is critical and if the inoculations are not performed correctly severe reactions and even death can occur.

A factor that further complicates the problem of immunity is the wide variation in susceptibility, to both natural infection and to the various vaccines, of different breeds of cattle and also between the same breed in different areas.

Despite the considerable work on vaccines carried out over the years, immunological investigations have been largely confined to the application of serological diagnostic techniques without the necessary understanding of the relevant immunological mechanisms. With a view to obtaining a better understanding of these mechanisms, an investigation of the in vivo antigenic components of Mycoplasma mycoides was undertaken using standard serological methods, with particular reference to the agar gel double diffusion precipitin test. This was followed by the isolation and tentative characterization of certain of the antigenic components with emphasis on their role in pathogenesis and immunity.

PART 1

EXAMINATION OF BODY FLUIDS FROM CASES OF CONTAGIOUS BOVINE PLEUROPNEUMONIA FOR ANTIGENS OF *MYCOPLASMA MYCOIDES*

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INTRODUCTION

Growth of a pathogenic organism in artificial culture modifies and alters it with respect to certain characteristics. Thus the alteration in morphology, colonial appearances and the decrease in virulence are well known when a pathogen is grown in unnatural conditions. It is at present impossible to reproduce *in vitro* the nutritional and environmental conditions appertaining to a normal physiological state, much less the conditions found in diseased tissue and fluids which may not be normal physiologically and may also be continually changing (Dubos, 1954). Conversely when a pathogenic organism is growing in the host body tissues and producing disease, we know that all compounds and processes involved in pathogenicity are being produced (Smith, 1958). Thus the increase in virulence by animal passage and the fact that in certain diseases only a viable vaccine has proved effective in preventing the disease, show that certain substances or processes associated with virulence and immunity are produced *in vivo* but not to any significant extent *in vitro*. It is also known that the defence mechanism of the host may influence the pathogen in various ways and certain virulence factors may well be produced only as a direct action of these defences, and furthermore some pathogenic substances may be actual host compounds which have been modified in some way by the pathogen. It is known too, as in anthrax, that the host tissues may protect the bacterial products from destruction by the producing organisms.

Thus there is abundant evidence that the complex metabolic activity of the pathogen producing disease under the cultural conditions of the host tissues is different from its metabolism *in vitro* in artificial cultural conditions and it seemed that serological, cultural and biological examinations of body fluids obtained from animals infected with *Mycoplasma mycoides* would be fruitful, as we could be certain that all compounds involved in pathogenicity and hence immunity had been produced in the animal and were probably present in the body fluids.

REVIEW OF THE LITERATURE

Contagious Bovine Pleuropneumonia (CBPP) is a disease virtually restricted to cattle, with only rare records of its occurrence in other animals, namely buffaloes, yaks, bison, reindeer and an antelope (Turner, 1959). The natural disease is a primary broncho-pneumonia with early secondary lymphatic involvement which leads to the characteristic necrosis and encapsulation (Turner, 1959). The pathological lesions are confined to the thoracic cavity, even though during the acute stage of the disease the causal organisms can be recovered from the blood. The disease may involve one or more lobes of one lung and rarely both lungs.

Artificial reproduction of contagious bovine pleuropneumonia

Cattle

In 1935 Daubney stated that all attempts to reproduce the pulmonary disease in cattle by inoculation of virulent thoracic lymph or pure culture had failed, whatever the method of inoculation employed, whether subcutaneous, intravenous, intratracheal, intraperitoneal, intrapleural or intracerebral. More recently, Turner (1959) stated that the pulmonary disease never follows simple subcutaneous, intradermal, intramuscular, intracerebral or intravenous inoculation of M. mycoides. Neither of these authors, unfortunately, quote any authorities when making these statements. Most of the work on the subject was carried out in the latter half of the last century or beginning of this century. Willems (1852) was apparently one of the first to describe in detail the effects of intradermal and subcutaneous inoculations of M. mycoides in cattle. He described how the inoculation of a large number of organisms produced a hot, painful, oedematous swelling at the inoculation site after 12-20 days. The swelling enlarged and, if the inoculation was performed on the thorax, behind the shoulder, the infiltration could involve the whole of the side of the thorax, extending proximally to the lowest part of the abdominal wall, anteriorly

to the base of the neck and dorsally to the withers. There was a rise in temperature, the appetite was lost and ruminations were irregular. Death was preceded by a period of depression and coma. On autopsy the hide was thickened and engorged, and the connective tissue was distended with a yellow fluid, which readily coagulated. Occasionally the oedema penetrated the chest cavity and produced a fibrinous exudative pleurisy. Reactions were less severe if the inoculations were performed at the extremities of the limbs or the tail. In these cases the reaction remained localised and Willems stated that this localised reaction conferred a solid immunity on the animals to a subsequent inoculation over the thorax and even to the natural disease. In young calves subcutaneous inoculation in the tail produced an insignificant local reaction but could be followed later by a general rheumatic arthritis.

Walker (1930) considered that the oedematous swellings, following subcutaneous inoculation of virulent culture or lymph, occurred 6-14 days after inoculation, and only in a varying percentage of susceptible cattle. In some cases the swellings extended considerably resulting in the death of the animals 10-15 days later. In other cases the swellings remained localised and gradually disappeared. He confirmed that inoculation of suckling-calves often produced specific lesions of the joints and tendons. The organisms become bacteraemic in the later stages of subcutaneous infection (Hall and Beaton, 1931) and, although at autopsy the organisms could be isolated from all organs including the lungs, pulmonary disease similar to the natural disease has not been observed in these cases (Mornet, Orue and Diagne, 1949).

Walker's observation that only a certain percentage of cattle were susceptible to the subcutaneous inoculation of M. mycoides was supported by Piercy and Knight (1958) who stated that a small proportion of cattle were naturally resistant to subcutaneous challenge. Out of 57 cattle they found that 29 were fully susceptible, 21 were partially resistant and 7 were

fully resistant. Furthermore they stated that reference to challenge results in control animals of workers in the Sudan and Australia revealed a similar picture of susceptible, resistant and semi-resistant groups. Mendes and Da Garca (1954) found that 4 out of 28 calves were resistant to subcutaneous challenge.

Nocard and Roux (1903, quoted by Nocard and Leclainche, 1903) showed that ingestion of large quantities of infected lung tissue and infected fluid did not cause infection. They also showed that intravenous inoculation of infected serous fluid or culture was without ill-effect, and this was later confirmed by Curasson (1935) using virulent lymph. The inoculation of cultures of M. mycoides into the pleural cavity was followed by an intense fibrinous exudative inflammation which could spread to the peritoneum. Intraperitoneal inoculation of culture was followed by a fibrinous peritonitis with massive exudation. Five heifers which were inoculated with culture in the anterior chamber of the eye did not develop any reaction (Nocard and Leclainche, 1903). Newing and MacLeod (1956) inoculated infected pleural exudate into the pleural cavity and "probably also into the lung parenchyma" of 17 cattle. Two became infected with classical CBPP, the lesions at autopsy being indistinguishable from those of the natural disease. Six had only a fibrinous pleurisy and the remainder either had no lesions or only a slight congestion on the inoculated side.

Intracerebral inoculation of culture was followed, after an incubation period of 6-14 days, by apathy and drowsiness interrupted by symptoms of cerebral irritation. Animals refused to eat and there was a rapid loss of condition; death occurred in a variable number of days (Nocard and Leclainche, 1903).

Turner, Campbell and Dick (1935) attempted to produce the characteristic lung lesions by carrying out a series of deep inoculations in the peritoneal fascia at its entrance to the chest. A very marked inflammatory oedematous reaction

followed at the site of inoculation and in many cases the infection proceeded along the peritracheal fascia into the chest, where it produced a characteristic sero-fibrinous pleuritis resembling very closely that found in the natural disease. In no instance, however, did the disease extend into the lungs to produce the characteristic pneumonia.

Chauveau is credited by Nocard and Leclainche (1903) with having successfully transmitted the disease from a sick to a healthy animal by connecting their heads with a long tube made of linen. Nocard and Roux (1901, cited by Nocard and Leclainche, 1903) attempted to transmit the disease by passing atomized cultures of the organisms into sacks surrounding the heads of cattle. Four out of the 5 animals so treated developed a transient pyrexia, but were apparently immunized, while the fifth animal died a month later after running a high temperature. At autopsy the lungs were said to have lesions resembling the early stages of pleuropneumonia.

Walker (1922) showed that contact experiments in the field, under natural conditions, were not very satisfactory as he exposed susceptible cattle in highly infected herds for periods of up to 7 months and only 41 per cent of the in-contact susceptible animals developed the disease though the remainder were subsequently shown to be still susceptible. Daubney (1935) placed 7 susceptible cattle in contact with 3 cattle chronically affected with pleuropneumonia in a large loosebox. The exposure lasted 10½ months after which time only 2 of the animals had become infected. In a further experiment 5 susceptible cattle were placed in contact with 4 acutely affected animals and the exposure lasted 10 days, by which time all the naturally affected animals were dead. Only 2 of the susceptible cattle developed the disease, one 27 and the other 30 days after the end of the exposure.

Campbell and Turner (1936) reported that out of 65 cattle placed in close contact with active cases of the disease,

22 developed clinical pleuropneumonia, 26 the subclinical form and 17 were refractory. Pulmonary lesions were produced in 5 out of 9 susceptible cattle by injecting into the jugular vein M. mycoides, as lymph or recently-isolated organisms in pure culture, in emboli of agar (Daubney, 1935). Turner, Campbell and Dick (1935) repeated Daubney's work and, in addition, they found that in the case of infected lung, with or without the addition of agar, lesions were produced in the lung closely resembling natural pleuropneumonia. Mettam and Ford (1939) later confirmed these findings and produced lung lesions in 14 out of 20 cattle inoculated by Daubney's method. From the descriptions of the lesions produced by the emboli methods of Daubney and Turner, Campbell and Dick, the majority of lesions appeared to be of the infarct type and did not resemble the acute lesions of CBPP.

Nocard and Mollereau (quoted by Nocard and Leclainche, 1903), did not observe any reaction after the inoculation of organisms intratracheally in 2 cows but Daubney (1935) managed to produce the pulmonary disease in 1 out of 24 cattle that he inoculated intratracheally.

Turner, Campbell and Dick (1935) attempted to produce the disease by injecting bronchial washings from natural cases of the disease, virulent pleural exudate or cultures, intratracheally without success, and Curasson (1935) found that intratracheal injections of virulent lymph failed to produce infection.

More successful were the endobronchial inoculations carried out by introducing cultures (Yamagiwa and Ito, 1932, cited by Yamagiwa, Ito and Niwa, 1941; Campbell, 1938; Yamagiwa, Ito and Niwa, 1941) or ground up CBPP lung material (Anon., 1960a) by a narrow catheter down the trachea until it impacted in a small bronchus. The disease produced was in every way similar to that occurring in natural outbreaks according to Campbell who managed to produce lung lesions in 6 out of 6 animals in this way. Using CBPP lung material the workers in Australia (Anon., 1960a) reported that 38 out of 47

animals became clinical cases of the disease 4-17 days after inoculation, and 14 died within 13-24 days after inoculation. Nine were subclinical cases. Unfortunately the endobronchial method of inoculation has not proved very successful at Muguga as, out of 34 cattle inoculated by this route with ground up CBPP lung material, only 1 has developed acute pleuropneumonia (Brown, 1962).

The technique most nearly approaching natural infection is the exposure of animals to aerosol cultures. By the use of this method, 74 out of 80 cattle revealed either after death or slaughter, conclusive evidence of having developed a specific pulmonary CBPP (Campbell, 1938). Of these, 33 developed clinical pleuropneumonia associated with extensive acute lesions. Forty-one developed only a mild, subclinical infection but revealed definite lesions when examined at autopsy, while 6 animals proved refractory.

Hyslop (1955) reported that, at Kabete, Kenya, all cattle exposed to aerosol infection developed clinical signs of CBPP and/or characteristic lesions at autopsy. He did not, unfortunately, quote any protocols.

The aerosol method of infection did not, however, prove equally successful at this laboratory (Newing and MacLeod, 1956). Four cattle were exposed and no clinical symptoms of infection were observed other than a slight transient pyrexia in one case. At autopsy all showed discrete pneumonic lesions and in 1 case the lesion had become encapsulated resembling a typical CBPP sequestrum. M. mycoides was isolated from this lesion. In a further experiment, with 4 cattle and the addition of Hyaluronidase to the aerosol, no clinical symptoms were observed and at autopsy only 1 had small patches of congestion in one lung.

Sheep and Goats

According to Campbell (unpublished, quoted by Turner, 1959) sheep and goats never contact natural CBPP and are not susceptible to inhalation of aerosol cultures. They are, however, susceptible to subcutaneous inoculation of cultures of M. mycoides and frequently die as a result (Turner, Campbell and Dick, 1935).

Willems (1852) found that sheep and goats did not react to inoculation of bovine lymph, and Nocard and Leclainche (1903) reported that sheep were refractory and that the transmission to goats had been attempted in vain by a number of workers; only Galtier had been successful using the oral route and Nocard and Leclainche appeared doubtful of the authenticity of this.

Walker (1930) reported that he was unable to produce a reaction in sheep injected by the subcutaneous as well as the intravenous route.

On the other hand, Dujardin-Beaumetz (1906) claimed that sheep and goats were susceptible to subcutaneous injection of large doses (e.g. 100 ml.) of M. mycoides grown in cultures enriched with sheep, goat or horse sera. Beller and Tahssin Bey (1926, cited by Dick, 1937) found that sheep and goats were susceptible to the injection of pleuritic exudate or broth cultures and concluded that sheep were slightly more susceptible than goats. This was borne out by Turner, Campbell and Dick (1935) who also reported that the subcutaneous inoculation of either pleuritic fluid or culture of the organisms into sheep or goats was followed by characteristic inflammatory oedematous swellings similar to those produced in cattle and in both animals the organisms became septicaemic. Tang, Wei, McWhirter and Edgar (1935) obtained similar results with goats. Campbell (1936) recorded the effects of subcutaneous injection of cultures of the organism into goats and the recovery of the organisms from the blood during the period of 8 days. Dick (1937) reported an age resistance in goats. Typical reactions resulted from the

subcutaneous injection of cultures in mature goats but the same dose produced no reactions in kids 3 to 4 months old. Beller and Tahssin Bey (1926) also reported that in pregnant animals the organisms can become localised in the decidua and the foetus, followed by abortion. This was confirmed by Turner, Campbell and Dick (1935).

Other Animals

Willems (1852) found that rabbits, dogs, fowls and swine did not react to the inoculation of bovine lymph, while Nocard and Leclainche (1903) stated that pigs, carnivores, guinea pigs and birds were refractory. Walker (1930) reported that he was unable to produce a reaction in mice, guinea pigs and rabbits inoculated by the subcutaneous and intraperitoneal routes, or in horses, camels and pigs inoculated by the subcutaneous method. Tang, Wei, McWhirter and Edgar (1935) report no reaction to the organism in white mice, hamsters, albino rats, guinea pigs, rabbits and cats after subcutaneous, intraperitoneal, intracranial and intravenous and, in some cases, intratesticular inoculations. According to Turner (1959) man, horse, pig, dog, fowl and turkey are quite refractory to the organism.

It seems, therefore, that laboratory animals are not susceptible to the simple inoculation of M. mycoides. However, Nocard and Roux (1898, quoted by Nocard and Leclainche, 1903) demonstrated that M. mycoides was able to multiply in the tissue fluids of the rabbit when they enclosed the organisms in collodion sacs inserted in the peritoneal cavity. Ono (1925) reported iritis and local survival of the organisms for 3 weeks after intra-ocular inoculation, and orchitis persisting for 2-3 weeks after intratesticular injection. Turner, Campbell and Dick (1935), however, were able to recover organisms from the internal organs of rabbits after an interval of 2 days but not 10 days, and also to isolate the organisms from the foetuses of pregnant rabbits following subcutaneous inoculation of the organisms. They also showed that following intracerebral inoculation into guinea pigs and rats the organisms could be

recovered from the brain for up to 8 days and occasionally from the blood and internal organs.

Sheriff (1951) and Hyslop (unpublished, quoted by Hyslop, 1958) employed the agar emboli method, that Daubney (1935) had used in cattle, to infect rabbits. Specific pulmonary lesions developed in a significant proportion of cases. Sheriff maintained one strain of M. mycoides for more than 22 serial passages in rabbits. Gambles (1956) reported that rabbits inoculated with West African strains developed complement fixing antibodies, although attempts by Mendes (1955) had met with little success using the subcutaneous route of inoculation primarily, although he also tried the intraperitoneal, intratracheal, intravenous and intrapulmonary routes. No protocols were given and it is not known whether he used the agar emboli technique.

Gerlach and Heikkila (1956) were able to passage M. mycoides in white mice by subcutaneous inoculation of the organisms incorporated in solid culture medium ground up with normal saline. Hyslop (1958) reported that rabbits, guinea pigs, golden hamsters and mice could be infected with M. mycoides by intramuscular, intrathoracic or subcutaneous injection of the organisms incorporated in an agar gel. Fifty subcutaneous passages were performed.

Apart from instances mentioned above where the incorporation of agar or culture medium was required to initiate growth, buffalo were shown to be susceptible to intrapulmonary or intrapleural injection of M. mycoides alone (Nocard and Leclainche, 1903) and it has also been shown that buffalo can be infected by the endobronchial inoculation technique (Anon., 1960b).

Tang, Wei, McWhirter and Edgar (1935) showed that the organisms would multiply on the chorio-allantoic membrane of 8-11 day chick embryos, producing diffuse oedematous lesions or white spots. In addition to the chorio-allantoic route of

inoculation, Sheriff and Piercy (1952) obtained more constant results by using the yolk sac route and deaths occurred regularly on the third and fourth days. Organisms to a high titre were demonstrated in embryo, yolk, allantoic fluid and chorio-allantoic membrane (Piercy and Knight, 1956). Elek and Cottew (1961) reported that the yolk sac, allantoic and amniotic routes of inoculation in the egg embryo were equally good.

Distribution of viable M. mycoides in the body fluids of infected animals

Following the first demonstration of the presence of the organism causing CBPP in the pleural exudate and lungs of infected cattle by Nocard and Roux, with the collaboration of Borrel, Salimbeni and Dujardin-Beaumetz in 1898 (cited by Nocard and Leclainche, 1903), it had been assumed that the causal organisms were confined to the obvious pathological lesions, namely the lungs, pleural exudate and associated lymph glands in natural cases of the disease and the local inflammatory lesions and affected regional lymph glands after experimental subcutaneous inoculation of virulent organisms. Later, however, Dujardin-Beaumetz (1913, quoted by Campbell, 1936), isolated the organisms from specific joint lesions in young calves. Nakamura, Futamura and Watanuki (1926) demonstrated M. mycoides in the circulating blood, liver, spleen, kidneys and lymph glands as well as the lungs, pleural exudate and thoracic lymph nodes. Daily blood cultures showed that the organism became bacteraemic during the height of the disease. Soon afterwards Beller and Tahssin-Bey (1927, cited by Campbell, 1936) recovered the organisms from the pleural, pericardial and peritoneal fluids of a lamb that died 12 days after birth, the mother having been inoculated subcutaneously with M. mycoides 107 days before parturition. From an aborted foetus of another ewe which had been inoculated 180 days before the abortion, the organisms were recovered from pleural and peritoneal fluids, but not from heart blood, spleen and stomach contents. They were also able to recover the organisms from the lymph glands, body fluids, spleen and kidneys of cattle

inoculated subcutaneously. The organisms were cultured from the urine of a calf for 7 months after subcutaneous inoculation, but they were unable to cultivate the organisms from liver, heart blood or bile obtained post mortem.

Yamagiwa, Itabashi and Ito (1930) obtained the organisms from the blood of 4 experimentally infected cattle for some 10 days before death; but they did not obtain organisms from the blood of any of the naturally infected cases.

Hall and Beaton (1931) demonstrated, by cultural methods, the presence of organisms in the circulating blood of subcutaneously inoculated cattle from the first day of thermal response, accompanied by a local reaction, until the local reaction began to undergo resolution and the temperature to drop. They cultivated the organisms from the spleen, prescapular lymph glands and nearly every organ of an animal that had died from artificial infection and from the spleen and lymph glands of all naturally infected animals but only if the lung lesions remained unencapsulated. They also cultivated the organisms from the blood of these naturally infected animals until the lung lesions became encapsulated. Campbell (1936) was unable to confirm the claim of Hall and Beaton (1931) that blood culture was a simple method for detecting and confirming a clinical diagnosis of contagious bovine pleuropneumonia (CBPP) as in only 14 out of 83 culture tests on 18 cattle were organisms recovered from the blood even though all the animals showed clinical signs of the disease and all were positive to the complement fixation test. By testing the blood of cattle, sheep and goats inoculated with pleural fluid from natural cases of CBPP or with broth cultures of M. mycoides, Campbell was only able to demonstrate organisms intermittently in the blood from 24 hours after inoculation until death or resolution of the local lesion commenced.

Cultural examinations on 23 cattle that had died from acute CBPP or had been destroyed in the early stages of the disease showed that organisms were always present in lung tissue, pleural exudate, mediastinal and bronchial lymph glands,

frequently present in the heart blood, liver, spleen and kidneys and in 4 cases also from the brain and cerebro-spinal fluid and inflammatory oedema in the epidural space (Turner and Campbell, 1935). Contrary to the findings of Beller and Tahssin-Bey, Campbell (1936) was unable to demonstrate the organisms in the urine of animals affected with acute CBPP or those inoculated subcutaneously with either virulent culture or pleural exudate, although he found them in the kidneys in 50 per cent of the animals. Campbell also demonstrated the organisms in amniotic fluid but not from the internal organs of 2 foetuses from naturally infected heifers, and from the internal organs of aborted twin foetuses from an experimentally infected heifer. At a post mortem examination of a further experimentally infected heifer he was able to demonstrate M. mycoides from the amniotic fluid, blood and liver of the foetus. He also recovered M. mycoides from subcutaneous lesions, heart blood, liver, spleen and kidneys of inoculated sheep, confirming the findings of Beller and Tahssin-Bey.

More recently, Mornet, Orue and Diagne (1949) reported that they cultivated the organisms from the blood, lymph, kidneys and all organs of cattle that had been inoculated subcutaneously with infected pleural fluid.

Distribution of antigens of M. mycoides in the body fluids of infected animals

Apart from the demonstration of viable M. mycoides (and hence antigens) in the body fluids already mentioned, the first demonstration of antigens of M. mycoides by serological means in the body fluids was by Dujardin-Beaumetz (1906), who demonstrated precipitinogen in sera obtained from affected animals. Beitzen (1919, quoted by Nakamura, Futamura and Watanuki, 1926) reported the specificity of the precipitin reaction and demonstrated precipitinogen in sera of affected animals. Nakamura, Futamura and Watanuki (1926) confirmed that precipitinogen could be demonstrated in sera of naturally or artificially infected animals and also in the infected as

well as normal parts of affected lungs and the regional lymph glands, using antisera prepared in rabbits.

Even before this, antigen had been demonstrated in body fluids by indirect means, as various workers had used inflammatory exudates as antigens in the complement-fixation test. Schochowsky (1912, quoted by Campbell and Turner, 1936) used extracts of inflammatory exudate, while Poppe (1913, quoted by Campbell and Turner, 1936) used the material from subcutaneous lesions produced in experimental animals as well as pleuritic inflammatory exudate from natural cases of the disease.

As recently as 1936, Campbell and Turner used extracts of pleuritic exudate or subcutaneous inflammatory exudate as antigen in the complement-fixation test.

In an incomplete paper, Dafaalla (1957) reported the examination of 31 sera for the presence of precipitating antigen; 18 of these were from clinical cases, presumably natural cases of the disease, 3 were "swelling (dying)" cases (sic), 5 were apparently recovered animals, presumably recovered from natural disease, and 5 were normal animals, and he showed that precipitating antigens occurred only in 12 of the clinical cases, in all 3 of the "swelling (dying)" cases and in 2 of the apparently recovered cases. Agglutinating antibodies as demonstrated by the slide agglutination test, of which no details are given, were present in only 3 of the 18 natural clinical cases and it is of interest to note that these 3 were the only sera to show no precipitating antigens. Complement fixing and precipitating antigens of M. mycoides were also demonstrated in lymph from swellings caused by the intramuscular injection of virulent culture or pleural exudate from natural cases of the disease using a known positive serum of which no details are given. White (1958) using the agar gel double diffusion precipitin test showed that sera of cattle dying of CBPP when tested against immune rabbit sera, prepared by the injection of washed organisms, often gave 1 or 2 bands of precipitation. When the immune rabbit sera were tested against material from infected tissues such as pleural exudate or saline extracts of lung lesions usually 3 bands of precipitation were apparent.

MATERIALS

Experimental Animals

Cattle.- Experimental cattle were crosses between East African Shorthorn Zebu and breeds of European origin, in which the latter dominated, commonly called Grades. These were obtained from farms in areas free of CBPP. Materials from cases of natural CBPP were obtained from Zebu cattle in the Masai area of Kenya.

Sheep.- Sheep were crosses of Somali fat-tail and breeds of European origin obtained originally from farms in areas free from CBPP and kept as a self-contained flock at the East African Veterinary Research Organization, Muguga (EAVRO).

Rabbits.- The rabbits used were F1 crosses of pure bred New Zealand White rabbits from Onderstepoort, South Africa, and the Chinchilla line from the small animal colony, EAVRO.

Mice.- White Swiss mice were from the small animal colony at EAVRO.

Strains of M. mycoides

T3 strain is a strain obtained originally as lung material from a natural case of CBPP (Piercy and Knight, 1956) and maintained here as lymph (inflammatory exudate from subcutaneous experimental lesions) stored at -25°C. This strain has undergone 8 subcutaneous passages in cattle (Piercy and Knight, 1957).

Oremit strains 1, 2, 3 and 4. These are strains obtained as pleural fluid, lung material and blood from natural cases of CBPP in the Oremit area of Ngong, in the Masai area of Southern Province, Kenya.

Suswa strains 1 and 2. These are strains obtained as pleural fluid, lung material and blood from natural cases of CBPP in the Suswa area of the Southern Province, Kenya.

Ndeiya strain. This is a strain obtained as pleural fluid, lung material and blood from a natural case of the disease in the Ndeiya location of Central Province adjoining the Masai area of Southern Province, Kenya.

Strain S1. This is a strain obtained as dried "body fluid" from Somaliland (Priestley, 1961).

KH3J strain (series 48). This is an avirulent strain, kindly supplied by Mr. E. P. Lindley, Veterinary Research Laboratory, Vom, Northern Nigeria, as a freeze-dried culture that was in its 85th generation.

Centrifugation

All routine centrifugations were performed in an M.S.E. medium centrifuge* to which a refrigeration unit was incorporated, and all manipulations were carried out at 10° - 15°C.

The heads in use were the 4 and 6 place swingout heads and the 20 place angle head giving maximum r.p.m. of 2200, 2900 and 4000 and maximum RCF values of 1150, 2240 and 2240 respectively under the loading conditions used. For centrifugations at higher speeds an International Portable Refrigerated Centrifuge model PR-2* with high speed attachment was used.

Ultracentrifugation

High speed runs were carried out in a Spinco model L ultracentrifuge•

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* Measuring & Scientific Equipment Ltd., Spenser Street, London, S.W.1, England.

* International Equipment Co., Boston, Mass., U.S.A.

• Beckman-Spinco, Stanford Industrial Park, Palo Alto, Ca., U.S.A.

Liquid medium for the growth of M. mycoides

Tryptose broth (Newing and MacLeod, 1956) with slight modifications was used. Glycerol 0.5 per cent. was added and dextrose 0.5 per cent. (instead of 0.2 per cent.) and Difco* yeast extract 0.1 per cent. (instead of 5 per cent. aqueous yeast extract) were used. After solution in water, 10 per cent. pig serum and 100 i.u. per ml. penicillin were added before filtration through a Seitz EK filter pad. Organisms grown in this medium were used as a source of antigen for immunization and serological purposes.

Solid medium for the growth of M. mycoides

Solid medium was prepared by adding 50 ml. of Seitz filtered pig serum (inactivated at 56°C. for 30 mins.), and 35 ml. of 10 per cent. Bacto-agar (Difco) to 150 ml. of Tryptose broth medium.

Agar medium used for the agar gel precipitin tests

The medium used was 1 per cent. (w/v) Special Agar-Noble (Difco) dissolved in veronal buffer (Mayer, Croft and Gray, 1948) of pH 7.3 - 7.4 and containing 0.04 per cent. merthiolate to inhibit bacterial and fungal growth.

Disintegration of bacterial cells

Disintegration of bacterial cells was carried out at 4°C. using either a Raytheon 50 watt, 9 Kc. magnetostriction oscillator, model S-102* at maximum power for 60 minutes, or an M.S.E. ultrasonic disintegrator, Cat. No. 3000* at maximum output for 60 minutes.

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* Difco Laboratories Inc., Detroit, Michigan, U.S.A.

* Raytheon Mfg. Co., Waltham, Mass., U.S.A.

* Measuring & Scientific Equipment Ltd., Spenser Street, London, S.W.1, England.

Freeze-Drying

An LT/5 centrifugal freeze-drier* was used and the samples were dried over P_2O_5.

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* W. Edwards & Co. (London) Ltd., Allendale Works, Worsley Bridge Road, London, S.E.26, England.

METHODS

Preparation of T3 Antigen

An ampoule of M. mycoides T3 strain lymph was thawed out and diluted in a tenfold series in tryptose broth medium. From the highest dilution showing growth of M. mycoides after 3 days, flasks containing 2 litres of tryptose broth medium, warmed to 37°C., were seeded. After 3 days when growth was good, the organisms were harvested using a Lister vaccine clarifier type A.P.S.M. 12* with a 5¾ inch cone running at 14,000 r.p.m. The organisms were scraped off the cone, resuspended in 500 ml. normal saline, mixed well in an M.S.E. Atomix* at 12,000 r.p.m. for 2 minutes and passed through the clarifier again. This was repeated twice more and finally after mixing in the Atomix the suspension was made up to an opacity of twice tube 10 using Opacity Tubes - Wellcome• with normal saline and dispensed in small amounts and stored at -25°C. The antigen was checked for viability and for contamination by other bacteria by serial dilutions in tryptose broth medium and by plating onto solid tryptose broth-serum-agar medium and ox-blood agar plates.

Collection of certain body fluids

Nine bullocks which were bled and found to be negative to the serum agglutination slide test (SAST) and the complement fixation test (CFT) were inoculated subcutaneously (s/c) behind the shoulder; 3 with first passage tryptose broth culture of M. mycoides strain T3, 3 with first culture passage of strain S1 and 3 with pleural fluid from a natural case of CBPP (Oremit 1). When the local lesions which developed at the site of injection became sufficiently severe to prevent the animals from rising and

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* R. A. Lister & Co. Ltd., Dursley, Gloucestershire, England.

* Measuring & Scientific Equipment Ltd., Spenser Street, London, S.W.1, England.

• Burroughs, Wellcome & Co. Ltd., London.

hence unable to survive they were shot. Natural cases of CBPP were also obtained (numbered Oremit 1, 2, 3 and 4, Suswa 1 and 2 and Ndeiya 1) and these were likewise destroyed. Oremit 1 was severely ill when destroyed and probably would not have survived for more than 24 hours. One entire lung was involved and a considerable amount of pleural fluid was present. Oremit 2 was also severely ill but would probably have survived for about 4-5 days more had it not been destroyed. Again one entire lung was involved and there was a fair amount of pleural fluid present. Oremit 3 was moderately ill and about 90 per cent. of one lung was involved. There was very little pleural fluid present. Oremit 4 was only slightly ill and only about 50 per cent. of one lung was involved and again only very little pleural fluid was present. In the two latter cases the paucity of pleural fluid may have been in part due to dehydration caused by the severe drought conditions at the time. Suswas 1 and 2 and Ndeiya 1 were severely ill and the whole of one lung was involved in each case with little pleural exudate. Immediately the animals were dead the skin over the jugular vein was reflected and the vein severed and blood collected for plasma, serum or blood cells, after which the other body fluids were obtained. Blood for plasma and blood cells was collected into sterile bottles and heparinized (10 i.u. per ml.)*. Blood for serum was collected in sterile serum jars. Urine and pleural fluid were obtained with sterile syringes and needles. Lung tissue was removed with aseptic precautions and placed in sterile containers. Lymph (subcutaneous inflammatory exudate) was obtained by reflecting the skin over the area of the subcutaneous lesion and the straw coloured fluid was collected with a 10 ml. sterile pipette.

Heparinized blood was centrifuged at 2,000 r.p.m. for 1 hour and the plasma removed aseptically. Blood cells were

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* "Liquemin" concentrated heparin solution. Roche Products Ltd., Welwyn Garden City, England.

washed 3 times in normal saline and packed by centrifugation at 2,000 r.p.m. for 30 minutes and then lysed by mixing 1 volume of packed cells with 2 volumes of distilled water. Blood for serum was left at room temperature for 2 hours and, when the clot was formed, put at +4°C. for 18 hours and the serum removed. Lymph was centrifuged at 2,000 r.p.m. for 30 minutes to remove fibrin strands and blood cells. Fluid drained from the infected lung tissue on standing was collected in sterile bottles. All fluids were distributed in small amounts and stored at -25°C.

Concentration of antigenic materials

Two electric hair driers (Pifco*) were used for concentration purposes. They were used without the heating element in action, i.e. they were essentially air blowers. The solution to be concentrated was placed in "Visking" cellophane tubing* 24/32" and, if the quantity was great, the tubing was suspended in such a way that the air from the driers could play on a number of coils at one time.

Concentration normally continued for about 18-20 hours by which time the material had become concentrated to about 1/6th of the original volume. The Visking tubing was carefully washed through with a small quantity of distilled water to remove the dried deposit which was generally adherent to the empty lengths of tubing, and added to the rest of the solution.

Qualitative agar gel double diffusion precipitin test (qualitative AGT)

Diffusion of antibodies and antigens was carried out by the modification of Ouchterlony's method as described by Mansi (1957) modified as described below to fit the requirements of the present work. The medium, 8 ml., was poured into flat-bottomed

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* Messrs. Pifco Ltd., Watling Street, Manchester 4, England.

* Visking Corporation, New York City, U.S.A.

petri dishes of 6.5 cm. internal diameter which had previously been treated with silicone*. If alternative petri dishes were used the medium was poured in to a depth of 2.5 mm. Wells were cut in the agar by means of either home-made cutters made from clinical thermometer case tops set in perspex or Feinberg Agar Gel Cutters*. The home-made cutters consisted of a centre well circled by 6 concentric circumferential wells, each well being 7 mm. diameter. Two separate sizes were used, one in which the distance between the centre and circumferential wells was 3 mm. and the distance between circumferential wells was 3 mm., and the other in which the distances between centre and circumferential wells was 4 mm. and between circumferential wells was 4 mm. Feinberg Agar Gel Cutters routinely used were Cat. No. 1802, which consists of a central well surrounded by 4 concentric circumferential wells, Cat. No. 1801, which consists of a central well circled by 6 concentric circumferential wells, and Cat. No. 1804, which consists of 4 wells placed at the corners of a square. In addition to the simple patterns cut with the above mentioned cutters, composite patterns were prepared for specific purposes by manipulating the above cutters in various ways as required.

Wells were filled with antisera or antigen materials by means of Pasteur pipettes just to the point where the meniscus flattens out and disappears (Feinberg, 1959) and placed at room temperature (21-22°C.) either in an incubator or perspex box containing water. Lines of precipitation which formed were viewed by transmitted light passing through the petri dish and medium at an acute angle and were recorded by means of accurate drawings as only the more distinct lines could be recorded by photograph. Certain reactions were preserved by drying the agar and staining the precipitin lines, details of which are given later. Plates were examined daily for up to 14 days after preparation.

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* Silicone "Repelcote" Water Repellent : Hopkins & Williams Ltd., Chadwell Heath, Essex, England.

* Shandon Scientific Co. Ltd., 6 Cromwell Place, London, S.W.7, England.

Quantitative agar gel double diffusion precipitin test (quantitative AGT)

Feinberg Agar Gel Cutter, Cat. No. 1812, a concentric quantitative pattern, was used. This consists of a very large central well surrounded by 6 concentric, minute circumferential wells. Serum, 0.2 ml., was placed in the central well and an antibody gradient was established (Feinberg, 1959) by pre-incubation for 72 hours at 37°C. in an enclosed container which held a little water to prevent excessive drying. After 72 hours, antigen was serially diluted and placed in the circumferential wells by filling until the meniscus just flattened out and disappeared. After a further 24 hours incubation at 37°C. the precipitin bands were examined and the first dilution at which a complete ring of precipitation enclosed the well was taken as the endpoint for that antigen. The endpoints were recorded and certain results were preserved by drying and staining the gels.

Preservation of agar gel precipitin bands

The method used for preserving the qualitative and quantitative AGT results was essentially that of Grabar (1959). When the precipitin bands were considered optimal the agar gel was washed in N.saline for 6 days with daily changes of saline. A piece of Whatman No. 4 paper soaked in saline was then applied to the gel, care being taken to avoid air bubbles under the paper. Small holes were made with a needle in the filter paper over the reservoirs and the agar placed at 37°C. to dry. When dry, it was found that the agar had become detached from the plate and the paper, moistened with saline, was easily removed.

After removal of the agar sheet from the plate the specimen was mounted on a 3 inch x 2 inch (76 mm. x 52 mm.) glass slide and finally, after staining, treated with Metoxy varnish* to protect it.

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* Leyland Paint and Varnish Co. Ltd., Leyland, Lancashire, England.

The precipitin bands were stained for protein by either amido black for 10 to 60 minutes, or azocarmine for 1 hour (Grabar, 1959). Precipitin bands were stained for the presence of lipid by the method of staining for lipoproteins of Grabar (1959), using Sudan black.

Photographs were contact prints using the mounted stained preparation as a negative.

Agglutination tests

Tube Agglutination test (AT)

Doubling dilutions of sera were prepared with N.saline. These were distributed in 0.5 ml. amounts in agglutination tubes and 0.5 ml. of washed whole M. mycoides grown in tryptose broth and preserved with 0.5 per cent. phenol at Brown tubes 3 opacity, were added. The mixture was incubated at 37°C. in a waterbath for 2 hours and then placed at 4°C. for 18 hours. Before reading the tubes were placed at room temperature for 1 hour. Antiserum titres are given as the highest dilution giving visible agglutination (+).

Slide Agglutination serum test (SAST)

The slide agglutination test first introduced by Priestley (1951) was carried out using Newing's antigen (Newing, 1955) but the dye used was alcian blue instead of the methyl violet as it was found to be more suitable as certain batches of methyl violet tended to cause auto-agglutination (MacLeod, 1958).

Complement fixation test (CFT)

The CFT was carried out by the method of Campbell and Turner (1953) using antigen prepared at Muguga by Campbell and Turner's method using the T3 strain of M. mycoides but grown in tryptose broth medium instead of "BVF-OS" (Newing and MacLeod, 1956). Veronal buffer was used as the diluent instead of N.saline.

Preparation of Antisera

Antisera were produced in sheep and cattle which had previously been shown to possess no antibodies against M.mycoides by the CFT and the SAST and no antigens of M. mycoides in the serum by the qualitative AGT. They were inoculated intravenously with T3 antigen on Tuesday, Wednesday and Thursday each week for 3 weeks and were then bled 4 days after the last injection. Antigen diluted 1 in 10 with normal saline was used for the first week and thereafter full strength antigen was used. Sheep were inoculated with 3 ml. and cattle with 10 ml. of the antigen each time. However, cattle were inoculated, 12 months prior to the immunization regime, with avianized CBPP vaccine (Piercy and Knight, 1956), strain T2/33, challenged with T3 by the subcutaneous method (Piercy and Knight, 1957), from which they survived after developing large local lesions at the site of inoculation which eventually regressed and disappeared.

Titration of viable M. mycoides in broth

Serial tenfold dilutions of the material to be tested were set up in triplicate in tryptose broth medium and the 50 per cent. endpoint dilution estimated by the method of Reed and Muench (1938). The endpoint was taken as the highest dilution showing growth of M. mycoides.

Preparation of serum Oremit 2 and lymph 6183 antigens

Serum and lymph were boiled (94°C.) for 10 minutes and in each case the solid mass of coagulated protein was chopped up into small pieces and then centrifuged at 17,480 r.p.m. (22,500 RCF) for 1 hour in the International centrifuge. The supernatant fluids were removed and passed through Millipore VM filters* (pore size 50 mμ ± 3 mμ). The filtrates constituted the antigens.

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* Millipore Filter Corporation, Bedford, Mass., U.S.A.

The effect of heat on the precipitating antigenic components

Seven ml. of serum, plasma and pleural fluid Oremit 2 and urine 6183 were heated in a boiling water bath (94°C.) for 60 minutes. After heating a slight flocculation was seen in the urine, while the serum and pleural fluid were almost completely solidified. The last two were therefore centrifuged at 3,000 r.p.m. for 60 minutes in the cold and the supernatant fluids removed. The precipitin bands produced by boiled and unheated materials were then compared in the qualitative AGT using sheep x serum. Normal and ultra-sonicated concentrated T3 antigens were treated in a similar manner and the precipitin bands produced by the heated and unheated materials compared in the qualitative AGT using sheep x' serum.

The effect of trypsin on the precipitating antigenic components

Two ml. of serum and pleural fluid Oremit 2 and 2 ml. of pooled urine were treated by adding 1 ml. of 0.2 M Na_2HPO_4 buffer (pH 8.5), 5 mg. trypsin* in 0.5 ml. N.saline and 0.15 ml. of Toluene. The mixtures were incubated at 37°C. for 2 days after which a further 5 mg. of trypsin was added and the incubation continued for a further 3 days. The mixtures were then dialysed against distilled water at 4°C. for 3 days, then shaken well with 2 ml. Chloroform and centrifuged at 2,500 r.p.m. for 30 minutes (Morgan and Partridge, 1940). The supernatant fluids were removed and the precipitin bands produced by these and untreated materials were compared in the AGT using sheep x serum.

Concentrated T3 antigen and the filtrate of ultra-sonicated T3 antigen were treated in a similar manner and the precipitin bands of the trypsinised and untreated materials were compared in the qualitative AGT using sheep x' serum that had been absorbed with normal pig and ox sera.

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* Trypure Novo Crystalline Trypsin stabalized :
Novo Industri A/S, Copenhagen, Denmark.

Absorption of sera

Sera were absorbed by adding the absorbing antigens to the serum, mixing well and then incubating the mixture in a waterbath at 37°C. for 2 hours followed by 4°C. for 18 hours. The mixture was then centrifuged at 3,000 r.p.m. for 30 minutes. Following separation of the supernatant fluids and deposits the supernatant fluids were examined in the qualitative AGT using Feinberg Agar Gel Cutter No. 1804. If specific antibody remained or if no excess antigen was demonstrated the procedure was repeated until removal of the homologous antibody was complete, indicated by the demonstration of excess antigen.

Site of the major precipitating antigens

Twentysix ml. lymph 6183 and 26 ml. pleural fluid Oremit 2 were centrifuged in the ultracentrifuge at 30,000 r.p.m. (average centrifugal force = 57,300 times gravity) for 60 minutes. The supernatant fluids and deposits were separated. The deposits were washed 3 times in 10 ml. N.saline by mixing the deposits thoroughly in the saline and passing the mixture through Millipore filters size VM (pore size 50 mμ $\pm$ 3 mμ). The final washed deposits were then treated with the Raytheon oscillator at maximum power for 60 minutes, and then made up to the original volume (26 ml.) with distilled water. The washing fluids were retained after each washing of the deposits and ultimately combined with the supernatant fluids. As the combined fluids passed only very slowly through Millipore VM filters they were concentrated, without filtration, by means of hair-driers to the original volume (26 ml.).

Quantitative AGT using sheep x serum were perfomed on the supernatant fluids + washings, the washed ultra-sonicated deposits and control untreated lymph 6183 and pleural fluid Oremit 2. Qualitative AGT using sheep x serum were also set up using the same materials but the washed ultra-sonicated deposits were concentrated nine times before use.

Relationship between the extracellular major precipitating antigens and the elementary bodies of M. mycoides

The supernatant fluids + washings were passed through Millipore VM type cellulose membrane filters. The filtrates were diffused against sheep x serum in the qualitative and quantitative AGT and the precipitin bands compared with those of the unfiltered materials.

Relationship between the intracellular major precipitating antigens and the cell debris

The ultra-sonicated deposits of lymph 6183 and pleural fluid Oremit 2, which had been concentrated nine times, were passed through Millipore VM filters in order to separate the soluble precipitating endoantigens from the cell debris. Before filtration the deposits were milky white but the resulting filtrates were colourless. The cell debris were then washed 6 times with 10 ml. distilled water, and finally made up in 0.3 ml. distilled water, i.e. concentrated a further nine times. The precipitin bands produced by the cell debris and filtrates were compared in the AGT using sheep x serum.

Site of the minor precipitating antigens

M. mycoides (strain T3) was grown in tryptose broth for 4 days and harvested in the Lister separator. The organisms were washed 3 times as for the preparation of T3 antigen and finally resuspended in N.saline to give an opacity of 4 times opacity tube 10. This material was labelled concentrated T3 antigen. Six ml. of the antigen were treated with the M.S.E. ultrasonic disintegrator. The sonicated material was then filtered through a Millipore VM filter and the filtrate retained. The deposit was resuspended in 6 ml. of distilled water of which 1 ml. was set aside and the remaining 5 ml. was washed 6 times with 15 ml. of distilled water by passing the material through a Millipore VM filter following thorough mixing using a homogenizer. The resulting washed debris was resuspended in

5 ml. of distilled water. The precipitin bands formed by the organisms before and after ultrasonication, the Millipore filtrate of the ultrasonicated organisms and the cell debris before and after washing were compared in the qualitative AGT using sheep x' and ox 6904 sera previously absorbed with normal pig and ox sera and pig serum respectively.

Examination of broth cultures for the presence of major precipitating antigens

In order to determine whether the major precipitating antigens elaborated by M. mycoides in vivo are also produced by the organisms when grown in artificial culture, 1 ml. of first passage broth culture of the T3 strain of organisms was sub-inoculated into 2 litres of tryptose broth medium. Samples of 1 ml. were removed every second day for 3 weeks from the same flask and stored at -25°C. These samples were then diffused in the qualitative AGT against sheep x serum and the precipitin bands produced by the different samples compared, and also the precipitin bands produced by certain samples were compared with bands produced by serum Oremit 2 and urine (pooled).

Comparison between the major precipitating antigens produced in culture by the virulent T3 and the avirulent KH3J strains of M. mycoides

Samples that had been collected from 10 day tryptose broth cultures of the T3 and KH3J strains of M. mycoides were diffused in the qualitative AGT against sheep x and x' sera and the precipitin bands compared.

RESULTS

Titration of hyperimmune sheep and ox sera

The results of complement fixation and tube and slide agglutination tests on various batches of hyperimmune sera are given in Table I.

Table I

Serological tests on sheep and ox hyperimmune sera : endpoint dilutions

Serum	CFT*	AT*	SAST*
Sheep 4	1:160	1:640	++++
" 5	1:160	1:1280	++++
" 6	1:80	1:640	++++
" x	1:160	1:1280	++++
" x' ✱	1:640	1:2560	++++
Ox 4460	1:160	1:640	++++
" 5888	1:640	1:2560	++++
" 6904	1:640	1:2560	++++

* CFT Complement fixation test
AT Agglutination test
SAST Slide agglutination serum test

✱ Sheep x' was reimmunized 5 months after the initial immunization

All sera possessed satisfactory CFT titres, with sheep x', ox 5888 and ox 6904 sera having the highest at 1:640. The tube agglutination titres were all higher than the CFT titres, sheep x', ox 5888 and ox 6904 sera again possessing the highest at 1:2560. Sheep 5 and sheep x sera had fairly high titres at 1:1280. All sera produced ++++ reactions in the SAST.

It was important to collect the serum samples 4 days after the last inoculation as samples collected 7 days after possessed considerably less precipitating antibody as judged by the qualitative AGT.

Examination of the precipitin bands produced by the various sera

All serum samples were examined in the qualitative AGT against lymph 6183, urine (pooled), pleural fluid and serum Oremit 2. The precipitin bands produced by the sheep sera were essentially similar in that they produced bands which joined up with one another. Differences were obvious, however, in that certain bands were more prominent with one serum than with another. This was particularly noticeable with sheep x and x' sera as they demonstrated a strong precipitin band close to the serum well that only showed up weakly with sheep 5 serum and not at all with sheep 4 (fig. 1) and sheep 6 sera.

Fig. 1. Demonstration of the additional precipitin band produced by sheep x serum

Wells	A	sheep 4 serum
	B	sheep x serum
	1,2	urine (pooled)
	3,4	lymph 6183

Sheep x serum appeared the most satisfactory with regard to the demonstration and separation of the bands. Sheep 5 serum was almost as good but failed to demonstrate the inside band as well. Sheep 4 serum produced poor separation of the bands, while sheep x' and sheep 6 sera produced precipitin bands too close to the central serum well for the bands to be distinguished easily.

The precipitin bands produced by the ox sera were less clearly defined, possibly due to the strong yellow colour of the serum. One of them, serum 5888, was particularly bad in this respect, showing only one large diffuse hazy band. Serum 4460 produced bands almost identical to those of sheep 5 serum, although one of the bands nearest the antigen well was slightly more prominent. Ox 6904 serum produced the sharpest of the ox serum bands, all of which joined up with the bands produced by sheep 5 serum. However, an inside band, that is one nearer the serum well, which was present with sheep 5 serum was absent from the bands produced by ox 6904 serum, and when this ox serum was compared with sheep x serum it was obvious that 2 inside bands were absent (fig. 2).

Fig. 2. Comparison between precipitin bands produced by sheep x and ox 6904 sera

Wells	A	sheep x serum
	B	ox 6904 serum
	1,2	lymph 6183
	3,4	urine (pooled)

Ox 6904 serum, while lacking in certain antibodies, produced an additional one to those present in sheep 5 and x sera. This addition consisted of a thick diffuse hazy band nearer the antigen well. It was not strong and its composition was difficult to determine and possibly consisted of more than one band.

Following the examination of the various batches of antisera, it was decided to use sheep 5 and sheep x sera for all routine work, as mixing the batches of sheep sera to form a pooled serum was not entirely satisfactory as the additional inside band demonstrated by sheep x serum did not show up well using the pooled serum.

Sheep x' serum occasionally produced, in addition to the bands already mentioned, a weak band on the antigen-well side of the main bands when it was diffused against lymph 6183 but not against any of the other body fluids. Furthermore, when this serum was diffused against concentrated T3 antigen, prepared for the investigation on the site of the antigens, at least 4 weak bands were seen on the antigen-well side of the main precipitin bands. These additional bands were termed minor precipitin bands for convenience. Ox 6904 serum also produced at least 4 minor precipitin bands when diffused against concentrated T3 antigen.

Examination of sheep antisera for anti-bovine and anti-porcine antibodies, and ox antisera for anti-porcine antibodies

It was necessary to examine the antisera for non-specific antibodies that might react with the bovine body fluids giving non-specific precipitin bands. If culture grown antigens were used, i.e. T3 antigen, it was also necessary to check for non-specific antibodies that would react with porcine antigens. This was necessary as the tryptose broth medium contained pig serum and organisms grown in this broth were used both for the production of antiserum as well as the antigen.

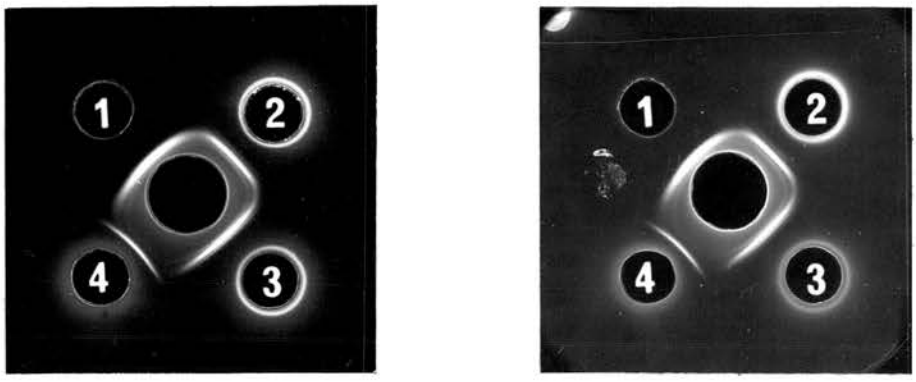
Two methods of examination were used. Firstly, the antisera were diffused against normal bovine or pig sera in the qualitative AGT and, secondly, 5 and 10 per cent. normal bovine or pig sera were incorporated in the agar during its preparation and an AGT performed using the sheep antisera diffused against the relevant body fluids. Control AGT using normal agar was performed simultaneously and the results of the two compared.

(a) Examination of antisera produced in sheep for anti-bovine antibodies

When the various antisera were diffused against normal ox serum in the qualitative AGT, no precipitin bands were produced by any of them except serum x' which gave a very faint band close to the antigen well.

When lymph 6183, urine (pooled), pleural fluid and serum Oremit 2 were diffused in the qualitative AGT in 5 and 10 per cent. ox serum agar and standard agar gel plates against sheep 5 and sheep x sera, the precipitin bands produced were identical. An example of this can be seen in fig. 3 where the body fluids were diffused against sheep x serum using standard and 10 per cent. ox serum agar plates.

/Fig. 3.....



(a) (b)

Fig. 3. Examination of sheep x serum for anti-bovine antibodies. Comparison between precipitin bands produced using:
(a) standard agar gel
(b) 10 per cent. serum agar gel

Centre well		sheep x serum
Wells	1	urine (pooled)
	2	pleural fluid Oremit 2
	3	lymph 6183
	4	serum Oremit 2

The bands formed in the 10 per cent. agar plate did not photograph as well as those in the standard agar plate due to the presence of residual serum which has not been completely removed by the washing process, and has stained slightly giving a darkened background.

When sheep x' serum was diffused against concentrated T3 antigen, Millipore filtrate and deposit of ultrasonicated T3 antigen, for the demonstration of minor antigens in 5 and 10 per cent. ox serum agar plates and normal agar plates, the precipitin bands produced were identical in the two ox serum plates but in the control normal agar plate there was an additional minor band near the antigen well in the case of the concentrated T3 antigen.

(b) Examination of sheep and ox antisera for anti-porcine antibodies

When sheep x' and ox 6904 sera were diffused against normal pig serum in the qualitative AGT, one distinct precipitin band was produced by sheep x' serum and 2 faint diffuse bands were produced by ox 6904 serum. None of the other sheep or ox sera produced any precipitin bands.

When sheep x' and ox 6904 sera were diffused against concentrated T3 antigen, Millipore filtrate and deposit of ultrasonicated T3 antigen in the qualitative AGT, for demonstration of minor antigens, in 5 and 10 per cent. pig serum agar and normal agar plates, the precipitin bands produced by sheep x' serum were identical in the pig serum plates but 1 minor band present in the normal plate was missing from the pig serum plates. Similarly the bands produced by ox 6904 serum were identical in the pig serum plates but the normal agar plate possessed 2 bands which were not present in the pig serum plates.

Examination of sheep and ox antisera for antibodies against medium constituents

This was a necessary control when culture grown antigens were used in the AGT and was performed by diffusing sheep x' and ox 6904 sera, that had been absorbed with normal pig serum, against concentrated yeast extract, tryptose broth before and after incubation at 37°C. for 3 days, and also against the minute deposit produced by passing the incubated broth through the Lister vaccine clarifier and taking the deposit up in the minimal quantity of N.saline. No precipitin bands were produced by any of these materials when diffused against the sera, although a slight haziness formed. Strong precipitin bands were produced by control lymph 6183 and T3 antigen.

Examination of fluids for viable M. mycoides

The fluids from 6 natural cases and 9 experimental cases of CBPP were examined, by serial dilution in tryptose broth, for viable M. mycoides. Details of the 50 per cent. endpoint dilution in broth obtained with each body fluid of each animal are given in tables II and III.

Table II

Titre* of M. mycoides in body fluids of natural cases of CBPP

Origin	Oremit				Suswa		Ndeiya
Number	1	2	3	4	1	2	1
Serum	ND	Nil	Nil	Nil	Nil	Nil	Nil
Plasma	ND	Nil	Nil	Nil	Nil	Nil	Nil
Blood cells	ND	Nil	Nil	Nil	Nil	Nil	Nil
Pleural fluid	9.76	7.76	5.24	5.50	5.76	2.76	ND
Urine	ND	Nil	Nil	Nil	Nil	Nil	Nil

ND Not done

* Titre expressed as the negative logarithm of the 50 per cent. endpoint dilution.

Table III

Titre* of M. mycoides in the body fluids of experimental cases of CBPP

Strain	T3			Orenit 1			S1		
Animal	6183	6501	6865	6930	6826	6748	7714	7785	7795
Serum	0.76	4.24	8.76	1.76	4.24	2.76	0.50	2.76	2.24
Plasma	1.0	4.24	8.76	Nil	4.24	5.24	0.24	3.0	2.24
Blood cells	Nil	0.24	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Lymph	9.0	7.76	9.5	5.5	4.59	7.24	6.0	7.0	3.24
Urine	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

* Titre expressed as the negative logarithm of the 50 per cent. endpoint dilution.

From the 6 natural cases of CBPP, viable M. mycoides were obtained from the pleural fluid only, while in the experimental cases the organisms were present in serum, plasma and lymph and in one case in lysed blood cells, but it is probable that insufficient washing of the cells was responsible for this result.

Examination of fluids for antibodies against M. mycoides

Each body fluid was examined for the presence of agglutinating, complement fixing and precipitating antibodies by the SAST, CFT and quantitative AGT respectively. Agglutinating antibodies were present in all the serum and plasma samples at +++ titre. Complement fixing antibodies were also present in all the serum and plasma samples at either +++ or ++++ titres as estimated by the method of Campbell and Turner (1953): in the natural cases all samples were ++++ except for the samples from Oremit 4, and in the experimental cases 6 of the samples were ++++. The qualitative AGT demonstrated no precipitating antibodies in any of the samples when diffused against T3 antigen.

Examination of fluids for precipitating antigens of M. mycoides

Each body fluid was examined for precipitating antigens of M. mycoides by the quantitative AGT using antisera prepared in sheep 5 and sheep x. On examination of the precipitin bands it was seen that at least 6 were present, some of which were much more prominent than others. This was particularly evident with sheep 5 serum, where 2 bands were very prominent. It was also evident that, with the dilutions used, a number of bands possessed identical endpoint titres. As, at this stage, an examination of the individual bands was not necessary but only an indication of the relative amount of total antigen present in each fluid, it was decided to limit the estimation to the two most prominent bands using sheep 5 serum. These 2 bands were present in all the fluids except urine, which possessed only one of them.

The results of the tests are given in tables IV and V.

Table IV

Quantitative AGT antigen titres* of body fluids from natural cases of CBPP

Strain	Oremit				Suswa		Ndeiya
Number	1	2	3	4	1	2	1
Serum	ND	8 16	N 8	N 2	32 64	16 32	8 32
Plasma	ND	8 16	N 8	N 2	32 64	8 32	8 32
Blood cells	ND	0	0	0	0	0	0
Pleural fluid	8 32	16 32	4 32	N 32	N 64	N 32	ND
Urine	ND	16	16	8	32	32	32

* Expressed as the reciprocal of the 50 per cent. endpoint dilution.

N Neat, i.e. undiluted

ND Not done

Table V

Quantitative AGT antigen titres* of body fluids of experimentally infected animals

Strain	T3			Oremit 1			S1		
Animal number	6183	6501	6865	6930	6826	6748	7714	7785	7795
Serum	16 64	128 256	32 256	64 128	32 128	64 256	8 64	16 128	32 128
Plasma	32 128	32 64	64 256	32 64	64 128	128 256	8 128	16 128	8 128
Blood cells	0	N 2	0	0	0	0	0	0	0
Lymph	32 128	16 64	32 64	32 256	32 64	128 256	32 256	32 256	16 64
Urine	128	512	16	512	64	1024	512	512	128

* Expressed as the reciprocal of the 50 per cent. endpoint dilution.

N Neat, i.e. undiluted.

Precipitating antigens were present in all fluids except lysed blood cells except in one instance when weak antigen was present, but this was probably due to plasma residue from insufficient washing. The precipitating antigens were present to higher titres in the fluids from the experimental animals except for those inoculated with the S1 strain. Antigen was consistently demonstrated in the urine of both the natural and experimental cases. In experimental cases, the titre of the antigen was particularly high, reaching 1:1024 in one instance.

Urine and serum samples were obtained from a further 52 cattle which had failed to withstand challenge by the subcutaneous method with T3 culture, 6 weeks after vaccination

with avianized CBPP vaccine, and had been destroyed in extremis. Only 2 samples of urine and 1 of serum failed to show precipitating antigens in the qualitative AGT.

An examination of the precipitin bands produced when the various body fluids were compared in the qualitative AGT against sheep 5 and sheep x sera indicated that at least 5 bands were present in urine (pooled). Two of the bands were only observed at certain dilutions of urine and were formed by a splitting of 2 bands which appeared as single bands in less diluted urine. Fig. 4 shows various dilutions of urine diffused against sheep x serum, the bands nearest the serum and antigen wells being the ones which split into 2 at certain dilutions.

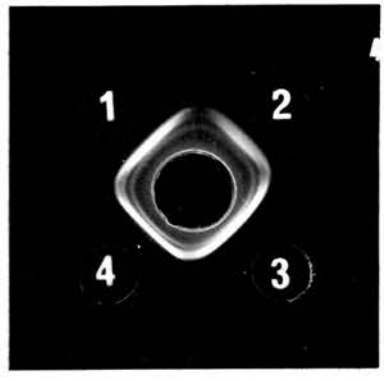


Fig. 4. Demonstration of precipitin bands produced by the diffusion of urine (pooled) at various dilutions against sheep x serum.

Centre well		sheep x serum		
Wells	1	urine (pooled)	diluted 1/4	
	2	"	"	" 1/8
	3	"	"	undiluted
	4	"	"	diluted 1/2

Identical precipitin bands were produced by all urine samples tested, from both natural and experimental cases of the disease. Examples of this can be seen in fig. 5.

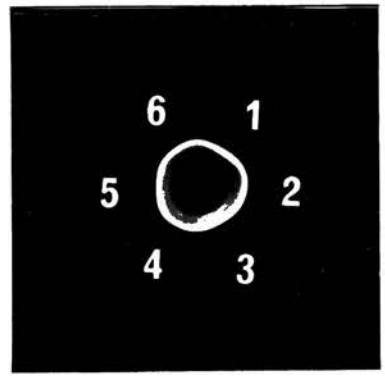


Fig. 5. Comparison between precipitin bands produced by urine from natural and experimental cases of the disease.

Centre well		sheep 5 serum
Wells	1	urine (pooled)
	2	" Oremit 2
	3	" Suswa 2
	4	" 6183 (T3)
	5	" 7785 (S1)
	6	" Ndeiya 1

Lymph and pleural fluid showed identical bands which included those present in urine and also an additional one nearer the antigen well (fig. 6).

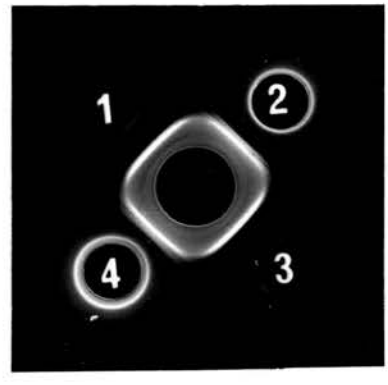


Fig. 6. Comparison between precipitin bands produced by urine, pleural fluid and lymph.

Centre well		sheep 5 serum
Wells	1	urine Suswa 2
	2	lymph 6183
	3	urine (pooled)
	4	pleural fluid Oremit 2

Serum and plasma of both experimentally and naturally-infected animals possessed bands identical to those of lymph and pleural fluid in most instances (fig. 7).

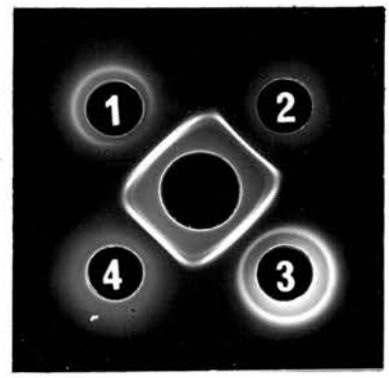


Fig. 7. Comparison between precipitin bands produced by serum, pleural fluid and lymph.

Centre well		sheep x serum
Wells	1	lymph 6183
	2	serum 6183
	3	pleural fluid Oremit 2
	4	serum Ndeiya 1

In certain sera and plasma, however, an additional band was evident nearer the antigen well. This band was first noted in the serum obtained from animal Oremit 2, and subsequently in various sera, namely 7795 and Suswa 2, but in these latter instances the band produced was weaker than in Oremit 2. An example of this extra band is shown in Fig. 8.

/Fig. 8.....

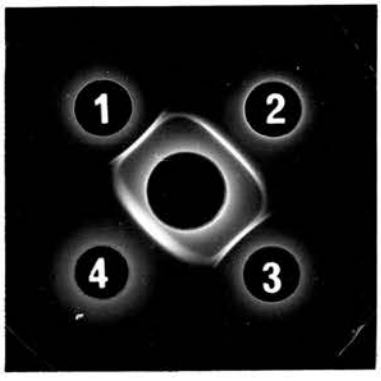


Fig. 8. Comparison between precipitin bands produced by different sera.

Centre well	sheep x serum
Wells 1	serum Oremit 2
2	" Ndeiya 1
3	" Oremit 2
4	" 7714 (S1)

The effect of heat on the major precipitating antigens

It proved unnecessary to use temperatures lower than 94°C. for 60 minutes as this combination had no apparent effect on any of the major precipitin bands. Figs. 9 and 10 show the effects of heat on these body fluids. The only difference noticed was that the precipitin band produced by heated serum Oremit 2 was slightly less dense than that produced by the unheated control material. Serum Oremit 2 unfortunately possessed insufficient antigen to produce precipitin bands near enough the serum well to cause them to join up with one another with the gel cutter in use. However, by using another cutter with wells closer together, the results still indicated that the precipitin bands were identical in the heated and unheated serum.

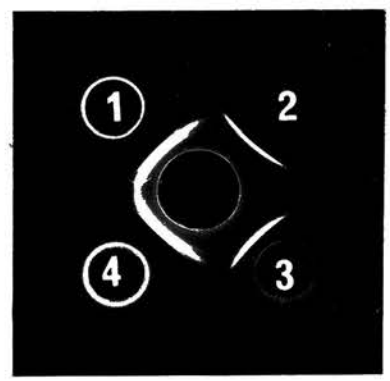


Fig. 9. Comparison between the precipitin bands produced by heated and unheated serum Oremit 2 and lymph 6183.

Centre well		sheep x serum
Wells	1	lymph 6183 unheated
	2	serum Oremit 2 unheated
	3	" " " heated
	4	lymph 6183 heated

/Fig. 10.....

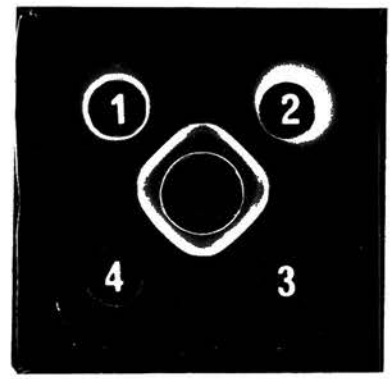


Fig. 10. Comparison between the precipitin bands produced by heated and unheated urine (pooled) and lymph 6183.

Centre well		sheep x serum
Wells	1	lymph 6183 unheated
	2	" " heated
	3	urine (pooled) heated
	4	" " unheated

The effect of heat on the minor precipitating antigens

The use of sheep x' serum, absorbed with normal pig and ox sera, in the qualitative AGT indicated that this temperature and time of heating destroyed the minor precipitating antigens present in lymph 6183 and concentrated T3 antigen. An example of this can be seen in fig. 11 where heated and unheated concentrated T3 antigen and heated and unheated ultrasonicated T3 antigen filtrate were diffused against absorbed sheep x' serum.

Fig. 11. The effect of heat on the minor precipitating antigens.

Centre well		sheep x' serum absorbed with normal pig and ox serum
Wells	1	concentrated T3 antigen unheated
	2	" " " heated
	3	filtrate of ultrasonicated antigen unheated
	4	filtrate of ultrasonicated antigen heated

The effect of trypsin on the major precipitating antigens

Trypsin appeared to have no effect on the precipitin bands produced in the AGT, except that the bands produced by serum Oremit 2 appeared less dense and those of urine appeared more distinct. Figs. 12 and 13 show the effect of trypsin on these body fluids.

Fig. 12. Comparison between the precipitin bands produced by trypsinised and untreated serum Oremit 2 and pleural fluid Oremit 2.

Centre well		sheep x serum
Wells	1	pleural fluid untreated
	2	" " trypsinised
	3	serum trypsinised
	4	" untreated

/Fig. 13.....

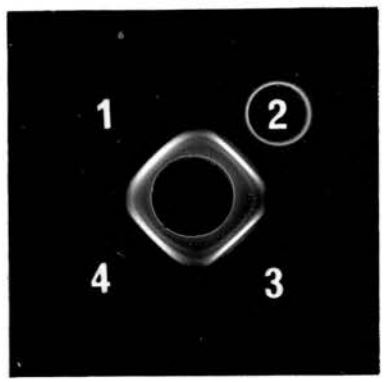


Fig. 13. Comparison between the precipitin bands produced by trypsinised and untreated urine (pooled) and pleural fluid Oremit 2.

Centre well		sheep x serum
Wells	1	urine untreated
	2	pleural fluid untreated
	3	" " trypsinised
	4	urine trypsinised

The effect of trypsin on the minor precipitating antigens

All but one of the minor precipitating antigens were destroyed by trypsin (fig. 14).

Fig. 14. The effect of trypsin on the minor precipitating antigens.

Centre well		sheep x' serum absorbed with normal pig and ox sera
Wells	1	concentrated T3 antigen untreated
	2	" " " trypsinised
	3	filtrate of ultrasonicated T3 untreated
	4	filtrate of ultrasonicated T3 trypsinised

Absorption of antiserum

Aliquots of sheep x' serum absorbed with normal pig and ox sera were further absorbed with concentrated T3 antigen, serum Oremit 2 antigen, lymph 6183 antigen and urine (pooled) and were examined by various serological tests, the results of which are given in Table VI.

Table VI

Absorption of hyperimmune sheep x' serum with concentrated T3 antigen, lymph 6183 antigen, serum Oremit 2 antigen and urine (pooled): results of serological tests

Serum	CFT*	AT*	SAST*	AGT*	
				Antigen present	Antibody present
Unabsorbed	1/320	1/640	++++	-	+
Absorbed T3	1/40	<1/4	-	+	-
" S 0.2	1/40	1/4	-	+	-
" U (P)	1/40	1/32	+	+	-
" L6183	1/40	<1/4	-	+	-

* CFT Complement fixation test
AT Agglutination test
SAST Slide agglutination serum test
AGT Qualitative agar gel test

The CF titres of the absorbed sera were lowered in all cases, from 1/320 to 1/40. The agglutination titres were reduced from 1/640 to 1/32 in the case of serum absorbed with urine, 1/4 for serum absorbed with serum Oremit 2 and less than 1/4 for sera absorbed with lymph and T3 antigens. In the SAST all the absorbed sera, except that absorbed with urine, were negative to

the test although the titre of the serum had initially been ++++. The serum absorbed with urine possessed a titre of +.

From these results it would appear that 87.5 per cent. of the CF antibody was removed from the serum by absorbing it with the antigens prepared from the 3 body fluids, and with concentrated T3 antigen. Almost all the agglutinating antibody was removed from the serum by absorbing with concentrated T3 antigen and lymph antigen, about 99.4 per cent. was removed by absorbing with serum antigen and about 95 per cent. by absorbing with urine antigen.

Examination of the various absorbed sera in the qualitative AGT against unheated homologous and heterologous antigens showed that T3 absorbed serum produced no precipitin bands against concentrated T3 antigen, urine (pooled), lymph 6183 or serum Oremit 2. Serum Oremit 2 absorbed serum produced no precipitin bands against serum Oremit 2 or urine (pooled), produced 1 band against lymph 6183 and 2 bands against concentrated T3 antigen. Urine (pooled) absorbed serum produced no bands against urine (pooled) or serum Oremit 2, produced 1 band against lymph 6183 and 2 bands against concentrated T3 antigen. Finally, lymph 6183 absorbed serum produced no bands against lymph 6183, urine (pooled) or serum Oremit 2 and 2 bands against T3 antigen. All the bands were faint compared to those produced by the unabsorbed serum and it was evident that they were minor antigens as they joined up with the minor antigen bands produced by concentrated T3 antigen when diffused alongside unabsorbed serum.

Site of the major precipitating antigens

The results of the qualitative AGT on the supernatant fluids + washings and deposits together with control untreated lymph 6183 and pleural fluid Oremit 2 are given in Table VII.

Table VII

Quantitative agar gel precipitin test titres of precipitin bands produced by supernatant fluid + washings, ultrasonicated deposits and control untreated lymph 6183 and pleural fluid Oremit 2 using sheep x serum.

Material	Titres* of precipitin bands				
Lymph untreated	8	32	64	128	128
Supernatant washings	4	16	32	64	128
Sonicated deposit	N	2	4	8	
P. fluid untreated	8	16	16	64	
Supernatant washings	8	16	32	64	
Sonicated deposit	N	2	4		

* Expressed as the reciprocal of the 50 per cent. endpoint dilution.

N Neat, i.e. undiluted.

The titres shown represent the titres of the recognisable precipitin bands, but those for one material cannot be correlated directly with those for another material. For example, the second titre for lymph 6183 is 1/32 whereas the second titre for the sonicated deposit of lymph 6183 is 1/2 but these need not necessarily represent the same precipitating antigen.

The number of bands or titres varied. Lymph 6183 untreated and its supernatant fluid + washings possessed 5 and pleural fluid Oremit 2 and its supernatant fluid + washings only 4. The sonicated deposits showed only 4 and 3 bands

respectively. Despite the limitations of the test as mentioned above, it can be seen that the titres for the pleural fluid supernatant fluid + washings are very similar to the control untreated material, while the titres for the lymph 6183 supernatant fluid + washings were slightly lower than the untreated material. The sonicated deposits showed 1 fewer precipitin band in each case and those produced were of a lower titre. The figures indicate that the greater part of the precipitating antigens are present in the supernatant fluid + washings and only a small amount in the deposits.

Whereas 4 and 3 precipitin bands were recognised with the sonicated deposits and 5 and 4 bands were recognised with the supernatant fluid + washings and untreated lymph and pleural fluid respectively, the qualitative AGT using sheep x' serum indicated that all the materials produced identical precipitin bands, as can be seen in figs. 15 and 16.

Fig. 15. Comparison between precipitin bands produced by supernatant + washings and ultrasonicated deposits of lymph 6183 and pleural fluid Oremit 2.

Centre well		sheep x' serum (absorbed with normal pig and ox sera)
Wells	1	lymph supernatant + washings
	2	lymph deposit
	3	pleural fluid deposit
	4	pleural fluid supernatant + washings

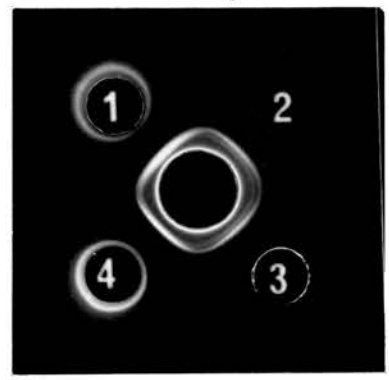


Fig. 16. Comparison between precipitin bands produced by ultrasonicated deposits and untreated lymph 6183 and pleural fluid Oremit 2.

Centre well		sheep x' serum (absorbed with normal pig and ox sera)
Wells	1	lymph untreated
	2	" deposit
	3	pleural fluid deposit
	4	" " untreated

It appears therefore that the absence of bands from the diffusion of any material in the quantitative AGT is purely a quantitative effect, the respective antigens being in insufficient quantity to produce precipitin bands even at the strongest concentration used.

From these results it appears that, in the body fluids, the greater part of the major precipitating antigens occur extracellularly. However, these identical precipitating antigens also occur in the bacterial cell but only in relatively small amounts.

Relationship between the extracellular major precipitating antigens and the elementary bodies of M. mycoides

The results of the quantitative AGT are given in Table VIII and these show that a considerable amount of the precipitating antigens are present in the filtrate.

Table VIII

Quantitative AGT titres of precipitin bands produced by Millipore VM filtered and unfiltered supernatant + washings of lymph 6183 and pleural fluid Oremit 2, using sheep x serum

Material	Titres of precipitin bands				
Lymph supernatant + washings	4	16	32	64	128
" filtered " "		8	32	64	128
P.fluid supernatant + washings	8	16	16	32	
" filtered " "	4	8	16	16	

The qualitative AGT, fig. 17, indicates that identical precipitin bands are produced by both the filtered and unfiltered fluids.

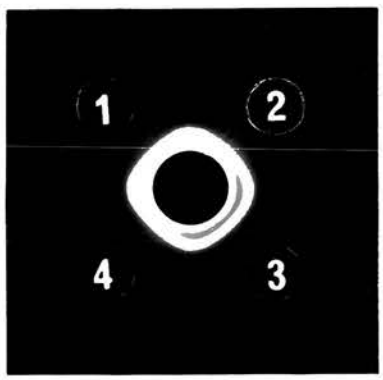


Fig. 17. Comparison between precipitin bands produced by Millipore filtered and unfiltered supernatant + washings of lymph 6183 and pleural fluid Oremit 2

Centre well — sheep x' serum absorbed with pig and ox sera

Wells
1 lymph supernatant + washings
2 pleural fluid supernatant + washings
3 " " filtrate
4 lymph filtrate

It appears, therefore, that a large part of the precipitating antigen is less than 50 mμ in size and as the elementary bodies of M. mycoides are 125-250 mμ in diameter (Freundt, 1958) it follows that these precipitating antigens are truly extracellular soluble antigens produced by the bacterial cell. There remains a certain amount of precipitating antigen which is associated with particles larger than 50 mμ and this may be contaminating deposit from the initial centrifugation or possibly elementary forms of the organism.

While the precipitin bands produced by the filtrates and unfiltered materials produced endpoint dilutions of very similar titre, the bands produced by the filtered materials were less dense and stained weaker with amido black.

Relationship between the intracellular major precipitating antigens and the cell debris

The washed and concentrated debris produced 2 good precipitin bands which, although weak, joined up with those produced by the filtrate (fig. 18).

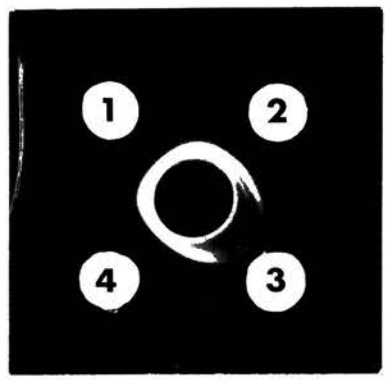


Fig. 18. Comparison between precipitin bands produced by washed and concentrated debris from lymph 6183 and the lymph filtrate.

Centre well		sheep x serum
Wells	1	urine (pooled)
	2	lymph filtrate
	3	washed and concentrated debris
	4	urine (pooled)

A third band was also present but was very weak. It appeared to join up also with those produced by the filtrate. At least 1 other band present in the filtrate did not occur in the debris in significant concentrations to produce a precipitin band. The 2 definite bands present in the debris also joined up with bands produced by urine (pooled) and therefore are not minor bands.

Site of the minor precipitating antigens

The qualitative AGT using sheep x' serum absorbed with normal pig and ox sera showed that the organisms before ultrasonication possessed at least 4 minor bands. The majority of these bands were faint, although the band nearest the antigen well became more hazy and intense the longer the diffusion proceeded until, after about 8 days, this band became so intense that it masked most of the other bands. After ultrasonication one very strong band was seen which apparently joined up with one of the weakest bands present in the untreated organisms. Also present with the ultrasonicated organisms were at least 5 weaker bands, the 2 nearest the serum well joining up with 2 in the untreated material while the other ones disappeared into the dense hazy band produced by the untreated organisms. Filtration of the ultrasonicated organisms removed all but 2 faint bands near the serum well. Washing of the deposit after filtration removed a considerable amount of the precipitating components. After concentration, however, it was seen that the debris contained, in addition to the expected major bands, 2 weak minor bands, possibly the ones that were present in the filtered ultrasonicated material. An example of the bands produced by the various materials diffused against sheep x' serum are given in fig. 19.

Fig. 19. Comparison between precipitin bands produced by concentrated T3 antigen before and after ultrasonication, the filtrate of the ultrasonicated antigen and washed and concentrated debris.

Centre well — sheep x' serum absorbed with normal pig and ox serum

Wells
1 ultrasonicated antigen
2 filtrate of ultrasonicated antigen
3 washed and concentrated debris
4 concentrated T3 antigen

The qualitative AGT, using ox 6904 serum, showed a similar picture. The initial material before ultrasonication possessed at least 5 precipitin bands, all of which were fairly distinct with one in particular being more prominent. After ultrasonication the bands produced were very similar although the 3 bands near the antigen well were stronger and the more prominent band in the untreated material had apparently split into two. The Millipore filtrate of the ultrasonicated organisms showed only 1 very hazy band apart from the major bands. The washed concentrated debris showed no minor bands. An example of the bands produced by the various materials can be seen in fig. 20.

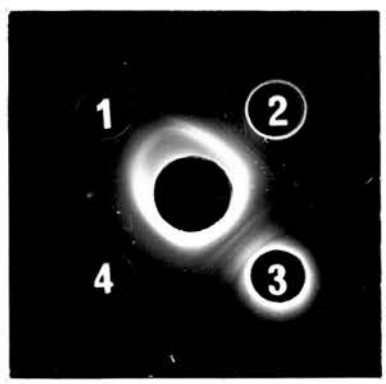


Fig. 20. Comparison between precipitin bands produced by concentrated T3 antigen before and after ultrasonication, Millipore filtrate of the ultrasonicated antigen and washed and concentrated debris.

Centre well		ox 6904 serum absorbed with normal pig serum.
Wells	1	concentrated debris
	2	concentrated T3 antigen
	3	" " " ultrasonicated
	4	Millipore filtrate

Examination of broth cultures for the presence of major precipitating antigens

Samples collected on the second day after inoculation gave weak precipitin bands. These became stronger in subsequent samples until the sixth day sample, after which the bands altered very little. An example of these bands can be seen in fig. 21.

Fig. 21. Comparison between precipitin bands produced by broth cultures of T3 M. mycoides after various lengths of incubation.

Centre well		sheep x' serum absorbed with normal pig and ox sera
Wells	1	day 14 sample
	2	" 21 "
	3	" 2 "
	4	" 6 "

Diffusion of 10 day T3 and KH3J samples against serum Oremit 2 and urine (pooled) showed that all the precipitating antigens present in the serum and urine were also present in the broth samples of both strains (fig. 22), indicating that the major precipitating antigens are produced in artificial culture.

/Fig. 22.....

Fig. 22. Comparison between precipitin bands produced by culture grown organisms and serum Oremit 2 and urine (pooled).

Centre well		sheep x serum
Wells	1	KH3J culture
	2	urine (pooled)
	3	T3 culture
	4	serum Oremit 2

Comparison between the major precipitating antigens produced in culture by the virulent T3 and the avirulent KH3J strains of M. mycoides

Comparison between the precipitin bands produced by 10-day samples of T3 and KH3J broth cultures showed that both strains produced identical precipitin bands (fig. 23).

Fig. 23. Comparison between the precipitin bands produced by the T3 and KH3J strains of M. mycoides grown in culture.

Centre well		sheep x serum
Wells	1	T3 culture
	2	urine (pooled)
	3	KH3J culture
	4	" "

SUMMARY

The agar gel double diffusion precipitin test was used to investigate the antigenic components of M. mycoides present in the body fluids. Since the bulk of the precipitating antigenic components were, however, apparently polysaccharide or polysaccharide-complexes in nature, its value was limited slightly by the fact that whereas protein antigens give sharp precipitin bands, polysaccharide antigens give more diffuse ones (Stacey and Barker, 1960). The precipitin bands were certainly rather hazy in outline which made the examination and evaluation of the results more difficult. However, by altering the concentrations of the various reagents a certain amount of separation could be observed especially at the extremities of the bands. Photographic reproduction of the bands proved slightly disappointing due to their hazy nature.

Sheep were used for the production of antisera for two reasons. Firstly as sheep are susceptible to infection with M. mycoides, it was thought possible that the antisera produced would contain antibodies against all antigens produced by the organisms growing in vivo and, secondly, for the ease of obtaining large quantities of serum.

Sheep injected intravenously with M. mycoides developed pyrexia and general malaise within a few days of the first injection but otherwise no other untoward effects were noticed. Whether this pyrexia was due to multiplication of the organisms or purely the effect of the inoculation of foreign material is unknown. Ox antisera were prepared with a view to comparing the precipitin bands produced by sheep and ox antisera as it was presumed that the antibodies produced in the ox would include the full complement of antibodies produced by in vivo growth in the natural host. The presence of the extra precipitin bands produced by the sera of sheep 5, x and x' and its absence from all other antisera, including ox, indicate, however, that the full complement of antibodies were not present in the ox

antisera, at least in sufficient quantity to be of use in the AGT. The rapid disappearance of precipitating antibody, as detected by the AGT, from the antisera could account for this situation as it was noticed during preliminary examinations of sera produced in sheep and cattle that the precipitating antibodies were present in the serum for only a limited time and samples collected 7 days after the last inoculation possessed considerably less precipitating antibody than samples collected 4 days after the last injection. As 12 months had elapsed since the challenge of the cattle and the undoubted growth of the organisms in the body and the subsequent inoculations for purposes of immunization, and as the route used for the inoculation of the organisms was the intravenous route, by which it is known that no ill effects are produced in cattle, it is probable that the antibodies present in the subsequent serum samples were not the products of in vivo growth as these had long ago disappeared but were the result of the intravenous inoculation of organisms which presumably did not multiply but were in a state of quiescence. It was not possible to overcome this disadvantage associated with the route of inoculation, as sera obtained from cattle naturally infected with M. mycoides possessed insufficient precipitating antibodies to be of use in the AGT even when the sera were concentrated or the globulins precipitated. Furthermore attempted immunization by the subcutaneous route failed to solve the problem as following the initial inoculation and subsequent swelling that formed, no further swellings were produced at the inoculation sites and the final serum possessed antibodies no different from those produced by the intravenous method and of lower titre.

The antisera produced in sheep appeared to detect all the major antigens detectable by the ox antisera and in the case of sheep 5, x and x' sera others as well, and it is possible in the case of sheep x that the organisms did in fact multiply in the animal but we cannot of course be certain. It is therefore evident that the antigens detected by either the sheep or ox antisera are not necessarily the full complement of antigens

involved in the *in vivo* growth of *M. mycoides*.

The major precipitin bands produced by the diffusion of sheep antisera against the various body fluids were evidently specific, in that the antisera were detecting antigens of *M. mycoides*. That these bands were not produced by bovine antigens reacting with anti-bovine antibodies in the sera was shown by the fact that normal ox serum diffused against the sheep antisera produced no precipitin bands and, furthermore, the diffusion of unabsorbed sheep antiserum in agar gels incorporating normal ox serum, or the diffusion of absorbed sheep antiserum in normal agar plates, produced no variation in the precipitin bands either qualitatively or quantitatively. A further indication that the major precipitin bands were not due to bovine factors was shown by the fact that the precipitin bands produced by the diffusion of sheep antisera against the body fluids joined up with bands produced by the diffusion of ox antisera against the same fluids. This did not of course apply in the case of the additional band near the serum well that was only demonstrated with sheep x, x' and 5 sera; but the other facts already mentioned apply equally to this band.

The specificity of the precipitin bands was shown by the fact that bands identical, in the sense that they joined up, to those produced by the diffusion of ox and pig absorbed sheep antisera against the body fluids were also produced by diffusion of the same sera against washed *M. mycoides*.

Viable *M. mycoides* were demonstrated only in the pleural fluid of natural cases of the disease, whereas in experimental cases viable organisms were demonstrated in serum and plasma as well as lymph of all cases except one where no viable organisms were detected in the plasma. In one instance some viable organisms were demonstrated in the blood cells, but it is likely that insufficient washing of the cells could have accounted for this. In the experimental cases there appeared to be no correlation between the number of organisms in the lymph and the number in the serum and plasma. It is of interest that no viable organisms were detected in the urine of either the

natural or experimental cases of the disease.

Precipitating antigen was present in all the body fluids in both natural and experimental cases of the disease, but none was present in the blood cells except in the one instance already mentioned in the case of viable M. mycoides. It was noted that the amount of antigen present, as estimated by the quantitative AGT, in the body fluids was much greater in the fluids from the experimental cases than from the natural cases except in the case of the cattle inoculated with S1 strain where the titres were not significantly different. The amount of precipitating antigen present in the different fluids of individual animals is of interest. In the natural cases generally speaking pleural fluid possessed the greatest amount of antigen, except in the cases of the Suswa animals where more antigen was present in the serum and plasma, although considerable antigen was also present in the pleural fluid. The urine possessed quite a considerable amount of one of the antigens. In the experimental cases, as already mentioned, the antigen titres were generally higher, and there appeared to be no correlation between the amount of antigen present in the lymph and the amount in the serum and plasma. The amount of antigen present in the urine of these animals reached a relatively very high titre in most instances, indicative of a concentration of the appropriate antigens, although there was no direct relationship between the amount of antigen in the urine and in the other fluids.

In certain of the body fluids, in particular urine, but also serum and plasma from the natural cases of CBPP, precipitating antigen was not associated with the presence of viable M. mycoides. This would indicate that either the antigen was associated with dead organisms and presumably the result of autolysis or was a soluble extracellular antigen. This point will be discussed later.

In the qualitative AGT, urine was seen to possess at least 5 distinct precipitin bands, lymph and pleural fluid at least 6 and serum and plasma at least 6 and sometimes 7. The 5 in urine were common to all the fluids, while the extra one in

lymph and pleural fluid was also present in the serum and plasma.

Antibodies demonstrated by the CFT and SAST were present in the serum and plasma samples of all the cattle, both naturally and experimentally infected. The qualitative AGT demonstrated that precipitating antibodies were not present in sufficient concentration to produce a precipitin band. The poor sensitivity of this test, however, would not detect small amounts of antibody if present. It is of interest that both precipitating antigen and antibody, as demonstrated by the CFT and SAST, were present in the serum and plasma at the same time. Furthermore, in the case of the experimental animals, viable organisms and precipitating antigens were also present in the serum and plasma together with antibody.

Absorption of sheep antisera with antigens prepared from the various body fluids showed that almost 100 per cent. of the agglutinating antibodies were removed by absorption with lymph 6183, 99.4 per cent. by absorption with serum Oremit 2 and 95 per cent. by absorption with urine (pooled), while all 3 antigens absorbed 87.5 per cent. of the complement fixing antibodies.

The major precipitating antigens were apparently resistant to boiling and trypsin and were, therefore, not protein in nature but probably polysaccharide, while the minor precipitating antigens were destroyed by boiling and all but one by trypsin and the majority, therefore, were probably protein.

The demonstration that considerable amounts of the precipitating antigens occurred in the supernatant fluid + washings of lymph 6183 and pleural fluid Oremit 2 indicated that the bulk of the precipitating antigens in these fluids were soluble and extracellular. The average centrifugal force was 57,300 g., and at this force the precipitation time for 100 mμ particles is 12.6 minutes, and the centrifugation was in fact carried out for 60 minutes, sufficient time presumably to deposit any particulate material. As the extracellular antigens were demonstrated in body fluids from animals severely

ill, it is impossible to say whether the antigens were the result of autolysis of the dead bacteria or truly extracellular products of intact bacteria, but presumably at the late stage of the disease many bacteria would be dead and lysed. We are, however, interested in the role the antigens play *in vivo* and whether they are autolysis products or not is immaterial. The demonstration that identical antigens to those in the supernatant fluid + washings were also present in the washed deposits shows only that autolysis could account for the presence of these antigens extracellularly. The results of the filtrations through the Millipore filters confirmed that the bulk of the antigens were soluble and also that they were not associated with the filterable form of the organisms, the so-called elementary bodies. The demonstration of the presence of at least 2 major precipitin bands associated with the cell debris after ultrasonication and washing showed presumably that these 2, at least, were probably associated with the structural framework of the organisms. The results of the AGT on the washed debris of the culture grown organisms indicated that all the major antigens were associated with the ultrasonicated washed debris.

There was apparently no difference between the major precipitating antigens produced by the different strains of *M. mycoides* when diffused against the sera used in this study. These sera were, of course, prepared from only one strain of the organism. Certainly the antigens present in the urine of all cases, both natural and experimental, were identical while the additional bands seen in lymph and pleural fluid were present in all such fluids. The additional band present in certain serum and plasma samples was not confined to animals infected with one particular strain but was associated with 3 different strains.

The minor precipitating antigens were only demonstrated with lymph from one strain of organism and against washed *in vitro* produced organisms of the same strain.

It was necessary to use organisms grown in artificial culture to demonstrate the minor antigens as insufficient

organisms were obtained from lymph or pleural fluid although a faint minor band was demonstrated occasionally with lymph 6183 using cutter No. 1802 or more consistently using cutter No. 1804, but as the wells were very close together using this cutter its uses were limited. The use of sera absorbed with the various body fluids facilitated the demonstration of the minor antigens using cutter 1804, and by this means it was seen that lymph 6183 possessed at least 1 minor antigen and concentrated T3 antigen possessed at least 2 minor antigens.

The use of organisms grown in culture added further complications to the serological picture as this introduced porcine antigens as the organism had of course been grown in broth culture incorporating pig serum. This complication did not arise when the antigenic materials were body fluids from cattle even though the antisera were produced by inoculation of organisms grown in the pig serum medium. The examination of antisera for anti-bovine antibodies was performed although the possibility of bovine antigens being inoculated into the sheep was very slight as this would have entailed carry over of bovine material from the original lymph used to inoculate the initial broth and although a precipitin band was produced by diffusion against ox serum it is probable that this band was in fact a band that is common to both ox and pig.

From the results of the examinations for anti-bovine and anti-porcine antibodies in the various sera, it was possible to conclude that for the demonstration of major antigens sheep x or sheep 5 sera should be used, bearing in mind the limitations of the latter, and they need not be absorbed with ox or pig sera provided culture grown organisms were not used as antigen. The demonstration of minor antigens could only be performed with sheep x' serum or ox 6904 serum, and it was necessary in both cases to absorb the sera with pig serum and in the case of sheep x' serum with ox serum also.

These minor bands were apparently associated with the organisms themselves as they were demonstrated with washed organisms. Whether these antigens also occur extracellularly

is unknown but they were not demonstrated with any of the body fluids except lymph 6183, which of course contained viable organisms. It was apparent that, with the sera used, the minor bands could only be demonstrated by the use of very concentrated suspension of the organisms and so it seems that the antigens are truly minor in that they are only present in small amounts.

At least 6 minor bands were demonstrated. One of them appeared to be associated with the contents of the cell as it was most distinct after ultrasonication. This antigen together with the 3 others located close to the antigen well were larger than 50 mμ as they failed to pass through a Millipore VM filter, while the 2 faint bands observed nearer the serum well were apparently smaller than 50 mμ as they were observed in the filtered material. As these minor antigens are not the object of further study in this thesis, it is proposed to leave more detailed examination of them to a later date.

All the major precipitating antigens were also produced by the organisms when grown in artificial culture medium, and those produced by fully virulent organisms were apparently identical to those produced by avirulent organisms.

We can conclude, therefore, that in this system urine possessed at least 5, lymph and pleural fluid at least 6 and serum and plasma at least 6 and sometimes 7 major specific precipitating antigenic components. The 5 in urine were common to all fluids, while the extra 1 in lymph and pleural fluid was also present in serum and plasma. In addition to the major precipitating components, minor ones, at least 6 in number, were also present and these appeared to be primarily associated with the organisms. The major precipitating antigens were probably polysaccharide in nature, while the majority of the minor antigens were probably protein.

Absorption of antisera with urine, which apparently contained only polysaccharide antigens, absorbed 87.5 and 95 per cent. of the antibodies responsible for the complement fixation and agglutination reactions respectively.

The major precipitating antigens were apparently predominantly extracellular with only small quantities present in the organisms. They were present in blood and inflammatory exudates in large quantities and the majority were excreted in the urine although 2 out of the 6 were apparently not, or only very slowly.

The major precipitating antigens were also elaborated by the organisms when grown in artificial culture medium, and those produced by fully virulent organisms were apparently identical to those produced by avirulent organisms.

PART 2

THE ISOLATION OF PRECIPITATING ANTIGENS FROM URINE

CONTENTS

INTRODUCTION

The previous work reported in Part 1 showed that specific precipitating antigens were present in the urine of cattle naturally and experimentally infected with Mycoplasma mycoides. These antigens were a product of the in vivo growth of the organisms.

The choice of urine for the initial antigenic isolation was governed by a number of factors. First, it was considered that urine would probably contain less non-specific contaminating material than, for instance, lymph or serum and this would therefore ease the task of purification. Second, the earlier work had shown that urine contained no minor antigens and 1 or 2 less major antigens, as estimated by the agar gel precipitin test, than any of the other body fluids and this would simplify the task of analysis. Third, it was apparent that the antigens present in the urine were of considerable serological significance, as they were able to absorb 87.5 per cent. and 95 per cent. of the complement fixing and agglutinating antibodies respectively from a hyperimmune serum. Fourth, the absence of demonstrable antibodies against M. mycoides in the urine precluded the possibility of subsequent isolation of complexes of antigen and antibody which otherwise might be a problem if other body fluids were used. Finally, consideration was given to the fact that type-specific polysaccharide material of considerable significance in pathogenesis, had been demonstrated in urine of patients with pneumococcal pneumonia, first by Dochez and Avery (1917) and later by Cruickshank (1938) and, as M. mycoides grown in vitro has been shown to contain a specific carbohydrate (Kurotchkin, 1937) and Buttery and Plackett (1960) isolated a galactan from washed suspensions of the organisms, it was thought that the material in the urine might possibly be of similar significance in the pathogenesis of M. mycoides as the soluble specific substance is in the pathogenesis of the Pneumococcus.

REVIEW OF THE LITERATURE

1. The use of body tissues and fluids for the study of bacteria and their products

Body exudates containing extracellular products of infecting pathogens were first studied by Bail and Weil (1911) who described the aggressive and immunizing properties of such fluids with particular reference to those from the anthrax bacillus. The first chemical fractionations of infected body tissues were carried out by Heidelberger and Avery (1923) who isolated the soluble specific substances of pneumococcus from urine of patients with type II pneumococcal infection. Later Cromartie, Watson, Bloom and Heckley (1947) and Watson, Cromartie, Bloom, Kegeles and Heckley (1947) prepared extracts of skin lesions from rabbits infected with anthrax and these were chemically fractionated for a protective antigen and an inflammatory factor. Similar extracts from streptococcal lesions in rabbits have also been studied (Watson and Cromartie, 1952). In all these studies interest centred on extracellular products of the infecting bacteria; the organisms themselves were not isolated from the body fluids and studied separately. In 1953, however, Smith, Keppie and Stanley isolated organisms from guinea pigs infected with anthrax and large quantities of body fluids were obtained which contained products excreted by the pathogen. The same method was used also with infections due to Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus aureus, Listerella monocytogenes and Pasteurella pestis (Smith, Keppie and Stanley, 1953; Keppie, Smith and Cocking, 1957). Similar work has also been carried out with the leprosy bacillus (Hanks, 1951; Gray, 1952), and Segal and Bloch (1956) obtained Mycobacterium tuberculosis in large numbers directly from the lungs of infected mice. Keppie, Fuller and Smith (1956, quoted by Smith, 1958) obtained sufficient Brucella abortus cells from infected bovine cotyledons for chemical work.

2. Isolation of bacterial polysaccharide antigens

Numerous methods have been used for the isolation of specific polysaccharides from bacteria. Often the isolation can be carried out very simply. Thus the nigeran of Aspergillus niger can be easily separated by cooling a boiling aqueous extract of the mould (Barker, Bourne and Stacey, 1953). The cellulose synthesised by Acetobacter acetigenum can be obtained by treating the membranes with water in a Waring blender, and treating the insoluble residue with 5 per cent. sodium hydroxide solution at 30°C. for 18 hours. After washing with water and acetic acid and then dialysing against water, the cellulose can be obtained in a very pure state (Barclay, Bourne, Stacey and Webb, 1954).

One of the earliest methods, devised by Boivin, Mesrobeanu and Mesrobeanu (1933), involved the extraction of acetone dried organisms with cold trichloracetic acid. This method has been used by Davies (1955) with many smooth forms of Gram-negative bacteria. He found that the extract was fairly pure and after ethanol fractionation consisted mainly of protein-polysaccharide-phospholipid antigen and free, specific, undegraded polysaccharide hapten which could be separated from one another in the ultracentrifuge at 100,000 g.

Morgan (1937) used diethylene glycol for the isolation of the somatic antigens of Shigella dysenteriae. The acetone treated organisms were extracted at 37°C. with diethylene glycol for 2 hours and then at 0°C. or 15-18°C. for 48 hours following vigorous shaking. The suspension was then centrifuged in a Sharples and the solution obtained was passed through a Berkefeld filter and dialysed against water. The resulting antigenic material was free from protein.

The isolation of the polysaccharides from Mycobacterium tuberculosis proved very difficult, principally due to the high lipid content of the organisms. As much fatty material as possible was extracted with organic solvents and then the residue was warmed with alkali to liberate the more firmly bound

lipids and enable the polysaccharides to pass into solution (Haworth, Kent and Stacey, 1948). Markowitz and Henderson (1958) used Trichlorotrifluoroethane, a water-immiscible reagent, to obtain the type-specific polysaccharide from Type I pneumococcus. The suspension of organisms was homogenised with the reagent and centrifuged. The upper aqueous layer which formed was treated three more times with fresh reagent and the polysaccharide recovered from the resulting material was free of protein.

Detergents have been used in the purification and separation of bacterial polysaccharides. Cetavlon (cetyltrimethyl ammonium bromide) was first used by Jones (1953) who showed that uronic acid containing polysaccharides was precipitated from aqueous solution on the addition of Cetavlon. Barker, Foster, Siddiqui and Stacey (1958) used this reagent in the purification of the acidic capsular polysaccharide of Aerobacter aerogenes.

Westphal, Luderitz and Bister (1952) devised a method for the isolation of specific lipopolysaccharides. The organisms were extracted with 45 per cent. phenol at 65-68°C. for 30 minutes and the solution cooled to 2°C. This brought about a separation of the aqueous and phenol phases. The lipopoly-saccharide plus some nucleic acid was present in the aqueous phase from which it was recovered by ethanol precipitation or by dialysis and freeze-drying. The nucleic acid could be removed by ammonium sulphate fractionation. The phenol layer contained mainly protein. This method was used by Buttery and Plackett (1960) for isolating the galactan from washed suspensions of Mycoplasma mycoides, more details of which will be given later.

Morgan and Partridge (1940) used two methods for the isolation of polysaccharide from an antigenic complex. In the first, the complex was treated with 1 per cent. (v/v) acetic acid at 100°C. for 4 hours in an atmosphere of nitrogen. The product of hydrolysis was then extracted with ether to remove

the phospholipid and the protein separated by centrifugation. After concentration the polysaccharide was precipitated with ethanol. In the second method, the antigenic complex was treated with formamide to extract the phospholipid component, and the protein part of the insoluble polysaccharide-protein complex was then destroyed by digestion with trypsin. Alternatively the protein component of the antigenic complex could be destroyed first with trypsin and the residual polysaccharide-phospholipid complex dissociated with formamide.

Miles and Pirie (1939) devised a method for removing the lipid-phospholipid mixture from the Brucella antigens by running a solution of the antigen into 10 times its volume of a mixture of equal parts of alcohol and ether containing 0.5 per cent. concentrated hydrochloric acid and the lipid-phospholipid free antigen was recovered by centrifugation.

This method was also used by Davies, Morgan and Mosimann (1954) with the 'Shiga' antigen.

The methods used for the isolation of the specific polysaccharides of the pneumococcus, apart from the method of Markowitz and Henderson (1958) already mentioned, are of interest in that the earlier methods used for the chemical fractionation were too severe for the isolation of the Type I pneumococcus polysaccharide in an undegraded form. These earlier methods (Heidelberger, Goebel and Avery, 1925) entailed concentration of the culture filtrates on a steam bath followed by alkali and acid treatment. Avery and Goebel (1933) showed that the alkali caused degradation of the acetyl groups in the polysaccharide and that the undegraded acetylated polysaccharide precipitated more antibody from horse anti-Type I serum which had already been absorbed with the degraded polysaccharide and in contrast to the degraded polysaccharide also induced active immunity in mice. Pappenheimer and Enders (1933) and Enders and Wu (1934) confirmed these findings by the use of an acid medium throughout the manipulations. Following this work less severe methods were employed for the isolation of these

polysaccharides. Heidelberger, Kendall and Scherp (1936) developed a method involving high speed centrifugation of the medium, precipitation of the polysaccharide from the concentrated solution by the addition of alcohol and sodium acetate and removal of protein by Sevag's method (1934), which entailed shaking with a chloroform-butanol mixture. Further purification was by precipitation with acidified saturated copper acetate as the copper salt to free it from contaminating glycogen. In 1950, Heidelberger, Macleod, Markowitz and Roe further improved the method of isolation. The organisms grown in broth culture were killed and precipitated by the addition of an equal volume of cold 95 per cent. ethanol. The sedimented organisms were then ground in acetate buffer pH 6.0 for 6 hours with stainless steel balls and the supernatant precipitated with 0.5 volumes of cold iso-propyl alcohol. The precipitate was dissolved in water and deproteinized by the Sevag method (Sevag, 1934), reprecipitated with iso-propyl alcohol and deproteinized again twice, dissolved in water, dialysed and finally lyophilized.

3. Isolation of the antigens of M. mycoides

Kurotchkin (1937) was the first to attempt the isolation of antigens of M. mycoides. He extracted 2 fractions from 5-day old cultures of the organisms, one a nucleo-protein and the other a carbohydrate. The organisms were collected by centrifugation for 2 hours at 3,000 r.p.m. in a centrifuge of which no details are given, and were resuspended in distilled water and left overnight in an ice-chest. Potassium hydroxide, 0.5 per cent., was added and the suspension which had become transparent and very slimy was recentrifuged. Glacial acetic acid was added to the clear supernatant and the floccular precipitate which formed was designated as nucleo-protein and was collected and dried. The supernatant fluid was then Seitz filtered, presumably through a sterilizing pad, and then precipitated with 5 volumes of 95 per cent. ethyl alcohol and the precipitate which formed was collected and dried. This was the carbohydrate fraction. No further purification processes were undertaken with either of the fractions.

Not until 20 years later was any further work done on the isolation of the antigens of M. mycoides. In 1957, Dafaala extracted two antigenic fractions (A and B) from organisms grown in culture for 4-7 days. The organisms were collected using a Sharples centrifuge and were resuspended in distilled water and stored in an ice chest pending extraction. In addition to fractions A and B, Dafaala also described a further product of the organisms, namely the residue which consisted of the cellular remnants after the extraction of fraction B. The residue was subjected to 2 successive washings, the first with 50 per cent. alcohol and the second with normal saline. The deposits were resuspended in normal saline and dilutions tested for complement fixation against a positive serum with negative results.

Fraction A was isolated by boiling the organisms in distilled water for 30 minutes. After cooling, the mixture was centrifuged at about 15,000 r.p.m. for 20 minutes and the clear supernatant was mixed with 2 volumes of absolute ethyl alcohol. The precipitate which formed (Fraction A) was collected by centrifugation and dissolved in normal saline, giving a white, translucent solution which precipitated when treated with alcohol or acetone. No details of the amount of alcohol or acetone are given.

Fraction B was isolated by mixing a cell suspension with 2 volumes of absolute alcohol (probably ethyl alcohol although it is not stated) and left at 37°C. overnight. The mixture was centrifuged until the supernatant was clear. The supernatant was then mixed with 3 volumes of acetone and left in the ice chest overnight. The precipitate which formed was mixed with 3 ml. of alcohol leading to the precipitation of contaminating fraction A which was removed by centrifugation. Fraction B was then reprecipitated from the supernatant with acetone and finally dissolved in normal saline.

Dafaala (1957) also performed serological tests on preparations obtained from lymph from swellings caused by intramuscular injections of virulent culture. The lymph (20 ml.)

was centrifuged at 15,000 r.p.m. for 30 minutes and the supernatant collected and the deposit resuspended in 20 ml. normal saline. The deposit was not washed. A further antigen was prepared from lymph by the addition of 2 volumes of alcohol, the precipitate being then dissolved in saline and boiled. Details regarding the type of alcohol, the volume of saline and the duration of boiling were not given. The coagulated protein was then removed on the centrifuge and the remaining fraction was concentrated by again precipitating with alcohol and redissolving in minimal quantities of saline. The actual volume of saline was not mentioned.

In this paper of Dafaala's no details are given of the complement fixation test nor of the positive serum used for the various tests. He also gave no details of the type of centrifuge nor the RCF values used.

Plackett and Buttery (1958) and Buttery and Plackett (1960) demonstrated a specific polysaccharide obtained from the 'V5' strain of M. mycoides. The organisms were grown in culture medium for 24-48 hours, washed in distilled water and then stored at -16°C. until required. After being subjected to 3 cycles of freezing and thawing, the organisms were centrifuged at 26,000 g. for 20 minutes. The deposit (cell residue) was washed and then heated with an equal volume of a phenol + water mixture (1:1) at 65°-70°C. for 45 minutes according to the method of Westphal, Luderitz and Bister (1952). After cooling the phases were separated by centrifugation and the phenol layer re-extracted with water at 65°-70°C. The phenol was removed by dialysis against distilled water. Removal of RNA was performed by ultra-centrifugation at 105,000 g. for 4 hours or by treatment with an ion-exchange resin. Earlier, washed suspensions of M. mycoides had been shown to contain about 10 per cent. by weight of carbohydrate and the only sugars detected on hydrolysis were galactose and ribose. The material extracted was shown to be a galactan. In a very recent paper, Villemot, Provost and Queval (1962) described some work carried

out with the galactan of Plackett and Buttery and also with an extract prepared by themselves from the T3 strain of M. mycoides. They state that they used a similar method of extraction as Plackett and Buttery, but gave no details except that the final centrifugation was at only 10,000 g. for 30 minutes. The pellet was freeze-dried and stored at -20°C. They also said that their extract was not purified as thoroughly as that of Plackett and Buttery and that it contained more ribo-nucleic acid.

MATERIALS

Urine

Urine was collected at autopsy from cattle which failed to resist challenge with first passage tryptose broth culture of T3 M. mycoides and which were killed in extremis. Six weeks before challenge the cattle had been injected with avianized CBPP vaccine. Each sample was first tested for the presence of precipitating antigen, by the qualitative AGT, and satisfactory samples were pooled. The quantitative AGT titre of the pooled urine using sheep 5 serum was 1/64, and using sheep x serum the 5 precipitin bands produced had titres of neat, 1/64, 1/64, 1/128 and 1/512.

METHODS

Dialysis

Dialysis was carried out at negative pressure in "Visking" cellophane tubing* 24/32" in at least 6 changes of distilled water at 4°C. over a period of at least 3 days. At each change of water the contents of the tubing were mixed. The volume of distilled water outside the sac was 100 times the quantity inside the tubing.

Demonstration of protein

Proteins were demonstrated by means of the Osborne modification of the Biuret test (A.O.A.C. methods of analysis, 1945).

Demonstration of Carbohydrates

Carbohydrates were demonstrated by means of the Molisch test (Hawk, Oser and Summerson, 1954).

Precipitation of Proteins from the Urine

Urine of ox 6183 (10 ml.) was dialysed and then saturated ammonium sulphate (at 4°C.) was added, with stirring, to the various concentrations, namely 30, 50, 58, 70 and 78 per cent. saturation. The mixture was left at 4°C. for 18 hours and then centrifuged at 2,000 r.p.m. for 45 minutes. The deposit was washed twice with the same concentration of ammonium sulphate as the final concentration in the urine, made up to 2 ml. with distilled water and finally dialysed against distilled water. The supernatant fluid remaining was used for the addition of further amounts of ammonium sulphate and the process repeated. Precipitates were formed by the addition of each concentration of saturated ammonium sulphate except the last when no visible precipitate was seen. Each deposit and the supernatant fluid after the removal of each deposit was examined in the

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* Visking Corporation, New York City, U.S.A.

qualitative AGT for antigen, using sheep 5 and sheep x sera. In addition, following the precipitation with 78 per cent. ammonium sulphate, the supernatant fluid was dialysed and concentrated to 10 ml., as it increased in volume considerably on dialysis, and tested by the Molisch and Biuret tests.

The Isolation and Purification of Polysaccharide Fractions

The method used for the isolation and purification of the polysaccharide fractions was essentially that of Heidelberger, MacLeod, Markowitz and Roe (1950) for the S1 fraction with modifications as proved necessary.

1. Preliminary Fractionisation

A 2,200 ml. volume of pooled urine was dialysed against distilled water and then concentrated by means of hair driers to 320 ml. It was then centrifuged at 2,500 r.p.m. for 60 minutes and the deposit discarded. The supernatant fluid was precipitated with 3 volumes of cold iso-propyl alcohol following the addition of 3 per cent. sodium acetate at pH 6.0. The alcohol was added with continual stirring and the mixture was then placed at 4°C. for 18 hours, after which the precipitate was removed by centrifugation. The supernatant fluid (A) remaining was stored at 4°C. The precipitate (precipitate A) was made up to 100 ml. with distilled water and deproteinized by the method of Sevag (1934) by the addition of chloroform and n-butanol to the solution in the proportion of 5:1:10 and the material mixed in an Atomix at 4°C. The resulting mixture was centrifuged at 2,500 r.p.m. for 60 minutes and the material was then seen apparently to consist of two layers, a greenish aqueous supernatant and a white semi-solid precipitate. The aqueous supernatant was removed and the remainder, however, was seen to consist of a large volume of white semi-solid material and under it a small volume of clear colourless liquid (chloroform). The chloroform layer was discarded. The emulsion (white semi-solid) layer was washed twice with 25 ml. of distilled water (Heidelberger, Kendall and Scherp, 1936) and stored at 4°C. The aqueous supernatant layer was deproteinized a further 14 times by the

same method, the emulsion layer being washed each time with the same washing water that had previously been used and stored at 4°C. This washing water itself was finally deproteinized 8 times by the same method, the emulsion layers produced being discarded however. Meanwhile the aqueous supernatant layer was reprecipitated with 3 volumes of iso-propyl alcohol after the addition of 3 per cent. sodium acetate. The supernatant fluid (B) resulting from centrifugation of the material was discarded. The precipitate (precipitate B) was deproteinized a further 3 times after the addition of the washing water from the previous deproteinization processes. This mixture was then reprecipitated by the addition of 1 volume of alcohol and the resulting precipitate, called C/1, removed. A further 1 volume of alcohol was then added to the supernatant fluid, giving precipitate C/2. The supernatant fluid from C/2 was then reprecipitated for the third time with a further 1 volume of alcohol and precipitate C/3 obtained.

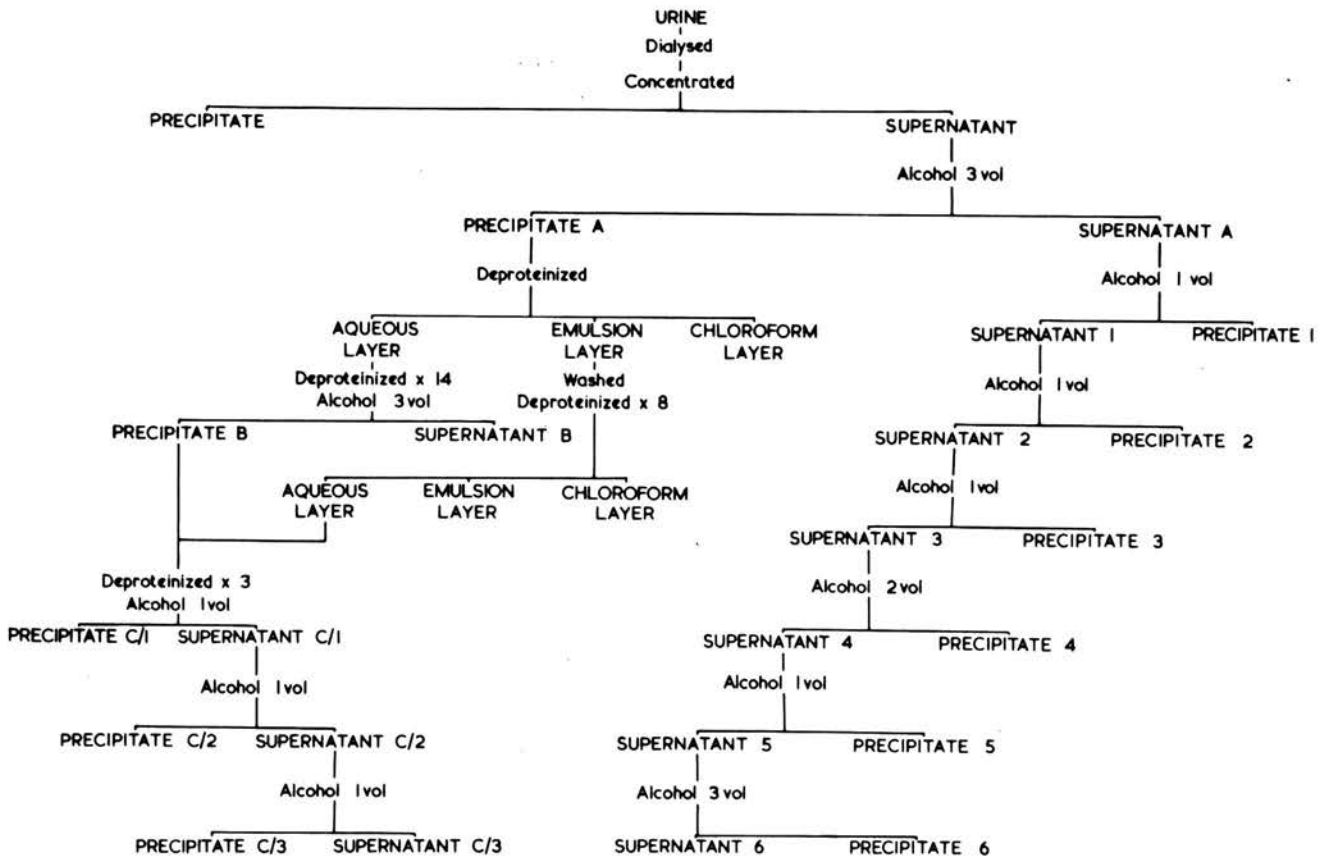
After numerous tests precipitate C/1, C/2 and C/3 were combined, deproteinized twice, dialysed and finally lyophilized, and labelled C/1 + C/2 + C/3.

Meanwhile supernatant fluid A, which had been stored at 4°C. was processed. A further 1 volume of cold iso-propyl alcohol was added and precipitate 1 was obtained. A further 1 volume of alcohol was added to the supernatant fluid from precipitate 1 and a further precipitate (2) was obtained. Precipitates 3 and 4 were obtained in an identical manner using 1 and 2 more volumes of alcohol respectively.

Each of the antigens (1, 2, 3 and 4) were deproteinized with chloroform and butanol until no emulsion layer formed, namely 3 times, twice, twice and once for each respectively. They were then dialysed and finally each was lyophilized.

The supernatant fluid from precipitate 4 was further precipitated (precipitate 5) with a further volume of alcohol giving a total of 8 volumes. The supernatant fluid from precipitate 5 was left standing for 3 weeks at 4°C. and was then mixed with a further 3 volumes of iso-propyl alcohol and a thick yellow floccular precipitate formed. A diagrammatic presentation of the process is given in fig. 24.

Fig. 24. Procedures in the isolation and purification of the polysaccharide fractions : Experiment 1.


URINE
Dialysed
Concentrated
PRECIPITATE
SUPERNATANT
Alcohol 3 vol
PRECIPITATE A
SUPERNATANT A
Deproteinized
Alcohol 1 vol
AQUEOUS LAYER
EMULSION LAYER
CHLOROFORM LAYER
SUPERNATANT 1
PRECIPITATE 1
Deproteinized x 14
Alcohol 3 vol
Washed
Deproteinized x 8
Alcohol 1 vol
PRECIPITATE B
SUPERNATANT B
SUPERNATANT 2
PRECIPITATE 2
AQUEOUS LAYER
EMULSION LAYER
CHLOROFORM LAYER
Alcohol 1 vol
SUPERNATANT 3
PRECIPITATE 3
Deproteinized x 3
Alcohol 1 vol
Alcohol 2 vol
PRECIPITATE C/1
SUPERNATANT C/1
SUPERNATANT 4
PRECIPITATE 4
Alcohol 1 vol
Alcohol 1 vol
PRECIPITATE C/2
SUPERNATANT C/2
SUPERNATANT 5
PRECIPITATE 5
Alcohol 1 vol
Alcohol 3 vol
PRECIPITATE C/3
SUPERNATANT C/3
SUPERNATANT 6
PRECIPITATE 6

2. Isolation and Purification of Fractions C/1, C/2 and C/3

In the second experiment, 2,200 ml. pooled urine was dialysed and concentrated as before. After precipitation with 3 volumes of alcohol the precipitate was removed, suspended in 400 ml. distilled water and deproteinized once by the Sevag method.

The solution was then reprecipitated with 1 volume of alcohol, and the precipitate resuspended in distilled water. This was then deproteinized 16 times, reprecipitated with 1 volume of alcohol and the precipitate resuspended and deproteinized a further 6 times. After one more reprecipitation with 1 volume of alcohol the precipitate was resuspended in distilled water, dialysed and finally lyophilized, and labelled C/1.

The supernatant fluid remaining after the removal of the precipitate formed by the addition of 1 volume of alcohol was treated with a further 1 volume of alcohol. The precipitate which formed was deproteinized 17 times, reprecipitated with 2 volumes of alcohol, resuspended in distilled water, dialysed and finally lyophilized and labelled C/2.

C/3 fraction was obtained by treating the supernatant remaining after the removal of C/2 with a further 1 volume of alcohol. It was only necessary to deproteinize the resuspended material twice before the fraction was reprecipitated with 3 volumes of alcohol, dialysed and lyophilized.

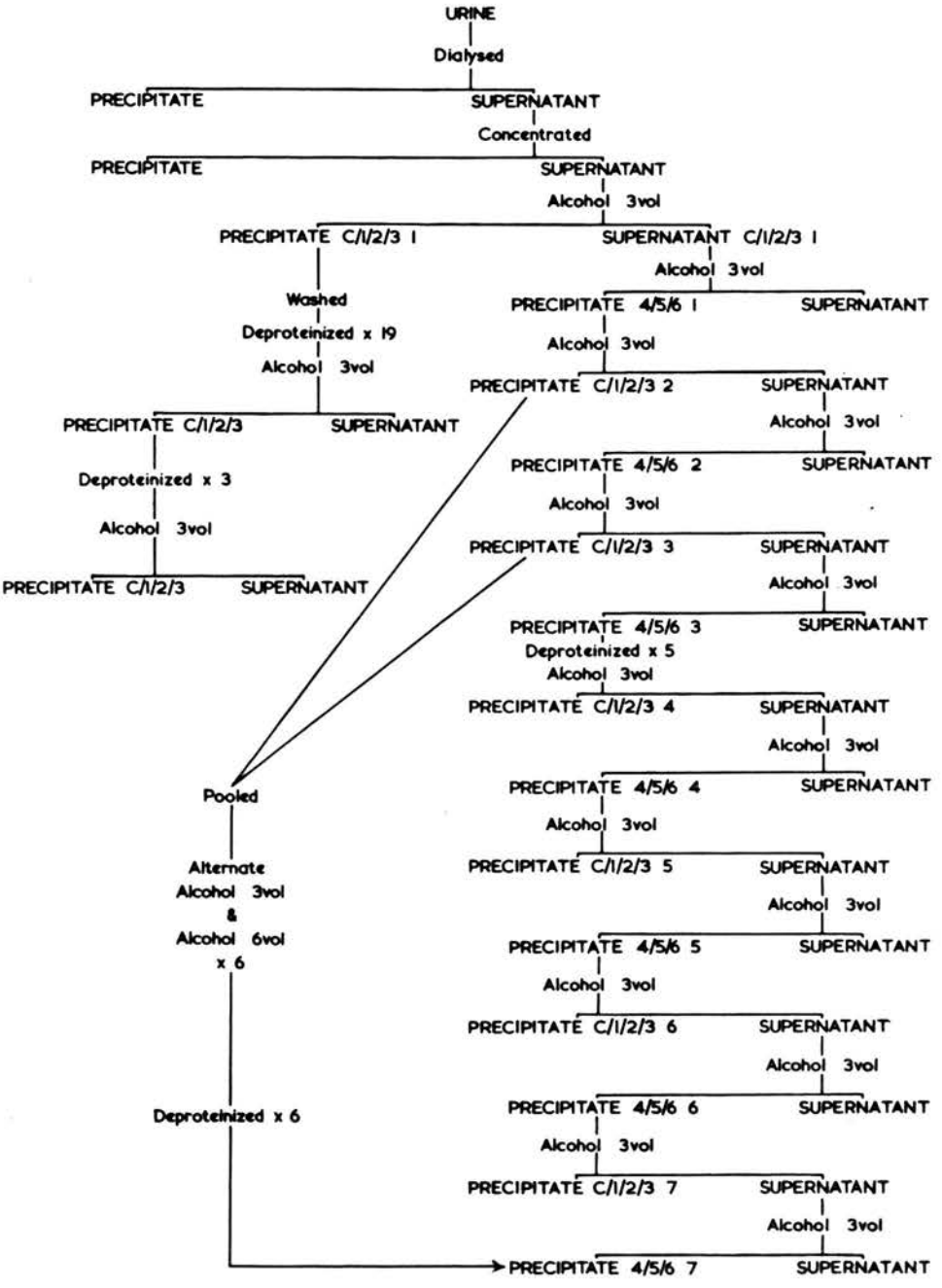
3. Isolation and Purification of Fractions C/1/2/3 and 4/5/6

In the third experiment, 2,200 ml. of pooled urine was processed in a similar manner. The urine after dialysis was centrifuged before concentration to remove the large amount of deposit that was present. This deposit was discarded. The supernatant fluid was concentrated to 325 ml. overnight and then recentrifuged, the precipitate being once again discarded. The supernatant fluid was precipitated with 3 volumes of cold

iso-propyl alcohol. The precipitate (C/1/2/3(1)) was washed twice in 3 volumes of alcohol in distilled water, then dissolved in 100 ml. distilled water and deproteinized with chloroform and butanol 19 times, reprecipitated with 3 volumes of alcohol, dissolved in 100 ml. distilled water, and deproteinized a further 3 times. The emulsion layers which had been washed as before and deproteinized 6 times were then added and the whole lot reprecipitated again with 3 volumes of alcohol. The resulting precipitate was deproteinized a further 4 times, concentrated from 200 ml. to 50 ml., dialysed and finally freeze-dried and labelled C/1/2/3.

The supernatant fluid from precipitate C/1/2/3(1) was precipitated with a further 3 volumes of cold iso-propyl alcohol and the supernatant fluid discarded. The precipitate (4/5/6(1)) was purified by dissolving it in 100 ml. distilled water and precipitating it with 3 volumes of alcohol, removing the precipitate which formed (C/1/2/3(2)) by centrifugation and reprecipitating the supernatant fluid with a further 3 volumes of alcohol (precipitate 4/5/6(2)). This process was repeated five more times giving fractions C/1/2/3(3), (4), (5), (6) and (7) and 4/5/6(3), (4), (5), (6), and (7), by which time no specific precipitate was formed on the addition of 3 volumes of alcohol. The final precipitate formed by 6 volumes of alcohol was deproteinized once with chloroform and butanol and then dialysed and finally freeze-dried and labelled 4/5/6(7). Precipitates C/1/2/3(2) and (3) were pooled and precipitated with alternate 3 and 6 volumes of alcohol 6 times, as above, deproteinized 6 times and added to the fraction 4/5/6(7) before dialysis. A diagrammatic representation of the process is given in fig. 25. Precipitates C/1/2/3(2) and (3) were dissolved in 20 ml. distilled water, precipitates C/1/2/3(4), (5), (6) and (7) were dissolved in 2 ml. distilled water. Precipitates 4/5/6(2) and (3) were dissolved in 100 ml. distilled water while 4/5/6(4), (5), (6) and (7) were dissolved in 20 ml. distilled water.

Fig. 25. **Procedures in the isolation and purification of the polysaccharide fractions : Experiment 3.**


URINE
Dialysed
PRECIPITATE
SUPERNATANT
Concentrated
PRECIPITATE
SUPERNATANT
Alcohol 3vol
PRECIPITATE C/1/2/3 1
SUPERNATANT C/1/2/3 1
Alcohol 3vol
Washed
Deproteinized x 19
Alcohol 3vol
PRECIPITATE 4/5/6 1
SUPERNATANT
Alcohol 3vol
PRECIPITATE C/1/2/3
SUPERNATANT
PRECIPITATE C/1/2/3 2
SUPERNATANT
Alcohol 3vol
Deproteinized x 3
Alcohol 3vol
PRECIPITATE 4/5/6 2
SUPERNATANT
Alcohol 3vol
PRECIPITATE C/1/2/3
SUPERNATANT
PRECIPITATE C/1/2/3 3
SUPERNATANT
Alcohol 3vol
PRECIPITATE 4/5/6 3
SUPERNATANT
Deproteinized x 5
Alcohol 3vol
PRECIPITATE C/1/2/3 4
SUPERNATANT
Alcohol 3vol
Pooled
PRECIPITATE 4/5/6 4
SUPERNATANT
Alcohol 3vol
Alternate
Alcohol 3vol
&
Alcohol 6vol
x 6
PRECIPITATE C/1/2/3 5
SUPERNATANT
Alcohol 3vol
PRECIPITATE 4/5/6 5
SUPERNATANT
Alcohol 3vol
PRECIPITATE C/1/2/3 6
SUPERNATANT
Alcohol 3vol
Deproteinized x 6
PRECIPITATE 4/5/6 6
SUPERNATANT
Alcohol 3vol
PRECIPITATE C/1/2/3 7
SUPERNATANT
Alcohol 3vol
PRECIPITATE 4/5/6 7
SUPERNATANT

Examination of fractions for antigens of M. mycoides

Fractions taken following each manipulation in the precipitation, isolation and purification processes were checked for antigens of M. mycoides by means of the qualitative AGT. The fractions were used either neat or following concentration and were put up against hyperimmune sheep 5 or sheep x serum.

Quantitative agar gel precipitin test

Polysaccharide fractions were titrated by the method given in Part 1 using sheep x serum and the initial concentration of each fraction was 1.0 mg./ml. except for fraction 4/5/6(7) which was 0.1 mg./ml.

Examination of fractions for precipitin bands produced by reaction with possible anti-bovine antibodies in the sheep sera

1. Fractions C/1, C/2, C/3, C/1/2/3 and 4/5/6(7) were diffused against sheep 5, x and x' in the qualitative AGT using 5 and 10 per cent. ox serum agar plates as well as normal agar plates as control and the precipitin bands compared.

2. Sheep x serum which had been absorbed with normal ox and pig sera was diffused against the various fractions in the qualitative AGT using normal agar plates.

Demonstration of the specificity of the polysaccharide fractions

M. mycoides (concentrated T3 antigen) and each of the polysaccharide fractions, at 0.1 mg./ml., were diffused against sheep x serum in the qualitative AGT and the precipitin bands produced by the fractions and the T3 antigen compared and examined for continuity.

RESULTS

Examination of the protein fractions

Precipitin bands were not produced by any of the protein fractions in the AGT. Precipitin bands were produced, however, by each supernatant fluid.

The final supernatant fluid, after concentration, was Molisch positive and Biuret negative.

Examination of the polysaccharide fractions

1. Preliminary Fractionisation

The addition of 1 volume of alcohol resulted in gross precipitation. Similarly with 2 and 3 volumes of alcohol. Very little precipitate formed, however, on the addition of the fourth volume and even less with the fifth and sixth. Eight volumes of alcohol brought down a considerable precipitate which was Molisch positive but only weakly antigenic. A thick yellowish precipitate formed with the addition of the twelfth volume of alcohol which was Molisch positive and Biuret negative but not antigenic in the qualitative AGT.

Each precipitate and supernatant fluid was diffused in the qualitative AGT against sheep 5 and sheep x sera. At least 4 precipitin bands were seen using sheep 5 serum and at least 6 bands using sheep x serum. These bands were labelled I, II, III, IV, V and VI respectively, numbered from the antigen well towards the serum well using C/1 and sheep x serum. Antigens I and II were present in precipitates C/1 and C/2 with a little of antigen II in C/3. Antigens III and IV were present in precipitates C/1, C/2, C/3, 1 and 2. Antigens V and VI were present in precipitates C/1, C/2, C/3, 1, 2 and 3 with a trace in precipitate 4. Antigens III and IV and antigens V and VI appeared to consist of single bands which on dilution split into two. The results of the qualitative AGT on the various precipitates are given in table IX.

/Table IX.....

Table IX

Precipitation of urine with iso-propyl alcohol - demonstration of precipitin bands formed by the various precipitates

Precipitate	Volume alcohol	Precipitin bands					
		I	II	III	IV	V	VI
C/1	1	+	+	+	+	+	+
C/2	1+1 = 2	+	+	+	+	+	+
C/3	+1 = 3	-	±	+	+	+	+
1	3+1 = 4	-	-	+	+	+	+
2	+1 = 5	-	-	+	+	+	+
3	+1 = 6	-	-	-	-	+	+
4	+2 = 8	-	-	-	-	±	±
5	+1 = 9	-	-	-	-	-	-
6	+3 = 12	-	-	-	-	-	-

An approximate estimation of the amount of precipitate C/1 + C/2 + C/3, after the separate precipitates had been combined, was 195 mg. and precipitate 1 was 84 mg. A little of these two materials was reconstituted at 11 mg. per ml. and was shown to be very strongly Molisch positive and Biuret negative.

2. Isolation and Purification of Fractions C/1, C/2 and C/3

The quantitative AGT using sheep x serum indicated that precipitate C/1 possessed 5 precipitin bands with the following titres: 1/4, 1/16, 1/32, 1/64 and 1/128. C/2 showed only 4 bands with titres of 1/32, 1/64, 1/128 and 1/1024 and C/3 possessed only 3 bands with titres of 1/4, 1/16 and 1/128.

Approximate estimations of the amount of the fractions obtained were C/1 90 mg., C/2 310 mg. and C/3 120 mg. of freeze-dried powder.

In the qualitative AGT using sheep x serum diffused against the various fractions at 0.1 mg./ml. concentration, C/1 was seen to consist of a single strong band which appeared to be split into two. C/2 produced a more diffuse band which was seen to be composed of at least 5 bands, while C/3 produced 5 distinct well separated bands. The bands produced by C/3 were examined carefully. The bands nearest the serum well, namely bands V and VI, were the most distinct and were seen to derive from the splitting of a single band. Bands III and IV were less strong and were very close together frequently giving the appearance of a single band, while a very faint band, band II, was seen on the antigen-well side of band III. Fig. 26 shows, not too clearly, the bands produced by these three fractions.

/Fig. 26.....

Fig. 26. Demonstration of precipitin bands produced by precipitates C/1, C/2 and C/3 diffused against sheep x serum.

Centre well		sheep x serum
Wells	1	C/1, 0.1 mg./ml.
	2	C/2, 0.1 mg./ml.
	3	C/3, 0.1 mg./ml.
	4	" " " "

With the bands produced by C/3 as the basis it was possible to conclude that fraction C/2 possessed at least 6 precipitin bands as it possessed all the bands present in C/3 as well as an additional one, band I. In the same way it was possible to say that fraction C/1 also possessed at least 6 bands as it possessed bands identical to C/2 although the different constituents were present in different concentrations, C/2 apparently possessing less of antigen I. On further dilution of fractions C/1 and C/2 separation of the various bands occurred and it was possible to confirm the conclusions reached above.

3. Isolation and Purification of Fractions C/1/2/3 and 4/5/6(7)

During the purification of fraction 4/5/6(7), the use of 3 volumes of alcohol removed antigens I and II as desired, but also unfortunately considerable amounts of the other antigens. Table X gives the details of the precipitin bands produced at different stages of the purification process.

Table X

Purification of fraction 4/5/6 by alternate 3 and 6 volumes of alcohol. Precipitating antigens present in the fractions

Alcohol 3 volumes	Alcohol 6 volumes	Precipitating antigens					
		I	II	III	IV	V	VI
C/1/2/3(1)		+	+	+	+	+	+
	4/5/6(1)	+	+	+	+	+	+
C/1/2/3(2)		+	+	+	+	+	+
	4/5/6(2)	+	+	+	+	+	+
C/1/2/3(3)		+	+	+	+	+	+
	4/5/6(3)	+	+	+	+	+	+
C/1/2/3(4)		+	+	+	+	+	+
	4/5/6(4)	±	±	+	+	+	+
C/1/2/3(5)		-	-	+	+	+	+
	4/5/6(5)	-	-	+	+	+	+
C/1/2/3(6)		-	-	-	-	-	-
	4/5/6(6)	-	-	+	+	+	+
C/1/2/3(7)		-	-	-	-	-	-
	4/5/6(7)	-	-	±	±	+	+

Following 5 precipitation processes the precipitate formed by 3 volumes of alcohol (C/1/2/3(5)) was found to contain no antigens I or II demonstrable by the qualitative AGT although traces of the other antigens were evident. The precipitate formed subsequently by 6 volumes of alcohol (4/5/6(5)) was found to be free of antigens I and II. Following 6 and 7 precipitations with 3 volumes of alcohol (C/1/2/3(6) and (7)), no precipitating antigens were demonstrated although a slight precipitate formed in both instances but they proved to be non-antigenic in the AGT. The precipitates formed by 6 and 7 precipitations with 6 volumes of alcohol, precipitates 4/5/6(6) and 4/5/6(7), proved to contain only antigens V and VI with traces of III and IV, and the purification process was therefore halted at this stage. Fraction 4/5/6(7) was freeze-dried and yielded 4 mg. of the dried powder. When this fraction was compared in the qualitative AGT with fractions C/1, C/2 and C/3 it was seen that it possessed only one strong precipitin band (fig. 27), which apparently consisted of antigens V and VI, although there were traces of antigens III and IV. These latter two were more clearly demonstrated using sheep x' serum.

Fig. 27. Comparison between precipitin bands produced by precipitates C/1, C/2, C/3 and 4/5/6(7), diffused against sheep x serum.

Centre well		sheep x serum
Wells	1	C/2, 0.1 mg./ml.
	2	C/1, " "
	3	4/5/6(7), 0.1 mg./ml.
	4	C/3, 0.1 mg./ml.

The quantitative AGT performed on fraction 4/5/6(7) showed only 2 precipitin bands with titres of neat and 1/2, but due to insufficient material this fraction was used at an initial concentration of only 0.1 mg./ml.

Precipitate C/1/2/3 consisted of approximately 175 mg. of dried powder and in the quantitative AGT produced 5 precipitin bands with titres of 1/4, 1/16, 1/32, 1/64 and 1/256.

Examination of fractions for precipitin bands produced by reaction with possible anti-bovine antibodies in the sheep sera

1. The precipitin bands produced in the 5 and 10 per cent. ox serum agar plates were identical to those produced in the control normal agar plates.

2. Sheep x serum absorbed with ox and pig sera produced precipitin bands identical to those produced by unabsorbed serum. An example of the bands produced by C/2 and C/3 using the absorbed serum can be seen in fig. 28.

Fig. 28. Comparison between precipitin bands produced by precipitates C/2 and C/3, urine (pooled) and T3 antigen.

Centre well		sheep x serum absorbed with normal pig and ox sera
Wells	1	C/3, 0.1 mg./ml.
	2	urine (pooled)
	3	C/2, 0.1 mg./ml.
	4	*M. mycoides* T3 concentrated antigen

Demonstration of the specificity of the polysaccharide fractions

The precipitin bands produced by the fraction were seen to join up with the major bands produced by M. mycoides (concentrated T3 antigen) as can be seen in fig. 28.

SUMMARY

Precipitation with cold iso-propyl alcohol and deproteinization with chloroform-butanol as a means of isolating the polysaccharide material from the urine was chosen because of its extreme mildness. It was hoped that the material so obtained would closely resemble the polysaccharide material as it existed in the animal host.

Precipitation with 1, 2 and 3 volumes of alcohol produced considerable precipitate while only very little formed with 4, 5 and 6 volumes. These precipitates contained material which specifically precipitated with sheep antiserum. Although considerable precipitates were again obtained with 8 and 12 volumes of alcohol, these materials proved to be either very weakly antigenic or not at all and presumably consisted mainly of non specific polysaccharide material excreted in the urine.

Diffusion of the fractions obtained from the urine against hyperimmune sheep sera in the qualitative AGT produced a total of 6 precipitin bands which presumably indicate that the polysaccharide material consists of at least 6 different antigenic materials. Certainly at least 3 different materials were indicated by the variation in precipitation with alcohol, as antigens I and II were precipitated by 1, 2 and 3 volumes, while antigens III and IV were precipitated by 1-5 volumes and antigens V and VI by 1-8 volumes. The demonstration of 6 precipitin bands in the isolated material and the demonstration of only 5 in the urine, as shown in Part 1, was probably a quantitative effect as it was shown that the precipitin bands produced by the various fractions joined up with those produced by urine, and that the fractions possessed no additional bands.

An attempt to obtain a fraction free from antigens I and II proved difficult as precipitation with 3 volumes of alcohol removed a considerable amount of the antigens we wished to retain as well as antigens I and II and the final relatively pure product weighed only 4 mg.

It was thought advisable to check once again the specificity of the precipitin bands produced. The fact that identical precipitin bands were produced in 5 and 10 per cent. ox serum agar plates and in control normal agar plates, and also by sheep antiserum that had been absorbed with ox and pig sera indicated that the precipitin bands produced by the diffusion of these fractions and the sheep antiserum were not due to the interaction of antibovine antibodies and bovine antigen and furthermore these bands were specific for M. mycoides as they joined up with bands produced by M. mycoides.

We can conclude by saying that by precipitation of urine with increasing volumes of iso-propyl alcohol and removing the precipitate at each step, deproteinization with chloroform and butanol and final lyophilization, a number of fractions were obtained which produced specific precipitin bands in the AGT when diffused against hyperimmune sheep sera, and were Molisch positive and Biuret negative. The number of precipitin bands produced by the different fractions depended on the amount of alcohol used for the precipitation. One and 2 volumes of alcohol produced fractions (C/1 and C/2) which gave 6 precipitin bands, while 3 volumes (C/3) gave 4 bands or occasionally 5, the latter being very weak. Four volumes (fraction 1) and 5 volumes (fraction 2) produced 4 bands, 6 volumes (fraction 3) gave 2 bands and 8 volumes (fraction 4) gave 2 very weak bands. Fraction C/1/2/3, the result of precipitation with 3 volumes of alcohol, gave 6 precipitin bands, whereas fraction 4/5/6(7), the result of precipitation with 6 volumes of alcohol followed by alternate precipitation with 3 and 6 volumes, gave 2 strong and 2 weak precipitin bands. For convenience these precipitin bands were numbered from I to VI from the antigen-well towards the serum-well respectively, using fraction C/1 and sheep x serum as the template.

PART 3

PROPERTIES OF THE MAJOR PRECIPITATING ANTIGENS ISOLATED FROM URINE

CONTENTS

INTRODUCTION

Polysaccharides may occur as separate entities in micro-organisms. They also occur in complexes, such as lipopolysaccharides, which are notable for their pyrogenic properties, or as antigenic complexes which are composed of firmly bound polysaccharide-protein-lipid.

The large majority of antigenic substances are protein in nature and it was earlier thought that substances other than protein could not function as antigens on their own but had to be in combination with protein to be antigenic. It is now known, however, that the purified type-specific capsular carbohydrates of the pneumococci can induce active immunity in mice (Avery and Goebel, 1933) and man (Francis and Tillet, 1930) although they are not antigenic in the rabbit (Avery and Goebel, 1933) or guinea pig (Maurier and Mausmann, 1958). Subsequent studies have also shown that dextrans (Kabat and Berg, 1952), levans (Allen and Kabat, 1957) and the Vi antigen of E. coli (Landy, 1954), all polysaccharides composed entirely of a single sugar, are also antigenic in man.

The importance of polysaccharides in immunochemistry was first shown by Heidelberger and Avery (1923) who demonstrated that virulent pneumococcal capsular material, which was responsible for both virulence and the specificity of the different types of pneumococci, was composed of polysaccharide material. They also showed that the polysaccharide differed for each of the numerous serological types of the organism. Following this work it was soon shown that polysaccharides were the determinant molecules responsible for the immunological specificity of many types of bacteria.

Polysaccharides and their complexes frequently exhibit biological activity. As already mentioned, lipopolysaccharides are notable for their pyrogenic properties. For example, the lipopolysaccharide of Pasteurella pestis (Davies, 1956) is

strongly pyrogenic, relatively non-toxic and is a hapten, but when it is combined with 'Shiga' conjugated protein, polysaccharide specific antibodies are produced. The lipopolysaccharides of the Entero-bacteriaceae (Westphal, Luderitz, Eichenberger and Keiderling, 1952) were also strongly pyrogenic and had a toxicity in mice of the order LD_{50} = 100 µg. The comparative toxicity of the polysaccharide from P. pestis was, however, LD_{50} = 5-10 mg. Davies, Morgan and Record (1955) obtained 3 polysaccharide preparations from Shigella dysenteriae, the degraded polysaccharide hapten was neither toxic nor antigenic but was pyrogenic in relatively large doses (2-5 µg/kg.). The undegraded polysaccharide was poorly toxic, the mouse LD_{50} being 400 µg., weakly antigenic and less active as a pyrogen than the lipopolysaccharide (0.05 µg/kg.). The lipopolysaccharide was toxic (mouse LD_{50} 80 µg) and was a strong heterophile antigen but weakly active in inducing the formation of specific agglutinating and precipitating antibodies in the rabbit. The lipopolysaccharide was a very powerful pyrogen active at 0.002 µg/kg. in the rabbit.

A further property or function of polysaccharide antigens is their aggressive action. Aggressins are generally soluble bacterial haptens or antigens, or similar components still attached to the bacterial cell, which act specifically by uniting with the corresponding antibody and so preventing the effective sensitization of the viable bacterial cell. Many of them are thermostable and are usually associated with the virulent form of the bacteria and are often derived from the bacterial capsule. The aggressive action of the pneumococcal polysaccharides in human infections was suggested by the work of Cole (1917) and Park and Cooper (1928), who recorded that a fatal outcome in cases of lobar pneumonia in man was frequently associated with the presence of specific soluble antigen in the blood in amounts greater than could be neutralized by the antibodies that the patient had produced. Nye and Harris (1937) calculated that the specific polysaccharide present in the lungs of certain patients dead of pneumococcal pneumonia

would require more than 60 litres of a good antiserum to neutralize it. Frisch, Tripp, Barrett and Pidgeon (1942) found an average of 0.033 g. of polysaccharide per 100 g. of lung tissue in areas of grey hepatization due to Types I, II, VII and VIII pneumococcus and in 10 cases of Type III pneumococcal pneumonia an average of 1.574 g. per 100 g., the maximum being 5.28 g. which corresponds to a total of 68.5 g. of specific polysaccharide in the lung of a fatal case. This amount is serologically equivalent to 750 g. of antibody nitrogen and it represents a reservoir of aggressin in the lesion of sufficient size to account for the failure of serum therapy in Type III pneumonia. In the case of CBPP it is of interest that Nakamura, Futamura and Watanuki (1926) found that both the normal and infected portions of CBPP lungs contained precipitating antigen.

The neutralization of protective antibody by soluble antigens is unlikely, however, to have any effect in susceptible animals until antibodies are produced in response to infection. A further action of soluble antigen is possible which may affect the very early stage of infection, namely the paralysis of antibody formation by excess and overwhelming amounts of antigen (Felton, 1949).

Extracellular bacterial polysaccharides possess certain properties which have a function in preserving bacteria in their natural habitat which do not depend on the fact that the polysaccharide is a hapten or complete antigen as the case may be. It is probable that the functions are related to two properties which the majority of these polysaccharides have in common, namely they are very hydrophilic and also they give the cell surface a characteristic charge (Wilkinson, 1958). Possible functions include protection against phagocytosis, amoebic attack and bacteriophage, protection against desiccation, a reserve carbon and energy source, an aid to the uptake of ions, an aid to dispersal and finally they may act as endotoxins increasing the invasiveness of the organism (Wilkinson, 1958).

The major precipitating antigens, isolated from the urine of cattle infected with M. mycoides as described in Part 2, were examined primarily from the biological aspect in an attempt to obtain information on the role, if any, that they may play in the disease process. Secondarily these antigens were submitted to a limited biochemical study in order to obtain some information on their structural characteristics.

REVIEW OF THE LITERATURE

Properties of the Antigens of M. mycoides

Kurotchkin (1937) was the first to isolate and characterise any antigenic components of M. mycoides. His nucleo-protein fixed complement but failed to give a precipitin reaction with antiserum against M. mycoides prepared in rabbits by the injection of washed organisms. The carbohydrate fraction gave negative Biuret and positive Molisch reactions and both fixed complement and gave a precipitin reaction. This carbohydrate fraction could be detected in the blood of naturally infected animals by the precipitin test. Moreover antibodies to it could also be detected (Kurotchkin and Bernaradsky, 1938).

Dafaala's fraction A (1957) precipitated but did not fix complement and was insoluble in alcohol. It was antigenic when injected into mice and there was some evidence that it neutralized the bactericidal action of immune serum. It occurred in the free state in pleural exudate and lymph and sometimes in the bloodstream of infected animals. Fraction B was always attached to the bacterial cell and did not occur free. It was alcohol soluble and specifically precipitated and fixed complement and it was antigenic when injected into rabbits. No chemical studies were made on either of these fractions.

White (1958) who first applied the agar gel double diffusion precipitin test to the study of M. mycoides showed that the 3 precipitin bands, demonstrated by him, were not destroyed by 5 per cent. formalin or 50 per cent. glycerin. He did not undertake any isolation, purification nor further characterization of these antigens.

The specific polysaccharide (galactan) isolated from M. mycoides by Plackett and Buttery (1958) and Buttery and Plackett (1960) has been extensively studied from the biochemical angle. In the first of their papers the authors reported that

the carbohydrate contained small and variable amounts of nitrogen (0.2-0.8 per cent.) and traces of phosphorus (<0.1 per cent.). After hydrolysis in 0.5N. sulphuric acid for 2 hours at 100°C., 85-89 per cent. of the material was accounted for as reducing sugar (calculated as anhydrohexose). Galactose was the only sugar detected on paper chromatograms. No antibody formation could be detected following injection of the galactan into rabbits. No details, however, were given regarding the dose or route of injection or the method of detecting antibody. In the second paper they reported that the final fraction contained less than 0.5 per cent. nucleic acid, was readily hydrolysed by acid releasing galactose and about 4 per cent. $CHCl_3$-soluble lipid. The intact polysaccharide showed a large negative rotation and the equilibrium value after acid hydrolysis and removal of the lipid fraction showed that at least 97 per cent. of the sugar was D-galactose. The polysaccharide was not pyrogenic when injected into rabbits at doses up to 1 μg/kg. body weight. It gave positive precipitin reactions with M. mycoides antisera, sensitised sheep erythrocytes to agglutination by the antisera, and itself inhibited the agglutination of sensitised erythrocytes by the antisera (Cottew, 1960). No pretreatment of the polysaccharide was required for sensitization of the sheep erythrocytes, while heating for 1 hour at 56°C. in 0.25N. NaOH destroyed the capacity to sensitize the cells as well as the capacity to inhibit the agglutination.

By means of cross sero-agglutination tests, agar precipitin tests and the study of the bactericidal action of antibodies, Villemot and Provost (1959a) reported that M. mycoides contains at least 3 distinct antigenic fractions, confirming the findings of White (1958). They also reported that M. mycoides possessed antigenic fractions common to M. capri, M. laidlawi, M. bovigenitalium and M. hominis. In a subsequent paper (Villemot and Provost, 1959b), they prepared antigenic formulae, similar to the Salmonella formulae, for the various species of Mycoplasma by means of the agar precipitin

test, the tube agglutination test and the agglutinin absorption test. M. mycoides, as they had previously shown, possessed 3 antigens, antigen 2 being present in the largest proportion, antigen 4 in the next largest proportion and antigen 1 in the least. In a later report, Provost (1960), it was stated that all the fractions gave rise to agglutinating antibodies, and antigen 2 was suspected of producing protective antibodies because M. laidlawi, which had antigen 2 in common with M. mycoides, if inoculated into cattle induced protection of the cattle to subsequent inoculation with infected CBPP lymph. The antigens described by Villemot and Provost were not isolated and therefore no chemical studies were carried out.

Villemot, Provost and Queval (1962) reported that their endotoxin induced pyrexia and leucopenia followed by leucocytosis in rabbits injected intravenously with doses varying from 1-500 µg. In cattle, fever and blood changes were reported and the intravenous injection of 2 mg. of the product produced sudden dramatic stress and collapse. No further details are given. These authors also reported that the galactan of Plackett and Buttery was pyrogenic in large doses (1 mg.) and a dose as small as 1 µg. induced a sharp leucocytic response. Both the endotoxin and the galactan were lethal to fowl embryos. Eleven-day-old embryos were inoculated by the chorioallantoic membrane route and deaths occurred within 48 hours and haemorrhagic lesions were prominent throughout. The LD_{50} titre of the endotoxin was 15 µg. while the titre of the galactan was 525 µg. The endotoxin failed to fix complement and one precipitin line was produced in the AGT when diffused against the same serum of which no details are given.

MATERIALS

Fractions used for study

The fractions used were obtained from the urine of cattle experimentally infected with Mycoplasma mycoides as described in Part 2. These fractions were namely C/1, C/2, C/3, C/1/2/3, 4/5/6(5) and 4/5/6(7). In addition to these fractions the following 2 materials were also used. The galactan (batch L9) (Buttery and Plackett, 1960), kindly supplied by Dr. P. Plackett, C.S.I.R.O. Animal Health Research Laboratories, Parkville, Victoria, Australia, and the endotoxin (Villemot, Provost and Queval, 1962) kindly supplied by the Director, Laboratoire de Farcha, Fort Lamy, Tchad.

Nitrogen determination

Digestions were carried out on a Thomas-Labconco High Temperature Kjeldahl Digesting Apparatus*.

Distillations were carried out using a One-piece model, micro-Kjeldahl distilling apparatus with Electric steam generator*.

Immunoelectrophoresis

Immunoelectrophoretic analysis was carried out using a Beckman-Spinco Model RD-2 Duostat regulated power supply unit*, with a Shandon Universal horizontal Electrophoresis tank after Kohn•.

Paper chromatography

Paper chromatography was carried out using a Shandon 12 in. Universal strip chromatank• on Whatman No. 1 paper.

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* Arthur H. Thomas Co., Philadelphia 5, Pa., U.S.A.

* Beckman-Spinco, Stanford Industrial Park, Palo Alto, Ca., U.S.A.

• Shandon Scientific Co. Ltd., 6 Cromwell Place, London, S.W.7, England.

Spectrophotometry

Measurements were carried out in a Beckman model DU spectrophotometer*, using fused silica cells and a tungsten lamp for measurements in the wavelengths from 3,200 - 10,000 Å and a hydrogen lamp in the wavelengths from 2,200 - 3,200 Å.

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* Beckman Instruments Inc., Fullerton, California, U.S.A.

METHODS

Nitrogen estimations

Nitrogen was determined by the Micro-Kjeldahl method. Digestion was carried out by the procedure recommended by Kabat and Mayer (1961). Nitrogen was then estimated using the one-piece model distillation apparatus recommended by Steyermark, Alber, Aluise, Huffman, Kuck, Moran and Willits (1951). Boric acid was used to receive the NH_3 (Eisner and Wagner, 1934) and titration of the ammonium borate which formed was carried out with exactly N/70 HCl. The alternative indicator mixture as recommended by Kabat and Mayer (1961) was used. Estimations were performed on replicate samples of 0.22 mg.

Phosphorus estimations

Phosphorus was estimated by the method of Fiske and Subbarow (1925).

Carbohydrate estimations

Carbohydrate was estimated by the α-Naphthol Reaction (Dische, 1955). Duplicate samples of 50 µg. of fractions C/1, C/2, C/3 and C/1/2/3 and 100 µg. of 4/5/6(7) were estimated. Readings were performed after 6 hours at 5500, 5600 and 5700 Å. The readings at 5600 Å were used for the estimation. Results were expressed in terms of galactose.

Hexosamine estimations

Hexosamine was estimated by the Elson-Morgan method modified by Belcher, Nutten and Sambrook (1954) as given by Kabat and Mayer (1961). The fractions were hydrolysed for 2 hours at 2 N.HCl. Readings were performed in the Beckman spectrophotometer at 5400 Å. Duplicate samples of 200 µg. of the fractions were estimated. Blanks and standard solutions containing 10, 40, 70 and 100 µg. of glucosamine hydrochloride were used to prepare the calibration curve from which the amounts of hexosamine were calculated. Known solutions of glucosamine

hydrochloride were hydrolysed and dried in the same manner as the unknown samples and these were compared with the standards.

Examination of the fractions for the presence of lipid

Fractions C/1/2/3 and 4/5/6(7) were examined for the presence of lipid by staining the precipitin bands produced by the fractions and antisera in the AGT by the method of staining for lipoproteins of Grabar (1959), using Sudan black in 60 per cent. alcohol.

After staining with Sudan black the precipitin bands were counterstained with Azocarmine (Grabar, 1959) in order to distinguish the other precipitin bands.

Examination of the fractions for the presence of nucleic acid

The fractions, dissolved in 0.05 N NaOH and distilled water, were examined for the presence of nucleic acid by preparing ultraviolet absorption spectra from 2200-3000 Å.

Paper Chromatography

Polysaccharide samples were hydrolysed by 3 different methods.

1. By a method used by Davies (1956). The samples were hydrolysed in sealed ampoules in a boiling water bath (94°C.) with $N.H_2SO_4$ (1 ml. per 10 mg. of sample) for 16-18 hours and neutralized with $Ba(OH)_2$. The neutral solution was centrifuged at 2500 r.p.m. for 45 minutes and the supernatant evaporated to dryness in vacuo over P_2O_5 using an LT5 freeze-drier* and redissolved in distilled water at suitable concentrations for application to paper chromatograms.

2. A modification of the method of Wilkinson, Dudman and Aspinal (1955) was used. This consisted of a preliminary hydrolysation in sealed ampoules in 90 per cent. Formic acid (1 ml. per 10 mg. fraction) in a boiling water bath (94°C.) for

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* W. Edwards & Co. (London) Ltd., Allendale Works, Worsley Bridge Road, London, S.E.26, England.

16-24 hours followed by removal of the acid by distillation *in vacuo*. The resultant syrup was further hydrolysed in sealed ampoules at 94°C. for 6 hours in $N.H_2SO_4$ (0.25 ml. per 10 mg. initial sample). The hydrolysate was neutralized with $Ba(OH)_2$ and the clear supernatant removed after centrifugation. This was concentrated to 1/3 volume *in vacuo* over P_2O_5 using an LT5 freeze-drier and made up to its original volume with methyl alcohol and kept at 4°C. for 18 hours. Following this it was recentrifuged and the supernatant used after being suitably diluted for application to paper chromatograms.

3. Fraction 4/5/6(7) was also hydrolysed by the method of Barker, Foster, Stacey and Webber (1958) for oligosaccharides modified as proved necessary. Fraction 4/5/6(7), 1 mg./ml., was hydrolysed for 40 hours at 94°C. (boiling water bath) in concentrated HCl using the proportions 7:3 (Fraction : acid).

Following hydrolysis, the hydrolysate was neutralized with methyl-di-n-octylamine.

Chromatograms on 9 in. wide Whatman No. 1 paper strips were carried out by the descending method using n-butanol-acetic acid-water (6:1:2, v/v) (Salton, 1960) and n-butanol-pyridine-water (6:4:3, v/v) (Jeanes, Wise and Dimler, 1951) as the solvent systems. Papers were sprayed with aniline hydrogen phthalate in water saturated butanol (AHP) for reducing sugars (Partridge, 1949), p-dimethylaminobenzaldehyde - acetyl acetone (PDMAB) for hexosamines (Partridge, 1948), 0.1 per cent. ninhydrin in butanol for amino acids and amines (Pratt and Auclair, 1948) and dihydroxynaphthalene-trichloroacetic acid for ketoses and uronic acids (Partridge, 1948).

Hydrolysed fractions were applied at 0.01 ml. per application. The spot was dried by means of a hair drier and a further 0.01 ml. applied if required, and so on.

The effect of heat and trypsin on the precipitating antigenic components

(a) Heat

Fractions C/1/2/3, 4/5/6(7), C/1, C/2 and C/3, at pH 7.2, were heated in a boiling water bath (94°C.) for 60 minutes. After heating, the fractions had remained completely clear. The boiled and unboiled control materials were then compared in the AGT.

(b) Trypsinization

13 mg. C/1/2/3 was made up to 1.5 ml. with distilled water. 1 mg. 0.2 M Na_2HPO_4 (pH 8.5), 5 mg. trypsin* in 0.5 ml. N.saline and 0.15 ml. Toluene were added. The mixture was placed at 37°C. Within 60 minutes a fluffy cloudy deposit formed. The mixture was incubated at 37°C. for 2 days then a further 4 mg. trypsin was added and the incubation continued for a further 3 days (Morgan and Partridge, 1940). The mixture was then dialysed against distilled water at 4°C. for 3 days and then 4 ml. of the mixture was treated with 2 ml. Chloroform and shaken well to cause the non-diffusible portion of the trypsin preparation and part of the polysaccharide to separate as an interfacial layer (Morgan and Partridge, 1940). This was then centrifuged and the supernatant removed. Agar gel tests were then set up using this supernatant and untreated C/1/2/3. As fraction 4/5/6(7) was very scarce only 1.5 mg. in 1 ml. distilled water was available and this was treated by adding 0.5 ml. Na_2HPO_4 0.2 M and 0.5 ml. trypsin (2 mg.) and incubated at 37°C. for 24 hours. AGTs were then set up using treated and untreated 4/5/6(7).

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* Trypure Novo Crystalline Trypsin Stabalized, Novo Industri A/s Copenhagen, Denmark.

Attempted separation of the precipitating antigenic components by ultracentrifugation

The possibility of separating the precipitating antigenic components by ultracentrifugation was investigated by the following method.

Fraction C/1/2/3, 9.0 mg., was made up in 13 ml. distilled water, mixed well and placed in 2 centrifuge tubes and centrifuged in the Spinco ultracentrifuge for 60 min. at 40,000 r.p.m. (average centrifugal force = 102,900 times gravity). One tube was then removed while the other one was centrifuged for a further 60 min. at the same speed. On removal of the tubes from the centrifuge they were placed carefully at -25°C. When the fluid in the tubes had frozen solid they were removed and sectioned. The first tube was cut up into 4 x 1.5 cm. sections while the second tube was cut up into 8 x 0.75 cm. sections. Each aliquot was placed in a separate bottle. These materials were labelled, from the top to the bottom of the tube, 1/1 - 1/4 and 2/1 - 2/8 respectively and they were each examined by the qualitative AGT using hyperimmune sheep serum.

Immunoelectrophoresis

The slide method of Scheidegger (1955) and Gall (1960), modified as necessary, was used.

Microscope slides, 3 in. x 1 in. (76 mm. x 26 mm.) previously coated with a thin film of 1% agar and dried to improve adherence of the gel were placed on a level surface checked by means of a spirit-level. A wall of Plasticine* was built around the slides to a height of about 10 mm. Sufficient agar, 1 per cent. in veronal buffer pH 7.2 or borate buffer pH 8 and 10, to give a depth of 2 mm. of agar on the slides was then poured on and when solidified the plasticine wall was removed and the slides divided from one another with a scalpel. Lint strips, 3 in. x 1 in. (76 mm. x 26 mm.) were then prepared

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* Harbutt's Plasticine Ltd., Bathampton, Bath, England.

and a piece about 5 mm. wide was bent over at right angles on one end of the strips. The slides were then placed in 4 rows of 3 on a piece of plate glass 9 in. x 8 in. on a raised surface and the lint strips were applied to each end of each row of slides and fixed in position with a little melted agar. When the agar had solidified and the strips become firmly adherent, the trough patterns were cut with a template and cutters as described by Gall (1960). The pattern consists of a small central transverse oval trough 5 mm. x 1,5 mm. to receive the antigen to be electrophorised and a flanking trough along the length of the slide 2 mm. wide with its inner edge 3 mm. from the central trough. A perspex template allows the central trough to be cut with a flattened cork borer and the lateral trough with a pair of scalpel blades bolted to a spacer.

The fractions to be examined were placed in the central wells and the slides placed in the electrophoresis tank, still on the piece of glass. The buffer compartments of the tank were filled with the same buffer as that used for preparing the agar. The lint wicks were dipped in and thoroughly soaked with the buffer. Runs were carried out at 43 m A constant current for 2 hours.

When the slides were removed from the tank, the longitudinal troughs were filled with hyperimmune sheep serum and the slides placed at room temperature in a moist atmosphere. The slides were examined daily for 3 days and drawings made of the precipitin bands formed. Control slides were included in each run. These consisted of bovine serum in the centre well and rabbit antibovine serum was used in the longitudinal troughs after electrophoresis.

Complement Fixation

(a) Examination of fractions for complement fixing properties.

Fractions C/1/2/3 and 4/5/6(5) were examined for complement fixing properties. It was necessary to use fraction 4/5/6(5) as there was insufficient fraction 4/5/6(7). Block titrations were performed using 10 doubling dilutions (1 + 1 = ½)* of antiserum and 10, 3 + 1 = $^{1}/_{1.333}$* dilutions of antigen. Serum and antigen controls were included, the antigen and serum respectively being replaced by veronal buffer.

The test followed the usual Campbell and Turner (1953) regime but the complement and antibody-antigen mixture was incubated overnight at 4°C. and then placed at 37°C. for 30 minutes before the addition of the haemolytic system. The test was then read following a further 30 minutes incubation at 37°C.

(b) Examination of sera for complement fixing antibodies.

The usual complement fixation test as described by Campbell and Turner (1953) with the modifications already mentioned in Part 1 was used. Dilutions of antisera, however, were taken by doubling dilutions to 1/640. Antiserum titres are given as the dilution of antiserum giving 50 per cent. lysis (++).

Pyrogenicity

Rabbits were injected intravenously with the material to be tested dissolved in pyrogen-free water and rectal temperatures were recorded at intervals using a clinical thermometer always inserted to the same distance. Control animals were injected with pyrogen-free water and their temperatures simultaneously recorded. Rabbits weighed 1.43 - 1.85 kg. and results are given in terms of temperatures for weight of polysaccharide injected per kg. body weight of rabbit.

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* Reactant + diluent.

Toxicity

Rabbits and mice were inoculated intravenously with the materials to be tested and were checked daily for signs of sickness or death.

Ten day old fowl embryos were inoculated by the allantoic route with fraction C/1/2/3 reconstituted in saline. Groups of 6 embryos were inoculated with varying amounts of the fraction in 0.03 ml. of inoculum. A control group was inoculated with saline alone. The embryos were examined daily for viability.

Two young adult cattle, weighing 290 and 312 lbs. respectively, were each inoculated intravenously with 2.0 mg. of fraction C/1/2/3 reconstituted in 2 ml. of N.saline.

Allergic reaction

Five cattle that had previously been inoculated with avianized CBPP vaccine and then challenged 3 months before by the subcutaneous route and had developed large swellings which had regressed and disappeared, and 5 control fully susceptible animals were inoculated with varying amounts of fraction C/1/2/3 reconstituted in N.saline by the intradermal route on the side of the neck. Each inoculum was 0.2 ml. Skin thickness measurements were recorded daily at the inoculation sites.

Two of the cattle received 0.02 mg. C/1/2/3, 2 received 0.2 mg. and the remaining one received 2.0 mg. The control cattle received identical doses. All immune animals had developed at some time after challenge CF titres of ++++, and all the control animals were negative to the CFT.

The Aggressive Action of Fraction C/1/2/3

In these experiments cattle were inoculated subcutaneously behind the shoulder with washed viable M. mycoides that had been grown in broth culture. On one side of the body the inoculum consisted of the organisms combined with fraction C/1/2/3 in N.saline and on the other side of the body the inoculum consisted

of the organisms plus a similar volume of N.saline. The cattle were examined daily for signs of swelling at the inoculation sites and the swellings measured. All cattle were bled before inoculation and the sera examined for antibodies by the CFT and SAST. In a preliminary experiment it was shown that there was no loss in titre of viable organisms in N.saline or phosphate buffer at 4°C. or at room temperature over a period of 90 minutes.

1. In the first experiment the T3 strain of M. mycoides was grown in tryptose broth medium for 3 days and then the medium was centrifuged at 27,000 r.p.m. (average centrifugal force = 65,900 times gravity) for 20 minutes in the Spinco ultracentrifuge. The deposited organisms were then washed 3 times in the Spinco at the same speed and length of centrifugation, using 38.5 ml. of N.saline each time, and finally resuspended in N.saline. The 50 per cent. endpoint dilution in broth of these organisms titrated immediately after the inoculations had been performed was $10^{3.75}$ organisms/ml. Four cattle were inoculated. Three of them had previously been inoculated with avianized CBPP vaccine and challenged by the subcutaneous route 3 months before and had developed large swellings which had regressed and disappeared and were termed immune, and 1 was a fully susceptible animal. Fraction C/1/2/3 was used at 4.6 mg./ml. concentration, and each inoculum consisted of 1 ml. of the washed organisms plus varying amounts of the antigen or N.saline, the mixtures being placed at 4°C. for 30 minutes before inoculation. The amount of antigen solution or N.saline contained in the inocula were as follows:

Ox 6173 - 0.1 ml. (0.46 mg.) C/1/2/3 & 0.1 ml. N.saline;
Ox 6436 - 0.5 ml. (2.3 mg.) C/1/2/3 & 0.5 ml. N.saline;
Ox 6333 - 1.0 ml. (4.6 mg.) C/1/2/3 & 1.0 ml. N.saline.

These 3 animals were immune. The susceptible animal, No. 7645, received 0.5 ml. (2.3 mg.) C/1/2/3 and 0.5 ml. N.saline.

2. The procedure in the second experiment was essentially the same as the first. The Lister vaccine clarifier was, however, used instead of the Spinco to deposit and wash the organisms. Three litres of N.saline were used each time to wash the organisms, the deposit being first mixed in an Atomix with 1 litre of the saline. The final saline suspension was very thick, having an opacity of 4 times opacity tube 10, although the 50 per cent. endpoint dilution in broth estimated after the inoculations had been performed was only $10^{3.24}$ organisms/ml. The mixtures of organisms and antigen or organisms and saline were left at room temperature for 60 minutes before inoculation. Fraction C/1/2/3 was used at 10 mg./ml. concentration. Four cattle were inoculated, 2 immune and 2 susceptible, and the inoculum in each case consisted of 1 ml. organisms and either 0.5 or 1 ml. of antigen or saline. One immune animal, No. 6456, and 1 susceptible, No. 7671, were inoculated with 0.5 ml. (5 mg.) C/1/2/3 and 0.5 ml. saline while the other immune animal, No. 6504, and the other susceptible animal, No. 7657, were inoculated with 1 ml. (10 mg.) C/1/2/3 and 1 ml. saline.

3. In the final experiment the organisms were grown as before and deposited using the Lister vaccine clarifier but all washings were performed with phosphate buffer at pH 7.4. The final deposit was resuspended in the buffer. The 50 per cent. end-point dilution in broth after inoculation was $10^{6.76}$ organisms/ml. Fraction C/1/2/3 was used at 5 mg./ml. Thirteen cattle, 8 immunes and 5 susceptibles, were inoculated subcutaneously with the same mixtures as before, that is, fraction plus organisms on one side and N.saline plus organisms on the other. The mixtures were placed at 4°C. for 60 minutes before inoculation. The immune cattle had not been subjected to avianized vaccines but had only been used as challenge controls and had been inoculated with T3 M. mycoides subcutaneously 6 weeks previously, had developed swellings in many cases and had all developed CFT antibodies, and the swellings had regressed and disappeared. All the immune animals

were given 2 ml. of organisms plus varying amounts of C/1/2/3 or N.saline. Ox 7817 and Ox 7824 received 0.8 ml. (4 mg.) C/1/2/3 or 0.8 ml. saline, Ox 7819 and Ox 7736 received 0.4 ml. (2 mg.) C/1/2/3 or 0.4 ml. saline, Ox 7829 and Ox 7740 received 0.2 ml. (1 mg.) C/1/2/3 or 0.2 ml. saline and Ox 7828 and Ox 7623 received 0.1 ml. (0.5 mg.) C/1/2/3 or 0.1 ml. saline. The susceptible cattle all received 0.4 ml. C/1/2/3 (2 mg.) or 0.4 ml. saline with varying amounts of organisms. Ox 8153 received 4 ml. of organisms, Ox 8154 and Ox 8155 received 2 ml. of organisms and Ox 8157 and Ox 8158 received 1 ml. of organisms.

Attempted Immunization of Rabbits and Mice with Fractions C/1/2/3 and 4/5/6(7) alone or combined with 'Shiga' conjugated protein

Inoculation regimes

Calves and Rabbits. Calves and young adult rabbits which had been shown to possess no agglutinating or CF antibodies to M. mycoides were inoculated intravenously with the fractions in N.saline on Tuesday, Wednesday and Thursday of each week for 3 weeks. The calves and rabbits were then bled for serum 4 days after the last injection.

Mice. Young adult mice were inoculated intravenously with the fractions in N.saline. They were inoculated in groups of at least 6 mice. All groups were inoculated 3 times on alternate days during the first week. Four days after the last injection one group of mice was killed and bled for serum while the remaining groups commenced the second series of injections. Again 4 days after the last injection another group was killed and bled while the remaining groups began the third series of injections. This regime continued until all groups had completed their course of injections and sera collected.

Sera collected were examined for antibodies against the respective fractions in the AGT and CFT against M. mycoides by the AGT, SAST and CFT using respectively T3 antigen, SAST antigen and CFT antigen.

(1) Attempted immunization with fractions C/1/2/3 and 4/5/6(7) alone

Rabbits

(a) Two rabbits were inoculated with a total of 0.66 mg. of fraction C/1/2/3. Examination of the sera by the AGT, SAST and CFT was carried out.

(b) Four of 6 rabbits which had been inoculated with fractions C/1/2/3 and 4/5/6(7) for a pyrogenicity experiment started a course for attempted immunization 4 days later, with the same fractions with which they had been initially inoculated. The 2 remaining rabbits received no further injections after their pyrogen dose and were bled 4 days after it. Details of the amount of each fraction given to each rabbit are shown in table XI.

Table XI

Details of the amounts of fractions C/1/2/3 & 4/5/6(7) given to each rabbit in the immunization procedures

Rabbit number	Fraction	Amount inoculated		Total amount inoculated mg.
		Pyrogenicity mg.	Immunizing mg.	
24	C/1/2/3	-	0.66	0.66
25	"	-	0.66	0.66
32	C/1/2/3	0.00106	1.166	1.167
33	"	1.060	1.166	2.22
34	"	5.0	-	5.0
37	4/5/6(7)	0.00120	1.342	1.34
36	"	1.20	1.342	2.54
35	"	4.90	-	4.9

Mice

Mice were inoculated in groups of 6. Seven groups were injected and individual mice in each group received the following total doses of each fraction.

C/1/2/3	-	0.3 µg. in 3 injections of 0.1 µg. each	
		0.6 µg. in 6 " " " " "	
		0.9 µg. in 9 " " " " "	
		3.0 µg. in 7 " , the first 3 being 0.2 µg. and the last 4 being 0.6 µg. each.	
		300 µg. in 3 " of 100 µg. each.	
4/5/6(7)	-	0.9 µg. in 3 " " 0.3 " "	
		300 µg. in 3 " " 100 " "	

(2) Attempted immunization with fractions C/1/2/3 and 4/5/6(7) combined with 'Shiga' conjugated protein

A second method of immunization was tried using the fractions combined with the conjugated protein component of the 'O' somatic antigen complex of Shigella dysenteriae (Morgan and Partridge, 1941), kindly supplied by Dr. D. A. L. Davies of the Microbiological Research Establishment, Porton, Nr. Salisbury, Wiltshire.

The method used for combining the protein was essentially that of Davies (1956). Fractions C/1/2/3 and 4/5/6(7), 1 per cent. w/v in distilled water were added to 1 per cent. w/v 'Shiga' conjugated protein in0.05 N.NaOH in the proportion 3:1, fraction to protein. The mixtures were allowed to stand at room temperature for 3½ hours, adjusted to pH 7 and made up to the required concentration with normal saline. This artificial complex was used to immunize rabbits and mice.

Rabbits

Two rabbits were inoculated with 0.25 mg. of C/1/2/3 conjugate each time during the first week, with 0.5 mg. each time during the second week, 0.75 mg. during the third week, 0.4 mg. during the fourth week and 1.0 mg. each time during the fifth

week, giving a total of 8.7 mg. of conjugate each. Insufficient 4/5/6(7) conjugate was available to inoculate rabbits.

Mice

Fraction C/1/2/3 conjugate was inoculated at 0.1 mg. conjugate per dose. Three groups of mice were inoculated with the following total doses: 0.3 mg., 0.6 mg. and 0.9 mg. in 3, 6 and 9 injections respectively. Due to scarcity of material, fraction 4/5/6(7) conjugate was only inoculated into 4 mice, at 0.09 mg. conjugate per dose. The total conjugate received by the 2 groups of two mice was 0.27 mg. and 0.54 mg. conjugate respectively.

Calves

Two calves, 12 months of age and weighing 252 and 332 lb. respectively, were inoculated intravenously with 1.0 mg. of C/1/2/3 conjugate each inoculum, giving a total of 9 mg. of conjugate each. Four weeks after the last inoculation the two calves were challenged, together with 2 control animals, by the subcutaneous route with T3 virulent culture.

Comparison of the precipitin bands produced by fraction C/1/2/3, the galactan of Buttery and Plackett (1960) and the endotoxin of Villemot, Provost and Queval (1962)

The 3 materials, each available as lyophilized powders, were reconstituted in distilled water at various concentrations and examined in the qualitative AGT against sheep x and sheep x' sera. Fraction C/1/2/3 and the galactan were found to dissolve very rapidly in the distilled water but the endotoxin contained certain insoluble material. The final concentrations chosen as the most suitable for demonstrating the various precipitin bands were C/1/2/3 0.1 mg./ml., galactan 0.1 mg./ml. and the endotoxin 2.0 mg./ml.

RESULTS

Nitrogen estimations

The results of the nitrogen estimations on the various fractions are given below.

C/1	36 µg./mg. or 3.6 per cent.
C/2	72 µg./mg. or 7.2 per cent.
C/3	81 µg./mg. or 8.1 per cent.
C/1/2/3	56 µg./mg. or 5.6 per cent.

Fraction C/1 contained the least nitrogen, 3.6 per cent. C/3 contained the most - 8.1 per cent., while C/2 contained an intermediate amount - 7.2 per cent. Fraction C/1/2/3 contained 5.6 per cent., rather less than an average figure for the three fractions.

Phosphorus estimations

Phosphorus estimations performed on fractions showed that C/1/2/3 contained 0.52 µg. phosphorus in 1 mg. of the fraction, while 4/5/6(7) contained 0.54 µg. phosphorus in 1 mg. of the fraction.

Carbohydrate estimations

The amount of carbohydrate (hexose, pentose and methyl pentose) present in the various fractions calculated from the calibration curve (fig. 29), expressed in terms of galactose, are given below:

Fraction	Carbohydrate (µg./100 µg.)
C/1	46.8
C/2	40.3
C/3	23.5
C/1/2/3	42.4
4/5/6(7)	18.2

Absorption maxima of all fractions except 4/5/6(7) were 5700 Å indicative of hexose. The absorption maximum of 4/5/6(7) was 5600 Å indicative of heptose and methyl pentose.

/Fig. 29....

Fig. 29. Carbohydrate estimation : calibration curve

0·3
0·2
0·1
Absorbance
0
2
4
6
8
10
12
14
16
18
20
22
μg. Carbohydrate (galactose)

Hexosamine estimations

The amount of hexosamine, expressed in terms of glucosamine hydrochloride, in the various fractions, calculated from the calibration curve (fig. 30) is given below:

Fraction	Hexosamine (µg./100 µg.)
C/1	9.0
C/2	9.9
C/3	14.75
C/1/2/3	11.9
4/5/6(7)	11.25

/Fig. 30....

Fig. 30. Hexosamine estimation : calibration curve

Examination of fractions C/1/2/3 and 4/5/6(7) for the presence of lipid

Of the bands produced by fraction C/1/2/3, one stained well with Sudan black. When the agar plate was counterstained with azocarmine, it was seen that the line that stained with Sudan black was central in position.

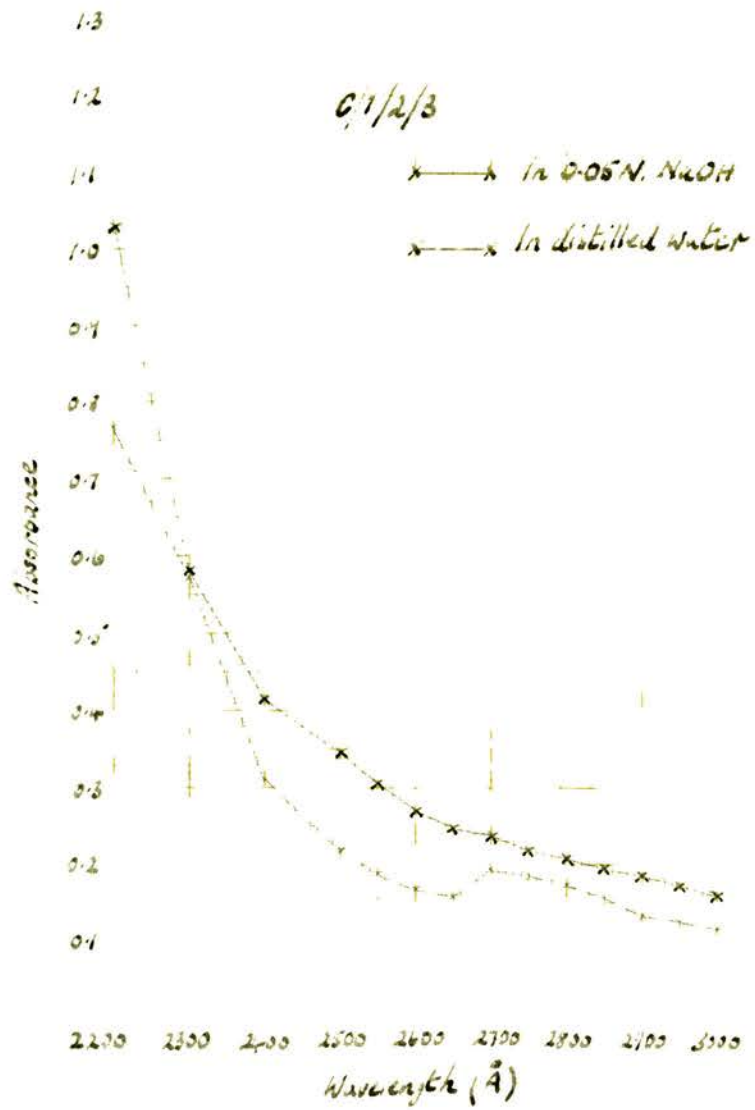
With fraction 4/5/6(7), one line stained with Sudan black which, on counterstaining with azocarmine, was seen to be the line nearest the serum well of the 2 lines present.

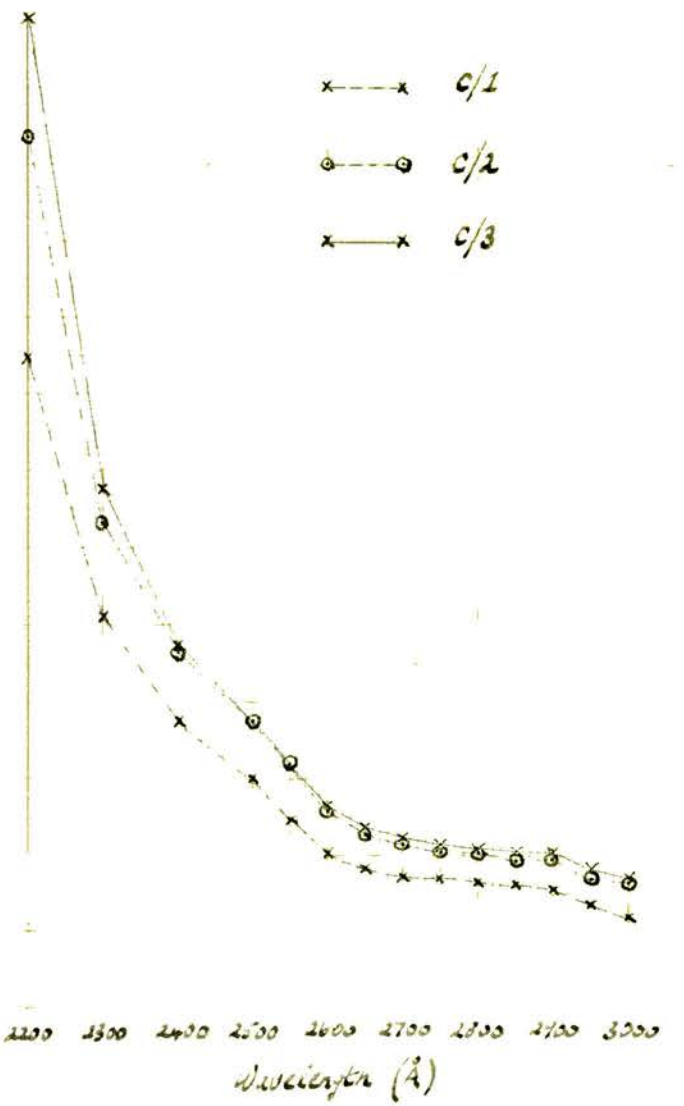
Examination of fractions C/1/2/3, C/1, C/2 and C/3 for the presence of nucleic acid

There was no indication of increased absorption at 2600 Å with any of the fractions. Fig. 31 shows the ultra-violet absorption spectra for the fractions.

/Fig. 31....

Fig. 31. Ultraviolet Absorption Spectra


C/1/2/3
In 0·05 N. NaOH
In distilled water
Absorbance
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2200 2300 2400 2500 2600 2700 2800 2900 3000
Wavelength (Å)


C/1
C/2
C/3
2200 2300 2400 2500 2600 2700 2800 2900 3000
Wavelength (Å)

Chromatographic examination of the fractions

1. Fractions C/1, C/2 and C/3 were hydrolysed by the N.H_2SO_4 method and chromatograms were prepared using n-butanol-acetic acid-water as the solvent and sprayed with AHP. Tables XII - XIV give the results of 3 chromatograms with the R_F-values of each spot produced by each fraction and the R_F-values of the control sugars.

The first spot had R_F-values less than that of raffinose and occurred in all three fractions. It was fairly strong, especially in C/1. The second spot appeared in only 3 instances, once in C/2 and twice in C/3. It was a weak spot and its R_F-values appeared to be close to the value of lactose, maltose and galacturonic acid of the controls used. Spot 3 produced very consistent R_F-values and appeared to correspond well with the R_F-value of galactose. This spot was demonstrated in all fractions and was the strongest one present. Spot 4, a weak spot, was only evident with fraction C/2. Its R_F-values were fairly close to that of arabinose, although that of mannose was also quite close.

/Table XII.....

Table XII

Chromatography of fractions, hydrolysed by the $N.H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 1			
	10. R_F-value of spots		
Fraction	C/1	C/2	C/3
1	41	45	
2		94	
3	155	155	154

10. R_F-value of Controls:-

Lactose	63
Galacturonic acid	97
Glucuronic acid	138
Galactose	153
Glucose	171
Sorbose	176
Mannose	205
Arabinose	217
Xylose	254
Ribose	284
Glucurone	334
Rhamnose	358

Table XIII

Chromatography of fractions, hydrolysed by the N.H_2SO_4 method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 2			
	10. R_F-value of spots		
Fraction	C/1	C/2	C/3
1	30	x	36
2			82
3	154	154	155
4		214	

10. R_F-value of Controls:-

Raffinose	55
Lactose	59
Maltose	103
Glucuronic acid	109
Galacturonic acid	111
Galactose	151
Sorbose	166
Sucrose	170
Glucose	175

x A very strong streak was present at the site of application of the material and extending some distance down the sheet.

Table XIV

Chromatography of fractions, hydrolysed by the N.H_2SO_4 method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 3			
	10. R_F-value of spots		
Fraction	C/1	C/2	C/3
1	33	x	27
2			64
3	158	163	157
4		224	

10. R_F-value of Controls:-

Raffinose	57
Lactose	68
Glucuronic acid	125
Galactose	168
Mannose	215
Arabinose	224
Rhamnose	365

x A very strong streak was present at the site of application of the material and extending some distance down the sheet.

2. Fractions C/2, C/3 and 4/5/6(7) were hydrolysed by the $H.COOH/H_2SO_4$ method and fraction 4/5/6(7) also by the concentrated HCl method. Chromatograms were prepared using n-butanol-acetic acid-water (6:1:2) and they were sprayed with AHP.

The results of 6 chromatograms are given in tables XV - XX with the R_F-values of each spot produced by the fractions and the control sugars.

The first spot occurred with each fraction, its R_F-value again being lower than any of the controls. This spot was fairly strong. Spot 2 occurred inconsistently in all fractions and was very weak which may account for this. Its R_F-value did not tie in with any of the controls. Spot 3, again weak, was seen only occasionally depending on the concentration of the fraction used. Its R_F-value did not tie in with any controls although it was nearest to galacturonic acid or glucuronic acid. The 4th spot was very strong and appeared to correspond with galactose in position and colour. Spot 5 was fairly strong and occurred in all fractions just below the possible galactose one. Its R_F-value was close to those of mannose and glucose.

Spot 6 occurred only with fraction 4/5/6(7). It was fairly strong and was pink in colour and its R_F-value corresponded well with arabinose in both position and colour.

The 7th spot was very weak and only showed up in 2 instances, with C/2, and the R_F-value corresponded closely with xylose.

Spot 8 was a fairly strong olive in colour and occurred with C/2 and C/3. Its R_F-value was closest to ribose of the controls used.

/Table XV.....

Table XV

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 5		
	10. R_F-value of spots	
Fraction	C/2	C/3
1		25
2		80
3	116	114
4	193	190
5	235	
6		311

10. R_F-value of Controls:-

Raffinose	86
Galactose	193
Mannose	242
Arabinose	255
Rhamnose	380

Table XVI

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 6			
	10. R_F-value of spots		
Fraction	C/2	C/3	4/5/6(7)*
1	40	37	33
2			75
3			129
4	149	149	
5	188	196	162
6			221
7	235		
8	283	287	

10. R_F-value of Controls:-

Raffinose	57
Galactose	158
Mannose	200
Arabinose	225
Rhamnose	349

* Hydrolysed by the conc. HCl method

Table XVII

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 7				
	10. R_F-value of spots			
Fraction	C/1	C/2	C/3	4/5/6(7)*
1	43	52	48	
2	91	87		69
3				
4	152	152	156	164
5	200	204	208	216
6				
7		247		
8		294	303	

10. R_F-value of Controls:-

Raffinose	60	Mannose	168
Lactose	65	Glucose	207
Dihydro-ascorbic acid	69	Sorbose	208
Maltose	69	Arabinose	216
Galacturonic acid	125	Xylose	251
Glucuronic acid	143	Ribose	280
Sucrose	164	Glucurone	329
Galactose	165		

* Hydrolysed by the conc. HCl method

Table XVIII

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 8			
	10. R_F-value of spots		
Fraction	C/1●	4/5/6(7)*	4/5/6(7)
1	33		
2	83	96	
3	128		
4	174	179	175

10. R_F-value of Controls:-

Raffinose	46
Lactose	50
Dihydro-ascorbic acid	54
Maltose	58
Galacturonic acid	108
Glucose	154
Sorbose	174
Arabinose	192

● Hydrolysed by the $N.H_2SO_4$ method

* " " " conc. HCl method

Table XIX

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 9		
	10. R_F-value of spots	
Fraction	C/3	4/5/6(7)*
1	27	
2	77	54
3		108
4	127	
5	177	173
6		204
7	269	

10. R_F-value of Controls:-

Raffinose	45
Lactose	48
Dihydro-ascorbic acid	58
Maltose	49
Galacturonic acid	113
Glucuronic acid	118
Glucose	153
Sorbose	176
Arabinose	200
Ribose	266
Glucurone	301

* Hydrolysed by the conc. HCl method

Table XX

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and sprayed with AHP

Chromatogram No. 10				
	10. R_F-value of spots			
Fraction	C/1•	C/2	C/3	4/5/6(7)
1	38	38	33	
2	84	74		74
3			102	115
4	133	138	135	
5	176	184	184	
6				207
7		242		
8		288	278	

10. R_F-value of Controls:-

Raffinose	54	Mannose	164
Lactose	59	Glucose	197
Dihydro-ascorbic acid	61	Arabinose	207
Maltose	64	Xylose	246
Sucrose	100	Ribose	269
Galacturonic acid	125	Glucurone	329
Glucuronic acid	136	Rhamnose	350

• Hydrolysed by the $N.H_2SO_4$ method

3. Fractions C/1, C/2 and C/3 were hydrolysed by the $H.COOH/H_2SO_4$ method and then chromatograms were prepared using the different solvent systems, namely n-butanol-pyridine-water (6:4:3) and then sprayed with AHP.

The results of 2 chromatograms are given in tables XXI and XXII.

Spot 1 occurred in all fractions and was not very strong but was strongest in C/1. Its R_F-value was very low and did not correspond with any control sugars, and was very similar to that obtained with the other solvent systems. Spot 2 occurred only with C/2 and was very weak. Again its R_F-value was lower than that of any of the control sugars. Spot 3 occurred with C/2 and C/3. It was also weak and its R_F-value did not correspond with any of the control sugars. The 4th spot occurred only with C/2 and C/3. It was a strong spot and olive in colour. Its R_F-value was not consistent with that of any of the controls used. Its position, however, just above the strongest spot (spot 5) and its colour suggested that it might be the same as spot 2 in table XIII. The R_F-value of spot 5, the strongest one, corresponded closely with the R_F-value for galactose. This presumably corresponds with spot 4 with the other solvent system. This spot was strongest with C/1 and C/2 and less strong with C/3.

Spot 6 was only observed with C/3 in one instance. It was weak and olive in colour. Spot 7 occurred with all fractions. It was strong with C/3, slightly less strong with C/2 and very weak with C/1. Its R_F-value corresponded closely with that of sorbose. Spot 8 was observed only with fraction C/3 and was rather weak and olive in colour. Its R_F-value corresponded closely with that of xylose but the colour was incorrect, xylose producing a red brown colour.

A photograph of chromatogram 14 can be seen in fig. 32 in which the stronger spots can be observed.

Table XXI

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-pyridine-water as solvent and sprayed with AHP

Chromatogram No. 14			
	10. R_F-value of spots		
Fraction	C/1	C/2	C/3
1	35	37	47
2		149	147
3		255	258
4	315	321	321
5	405	414	413
6			471

10. R_F-value of Controls:-

Raffinose	174
Galactose	327
Glucose	373
Arabinose	385
Sorbose	407
Xylose	470
Ribose	516

Table XXII

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-pyridine-water as solvent and sprayed with AHP

Chromatogram No. 16			
	10. R_F-value of spots		
Fraction	C/2	C/3	C/3
1		127	
2	214	206	
3	247	246	
4	310	294	306
5		334	
6	403	381	404
7	461	431	460

10. R_F-value of Control:-

Galactose 315

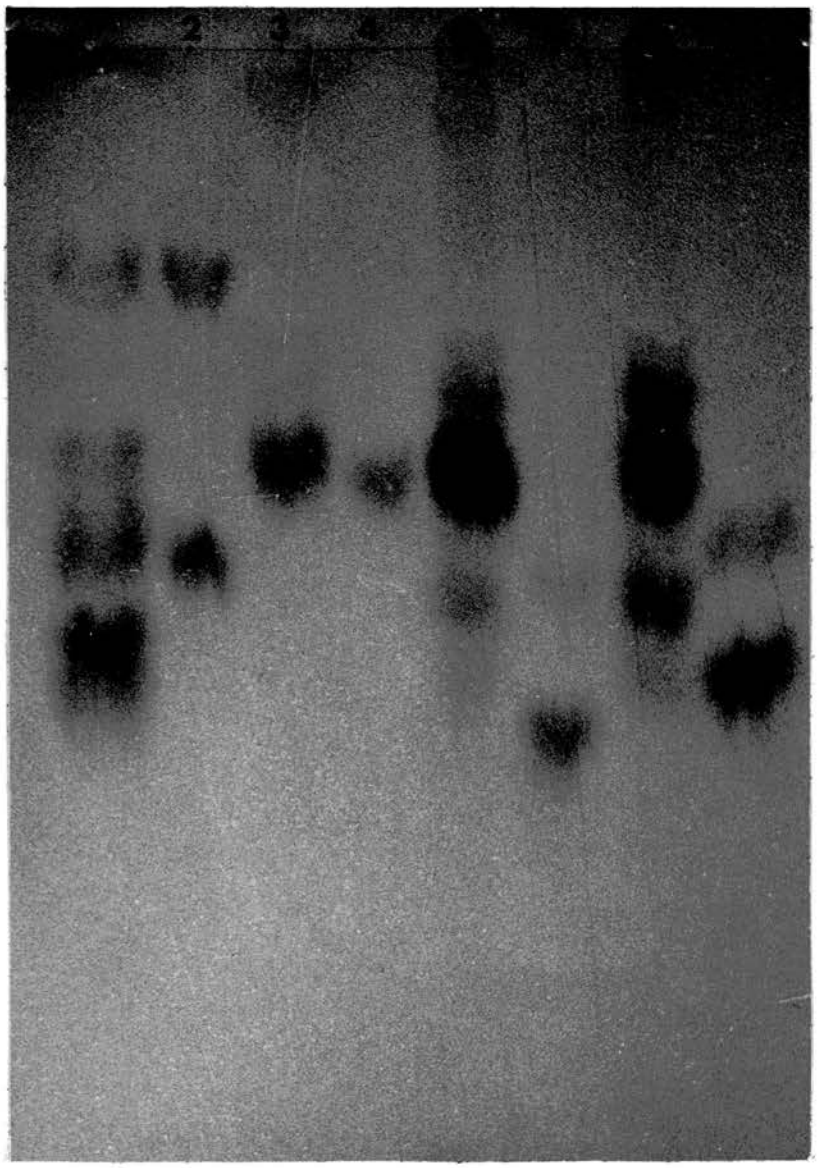


Fig. 32. Chromatogram 14. Fractions C/1, C/2 and C/3 hydrolysed by $H.COOH/H_2SO_4$, using n-butanol-pyridine-water as solvent and AHP as the spray.

Spots
1 raffinose, galactose, arabinose and xylose
2 raffinose and arabinose
3 fraction C/1
4 galactose
5 fraction C/2
6 sorbose and ribose
7 fraction C/3
8 glucose and xylose

4. Fractions C/1, C/2 and C/3 were hydrolysed by the $H.COOH/H_2SO_4$ method. A chromatogram was prepared using n-butanol-pyridine-water (6:4:3) and sprayed with PDMAB. Table XXIII gives the R_F-values of the spots obtained. Spots 1 and 2 were very weak and coloured an indefinite olive. Spots 3 and 4 were strong and pink. Spot 5 was the strongest and was violet or purple in colour. Spots 6 and 7 were rather weak and olive in colour.

Table XXIII

Chromatography of fractions, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-pyridine-water as solvent and sprayed with PDMAB

Chromatogram No. 15			
	10. R_F-value of spots		
Fraction	C/1	C/2	C/3
1	22		
2		148	139
3	212	216	220
4	244	250	256
5	283	301	302
6		404	405
7	453	468	476

10. R_F-value of Controls:-

Raffinose	165
Galactose	317
Glucose	360
Sorbose	386
Arabinose	392
Xylose	465
Ribose	489

5. Fractions C/1, C/2, C/3 and 4/5/6(7) were hydrolysed by the $H.COOH/H_2SO_4$ method and duplicate chromatograms were prepared using n-butanol-acetic acid-water (6:1:2) as the solvent. One chromatogram was sprayed with AHP and the other with PDMAB.

Tables XXIV and XXV show the results of two separate runs, one containing only C/1 and the other with C/1, C/2, C/3 and 4/5/6(7).

Spot No. 1 was a fairly strong spot with both sprays, its colour with PDMAB being a definite red. The 2nd spot was not so strong and was only evident in C/1 and C/2 and very weakly in 4/5/6(7). However, it only showed up with PDMAB in C/1 and C/2 when it was a pink colour. Spot 3 was only evident in C/3 and 4/5/6(7) and in these fractions it was fairly strong and pink in colour with PDMAB. The 4th spot was the strongest and occurred in all fractions except 4/5/6(7). The colour of this spot was purple. Spot 5 again occurred in all fractions except 4/5/6(7) and it was fairly strong and pink in colour. Spot 6 was only observed in fraction 4/5/6(7) and was fairly strong and pink in colour. Spot 7 only occurred spasmodically, in fractions C/1 and C/2. It was fairly strong in C/2 with AHP but only weak with PDMAB in both C/1 and C/2 and its colour was too weak to determine accurately. The 8th spot was only observed with AHP in C/2 and C/3 when it was olive in colour and fairly strong.

Table XXIV

Chromatography of fractions, hydrolysed by $H.COOH/H_2SO_4$, using n-butanol-acetic acid-water as solvent and AHP and PDMAB as the sprays

Chromatogram No. 8		
	10. R_F-value of spots	
Fraction	C/1	
Spray	AHP	PDMAB
1	33	27
2	83	78
3	128	123
4	174	173
5		255

Table XXV

Chromatography of fractions, hydrolysed by $H.COOH/H_2SO_4$, using n-butanol-acetic acid-water as solvent and AHP and PDMAB as the sprays

Chromatogram No. 10								
	10. R_F-value of spots							
Fraction	C/1		C/2		C/3		4/5/6(7)	
Spray	AHP	PDMAB	AHP	PDMAB	AHP	PDMAB	AHP	PDMAB
1	38	33	38	33	33	24		
2	84		74	79		89	74	
3					102	92	115	108
4	133	128	138	130	135	127		
5	176		184	179	184	178		
6							207	210
7			242	230				
8			288		278			

6. In order to obtain more definite evidence that the 5th spot observed with n-butanol-pyridine-water solvent was probably galactose, the following chromatogram was prepared.

Fraction C/2 hydrolysed with $H.COOH/H_2SO_4$ was placed on two spots side by side on the chromatogram; the second one, however, had galactose added to it. A third spot of galactose alone at the same strength as that in the second spot was placed alongside the other two. The chromatogram was run with n-butanol-pyridine-water and finally sprayed with AHP. Table XXVI gives the R_F-values of the spots produced and it can be seen that the R_F-values for spot 5, spot 3 in this case as the first two did not show up, corresponded very closely indeed with that of galactose and furthermore there was no indication of an additional spot in C/2 plus galactose and the only difference between C/2 and C/2 plus galactose was a slightly stronger and larger 3rd spot in the latter.

Table XXVI

Chromatography of fraction C/2, C/2 + galactose and galactose alone, hydrolysed by $H.COOH/H_2SO_4$ using n-butanol-pyridine-water as solvent and AHP as spray

Chromatogram No. 16			
	10. R_F-value of spots		
Fraction	C/2	C/2 + G.	G.
1	214	216	
2	247	246	
3	310	312	315
4	403	405	
5	461	460	

7. In order to check whether amino acids, peptides and amines or ketosugars and uronic acids were present in these fractions, duplicate chromatograms were prepared using n-butanol-acetic acid-water as the solvent and these were sprayed with ninhydrin and dihydroxynaphthalene respectively. The duplicates were sprayed with AHP.

Fractions C/2, C/3 and 4/5/6(7) hydrolysed by the $H.COOH/H_2SO_4$ method and fraction C/1 hydrolysed by the $N.H_2SO_4$ method were sprayed with ninhydrin. At least 12 spots were observed with C/2 and these were spread over an area roughly corresponding to the spots produced on the control AHP chromatogram and extending a short distance further down. The spots were confluent and in some cases were on top of one another or overlapping. The colours of the spots were various including violet, yellow, orange and pink. C/3 showed only about 10 spots, mostly violet, red and pink, while C/1 showed only 7 spots, mostly violet, yellow and orange. 4/5/6(7) showed 4 weak spots - violet, yellow and pink and one indefinite. As the spots in most instances were confluent, it was not possible in most cases to correlate the spots stained with AHP with the corresponding ones stained with ninhydrin but it did appear that spot 1 corresponded to a violet one with ninhydrin. Spot 2 possibly corresponded to a yellow one but this was less definite. The 4th spot appeared to be associated with an overlapping number of spots but predominantly violet and orange or red. Spots 6, 7 and 8 appeared to be associated with violet spots. The other spots did not correspond with any definite ones with ninhydrin.

Fractions C/1, C/2 and C/3 hydrolysed by the $N.H_2SO_4$ method were sprayed with dihydroxynaphthalene spray. Only one spot was observed after the first heating (for ketoses) and this was spot 1 of fraction C/1 and no further spots showed up on further heating for uronic acids.

8. Investigation of the specificity of the chromatogram spots obtained from the hydrolysed fractions

As the chromatogram spots observed may have been produced by hydrolysis of non-specific sugars in the urine of the cattle, the following experiment was undertaken.

Fractions C/2 (10 mg./ml.) and C/3 (6.7 mg./ml.) were each added to 20 ml. amounts of hyperimmune sheep serum until an excess of antigen was demonstrated in the serum by the AGT following incubation at 37°C. for 2 hours and 4°C. for 18 hours and removal of the precipitate by centrifugation. A total of 0.25 ml. C/2 (2.5 mg.) and 0.625 ml. C/3 (4.2 mg.) were ultimately added to each aliquot of serum. The same amounts of these fractions were also added to normal sheep serum and no precipitate formed following incubation.

The solid deposits following centrifugation were washed 6 times in 10 ml. N.saline, breaking up the deposit each time after centrifugation into the smallest fragments possible with a stirring rod. Finally the deposits were made up in 0.75 ml. 0.2 M Na_2HPO_4 buffer at pH 8.5 and 0.5 ml. trypsin* (10 mg./ml.) was added followed by 0.075 ml. toluene. These mixtures were incubated at 37°C. for 48 hours by which time the solid precipitates had become floccular. A further 0.5 ml. trypsin was added and the mixtures incubated for a further 48 hours, by which time the flocculation had become a cloudiness which deposited at the bottom of the tubes after shaking. This did not disappear following the addition of a further 0.2 ml. trypsin and incubation for a further 6 hours so the process was stopped and the mixtures were dialysed against distilled water for 48 hours under negative pressure. Chloroform, 2 ml., was then added and the mixtures shaken vigorously and centrifuged at 2500 r.p.m. for 30 minutes. A white interfacial layer formed and the supernatants were removed and dried over P_2O_5 in a desiccator. These materials were examined in the AGT and it

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* Trypure Novo Crystalline Trypsin Stabalized, Novo Industri A/s, Copenhagen, Denmark.

was seen that the lines produced by the specific precipitates joined up with lines produced by C/2 and C/3 fractions respectively. The specifically precipitated materials were then hydrolysed by the $H.COOH/H_2SO_4$ method and chromatograms were prepared using n-butanol-acetic acid-water and n-butanol-pyridine-water as the solvent systems and AHP and PDMAB as the sprays.

Hydrolysed specifically precipitated fractions and hydrolysed original fractions were placed side by side on the chromatograms and compared.

Table XXVII gives details of 3 chromatograms and the spots produced by C/2 following the use of n-butanol-acetic acid-water as solvent and AHP as the spray.

Table XXVII

Chromatography of specifically precipitated and original fraction C/2, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and AHP as spray

10. R_F-value of spots						
Chromatogram	11		12		13	
Fraction	C/2	SP	C/2	SP	C/2	SP
1	46	56	50		38	
2	72	83	83		73	73
3	97	99				
4	153	153	146	146	143	143
5	199	202	197	196	197	195
6	268	282				
7	319		296	292		305

The first spot was fairly strong and brown in colour in the control original fraction but was only observed as a very faint spot in one of the specifically precipitated (SP) fractions. Spot 2 was rather weak in the original material and was only evident in one of the SP fractions. Spot 3 was very weak and only observed in one instance in both the SP and original materials. Spot 4 was a strong spot and was observed in all materials; it was a deep brown colour. Spot 5 was fairly strong and again was observed in all cases. Spot 6 was rather weak but present in all SP chromatograms and absent in only one of the original ones. Spot 7 was seen only once in an original run and was rather weak.

In all instances the SP material was applied considerably weaker than the original material and therefore any differences between the two may be quantitative.

Table XXVIII gives details of 2 chromatograms and the spots produced by C/2 following the use of the same solvent but sprayed with PDMAB. Once more the SP material was applied weaker than the original. Twelve spots were evident with both the SP and original material in one of the chromatograms, and the R_F-values compared closely. In the 2nd chromatogram only spots 1 - 5 were evident with the original material and spots 2, 5 and 7 with the SP material. Again the R_F-values compared well.

Spots 1, 2 and 4 were red brown in colour. Spot 3 was yellow. Spots 5, 11 and 12 were pink. Spots 6, 7 and 10 were violet. Spot 9 was salmon coloured.

/Table XXVIII....

Table XXVIII

Chromatography of specifically precipitated and original fraction C/2, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-acetic acid-water as solvent and PDMAB as spray

	10. R_F-value of spots			
Chromatogram	11		13	
Fraction	C/2	SP	C/2	SP
1	38	27	38	
2	60	48	62	63
3	86	86	103	100
4	135	135	145	140
5	189	186	197	192
6	232	235		
7	295	295		298
8	327	330		
9	405	405		
10	511	514		
11	554	554		
12	589	589		

Table XXIX gives details of the spots produced by C/3, SP and original, following the use of n-butanol-pyridine-water and AHP spray. Spots 1 and 2 were very weak and the colour indefinite. Spot 3 was fairly strong and olive coloured. Spots 4 - 6 were all brown in colour, while spot 7 was olive.

Table XXIX

Chromatography of specifically precipitated and original fraction C/3, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-pyridine-water as solvent and AHP as spray

	10. R_F-value of spots			
Chromatogram	15		16	
Fraction	C/3	SP	C/3	SP
1			127	132
2			206	200
3			246	241
4	306	283	294	294
5			334	344
6	404	383	381	390
7	460		431	433

Table XXX gives details of the spots produced by C/3, SP and original, following the use of n-butanol-pyridine-water and PDMAB spray. Spots 1 and 2 were weak and the colour indefinite. Spot 3 was weak but the colour was pink. Spot 4 was pink with an orange centre. Spot 5 was violet. Spots 6 and 8 were olive and spot 7 was violet.

/Table XXX....

Table XXX

Chromatography of specifically precipitated and original fraction C/3, hydrolysed by the $H.COOH/H_2SO_4$ method, using n-butanol-pyridine-water as solvent and PDMAB as spray

	10. R_F-value of spots	
Chromatogram	15	
Fraction	C/3	SP
1	40	
2	140	
3	236	
4	250	256
5	287	296
6		333
7	390	387
8	463	459

From the results of these chromatograms it appears that the chromatogram spots produced by the fractions C/2 and C/3, and therefore probably also those produced by C/1 and 4/5/6(7), are specific in that identical spots are produced by specifically precipitated fractions.

9. From examination of the R_F-values of the various chromatogram spots, composite tables were prepared for each fraction for each solvent system. Tables XXXI - XXXIV for the n-butanol-acetic acid-water system and tables XXXV - XXXVII for the n-butanol-pyridine-water system.

From tables XXXI - XXXIV it can be seen that spot 1 was consistently present with fractions C/1, C/2 and C/3 but was only seen in one instance with fraction 4/5/6(7). It was, however, a fairly well defined spot in this one case. The second spot was weak and occurred spasmodically with all fractions. Spot 3 was also weak and was observed with fractions C/2, C/3 and 4/5/6(7) spasmodically. Spot 4 was the strongest and was observed with fractions C/1, C/2 and C/3 but not with 4/5/6(7). Spot 5 was present with all fractions and was fairly strong. Spot 6 was observed only with 4/5/6(7) and was fairly strong. Spot 7 was present in C/1 and in one instance with C/2. It was a weak spot. The eighth spot was seen with C/2 and C/3 and was moderately strong. Spots 9 - 13 inclusive only stained with PDMAB and in only one instance. These spots were not strong but were definite and corresponded roughly with certain violet spots produced by the ninhydrin spray.

Tables XXXV - XXXVII include only 3 of the fractions due to shortage of fraction 4/5/6(7). Spot 1 was present in all fractions and was rather weak. Spot 2 was present in C/2 and C/3 and was also weak. Spot 3 was observed with all fractions and was rather weak. Spot 4 was fairly strong in C/2 and C/3 but was only observed with C/1 when stained with PDMAB. The 5th spot was the strongest and was present with all fractions. Spot 6 was only observed with C/3 and was not very strong. Spot 7 occurred regularly with C/2 and C/3 and was fairly strong but was very weak with C/1. The 8th spot was seen with all fractions and was rather weak.

Table XXXI

Composite table of all chromatogram spots produced by fraction C/1, using n-butanol-acetic acid-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	10. R_F-value of spots							
		1	2	3	4	5	6	7	8
1	AHP	41			155				
2	"	30			153				
3	"	33			158				
7	"	43	91		152	200			
8	"	33	83		128	174			
8	PDMAB	27	78		123	173		255	
10	AHP	38	84		133	176			
10	PDMAB	33			128				

Table XXXII

Composite table of all chromatogram spots produced by fraction C/2, using n-butanol-acetic acid-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	No. R_F-value of spots							
		1	2	3	4	5	6	7	8
1	AHP	45	94		155				
2	"	*			154	214			
3	"	*			163	224			
5	"			116	193	235			309
6	"	40			149	188		235	283
7	"	52	87		152	204		247	294
10	"	38	74		138	184		242	288
10	PDMAB	33	79		130	179		230	
11	AHP	46	72	97	153	199		268	319
11x	"	56	83	99	153	202		282	
11	PDMAB	38	60	86	135	189		232	295
11x	"	27	48	86	135	186		235	295
12	AHP	50	83		146	197			296
12x	"				146	196			292
13	"	38	73		143	197			
13x	"		73		143	195			305
13	PDMAB	38	62	103	145	197			
13x	"		63	100	140	192			298

* A very strong streak was present at the site of application and extending some distance down the sheet.

x Specifically precipitated fraction.

Table XXXIII

Composite table of all chromatogram spots produced by fraction C/3, using n-butanol-acetic acid-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	10. R_F-value of spots							
		1	2	3	4	5	6	7	8
1	AHP				154				
2	"	36	82		155				
3	"	27	64		157				
5	"	25	80	114	190				311
6	"	37			149	196			287
7	"	48			156	208			303
9	"	27	77		124	177			269
10	"	33		102	135	184			278
10	PDMAB	24		92	127	178			
12	AHP	49	79		148	195			293

Table XXXIV

Composite table of all chromatogram spots produced by fraction 4/5/6(7), using n-butanol-acetic acid-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	10. R_F-value of spots							
		1	2	3	4	5	6	7	8
6	AHP	33	75	129		162	221		
7	"		69	129		164	216		
8	"		96			179			
8	"					175			
8	PDMAB		62	105		131	188		
9	AHP		54	108		173	204		
10	"		74	115			207		
10	PDMAB			108			210		

Table XXXV

Composite table of all chromatogram spots produced by fraction C/1, using n-butanol-pyridine-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	10. R_F-value of spots							
		1	2	3	4	5	6	7	8
14	AHP	35				315		405	
15	PDMAB	22		212	244	283			453

Table XXXVI

Composite table of all chromatogram spots produced by fraction C/2, using n-butanol-pyridine-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	10. R_F-value of spots							
		1	2	3	4	5	6	7	8
14	AHP	37	149		255	321		414	
15	PDMAB		148	216	250	301		404	468
16	AHP			214	247	310		403	461

Table XXXVII

Composite table of all chromatogram spots produced by fraction C/3, using n-butanol-pyridine-water solvent and AHP and PDMAB sprays

Chromatogram	Spray	10. R_F-value of spots							
		1	2	3	4	5	6	7	8
14	AHP	47	147		258	321		413	471
15[x]	"					283		383	
15	PDMAB		139	220	256	302		405	476
15[x]	"				256	296	333	387	459
15	"	40	140	236	250	287		390	463
16	AHP		127	206	246	294	334	381	431
16	"					306		404	460
16[x]	"		132	200	241	294	344	390	433

x Specifically precipitated fraction

10. From the information available it is possible to make a few tentative assumptions as to the possible class of substances producing some of the chromatographic spots. In some cases it is even possible to make tentative identification of the substances producing the spots.

The first spot was generally well defined and its R_F-values were similar with both solvent systems and did not correspond to any of the control sugars, being much less than raffinose which was the one with the smallest R_F-value. This spot gave a brown colour with AHP and a definite cherry red colour with PDMAB, indicating that the material could be an hexosamine, probably a free amine sugar. The violet colour given by ninhydrin to this spot would also indicate the presence of an amino radicle.

Spot 2 was weak and did not correspond with any of the control sugars used with either solvent system. It gave a brown colour with AHP and a pink colour with PDMAB which may indicate that this substance is also an hexosamine but the spot was really too weak for the colours to be identified accurately.

The 3rd spot with the acetic acid solvent which possibly corresponds to the 4th spot with the pyridine solvent was a strong spot and gave an olive colour with AHP and strong pink with PDMAB. Its R_F-values did not correspond with any of the controls used but its colour reactions would tend to indicate that it is not an aldopentose but may be one of a variety of substances which give an olive-brown colour with AHP including aldohexoses, deoxyaldohexoses, aldoheptoses, aldo-octoses and aldohexuronic acids (Partridge, 1949; Hough, Jones and Wadman, 1950).

The 4th spot with the acetic acid solvent and the 5th spot with the pyridine solvent appeared identical and it is probable that it is galactose as its R_F-values with both solvent systems corresponding closely with those of galactose. Also the evidence obtained when galactose was run together with the unknown further points to this being galactose. Its colour with AHP, brown (indicating aldohexoses etc.), also corresponds

closely to the colour produced by galactose. With PDMAB spray, however, this spot stained strongly purple, indicative of N-acetyl hexosamines. The ninhydrin spray indicated an overlapping of violet and orange or red spots at this position which may possibly mean that certain amino acids or peptides, with a similar migration in this solvent, are present in the fraction.

The 5th spot with the acetic acid spray, which probably corresponds to the 7th spot with the pyridine spray, had R_F-values corresponding closely with that of sorbose. The colour with AHP was brown and with PDMAB a weak violet/pink. Again this may have been due to contaminating amino acids.

Spot 6 with butanol-acetic acid-water, which was present only in fraction 4/5/6(7), may possibly be arabinose as its R_F-values correspond closely to those of arabinose and furthermore its colour with AHP, a definite pink, indicating an aldopentose (Partridge, 1949), also corresponds with the colour of the control arabinose.

The 7th spots with both solvents were very similar - both were brown with AHP and violet with PDMAB. The R_F-values and colour of the spot with the acetic acid solvent was close to that of xylose. The R_F-value of the spot using the pyridine solvent, however, did not correspond with any of the control sugars used and certainly not with xylose. The 8th spots with both solvents were also similar in colour, being olive with AHP and very faintly and indefinitely staining with PDMAB. In this case, however, the spot produced by the pyridine solvent had an R_F-value corresponding closely with that of xylose but the colour did not correspond, xylose being red in colour. The spot produced by the acetic acid solvent did not correspond with any of the sugars used. The identities of these spots therefore remain unknown. Also spot 3 with the pyridine solvent has not been correlated to any of the spots produced by the acetic acid solvent. Its colour, olive with AHP and pale pink with PDMAB, makes it possible that this one corresponds to the 8th spot with acetic acid solvent.

The effect of heat and trypsin on the precipitating antigenic components

a) Heat

It proved unnecessary to use temperatures lower than the boiling point of water (94°C.) and times less than 60 minutes as this combination had no apparent effect on the precipitin bands as can be seen in figs. 33 and 34.

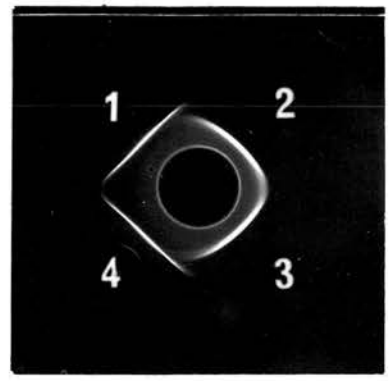


Fig. 33. Comparison between the precipitin bands produced by heated and unheated fractions C/1 and C/3.

Centre well		sheep x serum
Wells	1	unheated C/1
	2	unheated C/3
	3	heated C/3
	4	heated C/1

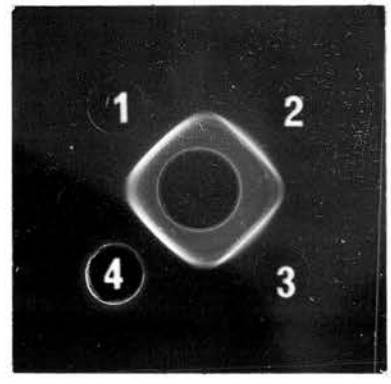


Fig. 34. Comparison between the precipitin bands produced by heated and unheated fraction C/2 and urine (pooled)

Centre well		sheep x serum
Wells	1	unheated urine
	2	unheated C/2
	3	heated C/2
	4	heated urine

b) Trypsin

The AGT indicated that there was no difference in the precipitin bands produced by trypsinised and untreated fractions. Fig. 35 shows trypsinised C/1/2/3 in wells 1 and 3 and the control untreated C/1/2/3 in wells 2 and 4 and no differences are apparent.

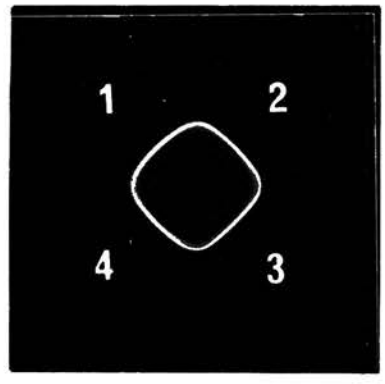


Fig. 35. Comparison between the precipitin bands produced by trypsinised and untreated C/1/2/3.

Centre well		sheep x serum
Wells	1	untreated
	2	trypsinised, diluted 1/2
	3	" " 1/8
	4	" " 1/4

Attempted separation of the precipitating antigenic components by ultracentrifugation

No separation of the antigenic components was seen as all aliquots gave identical precipitin bands as can be seen in fig. 36 in which the 1st, 3rd, 6th and 8th aliquots of the second tube are compared.

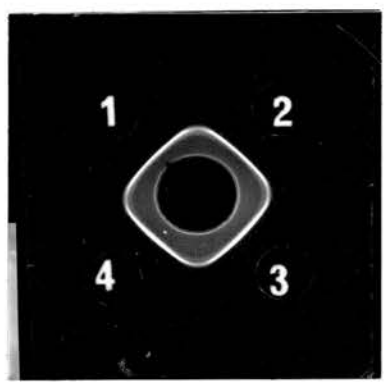


Fig. 36. Attempted separation of precipitating antigens by ultracentrifugation : Comparison between the precipitin bands produced by various aliquots.

Centre well		sheep x serum
Wells	1	aliquot 8
	2	" 1
	3	" 3
	4	" 6

Attempted separation of the precipitating antigenic components by immunoelectrophoresis

1. Fractions C/1/2/3 - 11 mg./ml., C/2 - 10 mg./ml., C/3 - 6 mg./ml. and 4/5/6(5) - 10 mg./ml. were electrophorised using veronal buffer pH 7.3. No separation of the precipitin bands was observed and they remained together but had migrated some distance towards the anode. The migration distances, measured from the lowest point of the arcs produced to a vertical line drawn through the centre of the transverse trough, were C/1/2/3 - 6.5 mm., C/2 - 6 mm., C/3 - 6 mm. and 4/5/6(5) - 5.5 mm.

2. Following the above results and the slight migration demonstrated but lack of separation of the precipitin bands using veronal buffer, the experiment was repeated using borate buffers pH 8.0 and 10.0 as migration of certain polysaccharide and carbohydrate derivatives in electrophoresis is facilitated by the formation of negatively charged complexes with the borate ions present in the alkaline buffer employed as the conducting solution.

In this experiment fractions C/1/2/3, C/2 and C/3 were run using borate buffered agar at pH 8.0 and pH 10.0. Again no separation of the precipitin bands occurred; they remained together, but had migrated further towards the anode. At pH 8.0 all the fractions had migrated 10 mm., measured as before from the lowest point of the arcs to the centre of the transverse trough. At pH 10.0, C/1/2/3 and C/3 had migrated 15 mm. and C/2 18 mm. An example of the migration without separation is given in figs. 37 and 38 where C/1/2/3 and C/3 were used with borate buffer pH 8.0.

Fig. 37. Precipitin bands produced following electrophoresis of fraction C/3 using borate buffer pH 8.0

Fig. 38. Precipitin bands produced following electrophoresis of fraction C/1/2/3 using borate buffer pH 8.0

Complement fixation

Both fractions C/1/2/3 and 4/5/6(5) fixed complement using hyperimmune sheep 5 serum. The results of the tests are given in tables XXXVIII and XXXIX. As can be seen the results show that the optimum concentration of C/1/2/3 was 0.74 µg./ml. and the antiserum 1/160 dilution, while the optimum concentration of 4/5/6(5) was 20 µg./ml. for the antigen and 1/160 for the antiserum.

Repeated tests with both fractions showed very similar results.

Table XXXVIII

Two-dimensional complement-fixation test with hyperimmune sheep 5 serum and fraction C/1/2/3 antigen

Fraction C/1/2/3 dilutions	Serum dilutions					
	1/20	1/40	1/80	1/160	1/320	1/640
20 µg./ml.	++++	++++	++++	-	-	-
6.6 "	++++	++++	++++	-	-	-
2.2 "	++++	++++	++++	++	-	-
0.74 "	++++	++++	++++	+++	-	-
0.247 "	++++	++++	++++	-	-	-
0.082 "	++++	++++	+++	-	-	-
0.027 "	++++	++++	++	-	-	-
0.009 "	+++	+++	-	-	-	-
0.003 "	-	-	-	-	-	-
0.001 "	-	-	-	-	-	-
V.B.	-	-	-	-	-	-

++++ Complete fixation (no haemolysis)
+++ Almost complete fixation (slight haemolysis)
++ Partial fixation (partial haemolysis - 50%)
+ Very slight fixation (not quite complete haemolysis)
- No fixation (complete haemolysis)
V.B. Veronal buffer

Table XXXIX

Two-dimensional complement-fixation test with hyperimmune sheep 5 serum and fraction 4/5/6(5) antigen

Fraction 4/5/6(5) dilutions	Serum dilutions					
	1/20	1/40	1/80	1/160	1/320	1/640
540 µg./ml.	++++	++++	++++	-	-	-
180 "	++++	++++	+++	-	-	-
60 "	++++	++++	+++	+	-	-
20 "	++++	++++	+++	++	-	-
6.6 "	++++	++++	+++	-	-	-
2.2 "	++++	+++	-	-	-	-
0.74 "	++	-	-	-	-	-
0.25 "	+	-	-	-	-	-
0.082 "	-	-	-	-	-	-
0.027 "	-	-	-	-	-	-
V.B.	-	-	-	-	-	-

++++ Complete fixation (no haemolysis)

+++ Almost complete fixation (slight haemolysis)

++ Partial fixation (partial haemolysis - 50%)

+ Very slight fixation (not quite complete haemolysis)

- No fixation (complete haemolysis)

V.B. Veronal buffer

Absorption of sera with the fractions

1. Three 1 ml. amounts of sheep x serum were absorbed with 1.1 mg., 0.55 mg. and 0.11 mg. of fraction C/1/2/3 in 0.1 ml., 0.05 ml. and 0.01 ml. distilled water respectively. Each absorbed serum was examined for residual homologous antibody and excess antigen by the qualitative AGT. Sera absorbed with 1.1 mg. and 0.55 mg. C/1/2/3 contained excess antigen but no homologous antibody, whereas the serum absorbed with 0.11 mg. antigen retained some homologous antibody and showed no signs of excess antigen.

The absorbed sera were examined in the qualitative AGT against urine (pooled), serum Oremit 2, pleural fluid Oremit 2, T3 antigen, fractions C/1/2/3 and 4/5/6(7) and ultrasonicated M. mycoides. Unabsorbed sheep x serum controls were also set up. The sera absorbed with 1.1 mg. and 0.55 mg. C/1/2/3 produced no precipitin lines with any of the materials except one line with serum Oremit 2 whereas very good precipitin bands were formed with the unabsorbed control serum. The serum absorbed with 0.11 mg. C/1/2/3, however, showed 2 lines with serum Oremit 2 and also weak lines with urine (pooled), pleural fluid Oremit 2, C/1/2/3, 4/5/6(7) and ultrasonicated M. mycoides. In the SAST the serum absorbed with 0.11 mg. antigen gave a strong positive reaction (+++) whereas the sera absorbed with 0.5 mg. and 1.1 mg. gave only a weak fine agglutination (+). Unabsorbed control serum gave a strong floccular reaction (+++). In the tube agglutination test, the titre of the hyperimmune sheep serum before absorption was 1/640. Following absorption with 0.11 mg. C/1/2/3, the titre was 1/320, while absorption with 0.55 mg. C/1/2/3 gave a lower titre, 1/80. This titre, however, did not drop further even when the serum was absorbed with 1.1 mg. C/1/2/3. The CFT produced a titre of 1/640 for the unabsorbed serum, 1/80 for the serum absorbed with 0.11 mg. C/1/2/3 and 1/40 for the two sera absorbed with 0.55 mg. and 1.1 mg. C/1/2/3, respectively. Details of the serological tests with these absorbed sera are given in table XL.

Table XL

Absorption of hyperimmune sheep x serum with varying amounts of fraction C/1/2/3. Results of serological tests

Serum	SAST*	AT* titre	CFT* titre	AGT*	
				Antigen	Antibody
Unabsorbed	+++	1/640	1/640	-	+
Absorbed 0.11 mg.	+++	1/320	1/80	-	+
" 0.55 mg.	+	1/80	1/40	+	-
" 1.10 mg.	+	1/80	1/40	+	-

* SAST Slide agglutination serum test
AT Tube agglutination test
CFT Complement fixation test
AGT Qualitative agar gel test

2. A different sheep serum, sheep 6, was absorbed with fractions C/1, C/2, C/3, C/1/2/3 and 4/5/6(7). Five 2 ml. amounts were absorbed with 0.3 mg. C/1 and 0.3 mg. C/2 each in 0.03 ml. distilled water, 0.4 mg. C/3 in 0.06 ml. distilled water, 1.32 mg. C/1/2/3 in 0.12 ml. distilled water and 0.4 mg. 4/5/6(7) in 0.04 ml. distilled water. In each case no homologous antibody remained and slight excess antigen was demonstrated in the AGT. Serological tests were carried out on each absorbed sera, details of which are given in table XLI.

Table XLI

Absorption of hyperimmune sheep 6 serum with fractions C/1, C/2, C/3, C/1/2/3 and 4/5/6(7). Results of serological tests

Serum	SAST*	AT* titre	CFT* titre	AGT*	
				Antigen	Antibody
Unabsorbed	++++	1/1280	1/80	-	+
Absorbed with:					
C/1	+	1/160	1/20	+	-
C/2	+	1/160	1/20	+	-
C/3	++	1/320	1/40	+	-
C/1/2/3	+	1/40	1/10	+	-
4/5/6(7)	+++	1/320	1/80	+	-

* SAST Slide agglutination serum test
AT Tube agglutination test
CFT Complement fixation test
AGT Qualitative agar gel test

The SAST and tube agglutination test results correlated fairly well. The unabsorbed serum was ++++ and 1/1280. When absorbed with C/1 it was + and 1/160, with C/2 it was also + and 1/160, with C/3 it was ++ and 1/320, with C/1/2/3 it was + and 1/40 and with 4/5/6(7) it was +++ and 1/320. The unabsorbed serum gave a titre of 1/80 in the CFT. When the serum was absorbed with C/1 the titre was 1/20, when absorbed with C/2 it was also 1/20, when absorbed with C/3 it was 1/40, with C/1/2/3 it was 1/10 and with 4/5/6(7) it was 1/80, the same as the unabsorbed serum.

Qualitative AGT with the absorbed sera showed that the sera absorbed with C/1 and C/2 did not produce any precipitin bands against the homologous nor the heterologous fractions, but the serum absorbed with C/3 produced a line with fraction C/1. There was unfortunately insufficient serum absorbed with fraction 4/5/6(7) for the qualitative AGT.

Pyrogenicity

Both fraction 4/5/6(7) and C/1/2/3 proved to be weakly pyrogenic. The body temperatures of 4 control rabbits observed during the tests did not rise above 101.8°C. As little as 0.67 µg./kg. of 4/5/6(7) gave a response, though only transient, the temperature rising to 102.4°C. in 2½ hours. Larger doses resulted in a higher and more rapid rise in temperature and the pyrexia was maintained for a longer time. A peak temperature of 104°C. in 1¾ hours was obtained with 3426 µg./kg. which returned to normal at about 4½ hours. A smaller dose, 660 µg./kg., gave an equally rapid rise to 103.7°C. and the pyrexia was maintained until 5½ hours.

With fraction C/1/2/3, 0.597 µg./kg. did not produce any significant pyrexia, but a dose of 707 µg./kg. gave a transient pyrexia of 102.6°C. at 2½ hours after an initial subnormal drop in temperature. A large dose, 3125 µg./kg., gave a rapid rise in temperature to 104.3°C. in 1¾ hours which slowly dropped but was still above normal after 6¼ hours when recording ceased. The temperature charts are shown in Figs. 39 and 40. The broken lines on the charts represent the maximum rise in body temperature of the control rabbits, i.e. 101.8°F.

Fig. 39. Effects of intravenous injections of fraction C/1/2/3 on the body temperature of rabbits.

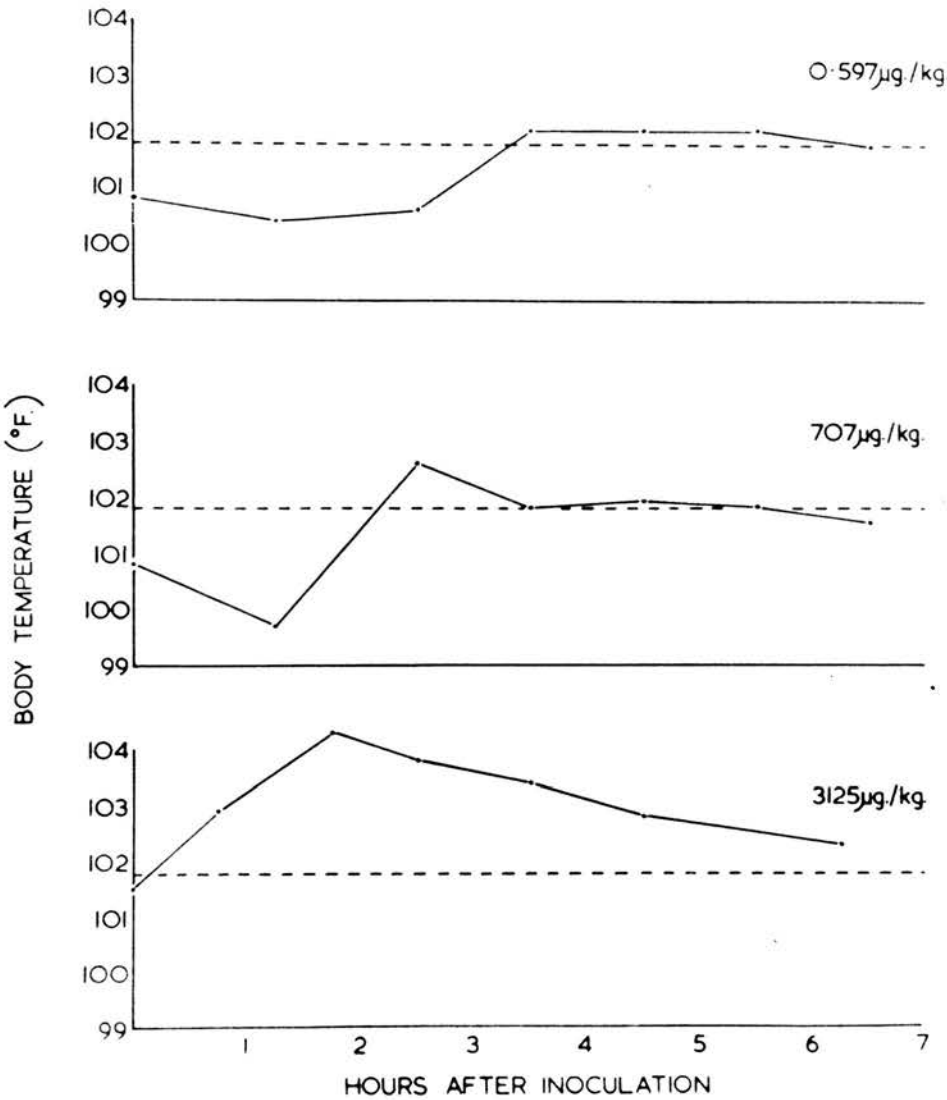
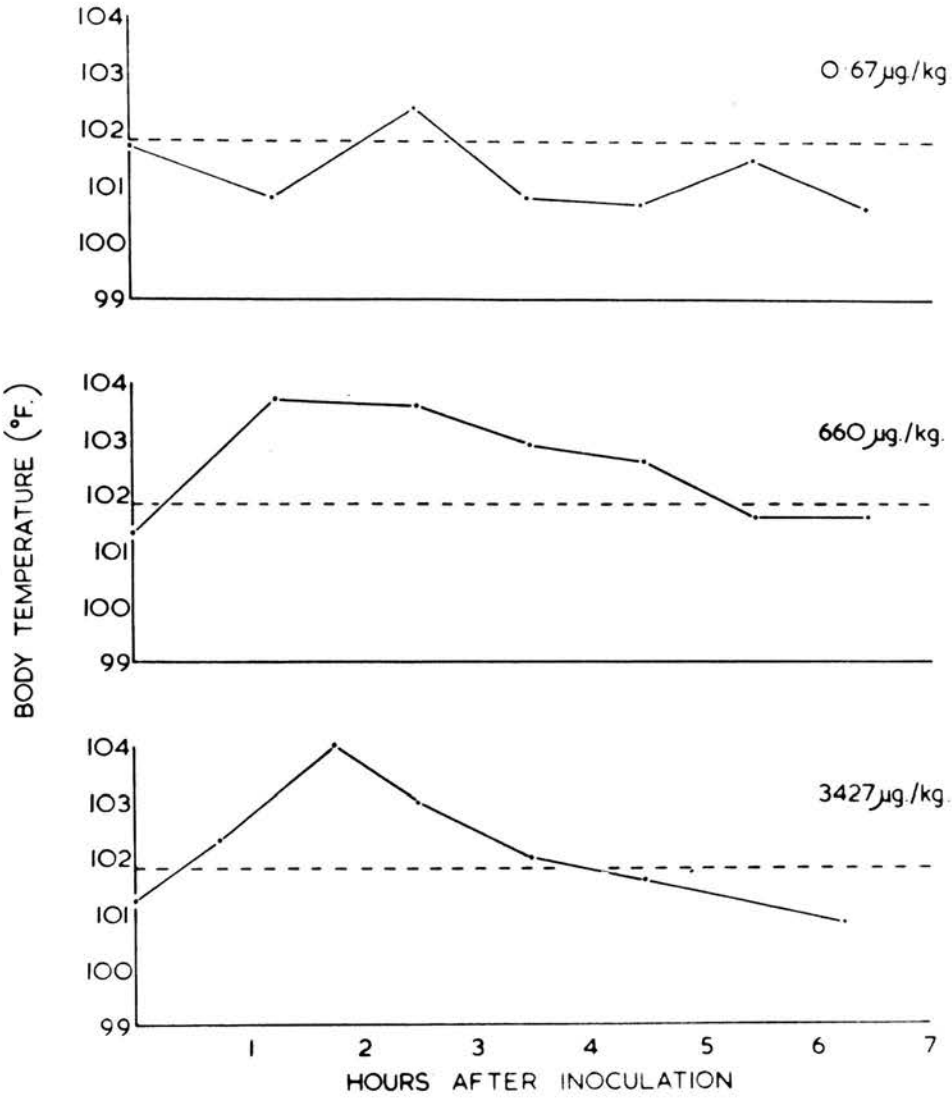

0·597µg./kg.
707µg./kg.
3125µg./kg.
104
103
102
101
100
99
BODY TEMPERATURE (°F.)
1
2
3
4
5
6
7
HOURS AFTER INOCULATION

Fig. 40. The effect of intravenous injections of fraction 4/5/6(7) on the body temperature of rabbits.


104
103
102
101
100
99
0·67µg./kg.
660µg./kg.
3427µg./kg.
BODY TEMPERATURE (°F.)
1
2
3
4
5
6
7
HOURS AFTER INOCULATION

Toxicity

Rabbits were inoculated intravenously with 5 mg. of C/1/2/3 and 4.9 mg. of 4/5/6(7) or 3.125 mg./kg. body weight and 3.43 mg./kg. body weight respectively without showing signs of sickness.

Mice were inoculated intravenously with 0.3 mg. of C/1/2/3 and 0.3 mg. of 4/5/6(7) also without showing any signs of sickness.

Fraction C/1/2/3 proved to be lethal to fowl embryos. The majority of deaths occurred between six and eight days after inoculation and examination of the dead embryos did not reveal any gross lesions. The LD_{50} dose of fraction C/1/2/3 calculated by the method of Reed and Muench (1938) was 179 µg. Details of the experiment are given in table XLII.

Table XLII

The inoculation of fraction C/1/2/3 into 10-day old fowl embryos by the allantoic route

C/1/2/3 inoculum µg.	Embryos inoculated	Deaths								Total
		Days Post-inoculation								
		1	2	3	4	5	6	7	8	
1000	6	0	0	0	0	0	1	3	2	6
500	6	1	1	0	0	0	1	1	0	4
250	6	0	0	0	0	0	1	2	0	3
125	6	0	0	0	0	0	0	0	0	0
62.5	6	0	0	0	0	0	1	0	0	1
31.25	6	0	0	0	0	0	1	2	0	3
15.6	6	0	0	0	0	0	0	0	0	0
7.8	6	0	0	0	0	0	0	0	1	1
3.9	6	0	0	0	0	0	1	0	0	1
Control	6	0	1	0	0	0	0	0	0	1
Total deaths		1	2	0	0	0	6	8	3	20

The intravenous inoculation of 2.0 mg. of fraction C/1/2/3 into the 2 cattle produced only some slight salivation and licking of the muzzle within a few minutes of inoculation otherwise no ill-effects were observed.

Allergic Reaction

There was no indication of an allergic reaction at the inoculation site in any of the immune animals. The 2 animals that received 0.02 mg. C/1/2/3 had maximum increases in skin thickness of 2 mm. and 1 mm. while the controls both had 1 mm. increases. The cattle that received 0.2 mg. had increases of 2 mm. and nil, and the controls 1 mm. and 2 mm. The remaining animal that received 2.0 mg. developed an increase of 2 mm. as did the control animal.

The Aggressive Action of Fraction C/1/2/3

1. In the first experiment only 2 animals produced any swellings. Ox No. 6436, an immune animal which possessed a CFT titre of + (weak positive) and a SAST titre of ++ before inoculation and which had been inoculated with 0.5 ml. (2.3 mg.) C/1/2/3, developed after 9 days a small nodule 3 cm. in diameter at the inoculation site on the side that the fraction had been inoculated. This nodule regressed rapidly to become a small fibrous nodule after 4 days. No swelling was seen on the other side. The other animal to develop a swelling was the susceptible one, No. 7645. This animal possessed no CFT or SAST antibodies, and it developed a swelling also on the side which received the fraction. This swelling first appeared 9 days after inoculation as a small nodule 3 cm. in diameter which slowly enlarged until it was extensive, that is over 25 x 25 cm. in area, after a further 12 days and had to be destroyed in extremis 6 days later. No swelling was seen on the other side.

The CFT and SAST titres of the 2 animals that did not produce swellings were: No. 6173, CFT ++ and SAST ++, No. 6333, CFT negative and SAST negative.

2. The results of the second experiment are given in table XLIII. In the case of the immune animals a very rapid reaction occurred, suggestive of an allergic response. This was particularly marked with animal 6504, and it is of interest that in the case of the organisms alone this swelling quickly regressed and had disappeared by the seventh day, whereas the swelling produced by the organisms plus C/1/2/3 regressed only very slowly and was still evident after 18 days. Ox 6456 produced a rapid reaction with the organisms alone which also disappeared rapidly, whereas the swelling produced by the organisms plus antigen was not observed until the second day and was relatively small and disappeared within 3 days. Only 1 of the susceptible animals reacted and the swelling was on the side that was inoculated with the organisms plus antigen. The swelling which was relatively large commenced on the sixth day, at about the time that most of the swellings in the immune animals had almost disappeared, and regressed only slowly, being still present on the eighteenth day.

Table XLIII

The Aggressive Action of Fraction C/1/2/3. Second Experiment

Ox No.	Status	Prebleed. CFT*	Prebleed. SAST*	Inoculum 1 ml. organisms plus	Increase in skin thickness in mm. Days after inoculation 1	2	3	4	6	7	8	9	10	11	13	14	15	16	17	18
6456	Immune	±	+	0.5 ml.C/1/2/3	-	4	4	5	-	-	-	-	-	-	-	-	-	-	-	-
				0.5 ml. saline	9	10	10	11	3	1	-	-	-	-	-	-	-	-	-	-
6504	"	+	+++	1.0 ml.C/1/2/3	17	16	8	9	8	8	6	5	5	4	5	2	2	2	2	2
				1.0 ml. saline	12	17	12	7	2	-	-	-	-	-	-	-	-	-	-	-
7671	Susceptible	-	-	0.5 ml.C/1/2/3	-	-	-	-	18	17	17	17	12	8	8	4	4	4	4	4
				0.5 ml. saline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7657	"	-	-	1.0 ml.C/1/2/3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
				1.0 ml. saline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* CFT Complement fixation test

SAST Slide agglutination serum test

3. Six of the 8 immune cattle reacted by producing swellings, and the first signs of swellings in all but 1 of these occurred on the third day after inoculation. Animal No. 7623 developed swellings on both sides within 24 hours of inoculation, which in the case of the organisms alone enlarged slightly before regressing slowly. The swelling produced by the organisms plus antigen was large on the first day but had disappeared on the second day. On the third day, however, a swelling was noted again which slowly regressed and disappeared at about the same time as the other swelling. It was noticed that the swelling on the first day was soft and oedematous whereas the swelling which appeared on the third day was firm. Four of the remaining animals developed swellings only on the side that had received organisms plus antigen. They all appeared first on the third day, enlarged slightly and then slowly regressed and had disappeared or formed a small nodule by the thirteenth day. The remaining animal, No. 7819, developed transient swellings on both sides on the fourth day. The swelling on the side inoculated with organisms alone had disappeared by the next day and the swelling on the other side had disappeared by the day after. Animal No. 7623 had previously undergone a severe reaction and the CFT and SAST results indicated a high antibody level. This applied equally to No. 7817 and No. 7829 which did not appear to develop allergic reactions. The results of this experiment are given in table XLIV.

The 5 susceptible cattle all developed swellings on both sides of the body. Nevertheless, in 2 animals, Nos. 8154 and 8158, the swellings developed earlier on the side inoculated with organisms plus antigen. The results of this experiment are given in table XLV.

Table XLIV

Aggressive Action of Fraction C/1/2/3. Third Experiment. Immune Cattle

Ox No.	Prebleed. CFT*	Prebleed. SAST*	Inoculum 2 ml. organisms plus	Size of swellings in cm. Days after inoculation 1	2	3	4	5	6	7	8	9	10	13	18
7817	+++	+++	4 mg. C/1/2/3	-	-	±	5x8	3x8	3x8	3x8	3x8	3x8	3x5	3x3	-
			0.8 ml.saline	-	-	-	-	-	-	-	-	-	-	-	-
7824	++	++	4 mg. C/1/2/3	-	-	1x1	5x5	8x8	8x6	8x6	8x6	8x6	5x5	3x3	-
			0.8 ml.saline	-	-	-	-	-	-	-	-	-	-	-	-
7819	++++	++++	2 mg. C/1/2/3	-	-	-	3x3	5x3	-	-	-	-	-	-	-
			0.4 ml.saline	-	-	-	3x3	-	-	-	-	-	-	-	-
7736	+++	+	2 mg. C/1/2/3	-	-	1x1	3x3	3x3	3x3	3x3	3x3	N	N	-	-
			0.4 ml.saline	-	-	-	-	-	-	-	-	-	-	-	-
7829	++++	++++	1 mg. C/1/2/3	-	-	5x5	5x5	8x5	5x5	5x3	3x3	3x3	N	-	-
			0.2 ml.saline	-	-	-	-	-	-	-	-	-	-	-	-
7740	++++	+++	1 mg. C/1/2/3	-	-	-	-	-	-	-	-	-	-	-	-
			0.2 ml.saline	-	-	-	-	-	-	-	-	-	-	-	-
7828	+	++	0.5mg.C/1/2/3	-	-	-	-	-	-	-	-	-	-	-	-
			0.1 ml.saline	-	-	-	-	-	-	-	-	-	-	-	-
7623	++++	++++	0.5mg.C/1/2/3	8x8	-	5x5	5x5	5x5	5x5	5x3	3x3	3x3	2x3	N	-
			0.1 ml.saline	3x3	5x5	8x5	5x5	5x5	5x5	3x3	3x3	2x3	N	N	-

N = small fibrous nodule

* CFT Complement fixation test

SAST Slide agglutination serum test

Table XLV

The Aggressive Action of Fraction C/1/2/3. Third experiment. Susceptible cattle

Ox No.	Inoculum		Size of swellings in cm.											
	Orgs.		Days after inoculation											
			1	2	3	4	5	6	7	8	9	10	13	18
8153	4 ml.	C/1/2/3	-	-	-	-	5x5	10x10	15x15	15x15	18x25	25x25	E	E
		saline	-	-	-	-	5x5	8x10	15x15	25x25	25x25	25x25	E	E
8154	2 ml.	C/1/2/3	-	-	-	±	5x5	8x8	15x15	10x13	10x15	10x13	10x13	10x13
		saline	-	-	-	-	-	10x10	15x15	25x25	15x20	10x15	E	E
8155	2 ml.	C/1/2/3	-	-	-	-	5x5	5x5	8x13	8x13	10x15	25x25	E	E
		saline	-	-	-	-	3x3	8x10	13x13	13x13	25x25	15x18	18x18	18x15
8157	1 ml.	C/1/2/3	-	-	-	-	-	-	3x8	3x8	N	N	-	-
		saline	-	-	-	-	-	-	5x5	8x8	10x10	8x8	8x8	5x5
8158	1 ml.	C/1/2/3	-	-	-	-	±	3x3	5x3	5x5	8x8	10x8	15x15	*
		saline	-	-	-	-	-	-	5x5	8x5	10x10	10x8	25x15	*

E = extensive swelling, i.e. larger than 25x25 cm.

N = small fibrous nodule

* = no measurements available

Attempted immunization of rabbits and mice with fractions C/1/2/3 and 4/5/6(7) alone or combined with 'Shiga' conjugated protein

(1) Attempted immunization with fractions C/1/2/3 and 4/5/6(7) alone

The sera obtained from rabbits and mice inoculated with these fractions alone contained no demonstrable antibodies to the respective fractions or M. mycoides by the AGT, SAST or CFT.

(2) Attempted immunization with fractions C/1/2/3 and 4/5/6(7) combined with 'Shiga' conjugated protein

Rabbits

Antibodies against fraction C/1/2/3 were demonstrated in the sera of the rabbits. In the qualitative AGT a very faint precipitin line was seen following the 3rd series of inoculations, when the rabbits had received a total of 4.5 mg. conjugate, when the sera were put up against the shiga conjugate but not against C/1/2/3 alone. At this stage the SAST was negative. After the fourth series of inoculations when the rabbits had received a total of 5.7 mg. conjugate, a stronger precipitin band was seen against the shiga conjugate and a very faint one against C/1/2/3 alone. The SAST was again negative. Following the fifth series of inoculations, total conjugate = 8.7 mg., the precipitin lines against C/1/2/3 alone were as strong as those formed against the conjugate and it was seen that apparently 2 precipitin lines formed with C/1/2/3 and probably 3 with the conjugate. When the serum of rabbit 2, the stronger of the two, was put up against fractions C/1/2/3, C/1, C/2, C/3 and 4/5/6(7) and serum Oremit 2, 2 lines were formed against C/1/2/3, C/2 and serum Oremit 2, but only one against the other materials. However, the CFT, SAST and tube agglutination test with the serum of rabbit 2 proved to be negative.

Mice

The sera obtained from mice contained no demonstrable antibodies to the respective fractions or M. mycoides by the AGT, SAST or CFT.

Calves

The sera obtained from 2 calves contained no demonstrable antibodies to fraction C/1/2/3 or to M. mycoides by the AGT, SAST or CFT.

Following subcutaneous challenge with T3 culture, the 2 calves inoculated with fraction C/1/2/3 and the 2 control calves all developed swellings by the sixth day which became extensive. One of the control animals had to be destroyed in extremis 14 days after the swellings first appeared, while the swellings on the other calves slowly regressed.

Comparison between the precipitin bands produced by fraction C/1/2/3, the galactan of Buttery and Plackett (1960) and the endotoxin of Villemot, Provost and Queval (1962)

The qualitative AGT showed that the precipitin bands produced by fraction C/1/2/3 (0.1 mg./ml.) and the galactan (0.1 mg./ml.) were very similar although quantitative differences were obvious. At this concentration antigen I, the one nearest the antigen well, was well defined in fraction C/1/2/3 but in the galactan it was seen as a faint, wide hazy band which appeared to be identical to antigen I when the galactan was diffused at a higher concentration, namely 1.0 mg./ml. The other precipitin bands all appeared to be identical to those produced by the galactan in that they joined together.

The endotoxin at 2.0 mg./ml. possessed 2 strong bands which were identical to those produced by the other two materials. The band nearer the antigen well was seen on careful examination to be composed of at least 3 bands. On the antigen-well side of this multiple band a very faint, hazy, diffuse band could be seen which did not join up with the bands produced by the other 2 materials possibly only because of its faintness. When the endotoxin was diffused against sheep x' serum at 5.0 mg./ml. concentration, the hazy band was seen to be composed of 1 band which joined up with bands produced by the other 2 materials. However, even at this strength there was no precipitin band corresponding to antigen I of C/1/2/3.

The precipitin bands produced by these various materials can be seen in fig. 41.

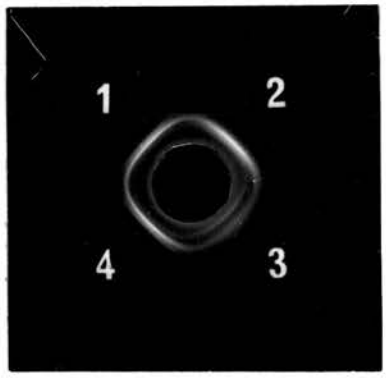


Fig. 41. Comparison between the precipitin bands produced by C/1/2/3, the galactan and the endotoxin

Centre well		sheep x' serum
Wells	1	C/1/2/3, 0.1 mg./ml.
	2	galactan 0.1 mg./ml.
	3	endotoxin 2.0 mg./ml.
	4	C/1/2/3, 0.1 mg./ml.

SUMMARY

The results of the various semi-quantitative biochemical estimations on each fraction are given in table XLVI.

Table XLVI

Composition of the various fractions isolated from urine

Fraction	Nitrogen %	Phosphorus %	Carbohydrate %	Hexosamine %
C/1	3.6	-	46.8	9.0
C/2	7.2	-	40.3	9.9
C/3	8.1	-	23.5	14.75
C/1/2/3	5.6	0.5	42.4	11.9
4/5/6(7)	-	0.5	18.2	11.25

The amount of Kjeldahl nitrogen increased with the increase in amount of alcohol used for precipitation. Unfortunately there was insufficient of fraction 4/5/6(7) for a nitrogen estimation to be performed. The percentage of carbohydrate on the other hand decreased with the increase in amount of alcohol used and fraction C/3, which possessed the highest amount of nitrogen, had only half as much carbohydrate as fraction C/1. Fraction 4/5/6(7) possessed even less carbohydrate than C/3. Hexosamine could account for some of the nitrogen as it also increased with the increase in amount of alcohol used, except in the case of fraction 4/5/6(7). A trace of phosphorus and an unknown amount of lipid were also present in the fractions. There was no indication of nucleic acid.

Information obtained from paper chromatography indicated that amino acids were present in the fractions as well as amino sugars. There is little doubt that galactose was present in all the fractions and was the most plentiful sugar judged by the

intensity of the spot produced. Sorbose and arabinose were also probably present, the latter apparently only in demonstrable amounts in fraction 4/5/6(7). It is probable that at least 2 of the chromatographic spots were due to hexosamines and 1 spot to the group of substances which give an olive-brown colour with AHP, which include aldohexoses, deoxyaldohexoses, aldoheptoses, aldooctoses and aldohexuronic acids. At least 2 other spots could not be identified. Examination of specific precipitates of fractions C/2 and C/3 by paper chromatography indicated that these spots produced by the various fractions were specific and were not caused by the hydrolysis of non-specific sugar in the urine.

The action of heat and trypsin on the major antigens isolated from the urine confirmed the findings reported in Part 1, that these antigens were relatively heat stable and were not affected by the action of trypsin.

Separation of the individual precipitating antigens demonstrated in the AGT was not possible by either ultra-centrifugation or by electrophoresis. In the former, however, insufficiently rapid freezing may have allowed convection currents to occur thus giving a false picture.

Fractions C/1/2/3 and 4/5/6(5) fixed complement in the presence of hyperimmune sheep serum.

When hyperimmune sheep x serum was fully absorbed by fraction C/1/2/3 and then diffused against body fluids from infected animals in the AGT, no precipitin bands were observed except when the serum was diffused against serum Oremit 2, when 1 band was seen. Fraction C/1/2/3 absorbed 87.5 per cent. of the agglutinating antibodies and 93.75 per cent. of the complement fixing antibodies from sheep x serum. The absorption of a different hyperimmune sheep serum by the various fractions indicated that C/1 and C/2 absorbed 87.5 per cent. of the agglutinating antibodies and 75 per cent. of the complement fixing antibodies from the serum. Fraction C/3 absorbed 75 per cent. of the agglutinating and 50 per cent. of the complement fixing antibodies, while fraction 4/5/6(7) absorbed

75 per cent. and none of the complement fixing antibodies. Fraction C/1/2/3 absorbed 96.9 per cent. of the agglutinating and 87.5 per cent. of the complement fixing antibodies.

Both fraction C/1/2/3 and 4/5/6(7) proved to be weakly pyrogenic which is not surprising as it had already been shown that these fractions contain a certain amount of lipid.

Fractions C/1/2/3 and 4/5/6(7) did not produce any obvious toxic symptoms in rabbits at 3.125 and 3.43 mg./kg. body weight respectively, or in mice with doses of 0.3 mg. Fraction C/1/2/3, however, proved to be lethal to 10 day old fowl embryos. These fractions were certainly not as toxic as the endotoxin of Villemot, Provost and Queval (1962) either to cattle or fowl embryos. The LD_{50} in fowl embryos was 179 µg., compared with the LD_{50} of the endotoxin of 15 µg. and of the galactan of 525 µg., and the lethal effects took longer after inoculation and also no haemorrhagic lesions were observed. The route of inoculation was, however, different from that used by the other workers. Inoculation of 2 mg. of fraction C/1/2/3 intravenously in cattle did not produce the dramatic collapse that the endotoxin was reported to induce, in fact no ill effect was seen.

The intradermal inoculation of fraction C/1/2/3 did not produce an allergic response in immune animals.

The recognised variability in resistance or susceptibility of individual cattle to the subcutaneous inoculation of viable M. mycoides made it necessary in the aggressin experiments to inoculate both the test and control materials in the same animal, so that each animal was essentially an individual experiment. The results of these experiments showed that the addition of fraction C/1/2/3 to the inoculum of viable organisms in immune animals produced a subcutaneous swelling, whereas the inoculation of the same number of viable organisms in the same animal without the addition of the antigen produced no swelling. There were 5 cases of this in the 3 experiments. These swellings were transient, remaining from 4 to 10 days, and did not become very large. We have already

seen that fraction C/1/2/3 did not induce an allergic response in immune animals and furthermore these swellings began usually on the third or fourth day after inoculation, whereas the allergic reactions seen in other cases began on the first day so it is unlikely that these swellings were the result of an allergic response and were presumably the result of multiplication of the organisms, in other words, small Willems reactions. These swellings evidently depended on the presence of this antigen in the inoculum and as the antigen was added to the organisms from 30 to 60 minutes before inoculation, it was possible that the antigen in some way protected the organisms during this period. If this was so it was likely to be of little significance as there was no loss of viability of M. mycoides at 4°C. and room temperature in N.saline or buffer over this period of time. It therefore appears that the antigen possessed a truly aggressive action either in enhancing the virulence of the organisms and so overcoming the humoral and cellular defences for a short time or in combining with available antibody and so temporarily inhibiting sensitization of the organisms. There was a definite indication of an allergic response in 3 animals, and this was not dependent on the presence of the antigen, in fact the presence of this antigen appeared to have a slight inhibitory effect on the response in one case (6456), and the important factor appeared to be the inoculation of a very concentrated suspension of organisms, as was the case in the second experiment. This allergic response was observed on the first day after inoculation and was maximal on either the first or second day. In one case (6504) the allergic response on the side incorporating the antigen appeared to proceed from an allergic response to a growth response, whereas the other side produced only an allergic reaction. Ox number 7623 developed an allergic response on both sides, which disappeared momentarily on the side with antigen, and both ultimately developed into growth swellings. There appeared to be no correlation between the CFT and SAST titres before inoculation and the development of growth or allergic responses.

For example, in the first experiment the immune animal which developed a transient swelling possessed a CFT titre of + and a SAST titre of ++ whereas the 2 immune animals which did not react had CFT titres of ++ and nil and SAST titres of ++ and again nil.

In susceptible cattle there were 2 instances where the addition of fraction C/1/2/3 to the inoculum produced a swelling while the other side, with no antigen, did not react. In the first case (7645) the swelling developed after 9 days and progressed to a typical Willems reaction from which the animal did not recover and had to be destroyed in extremis. The other instance (7671) resulted in a smaller swelling which slowly regressed and finally disappeared. Difficulty was encountered in judging the inoculum of organisms so that there were insufficient viable organisms to produce a swelling without the addition of the antigen, especially when the titre of the inoculum was only determined in retrospect after a week. Allergic reactions were another difficulty which was overcome by the use of a buffer to suspend the organisms as this maintained the viability of the organisms better enabling a less concentrated suspension to be used. The third experiment with susceptible cattle proved a failure for the reason mentioned above, in that the minimum inoculum used still produced swellings on both sides, although in 2 cases the addition of antigen produced an earlier reaction. The results of the inoculations into susceptible cattle suggest, therefore, that the addition of fraction C/1/2/3 enhanced the virulence of the organisms in some way.

Attempts to immunize rabbits and mice with fractions C/1/2/3 and 4/5/6(7) failed, indicating that these materials were complex haptens. The addition of 'shiga' conjugated protein made the fraction antigenic as precipitating antibodies against C/1/2/3 were produced in the rabbit. However, no antibodies were detected by the SAST or CFT, the reason for this being unknown. No antibodies, either precipitating, agglutinating or complement fixing were demonstrated in the

sera of mice or calves that had been inoculated with the 'shiga' conjugate and the calves subsequently were shown to possess no immunity to the subcutaneous inoculation of virulent M. mycoides.

The galactan of Buttery and Plackett (1960) and fraction C/1/2/3 appeared serologically very similar as demonstrated in the AGT.

The endotoxin of Villemot, Provost and Queval (1962) was very weakly antigenic when diffused in the AGT against our sheep serum, although it was evident that some of the same antigens were present in the toxin as in fraction C/1/2/3.

To summarise we can say that fraction C/1/2/3, which contained all the precipitating antigens demonstrable in the AGT, contained 5.6 per cent. nitrogen, 0.5 per cent. phosphorus, 42.4 per cent. carbohydrate (hexose, pentose and methyl pentose) and 11.9 per cent. hexosamine. In addition it contained an unknown amount of lipid but no nucleic acid. The sugars present in the fraction on hydrolysis were primarily galactose with probably sorbose and arabinose, 2 unknown hexosamines and probably one of the following substances: aldose, deoxyaldohexose, aldoheptose, aldooctose and aldohexuronic acid. In addition there were at least 2 other spots on the chromatograms that could not be identified. By specific precipitation methods it was shown that these sugars were specific and not the result of hydrolysis of non-specific sugars from the urine. The precipitin bands demonstrated in the AGT were not destroyed by heat (94°C. for 60 minutes) or trypsin. Separation of the individual precipitating antigens was not possible by either electrophoresis or ultracentrifugation. The fraction fixed complement in the presence of immune sheep serum, was weakly pyrogenic in rabbits and relatively non-toxic to mice and rabbits, but was lethal to fowl embryos. Fraction C/1/2/3 absorbed 87.5 and 96.9 per cent. of the agglutinating and 93.75 and 87.5 per cent. of the complement fixing antibodies from hyperimmune sheep x and sheep 6 sera respectively. Fraction

C/1/2/3 showed an aggressive effect when inoculated together with viable M. mycoides in both immune and susceptible cattle. The fraction proved to be non-antigenic in mice and rabbits although by conjugating it with 'Shiga' protein it was possible to produce precipitating antibodies against the fraction, in rabbits although not in calves. The galactan of Buttery and Plackett (1960) and the endotoxin of Villemot, Provost and Queval (1962) possessed some precipitinogens serologically identical to fraction C/1/2/3 although the endotoxin was serologically very much weaker.

GENERAL DISCUSSION

A total of at least 7 serologically distinct major precipitating antigens were demonstrated in the various body fluids by the qualitative AGT. Of these, 5 were present in the urine, a further one in the lymph and pleural fluid and all 7 in some samples of serum and plasma. White (1958) described only 1 or 2 precipitin bands in sera of cattle dying of CBPP and 3 bands in pleural fluid or extracts of infected lung, and Villemot and Provost (1959a) reported that culture grown M. mycoides contained at least 3 distinct precipitating antigens, substantiating the findings of White. The antigens described by these workers were presumably the same as our major precipitating antigens and the reason that only 3 were recognised may have been due to the antisera that they used, which was produced in rabbits and donkeys, or may simply have been due to lack of sensitivity of their test systems.

The presence of only 5 of the major precipitating antigens in urine presumably indicated that only those ones were readily excreted in the urine, the remaining 2 being either in very low concentrations in the urine and undetectable by the AGT, retained in the circulation, or excreted only after further breakdown in the body. It is difficult to explain the presence of the extra precipitating antigen in the serum and plasma of certain of the animals, especially as it was not demonstrated in the pleural fluid or lymph of those animals, unless it was an antigen that had become altered in some way after entering the circulation, or that its presence was a quantitative effect, the antigen being liberated in only very small amounts and accumulating in the circulation. This extra band was demonstrated in 3 animals, which included both natural and experimental cases of the disease.

These major precipitating antigens were present in the animal body in an extracellular form as well as being associated with the organisms. Whether they were excreted by or were the result of autolysis of the organisms, or a combination of the two, is unknown.

It had been hoped that, by using body fluids from cattle infected with M. mycoides and antisera prepared in cattle or sheep, it would ensure that the full complement of antigens associated with the disease process would be detected. However, it did not prove as simple as this as it was subsequently shown that certain precipitating antigens detected by the sheep antisera were not detected by the sera produced in cattle. The possible reasons for this are discussed at the end of Part 1. So despite the use of sheep sera for subsequent work it is not certain that we were dealing with the full complement of in vivo produced antigens. These antigens were, however, the products of in vivo growth and, as far as the serological picture is concerned, they appeared to form a major part of the antigenic moiety as they absorbed a considerable proportion of the complement fixing and agglutinating antibodies from hyperimmune sheep sera.

It was not surprising that we found no difference between the major precipitating antigens produced by the different strains of M. mycoides, as although Heslop (1924) reported that at least 2 agglutinogenic strains of the organism occurred in Australia this has never been confirmed (Turner, 1959) and all evidence points to the fact that there are no antigenic differences between strains (Tang, Wei, McWhirter and Edgar, 1935; Klieneberger, 1938) or between strains from Australia, Assam or Kenya (Shirlaw and Krishna Iyer, 1946).

No viable organisms were detected in the serum, plasma or blood cells collected from natural cases of the disease, substantiating the findings of Campbell (1936) that blood culture was not a simple method of detecting clinical cases of CBPP which had been put forward by Hall and Beaton (1931). Viable organisms were, however, detected in the serum and plasma of experimental cases, indicating presumably a variation in the ability of the subcutaneous and lung tissues to control the infection. No viable organisms were detected in the urine of natural or experimental cases of the disease which is in

agreement with the findings of Campbell (1936) and contrary to those of Beller and Tahssin-Bey (1927, cited by Campbell, 1936).

In the experimentally inoculated cattle it was shown that viable organisms, precipitating antigen and complement fixing and agglutinating antibodies, and in the natural cases only antigen and antibodies, were present in the serum and plasma at the same time. This situation is most interesting and is further complicated by the fact that antigens prepared from serum, lymph and urine by boiling, absorbed 87.5 and over 95 per cent. of the complement fixing and agglutinating antibodies respectively from hyperimmune sheep serum prepared by inoculating washed viable M. mycoides. It is not proposed to discuss this situation here as the possible explanations are numerous and, until further investigations are undertaken, conjecture is fruitless.

The absorption of hyperimmune sheep sera with the antigens prepared from the various body fluids, namely lymph 6183, serum Oremit 2 and urine (pooled), and also fraction C/1/2/3, removed a varying proportion of agglutinating and complement fixing antibodies and, furthermore, fraction 4/5/6(7) removed 75 per cent. of the agglutinating antibody but no complement fixing antibody. These results indicate that the agglutinating and complement fixing antibodies differ. As the urine antigens absorbed a considerable proportion of the antibodies responsible for the serological tests, it would seem that these antigens are the ones mainly responsible for the production of these antibodies, and also it is likely that the presence of these antigens in the circulation of infected cattle could account for the proportion of false negative results obtained with these tests. This would support the findings of Turner (1962) who reported that the presence of circulating antigen of M. mycoides during severe CBPP infection may produce the "eclipse" of serological activity to agglutination tests, precipitin tests for antibody and in rare instances to the CFT.

As already mentioned, fraction 4/5/6(7) absorbed no complement fixing antibody, which is surprising when it had already been shown that fraction 4/5/6(5) was complement fixing. The probable explanation of this is that fraction 4/5/6(5), which was used for the CFT, was less purified than fraction 4/5/6(7) used for the absorptions and probably contained a little contaminating antigens I and II which were not detectable by the relatively insensitive AGT but were by the more sensitive CFT, especially when it is seen that the optimal concentrations of the 2 fractions in the CFT were 0.74 µg./ml. for fraction C/1/2/3 and 20 µg./ml. for fraction 4/5/6(5). This explanation, together with the fact that fraction C/3 absorbed only 50 per cent. of the complement fixing antibodies while fractions C/1 and C/2 each absorbed 75 per cent., would also indicate that antigens I and II were the ones mainly responsible for complement fixation, as fraction C/3 contained considerably less of these antigens than the other 2 fractions as demonstrated in the AGT.

The presence of 5.6 per cent. Kjeldahl nitrogen in fraction C/1/2/3 may be accounted for by the presence of amino acids and amino sugars which were demonstrated by chromatography. Whether this nitrogen was part of the precipitinogenic complex or was merely contaminating material is impossible to say, although in the chromatography experiments amino acids were detected in the specific precipitates after acid hydrolysis. Approximately 60 per cent. of the weight of fraction C/1/2/3 can be accounted for by carbohydrate (42.5 per cent.), hexosamine (11.9 per cent.), nitrogen (5.6 per cent.) and phosphorus (0.5 per cent.). The composition of the remaining 40 per cent. is unknown, although it is known that there is a certain amount of lipid present. It is interesting that Plackett (1961) described a polyglycerophosphate compound from M. mycoides grown in partly defined medium. Plackett (1959) also reported on the probable absence of "mucocomplex" from the V5 strain of M. mycoides by the estimations of hexosamine and diaminopimelic acid which he found were only 0.03 and 0.02 per cent. respectively of the dry weight of the organisms. As fraction 4/5/6(7) possessed both

pyrogenic properties and a precipitin band that stained with lipid stain, it is obvious that the lipid fraction is associated antigens III, IV, V or VI, probably 2 of these antigens as it had earlier been shown that antigens III and IV, and V and VI were normally seen as only 2 bands, each of which split into 2 on dilution. Fraction 4/5/6(7) possessed 18.2 per cent. carbohydrate, 24.2 per cent. less than fraction C/1/2/3, indicating that antigens I and II accounted for at least this amount of carbohydrate.

Attempts to immunize rabbits and mice with fractions C/1/2/3 and 4/5/6(7) failed, indicating that these fractions were complex haptens as they possessed immunological specificity, combined with homologous antibody and formed a precipitate in vitro but did not stimulate antibody production in vivo. Whether these antigens were haptens in the animal body is impossible to say, but the method used for isolation was relatively mild and possibly would not be expected to alter the antigens significantly. Their size was indicated by their ability to pass through Millipore VM filters of 50 mμ pore size, but they were not dialysable and it is probable that they did exist as haptens in the circulation once they were liberated from the bacterial cells. As antibodies were produced against these antigens, it is therefore necessary to postulate that they were only antigenic when associated with the bacterial cell. The addition of 'shiga conjugated protein' to the antigens made them antigenic in rabbits, further supporting the contention that their inability to produce antibodies was one of molecular size. No antibodies were detected by the CFT or SAST in the rabbit serum, only by the precipitin test, which is surprising but suggests that the precipitating antibodies may differ from the CF and agglutinating antibodies. No antibodies, either precipitating, agglutinating or complement fixing were demonstrated in the sera of mice or calves inoculated with the conjugated antigens.

The galactan of Buttery and Plackett (1960) and fraction C/1/2/3 appeared serologically very similar as demonstrated by

the AGT. The chemical composition of these 2 materials was rather different, however. For example, the nitrogen percentage of the galactan was only 0.16 per cent., the reducing sugar was 89 per cent. and the phosphorus was 0.06 per cent. They isolated a lipid fraction but did not report any amino sugars, and the only sugar demonstrated by chromatography was galactose, although they had earlier reported the presence of ribose (Plackett and Buttery, 1958). The endotoxin of Villemot, Provost and Queval (1962) was very weakly antigenic when diffused against our sheep antiserum in the AGT, although it was also evident that most of the antigens present in fraction C/1/2/3 were also present in the endotoxin, even though those workers reported only 1 precipitin band in the AGT.

Villemot, Provost and Queval (1962) reported that 2 mg. of the endotoxin caused severe stress and collapse in cattle and that the LD_{50} in fowl embryos was 15 μg., indicating that it was considerably more toxic than fraction C/1/2/3.

The serological similarity between fraction C/1/2/3 and the galactan and, to a less extent, the endotoxin suggests that fraction C/1/2/3 is the in vivo stage of the polysaccharide that the other workers isolated from culture grown organisms. The endotoxin, which is apparently the most complex of these fractions, appears to contain a small amount of the antigens present in the galactan and fraction C/1/2/3 combined with a large proportion of other materials, which are presumably the portions of the endotoxin responsible for the toxicity.

Our principal interest in the in vivo antigens of M. mycoides is the role they play in the pathogenesis of the disease and immunity. It was shown in Part 1 that the major precipitating antigens were present in the blood and inflammatory exudates of infected animals. Nakamura, Futamura and Watanuki (1926) had also shown that precipitating antigens were present in the normal as well as infected areas of affected lungs. It is obvious, therefore, that these antigens are present throughout the body of an infected animal.

The results of the aggressin experiments with fraction C/1/2/3 showed that the addition of this fraction to viable organisms inoculated subcutaneously in immune cattle allowed the organisms to multiply and produce a swelling and, in susceptible cattle, enhanced the virulence of the organisms. These findings suggest that these antigens may play a part in the natural disease process by their aggressive activity, presumably by neutralizing the antibodies that are produced in response to infection and so preventing the sensitization of the organisms, and also by their ability to enhance the virulence of the organisms in some way. This enhancement of virulence probably has very little to do with toxicity, as these antigens in the form isolated by us were only weakly pyrogenic and relatively non-toxic. In addition to the aggressive and possibly protective functions of these antigens, the fact that they fix complement suggests that the antigen-antibody-complement complex might cause certain of the pathological changes associated with the disease, although there is no evidence of this at present, and also the presence of these antigens in large amounts in the circulation may produce some paralysis of antibody formation.

These antigens are in many ways analogous to the soluble specific substance of the pneumococcus and also to the polysaccharide antigens of many other organisms, for example Salmonella typhosa, the Neisseria group, especially the meningococcus, and the haemophilus group. It is tempting, therefore, to suggest that these antigens during elaboration may form a protective "capsule" round the organisms. The "capsule" in this case would probably be more in the nature of a loose slime-layer than a true capsule, as no capsule has yet been demonstrated around these organisms.

The presence of antigen in the blood that could neutralize antibodies and so account for the absence of immunity in some cases of CBPP was suggested by Kurotchkin and Bernaradsky in 1938. It certainly could be a possible explanation as to why methods of vaccination against CBPP have given on the whole poor results, as once a focus of infection is

set up in the lung, where possibly the immediate antibody level is not high, these antigens are elaborated and so protect the multiplying organisms from the subsequent antibody response of the host. To overcome this would necessitate a very high antibody level in the body, which might be difficult to attain by the use of killed or attenuated organism vaccines and also may be difficult to maintain for any length of time. These antigens presumably would not play a very significant part in the development of the initial focus of infection, although their protective capacity may be of use during the transmission stage of the process. The difficulty of producing the lung disease in experimental cattle suggests that some other factor or factors play a part in the natural spread of the disease. It has been shown, however, that M. mycoides alone can produce the disease as it has been produced by pure cultures of the organisms (Daubney, 1935; Campbell, 1938). The methods used, nevertheless, to produce the experimental disease with pure cultures are relatively drastic. For example, Daubney inoculated the organisms intravenously in an agar embolus which lodged in the lungs, and Campbell's method necessitated the inoculation into the lungs of large volumes of fluid. It is probable, therefore, that stress in some form or other is required to trigger off the infection. This stress could be bacterial or viral, but not necessarily any specific microorganism, or might be purely environmental. Whatever the stress, it would allow the organisms to gain a foothold and from there elaborate their antigens which, in turn, would play their protective role in assisting the growth of the organisms.

The demonstration that identical major precipitating antigens were elaborated by both virulent and avirulent strains of M. mycoides is of interest and further supports the idea that these antigens are not in themselves significantly toxic. The avirulent strain of M. mycoides certainly multiplies in the host when inoculated subcutaneously as demonstrated by the excellent serological response (Gourlay, unpublished) but in contrast to

the virulent strains does not cause any ill effects. It appears, therefore, that these antigens play a part in assisting the growth of the organisms in the host tissues and so allow the organisms, if virulent, to produce their harmful effects.

SUMMARY

Serum, plasma, lysed blood cells, urine and pleural fluid or lymph (inflammatory exudate from subcutaneous inoculation) were obtained from cattle naturally and artificially infected with Mycoplasma mycoides. These fluids were examined for antigens of M. mycoides by means of the agar-gel double diffusion precipitin test and the quantitative agar-gel precipitin test. They were also examined for antibodies against M. mycoides by means of the complement fixation and slide agglutination tests, and for viable M. mycoides by growth in broth cultures. Hyperimmune sera for use in these tests were prepared in sheep and cattle by the intravenous injections of washed organisms that had been grown in broth medium. From the natural cases of CBPP viable organisms were obtained from the pleural fluid only, while in the experimental cases the organisms were present in the serum, plasma and lymph. Antibodies against M. mycoides were demonstrated in sera and plasma samples of all cases. Specific precipitating antigens were demonstrated in all the fluids, urine possessing at least 5 serologically distinct antigens, lymph and pleural fluid at least 6 and serum and plasma at least 6 and sometimes 7 antigens. The 5 in urine were common to all fluids, while the extra 1 in lymph and pleural fluid was also present in serum and plasma. In addition to these so-called major antigens, minor ones, at least 6 in number, were also demonstrated, but these were apparently primarily associated with the organisms. The major precipitating antigens were predominantly extracellular with only small amounts present in the organisms. These major antigens were also elaborated by the organisms when grown in artificial culture medium, and those produced by fully virulent organisms were apparently identical to those produced by avirulent organisms.

Fractionation of pooled urine from the artificially infected cattle by precipitation with varying volumes of cold iso-propyl alcohol and deproteinization with a chloroform-butanol mixture was undertaken. A total of 6 serologically distinct

precipitating antigens were demonstrated in the AGT and separation of these antigens was possible to a limited extent by varying the volumes of alcohol used.

Fraction C/1/2/3, the fraction which contained all the antigens, was shown to contain approximately 5.6 per cent. Kjeldahl N, 0.5 per cent. P., 42.4 per cent. carbohydrate (estimated as galactose) and 11.9 per cent. hexosamine. One or possibly 2 of the precipitin bands was shown to contain lipid, but there was no indication of nucleic acid. By the use of paper chromatography, galactose was demonstrated and probably sorbose and arabinose, together with some amino acids. These antigens were resistant to a temperature of 94°C. for 60 minutes and to the action of trypsin. Separation of the individual antigens was not obtained by either ultracentrifugation or electrophoresis. The antigenic fraction fixed complement in the presence of hyperimmune sheep sera, and fraction C/1/2/3 absorbed 87.5 and 96.9 per cent. of the agglutinating antibodies and 93.75 and 87.5 per cent. of the complement fixing antibodies from hyperimmune sheep x and sheep 6 sera respectively. This fraction was pyrogenic in rabbits and relatively non toxic to cattle, rabbits and mice, but proved to be lethal to fowl embryos. The antigens were haptens in cattle, rabbits and mice, but precipitating antibodies were produced in rabbits when the antigens were combined with "shiga conjugated protein". Fraction C/1/2/3 possessed an aggressive action when inoculated together with viable M. mycoides in immune animals and appeared to enhance the virulence of the organisms in susceptible cattle.

It is suggested that these antigens play a part in assisting the growth of M. mycoides in the host tissues but are not in themselves significantly harmful.

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