The pathogenesis of infectious necrotic hepatitis (black disease) of sheep

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SUMMARY

The literature relating to infectious necrotic hepatitis is critically reviewed and discussed.

The bacteriological, parasitological and other techniques used in this study are outlined.

Surveys on five Scottish farms are described and the results analysed to show the correlation between the incidence of Cl. novyi type B in sheep livers and in soil from the pastures, both with each other, with the incidence of Fasciola hepatica in the livers of sheep and with the incidence of infectious necrotic hepatitis on these farms.

Infectious necrotic hepatitis was induced in laboratory guinea-pigs, rabbits and sheep. The spores of Cl. novyi type B were found to reach the liver within 24 hours after oral infection to guinea-pigs and sheep, but could be detected earlier in the mesenteric lymph glands. The possible pathways by which these spores reach the liver are discussed. Death from infectious necrotic hepatitis in guinea-pigs and rabbits occurred 4 - 6 weeks post-infection with Fasciola hepatica,
which corresponds with the critical period of maximal parenchymal damage in the liver caused by the flukes. In sheep the severity of the damage caused by the flukes in the liver was assessed by assaying the concentration of glutamic dehydrogenase (G.D.) in the serum and the host reaction to the infection by estimating the numbers of eosinophils in the plasma. All the sheep which developed infectious necrotic hepatitis died 6-8 weeks after infection with Fasciola hepatica; all these sheep showed high serum G.D. levels and high eosinophil counts during this period, which also coincides with the period of maximal parenchymal damage in this host species. This is believed to be the first report of the induction of experimental infectious necrotic hepatitis in sheep, using the natural infective agents.

The effect of putrefaction on the diagnosis of infectious necrotic hepatitis was investigated in experimental models and the significance of these findings is discussed in relation to the diagnosis of the disease in the field.
INTRODUCTION

Infectious necrotic hepatitis has long been considered to cause serious economic losses of sheep. In Australia, Turner (1930b) estimated the average annual losses of sheep from infectious necrotic hepatitis as £1 million. In Britain losses were reported to be severe in 1947 following an abnormal winter and a dry summer; losses in the autumn of 1954 and 1958 were again heavy (Roberts, 1959). However, the introduction of a vaccine against the disease, has reduced the incidence dramatically. A vaccine consisting of a formaline-treated whole culture of Clostridium novyi was used at first and its efficacy was demonstrated in the field by Turner (1930b). Alum precipitated vaccine is now widely used and is commercially available (Burroughs Wellcome); vaccination is carried out during the quiescent period preceding the expected appearance of the disease.

Since the association of the bacterium Cl.novyi and the fluke Fasciola hepatica in the causation of the disease was established (Turner, 1930b), research on infectious necrotic hepatitis has been devoted to
investigating the pathogenesis of the disease. Many workers have laid emphasis on the presence of \textit{Cl. novyi} spores in the livers of apparently healthy sheep (Edgar, 1928; Turner, 1930a; Jamieson, 1949). The introduction of the fluorescent antibody stain for the identification of \textit{Clostridia} species (Ratty and Walker, 1963) stimulated more workers to report the presence of \textit{Cl. novyi} in the livers of normal sheep and cattle at slaughter or post-mortem (Ratty \textit{et al.}, 1964; Corbould, 1966; Ratty \textit{et al.}, 1967; Thomson \textit{et al.}, 1968; Niilo \textit{et al.}, 1969; Roberts \textit{et al.}, 1970b & c). These results aggravate the doubts, which already exist, on the validity of bacteriological findings as evidence of infectious necrotic hepatitis and many workers have drawn the conclusion that greater care should be taken in the interpretation of findings of \textit{Cl. novyi} in pathological material. One factor which is important in the pathogenesis of the disease is its experimental reproduction. This has been attempted by only a few workers namely Turner (1930b); Jamieson, (1949) and Katitch \textit{et al.}, (1969b). No one has so far attempted to reproduce the disease in sheep using the natural and common infective agents, i.e. \textit{Cl. novyi} type B and \textit{Fasciola hepatica}. Many of these workers spoke of difficulties in obtaining enough metacercariae of \textit{F. hepatica} for use experimentally. On the other
hand in other countries workers such as Hasdemir and Gunel, (1968) in Turkey, have reported that it had been possible to investigate infectious necrotic hepatitis in that country until the establishment of a bacteriological laboratory. It seems likely, therefore, that research on infectious necrotic hepatitis has been hampered by a lack of the combination of bacteriological and parasitological laboratories. The close similarity of infectious necrotic hepatitis with acute fascioliasis and the world-wide distribution of fascioliasis suggests that the disease may occur in countries at present believed to be free of this disease.

The present work was designed to investigate some of the quantitative aspects of the disease and to develop experimental models that may elucidate the pathogenesis. Surveys of samples of soil and sheep livers have been made in Scottish farms with a history of infectious necrotic hepatitis and in similar farms without such a history. Procedures for the isolation and identification of Cl.novyi were evaluated and developed. Advantage was taken of an opportunity to use the available bacteriological and helminthological
facilities to study the disease in the natural host and in the guinea-pigs and rabbits. The results of these investigations are submitted to support the thesis that the correct diagnosis of infectious necrotic hepatitis depends upon accurate clinical, bacteriological, and helminthological observations. Such accurate diagnosis is essential for the prosecution of meaningful epidemiological studies that can contribute to the effective control of the disease.
CHAPTER II

LITERATURE REVIEW

(A) DEFINITION

Infectious necrotic hepatitis (black disease) is an acute toxaemic disease of sheep and cattle, which has sometimes reported in pigs (Batty et al., 1964; Bourne and Kerry, 1965; Corbould and Munday, 1966) with one authentic case in a horse (Dumaresq, 1939) and one in man (Mollaret, et al., 1948). The disease is caused by the elaboration in damaged liver tissue of the alpha toxin of Clostridium novyi type B ("Bacillus oedematis", Maligni" II, Novyi, 1894; "Bacillus oedematien", Weinberg and Seguin, 1915; "Bacillus gigas", Zeissler and Passfeld 1929 and Clostridium oedematien type B, Scott et al., 1934).

The initial liver damage in most field outbreaks is caused by the wandering immature liver fluke Fasciola hepatica (Turner, 1930b; Jamieson, 1949, and Williams, 1962 and 1964). In addition other causes of hepatic injury have been reported to give rise to the disease. In sheep these include the small liver
fluke *Dicrocoelium dentriticum* (Miessner et al., 1931; Cernaianu and Mihailesscu, 1935; Hasdemir and Gunel 1968; Katitch et al., 1969a); *Cysticercus tenuicollis* (Albiston, 1927; Hreczko, 1959), and *Echinococcus granulosus* (Wallace, 1964). *Ascaris suum* has been shown to cause the disease experimentally (Katitch, et al., 1969b). In rabbits *Cysticercus pisiformis* (Turner, 1930b) and in horses wandering strongylid larvae (Dumaresq, 1939) were reported to be associated with the disease. Turner (1930b) postulated that any parasite capable of producing liver necrosis might activate latent spores in the liver.

Chemicals and drugs which damage the liver cells have been reported to activate latent spores including the drug carbon tetrachloride used for the treatment of sheep against fluke infestation (Turner, 1930b), trichlorophon (Neguvon) used against warble flies in cattle (Hart, 1965) and detergent in polluted water drunk by cattle (Batty et al., 1964). Calcium chloride solution given intrahepatically has been used experimentally to cause the liver necrosis associated with the disease (Turner, 1930b).
Intensive barley feeding to cattle results in metabolic disturbances and liver damage and may predispose to the disease (Batty et al., 1964).

Although *Clostridium novyi* type B has been reported by many workers as the cause of infectious necrotic hepatitis in sheep, other species of clostridia have been incriminated as causing the disease, either alone or in association with *Clostridium novyi* type B. These include *Clostridium novyi* type A, *Clostridium septicum* and *Clostridium welchii* types A and C (Katitch et al., 1969b).

(B) **SYMPTOMS**

One of the outstanding features of the disease is the rarity with which one observes a sick sheep. The period between the first appearance of symptoms and death is usually only a few hours. The typical history is that of a sheep being found dead or recumbent on its brisket a short time before death. If one is able to observe an animal during the illness, the first sign of the disease is the tendency of the affected animal to lag behind the flock. A few hours later there is a disinclination to move and the animal quickly lies on its brisket. Respiration
becomes shallow and rapid and the body temperature is at first elevated to 104 – 107°C but drops suddenly to subnormal before death (Edgar and Rose, 1929; Turner, 1930b; Jamieson, et al., 1948 and Williams, 1962). The animal often shows hyperaesthesia of the head and ears in the form of spasmodic twitching which gradually subsides (Jamieson et al., 1948). Throughout the whole syndrome there is no evidence of pain and death usually occurs without a struggle, the sheep giving the appearance of having gone to sleep.

(C) POST-MORTEM LESIONS

The descriptive name of the disease "infectious necrotic hepatitis" advocated by Albiston (1927) is based on the lesions encountered in this disease in sheep. The rapidity with which the carcasses decompose is an important factor in the post-mortem findings. Jamieson et al., (1948) examined ten animals 30 minutes to 4 hours after death and noticed a frothy discharge from the mouth and nostrils. The carcass is usually in good condition and on removing the skin the subcutaneous blood vessels are engorged. This together with the extensive subcutaneous haemorrhagic oedema over the sternum, ventral abdominal muscles and inguinal region, accounts for the popular name "black disease".
(i) **Peritoneal Cavity**

On opening the peritoneal cavity varying amounts of ascitic fluid are found. There is usually about 500 ml of a clear, colourless fluid. The parietal surface of the peritoneum on the rumen, reticulum and omasum shows a varying degree of congestion (Jamieson et al., 1948).

(ii) **Thoracic cavity**

This usually contains a varying amount of clear colourless fluid. The lungs show no significant change. The heart however shows a characteristic pathological change in most cases, in which the pericardial sac is distended with a clear, colourless or straw-coloured fluid, the amount of which varies from 40ml to 250ml (Jamieson et al., 1948; Sinclair, 1956). The endocardium and myocardium may show haemorrhagic patches.

(iii) **Alimentary tract**

No abnormality is observed on the epithelial surface of the rumen, omasum or reticulum. Changes in the abomasum vary considerably. Often the wall is thickened and oedematous and the mucosa may be
covered with numerous punctiform haemorrhages (Williams, 1962). At the pyloric end there is usually an intense band of mucosal congestion with occasional small erosions. The only change which appears to be fairly constant in the intestine is a patchy congestion of the duodenal mucosa. The large intestine and caecum show little change.

The spleen is not usually markedly changed but may show a degree of congestion. The mesenteric lymph glands are generally normal but are occasionally slightly congested and oedematous.

(iv) The Liver

The changes in the liver are characteristic. The organ is dark brown, (Williams, 1962) sometimes blackish-red (Katitch et al., 1969b) and the gall-bladder is distended with bile. In most cases, the capsule and parenchyma show evidence of immature fluke infestation in the form of haemorrhagic tracts, although in some instances no evidence of immature fluke migration is found (Williams, 1962) and the liver damage may not always be seen at post-mortem examinations (Wallace, 1966). The infected necrotic areas are present in varying numbers. In many
instances they are not observable on the surface of the liver but are easily seen within the substance on incision. However, there are some cases in which no specific lesions were found (Batty, et al., 1964). When the lesions occur on the surface they are not elevated or depressed and there is no apparent break in the continuity of the organ (Jamieson, et al., 1948).

One or more sharply demarcated necrotic areas may be present. These vary in size; many are minute lenticular areas while others may be fairly large spherical areas up to 4cm in diameter. In some instances, the infected necrotic lesions appear linear, being as much as 8cm in length (Jamieson, et al., 1948). The necrotic areas are usually yellow in colour and are surrounded by a red, well-defined, intense zone of congestion. Diffuse areas of haemorrhage are wide-spread throughout the substance of the liver. Frequently under the capsule of the liver and sometimes in its substance, small, tortuous, yellowish bands not surrounded by a zone of hyperaemia are observed; these result from the passage of immature flukes.

(v) Histopathology of the Liver Lesion

The microscopical appearance of a liver lesion is regarded as being diagnostic. This consists of a
central zone of necrosis surrounded by a leucocytic barrier containing mainly polymorphonuclear leucocytes, with some large mononuclear cells and occasional lymphocytes. Within this leucocytic barrier large numbers of vegetative and sporulating forms of *Cl.novyi* are present.

The liver tissue immediately surrounding the lesion is intensely congested and the hepatic cells show degenerative changes in the form of cloudy swelling pyknosis and chromatolysis (Williams, 1962). However, there have been cases reported in which the organism could be isolated from the liver parenchyma in the absence of defined necrotic areas (Turner, 1930b).

According to Turner (1930b) some necrotic foci caused by the flukes only, unaided by *Cl.novyi*, may be present in these livers in addition to the infected necrotic areas that caused the death of the animal. These fluke necrotic areas do not show the characteristic leucocyte zone and may be replaced by fibrous tissue and remain as fibrous scar.

(D) **THE AETIOLOGY OF INFECTIOUS NECROTIC HEPATITIS**

Although the disease is now well known to have a dual causation namely a bacterium and a parasite, this
fact was not realized by early workers. The disease is known to have existed in Australia since 1880 (Roberts, 1959); Gilruth (1910) described the disease in Tasmania; Dodd (1918 and 1921) described the clinical character, epidemiology and pathology and noticed the presence of liver flukes. Albiston, (1927) identified the organism causing black disease as _Clostridioides_ but it was Turner (1930b) who finally demonstrated the true nature of infectious necrotic hepatitis, showing it to be the result of both bacterial infection and parasitic infestation. Further proof of the association of the parasite and bacterium in producing the disease was given by Jamieson (1949).

(a) _Clostridium novyi_ (_Clostridioides_)

This organism was first described under the name of _Bacillus oedematis maligni_ II by Novyi (1894) who recognized it as a cause of malignant oedema in guinea-pigs that had died following injection of casein. Migula (1900) renamed Novyi's bacillus _"Bacillus oedematiens"_, but the organism is now most correctly referred to in modern binomial nomenclature as _Clostridium novyi_ (Willis, 1969). Dodd (1918 and 1921) isolated an organism from cases of infectious
necrotic hepatitis and named it "black disease bacillus"; Albiston (1927) identified the black
disease organism as \textit{Cl. oedematiens} and this was later
confirmed by Turner and Davesne (1927); Zeissler and
Rassfeld (1929) isolated an organism similar to
\textit{Cl. oedematiens} from cases of "Bradsot" in Germany and
named it \textit{Bacillus gigas}. Dodd's "black disease bacillus", Albiston's \textit{Cl. oedematiens} or the German
\textit{B. gigas} are the causal organism of infectious
necrotic hepatitis and are now all known as
\textit{Cl. novyi} type B (Scott et al., 1934). Although in
the United Kingdom, this organism is still usually
known as \textit{Cl. oedematiens} type B, in the present work the
internationally accepted name \textit{Cl. novyi} type B (Breed,
Murray and Smith, 1957) is preferred.

\textit{Cl. novyi} type B is a large bacillus usually
4 to 8\textmu long and 0.8 to 1\textmu in breadth although larger
forms up to 18\textmu long occur. It is Gram-positive in
young cultures and Gram-negative after 48 hours. It
sporulates readily; the spores being oval, subterminal
and wider than the rod.

A characteristic feature of the culture is the
presence in it of a proportion of involution forms and
in particular of rods up to 18\textmu in length. The
organism is motile by means of a peritrichous flagella. It is a strict anaerobe and is fastidious in growth from small inocula. The optimum temperature for growth is 37°C, but growth occurs between 24°C and 43°C. The organisms produce a moderate turbidity, gas, a foul smell and pink coloration in the meat when grown in cooked-meat broth. Colonies on blood agar are flat plaques of fine filaments, scarcely raised above the surface and spreading; there is haemolysis which is quite characteristic, there being a clear zone of beta-type haemolysis underlying the areas of colony development surrounded by a very wide zone of diffused alpha-type haemolysis. On egg-yolk agar, small irregular, transparent colonies produce a wide (8mm. diameter), regular, sharply defined circle of precipitation. The bacterium ferments glucose, galactose and laevulose with production of acid and gas. The action on maltose and glycerol is variable. Gelatin is liquified and the reaction in litmus milk is variable. Toxin is produced readily in 48 hours in glucose broth if anaerobiasis is ensured. The toxin is lethal to mice within 24 hours when a dose of 0.001ml. is given intravenously. It is also lethal to guinea-pigs within 24 to 48 hours when given intramuscularly or subcutaneously in a dose of 0.02ml.
(b) *Fasciola hepatica* (Linnaeus, 1758)

Dodd (1921) noticed the close and constant association between the presence of immature wandering liver flukes and the occurrence of infectious necrotic hepatitis. Turner (1930b) was the first to establish the association of the liver fluke *Fasciola hepatica* and *Cl.novyi* in the causation of infectious necrotic hepatitis. A great deal of literature is now available about fascioliasis concerning both the causal agent and the disease but there is little information available about the relationship between fascioliasis and infectious necrotic hepatitis. This review will be confined mainly to a consideration of the degree of damage to the liver caused by the immature flukes during their migratory stage in the parenchyma and to the role of this damage in the causation of infectious necrotic hepatitis.

Infection of sheep with *Fasciola hepatica* occurs when metacercariae are ingested along with the herbage. The course taken by the juvenile flukes after they have excysted in the intestine has been investigated by many workers. Schumacher (1938) found that in guinea-pigs, rabbits and sheep most of the young flukes have assembled in the peritoneal
cavity about 24 hours after infection. They start to enter the liver after 48 hours and complete the migration within 4–6 days. Kendall and Parfitt (1962) working with sheep, gave times of 18 hours for the young flukes to group in the peritoneal cavity and 90 hours to reach the liver. Dawes (1961, 1962), working with mice, found that the young flukes assembled in the abdominal cavity in about 24 hours, that some flukes enter the liver during the next 2 days and that by 4–6 days the majority are in the liver tissue with only a few remaining in the abdominal cavity. The period during which the young flukes migrate in the liver parenchyma before entering the bile duct and reaching maturity is variable in different animals. Schumacher (1938) found the period to be 54 days in rabbits, 7 weeks in guinea-pigs and 8 weeks in sheep. Urquhart (1956) confirmed these results in rabbits and gave an average of 5 weeks. In mice the period is shorter, being about 24–29 days (Dawes, 1961, 1962).

The type of damage produced in the liver by immature flukes during their migratory activity was described by Dawes (1961, 1962). He concluded from his experiments in mice that immature flukes are tissue feeders and that they grow and thrive on hepatic
parenchymal cells until the critical period is reached after which their growth is inhibited if they remain within the parenchyma, as though by nutritional deficiencies. Sewell (1961) and Dawes (1963b) working with mice, showed that there is a critical time for both host and parasite at 4-5 weeks after infection, when many mice die. This critical period occurs about the same time in rats (Thorpe, 1965). In rabbits the period of most severe damage was shown by Urquhart, (1956) and Sewell (1961) to be from 6-7 weeks after infection. The studies of Ross (1965a, 1966) and Ross et al., (1966) in sheep and cattle, have indicated the presence of a localized phase in the migration of the immature parasites in the parenchyma of the liver of these animals between the 6th and 8th weeks, corresponding to the critical phase previously reported in laboratory animals (Dawes, 1963b). Infections with a "large" number of metacercariae result in overcrowding or competitive inhibition with consequent stunting of the parasite (Montgomerie, 1928; Sewell 1961; Ross et al., 1966 and Ross et al., 1967a). This stunting tends to prolong the period of migration of the young flukes in the liver and death of sheep from acute fascioliasis does not occur before the 7th or 8th week, (Boray et al.,
1965). The continuous feeding on the hepatic cells by the immature flukes, abrasion by their cuticular spines and possibly the effects of toxic or immunogenic secretory or excretory products results in mechanical injury and a necrotic process is set up followed by infiltration by eosinophils. The importance of this process depends mainly on the number of parasites present in the liver and this parameter, together with the time between infection and death, have been used by Ross et al., (1967a) to divide fascioliasis arbitrarily into acute, (Types 1 and 2), subacute and chronic syndromes. During this period of parenchymal damage, spores of *Cl.novyi* already present in the liver may germinate in the anoxaemic areas along the fluke tracts and produce the alpha toxin which has a specific necrotizing effect on the liver cells and results in the death of the animal. In the absence of such pre-existing necrosis the high oxygen tension in the liver prevents the germination of the spores.

The severity of the parenchymal liver damage caused by the young flukes can be estimated by assaying various serum enzyme activities. Boyd (1962) working on the experimental liver necrosis caused by carbon tetrachloride in sheep, calves and rats found that a serum glutamic dehydrogenase (GD) activity is a more sensitive
indicator of liver necrosis in sheep than is glutamic oxalacetic transaminase (GOT) or several other enzymes and that in these animals glutamic dehydrogenase is present in large amounts only in the cells of the liver. Sewell (1967) compared the two enzymes in sera obtained from sheep infected with a large number of Fasciola hepatica. He concluded that both the (GD) and (GOT) activities in the sera were elevated during acute fascioliasis but the difference between the normal and abnormal was shown much more clearly by the (GD) assay. Sewell (1967) also commented that for diagnostic purposes, serological methods would provide greater specificity but they give little indication of the severity of infection and he advocated the use of (GD) as an aid to prognosis during the acute stage of fascioliasis. Thorpe and Ford (1969) working with sheep infected with single and multiple doses of F. hepatica showed a significant increase in the serum levels of sorbitol dehydrogenase (SD), glutamic dehydrogenase (GD) and glutamic oxalacetic transaminase (GOT) in sheep given doses of 2000 metacercariae or 3 successive doses of 200 metacercariae.

Of the many circumstances in which an eosinophilia has been reported to occur parasitic infestation is
one of the most consistent (Jubb and Kennedy, 1963). Eosinophilia in the blood may reach very high levels in different types of parasitism, but it is only found where appreciable tissue damage is produced by the invading organism (Archer, 1965). The number of eosinophils in the blood varies at different stages of infection with *F. hepatica* (Ross et al., 1967a; Pullan, 1968) but it is common in all types of fasciolosis (Ross et al., 1967a), and is maximal during the acute phase of the disease, when the young flukes are migrating in the parenchyma.

Although the germination of *Cl. novyi* spores is usually associated with severe liver damage by *F. hepatica*, cases of black disease in which the livers were lightly infected with flukes and showed only slight damage have been reported, (Dodd, 1921; Williams, 1962). In addition there have been other cases in which the association with the immature liver flukes was not established (Williams, 1962; Batty et al., 1964).

(E) **THE GEOGRAPHICAL DISTRIBUTION OF INFECTION NECROTIC HEPATITIS**

This disease is known to have existed in Australia as early as 1880 and since then has been
reported by many workers in that country (Gilruth, 1910; Dodd, 1918 and 1921; Albiston, 1927; Edgar, 1928; Turner, 1930a&b). It has also been reported in New Zealand (Hopkirk, 1927); the United States of America (Shaw et al., 1939) and in many parts of Europe, namely Germany (Zeissler and Rassfeld, 1929; Miessner et al., 1931), France (Bruner and Gillespie, 1966), Rumania (Cernaianu and Mihaiescu, 1935), Poland (Jastrzebski et al., 1968) and Yugoslavia (Katitch et al., 1969a&b). In the United Kingdom the disease was first reported in the North of Scotland (Jamieson et al., 1948; Jamieson, 1948) and later in some parts of England (Thomson and Ross, 1948; Batty et al., 1964) and in Wales (Williams, 1962 and 1964). The disease has also been reported in Turkey (Hasdemir and Günei, 1968). In Africa there has been only one report of the disease in the "French Sudan", now known as Mali (Curasson, 1929), while in South America it has been reported in Chile (Bruner and Gillespie, 1966).

(F) THE MORTALITY OF INFECTIOUS NECROTIC HEPATITIS

The incidence of infectious necrotic hepatitis in a flock may vary within wide limits. Outbreaks with very heavy losses have been reported, varying
from 16% (Gilruth, 1910) to as high as 70% (Dodd, 1921), but Turner (1930b) gave the average in Australia as about 5%. Recently Williams (1962) suggested that the average flock mortality in Wales lies between 3 and 8% but at certain times may be as high as 15 to 20%. Because of the association of the disease with liver fluke a variation in incidence from year to year is to be expected.

The incidence of fluke infestation is also subject to climatic conditions, which controls the number of encysted metacercariae of *Fasciola hepatica* on the herbage. Turner (1930b) in Australia and Shaw et al., (1939) in the U.S.A. found a higher incidence of infectious necrotic hepatitis in dry periods, probably because at such times animals graze in wet areas not normally grazed. Jamieson et al., (1948) reported that an increased rainfall and a mild autumn coincided with an increase in the number of deaths due to infectious necrotic hepatitis. Ollerenshaw and Rowlands (1959) analysed climatic conditions and post-mortem diagnostic reports over a period of years and demonstrated that there was an increased incidence of both fascioliasis and black disease following a
Wet summer. Williams (1962) showed that the incidence of both fasciolasis and black disease in Wales found at autopsy in sheep over 6 months of age was much higher after a wet summer than after a dry summer. He also reported that in years when climatic conditions do not favour liver fluke development deaths due to infectious necrotic hepatitis are much less frequent.

It is generally agreed that the onset of frost or heavy snow falls bring the infectious necrotic hepatitis season to an abrupt close, possibly because of the low resistance of the encysted metacercariae to freezing (Turner, 1930b; Jamieson et al., 1948). However there have been reports of the disease occurring over a longer period which may extend into March and April (Ollereenshaw and Rowlands, 1959; Williams, 1962). Miller (1951) pointed out that cases of infectious necrotic hepatitis may occur any time as long as there are wandering, immature flukes in the liver and Osborne (1958) observed that in New South Wales the disease occurs in winter and not in mid-summer or autumn.
Breed susceptibility in sheep was not reported by Turner (1930b) who described the disease in Australia in Cross-bred, Comback and Dorset Horn sheep or by Jamieson et al., (1948) who reported the disease in Scotland in pure-bred Cheviots, cross-bred and Border Leicester sheep. Older sheep are reputed to be more susceptible to infectious necrotic hepatitis than young lambs. Turner (1930b) reported the disease in sheep over one year old and Jamieson et al., (1948) observed all his cases in sheep over three years old. However Dodd (1918) reported the disease in six-month-old lambs and Williams (1962) observed no significant increase in incidence with age, as more than half of the 113 animals he examined were under three years old and a number were only six to seven months old.

Sex appears to have no influence upon the incidence of the disease as cases have been observed in rams and ewes. However Parker (1948) described an outbreak of the disease in which deaths were more common amongst females.

A striking feature is that usually only animals in good condition die from this disease. Williams (1962) gave an explanation for this in that most of
the cases in Wales occur in lowland and valley flocks which have been on good grass for some months after weaning the lambs, so that the ewes dying from infectious necrotic hepatitis in the autumn and early winter are in good condition.

(C) THE RELATION OF INFECTIOUS NECROTIC HEPATITIS TO SIMILAR DISEASES

A group of sheep diseases have been reported under different names which seem to be similar to infectious necrotic hepatitis.

(i) Braxy

This is an acute, fatal disease of yearling sheep, characterised by sudden death, high mortality and haemorrhagic inflammatory lesions in the abomasum, which is always associated with Clostridium septicum (Vibrion septique). The disease has been known for some centuries in Scotland, Wales and Iceland (Roberts, 1959) and has long been confused with infectious necrotic hepatitis. It was not until Dodd (1918 and 1921) published his outstanding contribution on infectious necrotic hepatitis and defined the aetiology that confusion about the identity of the two diseases was clarified.
Even then Gaiger (1922) insisted that infectious necrotic hepatitis would yet be shown as identical with braxy. Turner (1930b) confirmed that braxy found in Britain and North-Western Europe was totally distinct from infectious necrotic hepatitis as found in Australia. Finally Jamieson et al., (1948) reported that the condition known for many years in Scotland as "Watery-braxy" was in fact infectious necrotic hepatitis.

(ii) "Swelled head" or "big head" in rams

Bull (1935) described a fatal disease in rams in Australia caused by localized infection with Cl. novyi. The disease is characterised by an enormous oedematous swelling of the head and neck. The sex incidence was explained by the fact that rams, especially young animals, are continuously butting each other resulting in wounds on the head which allow the entrance of the bacteria.

(iii) Bradso or "quick plague"

The term was applied to a disease of sheep occurring in Norway, Iceland, Northern Germany and Northern France (Turner 1930b). The symptoms, post-mortem appearance, histological features and epidemiology
are closely related to braxy. The causal organism of this disease was first reported to be *Clostridium septicum*, but Forget and Raffa Bey (1929) described an enzootic of "Bradsot" in Turkey and claimed that the causal organism was *Clostridium welchii*. Zeissler and Rassfeld (1929) described an atypical type of "Bradsot" in Germany, which was not associated with necrotic ulcers in the abomasum. This does not appear to have been infectious necrotic hepatitis as no mention was made to either necrotic areas in the liver or fluke infestation. The causal organism was claimed to be *Facillus gigas*, an anaerobe resembling *C.l.novyi* type B. However, *B.gigas* was later identified as a variant of *C.l.novyi* type B (Scott et al., 1934) and Roberts (1959) suggested that because of the fortuitous use by Zeissler and Rassfeld of certain media, the organism appeared larger to them than to the Australian workers.

(iv) Acute fascioliasis

Because of the association of infectious necrotic hepatitis with the liver fluke *F.hepatica* it is possible to encounter death from infectious necrotic hepatitis and from acute fascioliasis in the same flock. Acute fascioliasis is characterised by sudden
death, often without any symptoms (Taylor, 1964; Lapage, 1965). It develops quickly and appears in sheep that are apparently in good condition (Lapage, 1965).

However, Ross et al., (1967a) showed that affected sheep show weakness and anaemia for about 7 days before death. Sheep are often found in a characteristic position, similar to that seen in infectious necrotic hepatitis, lying on the brisket with the nose resting on the ground (Taylor, 1964). The abdomen is distended and while the sheep is alive, there is a variable degree of pain in the flank and retrosternal areas (Turner, 1930b; Jamieson et al., 1948 and Lapage, 1956). On post-mortem examination there is usually a blood stained serous exudate in the abdominal cavity and fibrinous peritonitis on the liver which is enlarged, friable in texture and congested with numerous haemorrhagic plaques (Taylor, 1964; Ross et al., 1967a).

Ross et al., (1967a) state that acute fascioliasis shows the general features of an acute haemorrhagic anaemia. The thoracic cavity may contain a large amount of clear straw-coloured fluid but in contradistinction to infectious necrotic hepatitis
the pericardial sac is not distended with clear fluid (Turner, 1930b). The histopathology of acute fascioliasis in sheep has recently been described by Ross et al., (1967a). Very few areas of the liver parenchyma escape damage from migrating parasites and sections of the liver show only remnants of the normal parenchyma, damaged tissue and evidence of a fibrotic reaction. Areas of total disruption, consisting of confluent haemorrhagic fluke tracts, are present. These tracts consist of a central area of haemorrhagic debris containing a few eosinophils, surrounded by a fringe composed of eosinophils and lymphocytes in a matrix of damaged hepatic cells.

(H) THE PATHOGENESIS OF INFECTIOUS NECROTIC HEPATITIS

The spores of *Cl. novyi* are usually found in soil (Zeissler and Rassfeld, 1929; Seddon and Edgar, 1930; Nishida and Nakagawara, 1964; Shinjo and Manabu, 1965; Jastrzebski et al., 1968) and if ingested by the animals on the pasture reach the liver and spleen, where they may remain innocuously for up to at least 9 months (Turner, 1930a&b) so that these animals may be considered as "carriers" of the organism.
The innocuity and latency of *Cl.novyi* spores in the animal body was proved experimentally by Turner (1930a&b) and Jamieson (1949), who showed that experimental animals, including sheep, could be artificially made to become "carriers" of latent spores in the liver, spleen and occasionally bone marrow, if these spores were injected by various routes or were fed by mouth. This carrier state in sheep on pasture was later confirmed by several workers who examined livers from apparently normal sheep in "black disease" areas. Thus Edgar (1928) found 24% of the livers he examined contained *Cl.novyi*; Jamieson (1949) examined livers of healthy sheep in "black disease" areas as well as in areas known to be free of the disease and he found that the percentage of livers containing *Cl.novyi* was 28.9% and 1.3% respectively; Williams (1962) examined random liver samples in Wales and revealed a carrier rate in sheep of 24%

Contamination of the pasture is usually caused by dead animals, the spores being washed by rain and floods to other areas, but less severe contamination may arise from the faeces of carriers. This will be aggravated by over-stocking of the pasture or by
imperfect disposal of carcasses. Contamination is probably also produced by infected wild animals and birds (Edgar, 1931; Jamieson, 1949).

The process by which ingested spores of Cl.novyi reach the liver has not been fully investigated. Dodd (1921) struck by the constant association of black disease with fluke infestation brought forward the hypothesis that the metacercariae are the actual carriers of the Cl.novyi spores. He suggested that metacercariae are contaminated with the spores either on the soil or in the alimentary canal of sheep. However, in view of the fact that later workers (Edgar, 1929; Turner, 1930 a&amp;b; Jamieson, 1949; Williams, 1962) reported that a high percentage of healthy sheep with no flukes carry spores of Cl.novyi in their livers, Dodd's theory is not necessary to account for infectious necrotic hepatitis. Albiston (1927) suggested that the organisms reach the liver via the portal circulation and then find a suitable nidus for development in the areas of tissue destruction caused by the flukes. Hopkirk (1927) considered that the organisms, having passed into the peritoneal cavity via the holes bored through the intestinal wall by the immature flukes, then grow along
the fluke tracks in the liver and so produces the disease.

Edgar (1928) postulated the possibilities of the spores of *Cl.novyi* reaching the liver from the intestines either via the portal vessels and along the portal vein to the liver or via the lymphatics (lacteals) to the general circulation and so to the liver via the hepatic artery. He emphasised that if the spores are carried by the lymphatics it should be possible to obtain the organism from the mesenteric lymph glands, lungs and kidneys as well as from the liver.

Turner (1930a&b) in reflecting on portals of entry of spores into sheep in pasture was attracted by the possibility of their absorption taking place during the eruption of teeth. He suggested that they would then pass to the liver via the general circulation. He concluded from his experiments in rabbits that extraction of teeth was not necessary, although in rabbits with extracted teeth, the spleen and liver regularly contained spores. As regards the time taken for the spores to reach the liver after ingestion, Turner (1930a) reported that *Cl.novyi* spores readily
enter internal organs even after ingestion, and he found the bacillus in the liver of rabbits within 24 hours of being swallowed.

The experimental production of infectious necrotic hepatitis has long attracted considerable interest; workers have attempted to prove that the disease involves the activation of latent spores of Cl. novyi in the liver by immature wandering flukes or other agents. Turner (1930b) was the first to attempt to reproduce the disease in guinea-pigs. He did not try to produce the disease in sheep as he found it impossible to obtain adequate supplies of metacercariae of Fasciola hepatica. In his experiments Turner infected 4 guinea-pigs intracardially with 7½ million spores of Cl. novyi each and 85 days later each was given 15 metacercariae of F. hepatica. The animals were still alive 22 days after feeding the metacercariae and Turner concluded that the spore population in the guinea-pigs was not adequate for the production of the disease and accordingly the animals were given more spores intraperitoneally. They all then died 3 days later with typical lesions of "black disease" and histological sections made from
necrotic areas were very similar to those of the natural disease. A control guinea-pig which had received 25 million spores showed some indisposition but later recovered. Turner was able to produce the disease in guinea-pigs by injecting them intrahepatically with a mixture of spores and calcium chloride solution and also in rabbits, naturally infested with the immature stage of *Cysticercus pisiformis*, by injecting spores intravenously.

Turner's experiments were criticised by Jamieson (1949) in that they did not prove that latent spores of *Cl. novyi* in the liver are activated by wandering liver flukes. In his experiments Jamieson was able to produce the disease experimentally in two guinea-pigs within 6 weeks after simultaneous interaperitoneal or intravenous administration of spores of *Cl. novyi* and feeding 25 metacercariae of *Fasciola hepatica* for each animal in gelatin capsules. He did not consider his attempts to produce the disease in rabbits to be successful, only one of three dying following intravenous injection of spores and feeding of metacercariae. In the rabbit which died, Jamieson did not observe any necrotic lesions but there was evidence of fluke infestation and the organism was
isolated in pure culture from the liver and spleen.

Recently Katitch et al. (1969b), claimed to have reproduced the disease in 7 groups of 12 guinea-pigs using washed vegetative organisms of \textit{Cl.novyi} type B, \textit{Cl.welchii} types A and C or \textit{Cl.septicum} orally and an unknown number of metacercariae of \textit{F.hepatica}. The animals either died or were killed 21 days after infection and it was possible to isolate the organisms used for infection by liver smears and blood cultures. In other experiments 4 groups of 10 guinea-pigs were used and each was given 6000 eggs of \textit{Ascaris suum} and 10 million vegetative organisms of \textit{Cl.novyi} type B, \textit{Cl.welchii} or \textit{Cl.septicum} orally. A control group of guinea-pigs received only the eggs of \textit{A.suums}. Some of the doubly infected guinea-pigs were killed and the bacteriological studies showed evidence in the liver of the bacteria used for infection. When the rest of these infected guinea-pigs died 21 to 29 days after infection, the organisms were isolated from the liver and blood culture. The controls were bacteriologically negative at autopsy. Katitch et al., also considered the possibility of causing infection in sheep with \textit{Cl.novyi} type B following the penetration
of the larvae of *A. suum*. They claimed to have produced the disease in 2 groups of 6 sheep by giving orally, at the same time, 20,000 larvae of *A. suum* and 5 million washed vegetative *Cl. novyi*. Control sheep which had 20,000 larvae of *A. suum* were included. The group infected with bacteria died between 4 and 9 days after infection with the clinical picture and post-mortem lesions of infectious necrotic hepatitis. All the sheep in the control group remained alive and infested with *A. suum*. Katitch et al. concluded that the occurrence of infectious necrotic hepatitis is always associated with liver lesions caused by various helminths and could be caused by other pathogenic anaerobic bacteria as well as *Cl. novyi* namely *Cl. welchii* types A and C and *Cl. septicum*. They suggested that the greater frequency of *Cl. novyi* type B is due to the high frequency of this organism in the digestive tract of the sheep, which is its normal host. However, these experiments by Katitch et al., (1969b) were criticised by Goret (1969) in that no control with the bacterial infection only, without the parasitic element, was included in any experiment. Under these circumstances Goret considered that the conclusions should not be accepted without reservation. Katitch et al., used vegetative
organisms of *Cl.novyi* for experimental production of the disease and this contradicts with the findings of early workers on the subject namely Turner (1930b) and Jamieson (1949) who had established that the disease is caused by the activation of latent spores of *Cl.novyi*. In addition *Cl.novyi* vegetative organisms are known to be very rare in normal animals (Thomson et al., 1968). Katitch et al did not use, in their experiments, the small liver fluke *Dicrocoelium dendriticum* which is very common in South East Europe and instead they used *A.suum*, a parasite of pig which only accidentally infest sheep.

(I) THE ISOLATION AND IDENTIFICATION OF ORGANISMS OF THE CLOSTRIDIUM NOVYI GROUP WITH SPECIAL REFERENCE TO *CL.NOVYI* TYPE B.

*Cl.novyi* is a fastidious "demanding" anaerobe, the absence of oxygen being a critical factor that influences the growth. The reasons why anaerobic conditions must exist before growth will occur are not fully understood. It may be that anaerobic organisms form peroxide in the presence of oxygen and that they lack a suitable enzyme, e.g. catalase, to remove this toxic material (Mcleod and Gordon, 1923).
Alternatively, free oxygen may irreversibly oxidise some of the enzymes of the metabolic pathways of the anaerobe. Willis (1964) stated that type B strains of *C.l.novyi* are more difficult to grow than type A strains, whilst type D are probably the most fastidious anaerobes known.

Various methods have been devised for cultivation of *C.l.novyi* and all of them are designed to exclude oxygen from the environment in which the anaerobe is growing (Willis, 1964). This was made possible by the introduction of the anaerobic jar (McIntosh and Fildes, 1916) the most significant recent modification of the anaerobic jar being the introduction of the room-temperature catalyst (Heller, 1954) which is now commercially available (Baird and Tatlock Limited).

Various liquid and solid media have been used for the cultivation of *C.l.novyi*. Robertson (1915) developed cooked-meat broth (CMB) which contains powerful reducing systems; it is a general purpose medium and it is still the most useful medium in anaerobic bacteriology. Rutter (1968) and Rutter and Collee (1969), incorporating Oxoid nutrient broth in this medium, found it to support consistently good
growth of types B or D strains of *Clostridium novyi* with adequate production of the recognized soluble antigens.

Brewer (1940) introduced his semi-solid medium, which consists of infusion broth as the basal medium with added small amounts of agar, sodium thioglycollate and dextrose; the last two ingredients providing reducing conditions within the medium. Keppie (1944) found Brewer's medium was the best that he tried for the growth of *Clostridium novyi*. However, Rutter (1968) reported that the addition of reducing agents such as thioglycollate or glucose did not ensure consistent growth of types B, C or D strains of *Clostridium novyi*.

Many workers (e.g. Turner, 1930b) have used shake-culture techniques to isolate *Clostridium novyi* type B and have described the different colony types that occur in this medium. However, the variation in the morphology of the colonies obtained using this type of technique, makes it of relatively little use. The difficulties of growing types B, C and D strains of *Clostridium novyi* in solid media have been encountered by many workers. As regards type B strains, Albiston (1927) was unable to grow the "black disease bacillus" on surface culture and Turner (1930b) observing that type B strains grow as a spreading film on blood agar, did not
recommend the medium. Keppie (1944) found that if plates were inoculated directly with infected tissue, they often fail to yield type B strains. Williams (1962 and 1964) used blood agar plates that contained 5% sheep blood and 3% agar for the isolation and growth of \textit{Cl.novyi}. He confirmed that the organism is very exacting and type B strains tend to spread on blood agar plates. Willis (1964) also observed the tendency for types A and B strains to spread over the surface of the medium. Rutter (1968) observed that the use of blood agar plates that contained 30% human group - O blood and 3-5% agar more consistently support growth of types B, C and D strains of \textit{Cl.novyi}. He also noted that egg-yolk agar usually fails to support adequate growth of types B, C and D strains but that when growth does occur, the type D strains produce a larger zone of turbidity than type B strains. A pearly layer was not produced by either of these types.

The isolation of \textit{Cl.novyi} from infected material

The paucity of present knowledge regarding the growth factors that are required by \textit{Cl.novyi}, and the lack of known biochemical or "metabolic character" that can be exploited in a specific enrichment medium
for \textit{C.l.novyi}, have often made the isolation of the organism from pathological material or soil difficult and slow.

(a) \textbf{Immunofluorescence procedures}

A recent aid to the identification of \textit{C.l.novyi} has been the development of a commercial fluorescent-labelled antiserum. Batty and Walker (1964) found that strains of \textit{C.l.novyi} representing all the types, fluoresce with an anti-serum prepared against a type B strain. However, Van Kampen and Kennedy (1968) reported failure to stain type B with \textit{C.l.haemolyticum} (\textit{C.l.novyi} type D) antibody conjugate. The fluorescent antibody technique has been used to demonstrate \textit{C.l.novyi} in liver and other tissues from cattle, sheep and pigs (Batty \textit{et al.}, 1964; Bourne and Kerry, 1965; Hart, 1965; Corbould, 1966; Batty \textit{et al.}, 1967; Thomson \textit{et al.}, 1968; Niilo \textit{et al.}, 1969; Roberts \textit{et al.}, 1970b).

Although the development of the immunofluorescent technique is of great value in the identification of \textit{C.l.novyi}, diagnosis of infectious necrotic hepatitis cannot be made on this basis alone, without considering associated relevant factors such as the clinical history,
the post-mortem findings and the state of
decomposition of the carcase (Batty et al., 1967).

(b) Heat treatment

Turner (1930b) showed that fully mature spores of
type B strains of *Cl. novyi* would resist heating at 100°C
for 10 minutes but are destroyed after 15 minutes.
Williams (1962) suggested that heating contaminated
cultures in liquid media at a temperature of 100°C for
periods of up to 3 hours can be of great value in
obtaining pure cultures of *Cl. novyi* type B.

Willis (1964) recommended that pathological
material may be heated at 80-100°C for 10-15 minutes
before culture, the spores of *Cl. novyi* being
unaffected by this treatment. Records and Vawter
(1945) stated that type D strains are less resistant
and none survive 95°C for 15 minutes. However, if
the pathological material is heated before sub-
culture, it is probable that the resulting bacterial
population is entirely derived from spores. Such a
population may have certain differences compared with
a population derived from vegetative organisms
(Nishida and Nakagawara, 1965).
(c) Selective agents

Willis (1964) reviewed a number of selective agents that may be incorporated in media to suppress the growth of facultative anaerobes and to facilitate the isolation of clostridia from pathological materials. These agents include gentian violet, sodium azide, sorbic acid (Emard and Vaughn, 1952) and antibiotics at various concentrations. Willis however concluded that none of them was ideal for the purpose. Rutter (1968) reviewed various selective agents suitable for the growth of Cl. novyi and suggested that the use of a mixture of neomycin sulphate (10µg/ml), sulphonamidimidine (1 in 4,000) and crystal violet (1 in 100,000) is useful for the selective isolation of Cl. novyi types A, B and D.

Typing of Cl. novyi strains

The most significant and important contribution to the identification of Cl. novyi was provided by Oakley et al., (1947) and Oakley and Warrack (1959). These workers identified antigenic components in toxic filtrates of Cl. novyi of various types; they demonstrated that each of the soluble antigens possessed one or more biological activities. They concluded that methods
based on the properties of these soluble antigens gave criteria for the identification of four types of *Clostridium novyi*, A, B, C and D (see Table 2.1). The alpha toxin is lethal and necrotizing, and is produced by strains of types A and B, but in greatest amount by type B. Following the subcutaneous injection of alpha toxin into mice, the animals die in 24–48 hours. This lethal activity can be neutralized by using specific antitoxin (Burroughs Wellcome). The beta and gamma toxins are both haemolytic lecithinases, and are of importance in typing strains of *Clostridium novyi* (Willis, 1964; Rutter and Collee, 1969; Roberts et al., 1970a and b). They both produce opalescence in egg-yolk emulsions at 37°C, and are hot-cold haemolysins, haemolysis develops only on cooling following incubation at 37°C. The beta toxin is produced by strains of types B and D, type D strains being more active. The gamma toxin, only consistently produced by type A strains. The production of opalescence in the (IV) test at 37°C and haemolytic activity (hot-cold), which is inhibited by appropriate antisera (Burroughs Wellcome) is indicative of beta or gamma activity. Strains of B and D are distinguished by injection of filtrates into mice; type B strains being lethal due to alpha toxin. This method of typing has been used to type organisms isolated from sheep (Williams, 1962; Roberts et al., 1970b&c) and cattle (Williams, 1964). The remaining toxins are of no great importance in typing strains of *Clostridium novyi*. 
<table>
<thead>
<tr>
<th>Activity</th>
<th>Designation</th>
<th>Presence in filtrate from <em>Cl.novyi</em> type</th>
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<td></td>
<td></td>
<td>A</td>
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<tr>
<td>Necrotizing, lethal</td>
<td>alpha</td>
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<td>Haemolytic, necrotizing, lethal, lecithinase</td>
<td>beta</td>
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<td>Haemolytic, necrotizing, lecithinase</td>
<td>gamma</td>
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<td>Oxygen-labile haemolysin</td>
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<td>Opalescence in egg-yolk emulsion ? pearly layer</td>
<td>epsilon</td>
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<tr>
<td>Haemolysin</td>
<td>zeta</td>
<td>-</td>
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<tr>
<td>Opalescence in egg-yolk emulsion ? lipase</td>
<td>theta</td>
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+, ++, +++ = increasing amount
- = none detected
? = doubtful results
tr = trace amount detected
MATERIALS AND METHODS

(A) EXPERIMENTAL ANIMALS

(a) White mice weighing approximately 30g supplied by Messrs. A. Tuck & Sons, Essex, England were used. These were kept in cages and fed on a commercial mouse diet (North Eastern Farmers Limited).

(b) Guinea-pigs weighing approximately 300g were supplied by the Small Animal Breeding Station, University of Edinburgh, and these were fed with pellets (Quayside), turnips and hay.

(c) Rabbits (Cross New Zealand white and Chinchilla) of 3 months of age were supplied by the Small Animal Breeding Station, University of Edinburgh and were fed with turnips and pellets (McGregor & Co., Leith).

(d) Cheviots and Black-face sheep, males and females of various ages ranging from 3 months to 1 year were supplied by the Easter Bush Farm, Veterinary Field Station, University of Edinburgh. These were brought
indoors and dosed with thiabendazole per kg live weight (Thibenzole, Merck Sharp & Dohme Ltd.,).
The sheep were fed on bruised oats, hay and water ad lib.

(B) **CLOSTRIDUM NOVYI** (Novyi, 1894)

(a) Strains.

Six strains of *Cl.novyi* type B have been used in the present work and were identified as follows:
Two strains were isolated from the livers of two sheep that had died from infectious necrotic hepatitis; the livers were kindly supplied by the Veterinary Investigation Department, West of Scotland Agricultural College and the Veterinary Laboratory, Merrythought, Penrith. These strains were designated B1B/68 and B2B/68 respectively.

Two strains of *Cl.novyi* type B were obtained from the National Collection of Type Cultures (N.C.T.C., Cat.No. 6577, Batches 2&3). Two strains designated GRIB & GR2B were from the collections of the Department of Bacteriology, Medical School, University of Edinburgh.

All the strains were grown anaerobically in cooked-meat broth (CMB), in McCartney bottles for 24 hours at 37°C and were kept subsequently at room temperature in
the dark. Checks were made at monthly intervals by growing the strains on sheep blood agar and egg-yolk agar plates, by immunofluorescent staining and by the haemolysin (HL) and lecithovitellin(LV) neutralization tests with the appropriate commercial antisera (Burroughs Wellcome).

(b) Anaerobic Jar Procedure

Anaerobic jars with a room-temperature catalyst (Baird and Tatlock Ltd., Chadwell Heath, Essex) were used throughout and were evacuated as recommended by Rutter (1968). With the inlet (H) closed and the inlet (V) open and connected to a pump (Speedivac, Edwards High Vacuum Limited, Essex, England), the jars were evacuated to a vacuum of about 660mm Hg. Carbon dioxide was added through inlet (H) from a bladder until the vacuum fell to about 600mm Hg (i.e. 10% of the vacuum) and thereafter hydrogen was run in from a separate bladder to fill the rest of the jar. Both inlets were then closed and the jars were left on the bench for 10 minutes before being checked with a mercury manometer to ensure that a secondary vacuum of at least -20mm Hg had been produced. More hydrogen was then admitted. The jars were then incubated in a constant - temperature room at 37°C. All useful checks to avoid failure of anaerobiosis were made together with proper greasing of valves and cleaning of the jars.
(c) Culture Media

A variety of liquid and solid media were evaluated for their ability to support regular growth of \textit{Cl. novyi}. The aim was to select the most reliable and simple media for routine work.

\textbf{Cooked-meat broth (CMB)} as described by Cruickshank (1965, p. 755), was routinely used except that nutrient broth (Oxoid) replaced peptone infusion broth in the preparation of this medium. Dehydrated Cooked-Meat Broth (Oxoid) was also used in the early stages of the work.

\textbf{Blood agar medium} was prepared from blood agar base (Oxoid) and defibrinated sheep blood and horse blood (Burroughs Wellcome) or occasionally citrated human group-O blood from the Blood Transfusion Department, Edinburgh Royal Infirmary.

\textbf{Selective blood agar medium}. This was used routinely for the isolation of \textit{Cl. novyi} from infected contaminated material as described by Rutter (1968). It contained sulphamethazine - sulphadimidine (1 in 4,000), neomycin sulphate (10 \(\mu\)g per ml) and crystal violet (1 in 100,000). Plates of this medium were usually seeded and incubated for 48-72 hours.
Enriched egg-yolk medium. This medium was prepared as described by McClung & Toabe (1947). To prepare 1000 ml of the medium, the following ingredients were mixed:

- Proteose Peptone (Oxoid) 40g
- Disodium Hydrogen Phosphate 5g
- Sodium Chloride 2g
- Magnesium Sulphate 0.01g
- Glucose 2g
- Agar (Davis) 30g
- Distilled Water to 1 litre

The pH was adjusted to 7.4 and the mixture was sterilized at 15 p.s.i. for 15 minutes. A 100ml volume of sterile egg-yolk emulsion (Oxoid) was added before pouring the plates.

Heated "Chocolate" blood agar. This was prepared by heating sheep blood agar medium at 80°C for 10 minutes before pouring the plates.

Addition of iron-filings. Sprinkling sterile iron-filings on blood agar plates after inoculation was used as described by Collee, Rutter and Watt (in press). The medium improves growth of Cl. novyi type B, but the results were variable.
Dithiothreitol (DTT) medium, Moore (1968). During the progress of the present work, Moore (1968) emphasised the need to have available a plating medium that could be made simply and reproducibly and could be relied upon to support luxuriant growth of Cl.novyi type B. This medium was used successfully in the present work. However, the cost of dithiothreitol (Cal. Biochem. Ltd.,) and the sterilization of the basal medium by Millipore G.S. Membrane made it impractical for routine work. Later in the work, it was confirmed that the use of L-tryptophan at 1mg/ml final concentration and L-cysteine at a concentration of 500-1000mg/ml (Moore, 1969 - personal communication) was very stimulatory and these can be added to the base and autoclaved.

(d) Isolation and identification of Cl.novyi type B

The presence of organisms similar to Cl.novyi in infected or contaminated material was first detected on either direct smears or on smears made from growth on liquid or solid media, by morphological staining with Gram's stain. The presence of the organism was then confirmed by immunofluorescent staining (Batty & Walker, 1964) using a commercial stain (Burroughs Wellcome).
Contaminated material was usually inoculated into freshly steamed cooked-meat broth and the inoculated medium was steamed at 100°C for 10 minutes before anaerobic incubation at 37°C. The presence of gas, the smell, and the change in the colour of the meat particles caused by the growth on cooked-meat broth were particularly noted.

The colonial morphology of the organism was studied by culturing contaminated material either directly or following growth on cooked-meat broth, on selective sheep blood agar or egg-yolk agar plates. Following growth on sheep blood agar, the presence and the extent of haemolysis was particularly noted and on egg-yolk agar the presence of opalescence around the colonies was also noted. Colonial morphology was also studied on "chocolate agar".

Pure cultures of the isolated strains of Cl.novyi were then grown in cooked-meat broth for typing.

(e) Typing of Cl.novyi strains

The strains of Cl.novyi isolated from livers, other infected organs or from soil samples were typed by the detection of soluble antigens (Oakley et al., 1947). Organisms in pure culture were grown in cooked-meat broth for 72 hours at 37°C anaerobically.
(1) Neutralization test for haemolysin and lecithovitellin activities

**Red Cell Suspension**

Defibrinated sheep blood (Burroughs Wellcome) was used routinely though occasionally defibrinated horse blood (Burroughs Wellcome) or human blood group-0 were used. A fresh sample of blood (1-5 days after dispatch) was washed 3 times by centrifugation with sterile physiological saline and washed cells were made up in a 2% (v/v) suspension in cagsal solution (see below). The cell suspension was then stored at 4°C and was used within 4-5 days.

**Lecithovitellin (LV) suspension**

The LV substrate was a commercially available egg-yolk emulsion (Oxoid). A 10% solution of this substrate was made in cagsal solution, stored at +4°C and used within 7 days.

**Calcium gelatin saline (Cagsal)**

This was used as diluent in the haemolysin and lecithovitellin tests and was prepared as follows (Brooks et al., 1957):-

- 1% Calcium Chloride in water: 227.5ml
- Sodium Chloride: 45g
- 5% Gelatin (BDH): 200g
- Phenol: 5g
The mixture was made up to 5 litres with distilled water and autoclaved for 10 minutes at 10 p.s.i.

**Michaelis Buffer**

Barbital sodium/sodium acetate, hydrochloric acid, pH 6.8 (see Documenta Geigy, 1962 p. 314), was used in most of the work. The formula used was:

Solution A (19.43g C₂H₂O₂·3H₂O+29.43g barbital sodium in 1 litre) 50ml  
Solution B (0.1 N HCL) 64.0ml  
8.5% sodium chloride 20ml

The mixture was made up to 250ml and pH adjusted to 6.8.

**Neutralization tests**

The two tests (i.e. HL and LV) were performed in parallel and different strains of *Cl. novyi* types A&B could be readily distinguished (Rutter, 1968; Rutter and Collee 1969). The test procedure employed one volume (0.5ml) of a 1 in 2 dilution of the test culture supernate in Michaelis buffer to which was added the antitoxin (Burroughs Wellcome) – approximately 0.02ml, delivered from a dropping pipette. After 30 minutes to allow neutralization, 0.5ml of the substrate i.e. 2% red cells suspension or 10% L.V. suspension was added to the test mixture and the tests were incubated for one hour at 37°C in a water bath and then chilled overnight at 4°C. In the case of the haemolysin test,
the results were estimated visually from + (25% lysis) to 3+ (complete lysis). Tests to demonstrate the neutralization of LV activity with commercial \textit{Cl. novyi} diagnostic anti-sera (Burroughs Wellcome) gave cross neutralization test reactions with cultures of both type A & B strains of the organism; it was necessary to dilute the antisera 1 in 10 in order to obtain the expected neutralization pattern.

(ii) \textbf{Demonstration of lethal activity}

The soluble lethal factors in culture products were estimated by intravenous injection of 0.1ml volumes of suitable dilutions of the material in saline into white mice. The results were recorded at 48-72 hours. Occasionally the intraperitoneal route was employed. Neutralization of lethal activity was performed by adding 2 drops of antiserum (Burroughs Wellcome) using a dropper to 0.5ml of the test material and leave it for 30 minutes before the injection of 0.1ml intravenously into mice. The mice were restrained in a small cylindrical plastic tube during the injection procedure and warm water was used as a vasodilator. Injections were made in the tail vein with 1ml syringes and a fine needle (Gillette Scimitar size 26g x \nfrac{3}{8}'' ID.).
(f) Production and counting of spores of Cl. novyi
for experimental work

Suspension of the spores Cl. novyi were produced in
bulk by growing the organism in the following medium
(Colle and Rutter, 1968 - personal communication):

To prepare 1 litre of the medium:

- \( \text{Na}_2\text{HPO}_4 \) 5g
- Peptone 30g
- L-Cysteine (BDH) 0.5g
- Maltose 10g
- Distilled water up to 1000ml
- Dry cooked-meat 5% (W/V)
- particles of broth

Adjust the pH to 7.4

The centrifuged deposit of a pure culture of Cl. novyi
type B in cooked-meat broth was inoculated deep in the
meat of 500ml bottles of the medium. The bottles
were then incubated anaerobically for 4 days at 37°C
and thereafter left for 14 to 20 days at room
temperature in a dark place. Samples were taken
every 3 days and stained with a spore stain
(Cruickshank, 1965). Samples taken after 14 to 20
days at room temperature showed a high proportion of
spores. The fluids were then decanted and strained
through cotton gauze to separate the meat particles. The meat particles remaining in the bottles were washed once with sterile normal saline which was also strained off. All fluids were pooled and centrifuged at 3000 r.p.m. for 20 minutes; the supernate was discarded and the sediment (containing the spores) was resuspended in sterile normal saline. This washing was repeated 3 times and finally the deposit was suspended in saline to a density equal to Brown's Opacity Tube No. 8 (Burroughs Wellcome). The suspension was then heated in a water bath at 80°C for 30 minutes to kill all vegetative organisms, stored at +4°C and used within 3 months.

The total number of spores per ml of suspension was then estimated by a method modified from that described by Prescott & Breed (1910) using a standard loop delivering approximately 0.01ml of spore suspension and the spore stain. Counts of the number of viable spores were done by a pour plate method using Reinforced Clostridial Medium, (RCM, Oxoid) to which was added 1% agar (Analar). This basal RCM medium containing the agar was prepared and distributed in 20ml amounts in McCartney bottles and then sterilized at 10 p.s.i. for 20 minutes. Before the plates were poured, 5%
defibrinated sheep blood (Burroughs Wellcome) and serial ten-fold dilutions of spore suspension previously prepared in Wasserman tubes (3" x ½") in sterile normal saline were mixed with aliquots of the molten ROM medium. The poured plates were then incubated for 48 hours at 37°C. The number of haemolytic colonies in each plate was then counted and the concentration of the viable spores in the initial suspension could thus be determined. The pathogenicity of the spore suspension was frequently checked by injecting 0.5ml of a mixture of equal volumes of 2.5% calcium chloride solution and spore suspension intramuscularly into the thigh of guinea-pigs; control animals were given only 2.5% CaCl₂. The guinea-pigs that had CaCl₂ plus spores always died within 24-48 hours and the organism could be isolated in pure culture from the site of injection in each case.

Fluorescent Antibody Techniques & Photomicrography

The direct fluorescent antibody technique was used extensively for the demonstration of Cl. novyi in liquid and solid culture media and in animal tissues. Commercial conjugated anti-Clostridium oedematiens globulin (Burroughs Wellcome) was used routinely, and
in addition anti-Cl.novyi sera conjugated with Fluorescein isothiocyanate - "F.I.T.C.". (Gurr's Limited) was prepared as follows:

(a) **Preparation of antigen.** Cl.novyi type B (GRIB strain) was grown anaerobically in Glucose CMB (1% glucose) for 20 hours at 37°C. Smears taken at that time showed predominantly large Gram-positive rods. The culture was then centrifuged and the deposit was washed 6 times with sterile normal saline and finally suspended in saline to an opacity equal to Brown's tube No. 8 (Batty and Walker, 1963). These vegetative organisms were killed by autoclaving at 10 p.s.i. for 20 minutes before being used for immunization.

(b) **Preparation of antisera.** A suspension of heat-killed organisms was injected intravenously into two 3-month-old rabbits according to the following schedule: (Table 3.1.)
Table 3.1.

Schedule of injections of rabbits for the production of Cl. novyi antiserum

<table>
<thead>
<tr>
<th>Day of Injection</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>13</td>
<td>2.0</td>
</tr>
<tr>
<td>16</td>
<td>2.0</td>
</tr>
<tr>
<td>19</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The rabbits were then bled 7 days after the last injection and the serum separated.

(c) Conjugation of antisera. This was performed as described by Holborow and Johnson (1967). The globulin fraction of the antiserum was prepared by mixing the chilled antiserum with an equal volume of a chilled aqueous solution of saturated ammonium sulphate. The mixture was kept at +4°C for 30 minutes and then centrifuged at +4°C in an MSE refrigerated centrifuge for 1 hour at 3000 r.p.m. The precipitate was
redissolved in the minimum volume of 0.15M saline necessary to enable it to be transferred to a dialysis sac. Dialysis was then carried out against normal saline for 18 hours at 4°C with two changes of saline. A magnetic stirrer (Gallenkamp), working at its minimum speed, was used as a mechanical aid to dialysis. The protein content of the globulin solution was then determined by the Biuret reaction and its volume measured. Conjugation with F.I.T.C. was carried out using the following conjugate mixture:

Globulin solution containing  
20 mg protein/ml .....................5ml

0.15 sodium chloride solution .............4ml

Carbonate-bicarbonate buffer pH 9.0  
(3.7g, Na HCO₃ + 6g, Na₂CO₃ - anhydrous,  
made up to 100 ml with distilled water).1ml

F.I.T.C. powder (which had been stored  
in a desiccator over CaCl₂ overnight).5mg

The above mixture was chilled in an ice-bath before adding the dry F.I.T.C. powder, and then left at 4°C for 18 hours using the magnetic stirrer to give gentle mixing without frothing. The mixture was then dialysed against several changes of phosphate buffered saline at pH 7.0 until the dialysing fluid no longer showed fluorescence. In some cases, removal of untreated fluorescent material was done by gel
filtration in phosphate buffered saline using column of Sephadex G25 (Pharmacia, Sweden). The conjugated serum was then absorbed with sheep liver tissue homogenate to eliminate non-specific fluorescence as described by Nairn (1969). The stock conjugate was finally dispensed in 1-2ml aliquots in small sterile plastic bottles and stored at -20°C.

Direct fluorescent staining procedure

Clean microscope slides, 0.8 - 1.0 mm in thickness, were used and the area for staining was ringed and the slide labelled with a diamond pen to permit ready identification. Smears of a portion of a liver lesion, of liquid or solid media were made on the slides and left to dry in air. The slides were fixed by gentle flaming or by immersing them in anhydrous acetone for 10 minutes, the former technique being preferred as it gives better results (Butler, 1968). A drop of the fluorescent stain was then placed on each of the ringed areas of the slides and the slides were then placed for 30 minutes at room temperature in a plastic box acting as a moist chamber. The excess of uncombined reagents was then washed off with phosphate-buffered saline at pH 7.1.
(0.15M sodium phosphate in physiological saline) and finally washed for 10 minutes with 2 changes of buffered saline. The slides were then left to dry for 10 minutes before being mounted under a coverslip No. 22 (Chance) in buffered glycerin consisting of nine parts of glycerin and one part of carbonate-bicarbonate buffer. The slides were then examined immediately under the fluorescent microscope.

**Frozen sections for fluorescent antibody staining**

Small pieces of infected liver tissue, 5mm thick and about 5-8mm square, were snap frozen with a slurry of liquid nitrogen and isopentane (Maxwell et al., 1966) which has a temperature of -160°C; sections were then cut in a cryostat and placed on slides. Alternatively frozen blocks of infected liver were treated by freeze-substitution and the blocks stored at 4°C before cutting as described by Taffs and Voller (1964). Sections were stained immediately after cutting, to demonstrate of Cl.novyi in the tissue, using the same procedure for direct staining as for smears except that the stain was left longer on the slides before washing (about 40 min.).
Fluorescent microscopy

A Vickers Patholux Microscope (Vickers Instruments Limited, York, England) was used with the fluorescent attachment for use with ultraviolet light or blue light. The primary filters used include the BG-38 in front of the Superpressure Mercury Vapour Lamp to absorb heat and suppress the red emission; a filter for transmitting ultraviolet light alone (U.G.2.). Barrier filters which can be used alone or in combination are also fitted. Either a bright field condenser or a dark ground condenser was used. The objectives used were a X10 flat field (Microplan), a X20 (Apochromatic), a X50 (Fluorite, oil immersion) and a X100 (Apochromatic, oil immersion). A X10 wide field (Complan) monocular eye piece was used; the object being to allow maximum emitted light to reach the film for microphotography.

Fluorescent microphotography

The Vickers 35mm camera was used throughout the work. Because of the fading image, there is a need to use a high-speed colour film. The use of high-speed Ektachrome film with a time exposure of
2 minutes gave variable and disappointing result in the early stages of this work. Few transparencies showed the organism fluorescing brightly with a dark background, others showed very pale green organisms on a pale blue background and many transparencies were completely blank. These poor results were attributed to two factors: (a) bright light being reaching the film through the viewing eyepiece attached to the camera, (b) slight vibration of the microscope during photography may change the focus of the fluorescent image under oil-immersion during the exposure time.

An improvement in the quality of the transparencies was obtained by reducing the above factors to a minimum, but there remained a major difficulty in obtaining adequate prints from apparently suitable coloured transparencies. In the absence of colour printing facilities, it was necessary to depend on commercial processing but as a result most of the prints were of very poor quality and of low contrast (Figures 3.1, 3.2, 3.3).

For black and white fluorescent on microphotography, the use of Ilford FP4 (125 ASA) gave the best results in preparations from cultures or frozen tissue sections and the prints were clearly defined (Figures 3.4 and 3.5).
**Fig. 3.1.**

Frozen section from liver of sheep number 22 (see results) stained with fluorescent anti-Cl.oedematiens globulin (Burroughs Wellcome). X 1,200.

**Fig. 3.2.**

Smear from the growth of Cl.novyi type B (strain BIG/68) in cooked-meat broth (18 hours), stained with fluorescent anti-Cl.oedematiens globulin (Burroughs Wellcome). X 1,200.

**Fig. 3.3.**

Smear from a lesion in the liver of sheep number 32 (see results) stained with fluorescent anti-Cl.oedematiens globulin (Burroughs Wellcome). X 1,200.
**Fig. 3.4**

Smear from a lesion in the liver of sheep number 32 (see results) stained with fluorescent anti-*Cl. oedematiens* globulin. X 1,800.

**Fig. 3.5**

Frozen section from the liver of sheep number 42 (see results) stained with fluorescent anti-*Cl. oedematiens* globulin. X 1,500.
(C) FASCIOLA HEPATICA (Linnaeus, 1758)

The techniques used for the production of metacercariae of *F. hepatica* for the experimental work, were those described by Sewell (1961) with the modifications described by Pullan (1968). The snails, *Lymnaea truncatula*, the intermediate host of *F. hepatica* and the algal food for these snails, mainly *Oscillatoria* spp., were available from a stock colony at the Helminthology Unit, Centre for Tropical Veterinary Medicine, University of Edinburgh.

**Preparation of algal cultures**

A bucket of dry top soil containing the minimum number of stones and vegetable detritus was collected. The soil was passed through a large sieve with a \( \frac{1}{4} \)" mesh to remove any stones and other debris and was then sterilized by autoclaving at 15 p.s.i. for 20 minutes. After cooling, distilled water was added to the soil and mixed with a spatula to produce mud of about the consistency of dough. The mud was then smoothed into the bottom of a plastic sandwich box (Stewart Plastic Products, size 18cm x 11.5 cm x 4cm) to a depth of about 1.5cm, taking care to produce a very flat surface on which the algae could spread. A small amount of algae from an old green culture was
then placed in the middle of the fresh mud and the whole surface was very gently wetted with distilled water. The box was covered with its lid; great care being taken at all times to avoid dirtying or scratching the lid. The prepared boxes were labelled with the date of preparation and placed in a warm room at 37°C, 15 inches beneath two 30 watt Osram, warm-white, fluorescent, strip-lights, six inches apart. The cultures were examined daily and damped with water, taking care to avoid flooding them. When the algae had covered the whole surface of the mud with a dark green layer, the cultures were ready for use. They were usually stored at room temperature in the day light with the lid on until used.

The Management of the stock of Lymmaea truncatula

The snails were grown on algal cultures in an incubator at 23°C. As many as 30 adult snails or many more young snails were kept on a single culture. Soon after the snails had laid egg masses and had almost eaten the algae off the culture, they were transferred onto a fresh culture and either left in the incubator or replaced in the cold room, depending
on the number of young snails that were required. The boxes bearing the egg masses were labelled, dated and placed in the window at room temperature for the young snails to hatch and feed. The snails (young, growing or infected) were transferred to a new culture as soon as they had eaten the algae off the older culture.

Culture of fasciola eggs

Eggs of *Fasciola hepatica* were obtained from the gall-bladders of infected sheep at Gorgie abattoir, Edinburgh. The bile was washed through a 200 mesh sieve and into a 300 mesh sieve in which the eggs were trapped. The washed eggs were kept in distilled water in the dark; (in an amber bottle in a light-tight box) in the incubator at 23°C. The distilled water was changed daily for seven days after which the eggs were left in the dark until the miracidia were required. Miracidia would hatch on exposure to light by 14 days after setting up the culture and were used within a few hours to infect the snails. Clean *Fasciola hepatica* eggs can be kept for 3 months in the cold room in water and will then develop normally in the incubator.
Infection of *Lymnaea truncatula* with *Fasciola hepatica*

Snails, 2-5mm long, were placed individually into the cells of a perspex haemagglutination plate, each of which contained a little distilled water into which had been placed 5-10 miracidia. The plates were then covered and left overnight at room temperature for infection to occur. Infected snails were maintained in the incubator on good cultures which were changed regularly. By four weeks post-infection the rediae and cercariae can be clearly seen inside the infected snails if they are turned onto their backs and examined with a stereoscope in the area immediately behind the shell opening. Shedding commences about five weeks post-infection. Shedding was induced by placing all the infected snails together into a small polythene bag half filled with distilled water at 10°C. The polythene bag was supported in a beaker and spring clip was used to close the top. The snails were removed after 5 to 6 hours and placed onto fresh algal cultures in the incubator. The bag containing the shed metacercariae was then reclosed and left at room temperature overnight. The water
in the bag was poured away into a beaker and any debris was washed off the polythene bag into the same beaker, this water being boiled to kill any metacercariae in it. The bag was then placed in a four ounce, screw topped bottle and filled with distilled water. The bottle was sealed and labelled. Bottles containing metacercariae were left at room temperature for one week and then transferred into the cold room at 10°C for further storage.

Infection of animals with metacercariae of Fasciola hepatica

(a) Sheep

The technique used for infecting sheep was similar to that described by Pullan (1968). The metacercariae (all of which had been shed within the previous 3 months) were scraped off the polythene and washed into a large beaker of water. Here the metacercariae sedimented and could be concentrated by pouring off some of the supernatant water. The water containing the metacercariae was transferred into a measuring cylinder. After thorough mixing to evenly suspend the cysts, 1ml aliquots were rapidly taken with a wide bore pasteur pipette and transferred to a petri dish. The pasteur pipette
was siliconed (Ropelcote, Hopkins & Williams Ltd.,) to prevent the metacercariae from sticking inside it. Using a stereoscope, the number of apparently viable cysts in at least six aliquots of metacercariae was assessed, so that it was possible to calculate the number of viable metacercariae in the measuring cylinder.

After further thorough mixing, aliquots containing the required number of viable metacercariae were poured on to filter paper in a Buchner funnel and the water was sucked away through the paper by gentle vacuum. The filter paper retained the metacercariae and was carefully folded so as to ensure that they were not lost. The paper was folded to a size that could be administered to the sheep with a balling gun.

(b) Laboratory animals

For the infection of guinea-pigs and rabbits the metacercariae were scraped off a portion of a polythene bag and washed into a large petri dish with water. Using a stereoscope and a siliconed pasteur pipette, the appropriate number of viable cysts was transferred into a small petri dish. The metacercariae were given to both guinea-pigs and
rabbits by putting them directly at the back of the throat using a wide-bore, strong pasteur pipette with a blunt end or by first putting the metacercariae on a small piece of filter paper which was placed at the back of the mouth with a blunt pair of forceps. Alternatively metacercariae were put directly into the stomach using a Nelaton Catheter (Portex Limited, Kent), and a syringe. In all cases rabbits and guinea-pigs were restrained by a gag.

(D) SURVEYS

(1) The incidence of *Cl.novyi* and *Fasciola hepatica* in ovine livers at slaughter

(a) Selection of farms

Two farms with a history of infectious necrotic hepatitis and another two that had no such history of the disease for at least 3 years prior to the survey were selected in each of the South West and South East of Scotland. In addition one farm in Central Scotland, which had no history of fascioliasis or infectious necrotic hepatitis was included in the survey for a comparison (see Table 3.2).
Table 3.2

Location of and history of the farms studied

<table>
<thead>
<tr>
<th>History of infectious necrotic hepatitis in the farm</th>
<th>Geographical position in Scotland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recorded positive</td>
<td>South East</td>
</tr>
<tr>
<td>Farm (IE) .hashCode</td>
<td>Central</td>
</tr>
<tr>
<td>Dryden</td>
<td>South West</td>
</tr>
<tr>
<td>Estate</td>
<td></td>
</tr>
<tr>
<td>Selkirk</td>
<td>Farm (IW) .hashCode</td>
</tr>
<tr>
<td></td>
<td>Cornberry</td>
</tr>
<tr>
<td></td>
<td>Farm</td>
</tr>
<tr>
<td></td>
<td>Cumnock</td>
</tr>
<tr>
<td></td>
<td>Ayrshire</td>
</tr>
<tr>
<td>Presumed negative</td>
<td></td>
</tr>
<tr>
<td>Farm (2E) .hashCode</td>
<td></td>
</tr>
<tr>
<td>Burnfoot Ale</td>
<td></td>
</tr>
<tr>
<td>Cadzo Ltd.</td>
<td></td>
</tr>
<tr>
<td>Fisherton</td>
<td></td>
</tr>
<tr>
<td>Farm (IC) +</td>
<td></td>
</tr>
<tr>
<td>Glendevon</td>
<td></td>
</tr>
<tr>
<td>Ayrshire</td>
<td></td>
</tr>
<tr>
<td>Farm (2W) .hashCode</td>
<td></td>
</tr>
</tbody>
</table>

hashCode = Flukes known to affect sheep on these farms.
+ = No history of fluke infestation of sheep.

The disease recorded on these farms had been diagnosed and confirmed at the Veterinary Investigation Department, West of Scotland Agricultural College (South West) or the Veterinary Investigation Laboratory, St. Boswells (South East).
(b) **Collection of samples**

Arrangements were made to examine the livers of normal animals from these farms when taken to slaughter. Animals from the South West were slaughtered at Ayr or Biggar slaughter houses, those from the South East were slaughtered at the Scottish Border's Abattoir in Galashiels, and those from Central Scotland were slaughtered at Linlithgow slaughter house. A form was drawn up to cover all the relevant information for each group of sheep, including the area, the name of the farm, the owner, any pathological changes in the livers and the presence or absence of flukes in the livers. Immediately after slaughter, a thorough examination of each liver was made and the relevant information recorded. A piece of each liver (4" x 2" x 2", weighing approximately 50g) was taken, placed in a self-sealing polythene envelope (Gallenkamp) and labelled. Samples were then transported in ice to the laboratory where they were stored at -30°C until examined within 3 to 5 weeks after collection. After thawing the sample, a piece from each liver (2" x 2" x 2") was seared on all sides, placed in a sterile disposable petri dish (Oxoid) and then incubated.
anaerobically with 10% CO₂ at 37°C for 48 hours. The object of this procedure is to allow any spores of Cl. novyi present in the liver to proliferate, so that they can easily be detected by the fluorescent anti-body technique (Corbould, 1966; Batty et al., 1967). After incubation, three smears were made from a freshly cut area on each sample, one smear being stained with Gram's stain and the other two with Fluorescent anti-Cl.oedematiens globulin (Burroughs Wellcome). Samples which were considered positive after fluorescent staining were recorded, subcultured in selective sheep blood agar and later typed as previously described.

(2) The examination of soil samples for the presence of Cl.novyi

Samples of top soil were collected into sterile Bijou bottles from various sites on the surveyed farms and brought to the laboratory. They were then examined for the presence of Cl.novyi by a method derived from that of Nishida & Nakagawara (1967). A small portion of each soil sample (pea size) was suspended in 20ml of sterile nutrient broth (Oxoid) in McCartney bottles. Using sterile pasteur pipettes, 2 drops of each suspension were transferred into 20ml of liver broth (Oxoid) in McCartney bottles; each sample was then heated at 100°C for 10 minutes and after cooling they were
incubated anaerobically with 10% CO₂ at 37°C for 3 days. Evidence of presence of _Clostridium novyi_ was sought on the 3rd day by fluorescent staining and positive samples were further identified by subculturing in selective sheep blood agar and later typed.

(E) **THE BEHAVIOUR OF SPORES OF CLOSTRIDIUM NOVYI GIVEN ORALLY TO ANIMALS**

A suspension of spores of _Clostridium novyi_ (strain GR2B) was produced and the concentration of viable spores was estimated. Guinea-pigs weighing approximately 300g and Cheviot lambs aged 4 months were used for the experiments. The animals were deprived of food and water before they were infected orally with the spore suspension. Sheep were given 500ml each of spore suspension (approximately 50 million viable spores) using a stomach tube and the guinea-pigs were given 10ml each (approximately 1 million viable spores) using Nelaton Catheter (Portex Limited, Kent). The challenged animals were killed together with uninfected controls at various times. The guinea-pigs were killed by ether and the sheep with a humane killer.

Isolation of _Clostridium novyi_ was attempted from the stomach (rumen, reticulum, omasum and abomasum in
sheep), small intestine, large intestine, spleen, liver, mesenteric lymph glands (duodenal), kidneys, heart blood and hepatic portal vein.

An aseptically dissected portion of each organ was cultured in McCartney bottles with freshly steamed cooked-meat broth and incubated anaerobically for 48 hours. Each organ was then examined for the presence of Cl. novyi from a centrifugal deposit of the growth on the cooked-meat broth. Positive samples were further identified by growth on selective sheep blood agar and were confirmed by typing.

(F) EXPERIMENTAL INFECTIOUS NECROTIC HEPATITIS

Experimental infectious necrotic hepatitis was induced in laboratory guinea-pigs, rabbits, and in sheep by the oral administration of Fasciola hepatica, followed by a known number of viable Cl. novyi spores in suspension by the same route 2 weeks later. In laboratory animals the disease was also produced by injecting 2.5% CaCl₂ intrahepatically to cause the necrosis similar to that associated with the disease, spores being given to those animals by the oral, intravenous and intraperitoneal routes.
The following techniques were carried out mainly in sheep:

(1) **Examination of faeces for Fasciola hepatica eggs**

Sheep for experimental work were usually examined for the presence of *Fasciola hepatica* eggs immediately after they were brought indoors. They were again examined 6 weeks after administration of the metacercariae. The method used for examination was the direct differential centrifugal floatation (D.D.C.F.) procedure as described by Pullan (1968).

(2) **Haematology**

Blood was collected weekly in 2.5 ml aliquots in small plastic bottles using E.D.T.A. as an anticoagulant. The blood was then mixed using a Matburn cell mixer (Matburn Ltd.) and absolute eosinophil counts were done, using an improved Neubauer chamber, by the method of Dacie and Lewis (1963) except that the total volume was altered, 0.1 ml of blood being added to 0.8 ml of eosinophil diluent (Pullan, 1968).
(3) Serum Biochemistry

Glutamic Dehydrogenase (G.D.)

Serum glutamic dehydrogenase (GD) assays were performed by the method described by Ford & Boyd (1962). Blood for the test was collected weekly in 15ml amounts in sterile universal bottles. It was then left to clot at room temperature for six hours and the serum was separated by centrifugation at 3,000 rpm for 1 hour.

An ultra-violet spectrophotometer (Uvispek, Hilger & Watt), with a constant temperature housing was used to measure the enzyme activity. The settings for the spectrophotometer were slit widths 12mm, wavelength 340 mp and a photocell operating in the ultra-violet range. The assay was carried out at a temperature of 25°C, in a 3ml quartz cuvette, of 1cm light path, with 15 minutes preincubation. As a first approximation the loss in optical density between 1 and 6 minutes after starting the reaction was used to determine the drop in optical density per minute. A reduction of optical density of 0.001 on the spectrophotometer is equivalent to the oxidation of $4.83 \times 10^{-4}$ µ moles of diphosphopyridine nucleotide (DPNH). Hence the activity of the serum in µ moles/litre/min., (i.u.s.) could be calculated
as $2.415X$, where $X =$ the number of 0.001 divisions drop in optical density per minute.

(4) **Post-mortem procedure**

Post-mortem examinations were carried out on sheep and laboratory animals as soon as possible after they had died or been humanely destroyed.

In sheep, the time elapsed since death, any discharges from the natural orifices and the colour of the subcutaneous tissues were noted. When opening the peritoneal cavity, the amount of fluid was roughly estimated and its colour noted. The internal organs, namely the liver, spleen, kidney, mesenteric lymph glands (duodenal), lungs, bone marrow and abomasum were carefully examined and whenever bacteriological work was indicated, a piece of the organ was removed carefully and put in a sterile disposable petri dish (Oxoid).

Blood, peritoneal or pericardial fluids for bacteriological examination were collected in sterile McCartney bottles. Samples of tissues for histopathology were fixed in 10% formal saline and were later stained with haematoxylin and eosin or Gram's stain. When required, frozen sections were
prepared from unfixed tissues in liquid nitrogen. The liver was examined for colour, size consistency and lesions and then photographed. The whole organ was then removed for further examination. The number of immature flukes in the hepatic parenchyma was estimated by cutting the liver into slices 1 to 2 cms thick and taking a random sample of these slices, which together constituted a known proportion of the weight of the liver (usually about a tenth). This sample was squeezed in warm saline and left, with periodic further squeezing, for about 4 hours at 37°C to allow flukes to migrate out. The liver sample was then passed through three sieves. The first sieve (10 mesh per inch) kept back most of the liver matrix. Most of the flukes were retrieved from the next sieve (30 mesh per inch) but some very small ones and some pieces of flukes were collected on the final sieve (60 mesh per inch). The flukes were washed from the sieves into a plastic tray and thence poured into a large beaker in which they were allowed to sediment. Excess water was decanted off and the flukes were counted under a stereoscope.

When examining the thoracic cavity, any changes were noted. The amount of pericardial fluid and its colour were particularly noted.
THE EFFECT OF PUTREFACTION ON THE ISOLATION OF CLOSTRIDIUM NOVYI

Introduction

Livers received for confirmation of infectious necrotic hepatitis are often putrefied and this might lead to false positive confirmation. In the field, animals not dying from black disease may harbour Cl.novyi spores in their livers (Edgar, 1928; Turner, 1930a; Jamieson, 1949), and this may not be apparent in smears of livers from fresh carcasses but if the dead animal is left in the field, the spores may proliferate rapidly under suitable conditions and then may well be detected by fluorescent staining (Corbould, 1966; Batty et al., 1967). The growth and proliferation of Cl.novyi type B spores in infected livers results in the formation of the alpha toxin (lethal toxin) and this performed alpha toxin could be detected in infected liver tissue (Williams, 1962). Accordingly, relationship between the degree of putrefaction, the temperature at which the carcass or an infected liver are kept, and the chances of detecting Cl.novyi or its performed toxin from those carcasses or livers has been studied in laboratory animals and sheep.
Two approaches were used:

(1) Detection of the extent of multiplication of *Cl.novyi* in infected carcasses by counting the number of fluorescing organisms in impression smears of livers and spleens from these animals.

Groups of 4 guinea-pigs, each approximately 300g were each infected orally with a 10ml dose (1 million viable spores) of a spore suspension of *Cl.novyi* type B (N.C.T.C., Batch 2). These animals were all killed 24 hours later, together with a group of uninfected controls. They were then placed in thin polythene bags (4 in one bag) in an incubator at 23°C. Four infected animals and one uninfected control were examined on each occasion after 6, 12, 24, 48, 60, 72 and 96 hours of incubation. In each case, the livers and spleens of the animals were removed aseptically and placed in sterile labelled petri dishes. Impression smears were made from a freshly cut surface and stained with fluorescent anti-*Cl.cedematians* globulin (Burroughs Wellcome). The numbers of fluorescing organisms in ten x 50 fields were counted for each organ and a mean count per field calculated. The mean of the counts on the 4 infected guinea-pigs was then related to the time of incubation.
(2) Detection of the multiplication of Cl.novyi spores by demonstration of alpha toxin (lethal toxin) produced in infected liver tissues.

A group of guinea-pigs were challenged orally with a 10ml dose of spore suspension of Cl.novyi type B as above. They were then killed 24 hours later together with uninfected controls by a blow on the head. The livers from these animals were immediately removed aseptically and placed in sterile labelled petri dishes (Oxoid). These livers were cut in half and each half was incubated anaerobically in parallel in an anaerobic jar with 10% CO₂ at 23°C or at 37°C for comparison. After incubation for 6, 12, 18, 24, 48, 60 and 72 hours, a piece of each liver (10g) was macerated in a sterile tissue grinder (Griffins) with 10ml of sterile normal saline and centrifuged; 0.2ml of the supernate was injected into each of 4 white mice intravenously in the tail vein and these animals were observed for 48 hours. The number of dead mice out of four injected was recorded. Whenever death had occurred, neutralization of the lethal agent was confirmed by observation of a simultaneously challenged mouse that
had been given a mixture of the macerate and Cl. oedematiens type B anti-serum (Burroughs Wellcome).

The number of deaths were related to the times of incubation at the different temperatures (23°C and 37°C).

Livers from sheep, orally infected with spore suspension and killed 24 hours later as described earlier in method (E) were also treated by the same procedure for the detection of the alpha toxin.
CHAPTER IV

RESULTS

THE LABORATORY CULTURE AND IDENTIFICATION OF
CLOSTRIDIUM NOVYI TYPE B

Growth on fluid media

Freshly prepared cooked-meat broth was found to support consistent growth of Cl.novyi type B from infected tissues, soil or solid media. The unconcentrated supernatant of a pure culture of type B strains grown anaerobically overnight in this medium contained sufficient soluble antigens for typing. The use of commercial CMB (Oxoid) however, gave variable results and sometimes poor growth of Cl.novyi type B strains.

Growth on solid media

Cl.novyi type B strains showed variable degrees of growth on the various solid media used in this work. Generally the freshly prepared media gave better growth of the organism than those prepared and stored at +4°C and it was found that to obtain a consistent growth of this organism it was essential
to dry the plates before inoculation. Type B strains required at least 48 hours incubation for adequate growth.

Sheep blood agar containing 3-4% agar and 10-20% defibrinated sheep blood (Burroughs Wellcome) consistently supported good growth of type B strains. On this medium the strains usually grow as flat spreading films but sometimes formed discrete raised grey colonies with an irregular outline. Haemolysis was distinct following incubation of the plates for 48 hours (Fig. 4.1).

If sterile iron filings were sprinkled on the sheep blood agar after inoculation, *Cl. novyi* type B strains usually grew as a spreading film in areas where the iron filings were numerous (Fig. 4.2). However, sprinkling these iron filings sometimes only yielded poor growth.

Dithiothreitol medium (Moore, 1968) supported good growth of *Cl. novyi* type B strains and when the agar concentration was raised to 3%, the colonies grew as a greyish spreading film with slightly raised centres (Fig. 4.3). This medium was used in the
Colonial morphology of Cl. novyi type B on solid media

**Fig. 4.1.**

Strain N.C.T.C. (2) on sheep blood agar (20% sheep blood and 4% agar) - 48 hours after incubation anaerobically at 37°C. X 10.5.

**Fig. 4.2.**

Strain GRIB on sheep blood agar (20% sheep blood and 3% agar) plus sterile iron filings, 48 hours after incubation anaerobically at 37°C. X 10.5

**Fig. 4.3.**

Strain N.C.T.C. (3) on Dithiothreitol (DTT) medium (3% agar) 48 hours after incubation anaerobically at 37°C. X 2.4.
later stages of the work but because of difficulties in preparation and the cost of the ingredients it was not used routinely.

The enriched egg-yolk medium of McLung and Toabe (1947) supported good and consistent growth of type B strains when using concentrated egg-yolk emulsion (Oxoid). The organism grew as a spreading film with opalescence around the colonies.

**The production of Cl.novyi type B spores**

No previously published medium is suitable for the production of spores of this organism but a medium recommended by Collee and Rutter (1968) gave a high yield of Cl.novyi type B spores after incubation for at least 14 days. The concentration and viability of the spores produced by three strains of Cl.novyi type B growing in this medium for 18 days are shown in Table 4.1, which shows that type B strains of Cl.novyi produce more spores as the culture medium ages.
Table 4.1
Spore production by Cl. novyi type B

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>Estimated total number of spores per ml $\times 10^4$ from strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GR2B</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>82</td>
</tr>
<tr>
<td>18</td>
<td>120</td>
</tr>
<tr>
<td>Estimated percentage viability after 18 days</td>
<td>75</td>
</tr>
</tbody>
</table>

Immunofluorescence

This technique was extensively used for the rapid identification of Cl. novyi in fluid or solid media, infected animal tissues or soil samples.

Thin smears gave bright fluorescence, but in thick ones there was a fluorescent haze and some organisms appeared out of focus. The thickness of
the coverslip on the stained preparation may affect the fluorescent image but it was found that the use of a No.22 (Chance) coverslip gave good results with both smears and sections. On mounting the coverslip in buffered glycerine the minimum amount of mountant should be used as large amounts may cause hazy fluorescence.

The degree of fluorescence depends on the source of the stained preparation. Smears prepared from overnight culture in CMB fluoresced more brightly than smears from old culture in the same medium or smears from colonies on sheep blood agar after 48 hours of incubation. However, organisms in frozen liver sections, or smears from infected tissues or soil samples showed even brighter fluorescence. The degree of fluorescence of Cl.novyi type B in various preparations is shown in Table 4.2.
Table 4.2

Fluorescence by Cl._novyi type B

<table>
<thead>
<tr>
<th>Source of preparation</th>
<th>Degree of fluorescence estimated by brightness</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMB (18 hours)</td>
<td>++</td>
</tr>
<tr>
<td>CMB (36 hours)</td>
<td>+</td>
</tr>
<tr>
<td>CMB (3 days)</td>
<td>+</td>
</tr>
<tr>
<td>Sheep blood agar (48 hours)</td>
<td>+</td>
</tr>
<tr>
<td>Smears from infected tissue</td>
<td>+++</td>
</tr>
<tr>
<td>Frozen section (liver)</td>
<td>+++</td>
</tr>
<tr>
<td>Soil sample in liver broth (48 hours)</td>
<td>++</td>
</tr>
</tbody>
</table>

+, ++, ++++ = indicate increased brightness
THE BEHAVIOUR OF ORALLY ADMINISTERED CLOSTRIDIUM NOVYI TYPE B SPORES

(1) In guinea-pigs

The process by which spores of *Clostridium novyi* type B reach the liver was first studied in sixteen guinea-pigs in eight groups of two. Each animal in groups of seven were challenged orally with approximately one million viable spores of *Clostridium novyi* type B and one group was kept as a control. The infected animals were killed after previously determined times and various organs from these and the controls were examined for the presence of *Clostridium novyi* type B. The preliminary results from this experiment indicated that the organism could be recovered from the livers and spleens of infected guinea-pigs within 24 hours of oral challenge.

The experiment was then repeated in thirty two further guinea-pigs in eight groups of four. Seven groups were infected and one group was kept as an uninfected control. The summated results from both experiments are shown in Table 4.3.
<table>
<thead>
<tr>
<th>Organs</th>
<th>Time killed (hours)</th>
<th>0 (uninfected control)</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>42</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large intestine</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric lymph glands</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peritoneal washing</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart blood</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic portal vein</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

N.B. 48 guinea-pigs were used in all, six being killed after each time interval.
It is clear from the table that *Cl. novyi* type B was present in the mesenteric lymph glands of 4 out of 6 infected guinea-pigs (66.7%) 12 hours after oral challenge, but no organisms could be detected in either the livers or the spleens of those animals at that time. By 24 hours, however, the livers of all the infected guinea-pigs contained the organism and in addition 5 out of 6 spleens (83%) and 5 out of 6 mesenteric lymph glands (83%) from the infected guinea-pigs also contained the organism.

In the preliminary experiment, organisms were isolated at 12 hours after oral challenge from the peritoneal washings of one infected guinea-pig. However, when the experiment was repeated no organisms could be detected in the peritoneal washings from any of the infected guinea-pigs. No organisms were isolated from the heart blood, kidneys or the hepatic portal vein of any of the guinea-pigs. No *Cl. novyi* type B was isolated from any of the organs of the uninfected control guinea-pigs.
(II) In sheep

The same experiment was repeated in sheep, first using five 4-month old Cheviot lambs. Four lambs were challenged orally with approximately 50 million viable spores of \textit{Clostridium novyi} type B and one lamb was kept as an uninfected control. The results obtained again showed that the organism is present in the mesenteric lymph glands by 12 hours and in the liver and spleen by 24 hours after oral challenge. As 12 and 24 hours after oral challenge appeared to be the most significant times, the experiment was repeated in four 13-month old ewes, each of which was challenged orally with approximately 50 million viable spores. Two ewes were then killed after 12 hours and the other two after 24 hours.

The organs from the sheep which contained \textit{Clostridium novyi} type B at the various times in both experiments are shown in Table 4.4. No \textit{Clostridium novyi} type B organisms were isolated from the hepatic portal vein, heart blood or kidneys of the infected sheep and none were isolated from any of the organs of the uninfected control sheep.
Table 4.4
Number of sheep containing Cl. novyi type B in various organs after oral administration of spores

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time killed (hours)</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of sheep used</td>
<td></td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(uninfected control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rumen</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Reticulum</td>
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<td></td>
</tr>
<tr>
<td>Omasum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasum</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
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<td></td>
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<tr>
<td>Large intestine</td>
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<td></td>
<td></td>
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<tr>
<td>Liver</td>
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<tr>
<td>Spleen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric lymph glands</td>
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<td></td>
</tr>
<tr>
<td>Peritoneal washing</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Heart blood</td>
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<td></td>
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<tr>
<td>Hepatic portal vein</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
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</tr>
</tbody>
</table>
The incidence of \textit{C1.novyi} type B and \textit{Fasciola hepatica} in the livers of sheep at slaughter

The farms used in the survey were selected because their histories were known in the veterinary investigation centres in the areas and because the necessary facilities were available for the survey to be carried out. The number of liver samples examined from various farms were variable due to variation in the number of sheep available for slaughter from the farms at the time of the survey.

A total of 646 livers were examined from 5 farms and the incidence of \textit{C1.novyi} type B and flukes in sheep from each farm are shown in Table 4.5.

Of all the livers examined during the survey 53.4\% contained organisms which stained with the fluorescent anti-\textit{Clostridium oedematiens} globulin. However, only 14.7\% contained such organisms, which were also resistant to differential heating and 12.7\% contained organisms shown to be \textit{C1.novyi} type B following typing. Only these latter strains will be considered in the present studies.
Table 4.5 shows that sheep from the two farms which had a history of infectious necrotic hepatitis harboured a high percentage of \textit{Cl. novyi} type \( B \) in their livers, the mean incidence being 20.3\%. In the other three farms which had no history of the disease \textit{Cl. novyi} type \( B \) can still be detected in the livers of the sheep but the mean incidence of 6.8\% is much lower.

Furthermore the table shows that the incidence of infectious necrotic hepatitis is also positively related to the distribution of \textit{Fasciola hepatica}. On farms with a recorded history of infectious necrotic hepatitis, the mean incidence of fluke infection in the sheep was 56.2\% whereas in farms with no history of the disease the average incidence of fluke infection was only 13.7\%.

\textbf{(II) The incidence of \textit{Cl. novyi} type \( B \) in soil}

A total of 310 soil samples from the five farms were examined for the presence of \textit{Cl. novyi} type \( B \). The incidence of these organisms on each farm is shown in Table 4.6.
Of all the soil samples examined 19.6% contained organisms which stained with the fluorescent anti-Clostridium oedematiens globulin. However, only 14.1% contained organisms showed to be Cl.novyi type B following typing.

Table 4.6 shows that the incidence of Cl.novyi type B was relatively high in farms with a recorded history of infectious necrotic hepatitis, the mean incidence being 15.3%, whereas on farms with no history of the disease the mean incidence of Cl.novyi type B in the soil was only 5.6%.

A statistical analysis of the survey data showed that there is a significant positive correlation between the incidence of Cl.novyi type B in the soil and its incidence in livers of sheep on the same farms ($r = +0.927^*$) (Graph 4.1). When the incidence of Cl.novyi plus flukes in the livers was compared with the incidence of Cl.novyi type B in soil from the same farms, the positive correlation was highly significant ($r = +0.979^*$) (Graph 4.2) and again there was a significant correlation ($r = +0.931^*$) (Graph 4.2) between the incidence of Cl.novyi type B plus flukes in the livers and the incidence of flukes alone in the livers from sheep on the same farms.
Table 4.5

The incidence of *Clostridium novyi* type B and flukes in livers of sheep from five farms at slaughter

<table>
<thead>
<tr>
<th>Geographical position in Scotland</th>
<th>South West</th>
<th>South East</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm number</td>
<td>1W 2W 1E 2E 1C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of infectious necrotic hepatitis</td>
<td>+ - + - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of livers examined</td>
<td>131 90 105 106 164</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage with</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium novyi</em> type B</td>
<td>21.6 7.8 19.1 7.6 4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flukes</td>
<td>68.6 28.9 43.3 12.3 nil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>19.9 1.1 11.4 2.8 nil</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium novyi</em> type B but no flukes</td>
<td>1.6 6.7 8.6 4.7 4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flukes but no <em>Clostridium novyi</em> type B</td>
<td>48.6 26.7 32.4 9.4 nil</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* + = Infectious necrotic hepatitis recorded and confirmed
  - = Infectious necrotic hepatitis not recorded
Table 4.6

The incidence of *Clostridium novyi* type B in soil samples from five farms

<table>
<thead>
<tr>
<th>Geographical position in Scotland</th>
<th>South west</th>
<th>South east</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm number</td>
<td>1W</td>
<td>2W</td>
<td>1E</td>
</tr>
<tr>
<td>History of infectious necrotic hepatitis</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Number of soil samples examined</td>
<td>109</td>
<td>39</td>
<td>50</td>
</tr>
<tr>
<td>Percentage of soil samples containing <em>Cl. novyi</em> type B</td>
<td>26.6</td>
<td>7.7</td>
<td>14.0</td>
</tr>
</tbody>
</table>

* + = Infectious necrotic hepatitis recorded and confirmed

* - = Infectious necrotic hepatitis not recorded*
Graph 4.1.

Regression of the incidence of Cl. novyi type B in sheep livers on its incidence in the soil for 5 farms.
\( y = 0.75x + 3.58 \)

\( r = +0.927^* \)
Graph 4.2.

Regression of the incidence of *Cl.novyi* type B plus flukes in the liver of sheep from 5 farms on the incidence of *Cl.novyi* type B in soil and the incidence of flukes in sheep livers from the same farms.
Cl. novyi in soil
- Flukes in livers

\[ y = 0.89X - 3.19 \quad r = +0.979^{**} \]
\[ y = 0.29X - 1.86 \quad r = +0.931^{*} \]
Graph 4.5.

Regression of the incidence of *Cl.novyi* type B in sheep livers and the incidence of *Cl.novyi* type B in soil on the incidence of sheep livers containing flukes from the same 5 farms.
Cl. novyi in livers

Cl. novyi in soil

**Soil**

**Livers**

\[ y = 0.26x + 4.20 \]

\[ r = +0.937^* \]

\[ y = 0.33x + 1.37 \]

\[ r = +0.966^{**} \]

Incidence of Livers with Flukes (%)
Finally when the incidence of flukes was correlated with the incidence of _Cl.novyi_ in the livers of sheep and in the soil on the same farms, there was a significant correlation, \( r = +0.937 \)\(^*\) in the first case and a highly significant correlation, \( r = +0.966 \)\(^*\) in the second case (Graph 4.3). When these last two regressions were compared, it was found that they were statistically indistinguishable as regards both slope and position \( F = 0.04 \) and \( F = 0.05 \) respectively; degrees of freedom 1, 6).

**EXPERIMENTAL INFECTIOUS NECROTIC HEPATITIS**

The production of experimental infectious necrotic hepatitis has been attempted in guinea-pigs, rabbits and in sheep. In all these animals metacercariae of _Fasciola hepatica_ were given orally followed two weeks later by a known dose of spores of _Cl.novyi_ type B by the oral route for sheep and by the same route, together with the subcutaneous, intraperitoneal, intramuscular and intravenous routes for guinea-pigs and rabbits.
A. Guinea-pigs

A total of 52 guinea-pigs was used, 36 infected and 16 controls, in 5 experiments. The dose of metacercariae of *Fasciola hepatica* given to these animals varied from 15 to 25, the object being to induce various grades of liver damage which might stimulate the growth of *Gl. novyi* type B spores. The details of the infections in these animals are shown in Table 4.7.

Of 36 infected guinea-pigs, 25 died naturally with the disease. Most of these animals died suddenly during the night and were autopsied 12-15 hours after death. The animals which were given spores by the intraperitoneal route all died 20-28 days following the onset of the fluke infection. Those which received the spores orally died after a longer period. The administration of the spores by the intramuscular and the subcutaneous routes did not produce the disease and although the organisms were isolated from the site of injection, they were not found in the liver, spleen or bone marrow.
The post-mortem lesions and bacteriological findings in the infected guinea-pigs are shown in Table 4.8. None of the control guinea-pigs infected with only one of the pathogenic agents died naturally, although those which received metacercariae contained flukes in their livers and *Cl.novyi* type B was isolated from the livers and spleens of those guinea-pigs which had been dosed with spores alone by the oral and intraperitoneal routes.

B. **Rabbits**

A total of 22 rabbits was used in 5 different experiments; 12 rabbits were infected with spores of *Cl.novyi* and metacercariae of *F.hepatica* and 10 were kept as controls being infected with either flukes or spores.

The details of the infections are shown in Table 4.9 and the post-mortem lesions and bacteriological findings in Table 4.10. Table 4.9 shows that of 12 infected rabbits, 6 died of the disease and that these deaths occurred only after intravenous or oral administration of spores. However, the organisms were also isolated from the liver and spleen in rabbits, infected by the
<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Number of animals used</th>
<th>Dose of viable spores of Cl.novyi (million)</th>
<th>Route</th>
<th>Dose of metacercariae of F.hepatica</th>
<th>Number of animals infected with both Cl.novyi and F.hepatica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Control</td>
<td>Died</td>
<td>Killed</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>4</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* In each case half the control group was infected with spores only and the others with flukes only.
Table 4.8

Post-mortem lesions and bacteriological findings in guinea-pigs infected with both Cl. novyi type B and Fasciola hepatica

<table>
<thead>
<tr>
<th>Experiment of</th>
<th>Number of animals infected</th>
<th>Time from infection to death</th>
<th>Presence of Cl. novyi type B in liver</th>
<th>Presence (+) or absence (-) of Cl. novyi type B in</th>
<th>Site of injection of spores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>in liver areas</td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>nil (killed)</td>
<td>55</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
<td>24 ± 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>nil (killed)</td>
<td>50</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>7</td>
<td>34 ± 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>8</td>
<td>30 ± 2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NS = No site, orally administered
### Table 4.9

**Experimental infectious necrotic hepatitis in 22 rabbits**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Number of animals used</th>
<th>Dose of viable spores of Cl.novyi (million)</th>
<th>Route</th>
<th>Dose of metacercariae of F.hepatica</th>
<th>Number of animals infected with both Cl.novyi and F.hepatica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>l/p</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>s/c</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>l/v</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>oral</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>oral</td>
<td>50</td>
</tr>
</tbody>
</table>

*One animal infected with spores only and the other with flukes only in each control group*
### Table 4.10

**Post-mortem lesions and bacteriological findings in rabbits infected with both Cl.novyi type B and Fasciola hepatica**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Number of dying animals</th>
<th>Number of Fasciola infection to death (days)</th>
<th>Presence of flukes in liver</th>
<th>Presence of defined necrotic areas in liver</th>
<th>Presence (+) or absence (-) of Cl.novyi type B in</th>
<th>Liver</th>
<th>Spleen</th>
<th>Bone</th>
<th>Marrow</th>
<th>Abdominal fluid</th>
<th>Kidneys</th>
<th>Site of injection of spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>nil</td>
<td>49</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>nil</td>
<td>47</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>29-31</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>35-36</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2</td>
<td>32-37</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NS** = No site - oral or i.v. administration of spores
intraperitoneal route although there were no defined necrotic areas in the livers of these rabbits. The necrotic lesions in the rabbits that died were variable in size and shape. Some were small, circular and yellowish-brown in colour (Fig. 4.4) while others were linear (Fig. 4.5) and may be associated with old fluke tracts.

*Cl.novyi* type B could be isolated from these liver lesions as well as from other organs as shown in Table 4.10. A frozen section from a liver lesion stained with conjugated anti-*Cl.oedematiens* serum (Burroughs Wellcome) shows the organism fluorescing in the liver tissue (Fig. 4.6).

C. Sheep

A total of 12 Cheviot lambs and gimmers were used in 3 experiments for the production of the disease. The animals were examined for the presence of *F.hepatica* eggs in the faeces immediately after they were brought indoors. No such eggs were found in any of the animals. The doses of metacercariae of *F.hepatica* and spores of *Cl.novyi* type B given orally to these animals varied in each experiment, the details being shown in Table 4.11.
Liver from a rabbit which died following infection with 50 metacercariae of *F. hepatica* orally and 2 million viable spores of *Cl. novyi* type B intravenously.

**Fig. 4.4.**

Liver from a rabbit which died following oral infection with 50 metacercariae of *F. hepatica* and 5 million viable spores of *Cl. novyi* type B.

**Fig. 4.5.**
Fig. 4.6. Frozen section from the liver in Fig. 4.5, stained with fluorescent anti-Cl.oedematiens globulin (Burroughs Wellcome). X 1,500.
<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Sheep number</th>
<th>Age (months)</th>
<th>Sex</th>
<th>Dose of viable metacercariae of F. hepatica (million)</th>
<th>Dose of viable spores of Cl. novyi (million)</th>
<th>Time from F. hepatica infection to death (weeks)</th>
<th>Flukes recovered from the liver</th>
<th>Number</th>
<th>Percentage of initial dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>4</td>
<td>Male</td>
<td>2,000</td>
<td>100</td>
<td>10 (killed)</td>
<td>644</td>
<td>32.2</td>
<td>32.2</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>4</td>
<td>Female</td>
<td>2,000</td>
<td>100</td>
<td>8 (died)</td>
<td>617</td>
<td>30.8</td>
<td>30.8</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>4</td>
<td>Female</td>
<td>2,000</td>
<td>nil</td>
<td>10 (killed)</td>
<td>590</td>
<td>29.5</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4</td>
<td>Male</td>
<td>nil</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>16</td>
<td>Female</td>
<td>3,000</td>
<td>100</td>
<td>9 (killed)</td>
<td>760</td>
<td>25.3</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>16</td>
<td>Female</td>
<td>3,000</td>
<td>100</td>
<td>6 (died)</td>
<td>1,060</td>
<td>35.3</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>16</td>
<td>Female</td>
<td>3,000</td>
<td>nil</td>
<td>7½ (died)</td>
<td>1,120</td>
<td>37.3</td>
<td>37.3</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>16</td>
<td>Male</td>
<td>nil</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>8</td>
<td>Female</td>
<td>4,000</td>
<td>100</td>
<td>6 (died)</td>
<td>1,220</td>
<td>30.5</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>8</td>
<td>Female</td>
<td>4,000</td>
<td>100</td>
<td>6½ (died)</td>
<td>1,452</td>
<td>35.8</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>8</td>
<td>Male</td>
<td>4,000</td>
<td>nil</td>
<td>9 (killed)</td>
<td>1,164</td>
<td>29.1</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>8</td>
<td>Male</td>
<td>nil</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Experiment no. 1

The sera from the three animals infected with *F. hepatica* showed an increased glutamic dehydrogenase (GD) activity, which reached a peak about 8 weeks post-infection and then started to drop gradually. However, in sheep number 24 the GD activity remained low throughout the experiment (Appendix Table 1 and Graph 4.4).

The absolute eosinophil counts rose in the sheep infected with *F. hepatica* reaching a peak between 4 and 6 weeks post-infection and dropping gradually thereafter. Sheep 24 showed a low eosinophil count throughout the experiment (Appendix Table I and Graph 4.5).

All the animals except sheep 22 remained alive until they were killed 9-10 weeks after the start of the experiment. However, sheep 22 died suddenly during the night without previous symptoms, 58 days after infection with *F. hepatica* and 43 days after infection with spores of *Cl. novyi*. Prior to death the animal was in good condition and eating well. Autopsy was carried out within 10 hours of death.
Graph 4.4.

Serum glutamic dehydrogenase (GD) activities in 4 sheep used in Experiment no. 1.

Graph 4.5.

Absolute eosinophil counts in 4 sheep used in Experiment no. 1.
Serum Glutamic Dehydrogenase

Spore infection (Nos. 21 22 24)

Details:
- Fluke infection (Nos. 21 22 23)
- Weeks Post Infection

Eosinophil Count

Details:
- Fluke Infection (21 22 23)
- Weeks Post Infection
Experiment No. 2

The experimental design was similar to that in experiment No. 1 except that the dose of metacercariae given to each animal was increased to 3,000.

The serum GD activities of these sheep are shown in (Appendix Table I and Graph 4.6). Sheep 31, 32 and 33 showed an increased G.D. activity following infection with metacercariae. Sheep 32 and 33 showed a sharp rise in G.D. activity post-infection with a peak at the 3rd and 4th week respectively and then gradually dropped. The G.D. activity in sheep 31 did not show a similar peak, but rose steadily throughout the experiment while that in sheep 34 remained very low throughout.

The absolute eosinophil counts for these sheep are shown in Appendix Table I and Graph 4.7. There was a sharp rise in the eosinophil counts per c.mm of blood in sheep 31, 32 and 33 by 3-4 weeks post-infection with metacercariae, but the counts from sheep 34 remained relatively low throughout, apart from a transient peak 3 weeks after the beginning of the experiment.
Graph 4.6.

Serum glutamic dehydrogenase activities in 4 sheep used in Experiment no. 2.

Graph 4.7.

Absolute eosinophil counts in 4 sheep used in Experiment no. 2.
Serum Glutamic Dehydrogenase

Fluke Infection (31 32 33) Weeks Post Infection

Eosinophil Count

Fluke Infection (31 32 33) Weeks Post Infection
All the animals remained alive and in good condition until the sixth week after infection with metacercariae. Sheep 32 then died suddenly without previous symptoms 43 days after infection with flukes, 28 days after infection with spores of Cl.novyi. Sheep 33 (fluke control) showed anorexia and clinical symptoms of weakness for 3 days beginning 49 days after infection. It tended to lie on its brisket with laboured respiration and died 52 days after infection. Autopsies on sheep 32 and 33 were carried out within 8-10 hours of death.

**Experiment no. 3**

In this experiment the dose of metacercariae of *F.hepatica* given to each animal was increased to 4,000.

Analysis of the G.D. activity in the sera of these sheep (Appendix Table I and Graph 4.8) showed that in the sheep infected with *Fasciola hepatica* the G.D. activity rose sharply from the first week post-infection with metacercariae to the fifth week and then dropped. The G.D. activity in sheep 44 remained very low.
Graph 4.8.

Serum glutamic dehydrogenase activities in 4 sheep used in Experiment no. 3.

Graph 4.9.

Absolute eosinophil counts in 4 sheep used in Experiment no. 3.
Serum Glutamic Dehydrogenase

Weeks Post Infection

Fluke infection 41, 42, 43.
Spore infection 41, 42, 44.

Eosinophil Count

Weeks post infection
Fluke infection 41, 42, 43.
The three sheep infected with *F. hepatica* also showed an increased absolute eosinophil counts with a peak at the fourth week post-infection with metacercariae the counts in sheep 44 remained low throughout (Appendix Table I and Graph 4.9).

Sheep 41 died suddenly overnight without previous symptoms 44 days after infection with metacercariae, 29 days after infection with spores. Autopsy was carried out within 10 hours of death.

Sheep 42 also died suddenly 47 days after infection with metacercariae, 32 days after infection with spores. Autopsy for this sheep was carried out within 6 hours of death.

**Post-mortem and bacteriological findings from the 12 sheep used in 3 experiments**

I. Sheep 22, 32, 41 and 42

These were the animals infected with metacercariae of *F. hepatica* and spores of *Cl.novyi* type B, which died naturally of the disease.

All the animals showed general emphysema and froth from the mouth and nose. The subcutaneous tissue
was dark reddish-purple in colour. On opening the abdominal cavity the stomach and intestines of sheep 22 and 32 were empty and full of gas. The abdominal cavity contained straw-coloured, blood-stained fluid (Fig. 4.7), the volume of which was about 500ml in sheep 41 and 42 and 1 litre in sheep 22 and 32.

The spleen in all the sheep appeared grossly normal, the kidneys showed a mottled appearance, the hepatic and mesenteric lymph glands were enlarged and oedematous. In the thorax the pericardial sac was distended with clear, straw-coloured fluid.

The livers in all these sheep were enlarged and friable with a fibrinous covering and dark reddish-purple to reddish-brown in colour, although the livers from sheep 22 and 42 were the darkest. On external examination of these livers, numerous dark fluke tracts and small brownish necrotic areas were noticed in the livers of sheep 32 and 42 but were less obvious in the livers of sheep 22 and 41; in the latter these lesions were best seen when the organs were dissected open. The livers of sheep 22, 32, 41 and 42 are shown in figures 4.8, 4.9, 4.10 and 4.11 respectively.
Fig. 4.7.

Post-mortem lesions of sheep 42 showing the blood stained fluid in the abdominal cavity.
Fig. 4.8.

Liver of sheep 22 that died following oral infection with 2,000 metacercariae of *F. hepatica* and 100 million viable spores of *C. novyi* type B.

Fig. 4.9.

Liver of sheep 32 that died following oral infection with 3,000 metacercariae of *F. hepatica* and 100 million viable spores of *C. novyi* type B.
Liver from sheep 41 that died following oral infection with 4,000 metacercariae of *F. hepatica* and 100 million spores of *Cl. novyi* type B.

Liver from sheep 42 that died following oral infection with 4,000 metacercariae of *F. hepatica* and 100 million spores of *Cl. novyi* type B.
Smears made from the areas of the lesions in all the livers and stained with Gram's stain showed large Gram-positive bacilli. Some smears were also stained with the fluorescent stain and showed large and small, brightly fluorescing bacilli (Fig. 4.12). Frozen sections of the liver lesions from all sheep stained with the fluorescent stain also showed brightly fluorescing bacilli in the liver tissue (Fig. 4.13). Sections of the livers in the area of the lesions, stained with Gram's stain, showed Gram-positive bacilli on the edge of the necrotic area with cellular infiltration further out (Fig. 4.14).

*C. novyi* type B was isolated in pure culture from the lesions in all the livers as well as from the following organs of these sheep:— Spleen, mesenteric lymph glands (sheep 22, 41 and 42), abdominal fluid, bone marrow (sheep 22 and 42), and kidneys (sheep 42 only). No *C. novyi* type B organisms were isolated from the lungs, heart blood or pericardial fluid of any of the animals.

Injection of 0.2ml of the supernate of the abdominal fluid, after centrifugation, intravenously into mice was lethal in about 24 hours; the
Fig. 4.12.

Smear from a lesion in the liver of sheep 42 stained with fluorescent anti-Clostridium edema globulin (Burroughs Wellcome). X 1,950.
Fig. 4.13.

Frozen section from liver of sheep 32 stained with fluorescent anti-
Cl.oedematiens globulin (Burroughs Wellcome).  X 1,500.

Fig. 4.14.

Section of a lesion from the liver of sheep 42 stained with Gram's
stain.  X 750.
lethality was neutralized by the injection of both the supernate and a few drops of *Cl. oedematiens* type B antiserum (Burroughs Wellcome).

The total number of flukes recovered from the bile ducts and parenchyma of the livers from these sheep is shown in Table 4.11 (above).

II Sheep 21 and 31

These were the animals which were infected with metacercariae of *F. hepatica* and spores of *Cl. novyi* type B which remained alive until they were killed 10 weeks and 9 weeks post-infection with *Fasciola hepatica* respectively.

Both animals were in good condition at autopsy. The abdominal cavity contained straw-coloured fluid, the volume of which was about 250ml. The spleen in both animals was grossly normal, the mesenteric and hepatic lymph glands being enlarged. The kidneys were grossly normal in sheep 21 but showed a mottled appearance in sheep 31. In the thorax, the pericardial sac was not distended with fluid in either animal.
The livers from these sheep were enlarged and friable, dark purple (sheep 21) or red (sheep 31) in colour. Numerous small fluke tracts were seen in the liver from sheep 31 (Figures 4.15 and 4.16).

*Cl.novyi* type B was isolated in pure culture from the livers of these 2 sheep and also from the spleen, kidneys, and mesenteric lymph glands (sheep 31 only). No *Cl.novyi* organisms were isolated from the lungs, heart blood, bone marrow, abdominal fluid or pericardial fluid of either animal. Injection of abdominal fluid into mice was not lethal.

The numbers of flukes recovered from the bile ducts and parenchyma of the livers from both sheep are shown in Table 4.11, (above).

**III  Sheep 33**

This animal was used as a fluke control in experiment no. 2 and was given 3,000 metacercariae of *F.hepatica*. The animal died after clinical symptoms of weakness for 3 days beginning 49 days after infection with metacercariae.
Liver of sheep 21 which was killed 10 weeks after infection with 2,000 metacercariae of Fasciola hepatica, 8 weeks after infection with 100 million viable spores of Cl. novyi type B.

Liver of sheep 31 which was killed 9 weeks after infection with 3,000 metacercariae of Fasciola hepatica, 7 weeks after infection with 100 million viable spores of Cl. novyi type B.
The condition of the animal was good and there were no discharges from the mouth or nose. The colour of the subcutaneous tissue was dark-red. The abdominal cavity contained about 500ml of clear, straw-coloured fluid. The spleen was grossly normal, the kidneys showed a mottled appearance and the mesenteric lymph glands were enlarged.

The liver was enlarged and friable with a fibrinous covering, yellowish-red in colour; the gall-bladder was distended with bile. There were numerous fluke tracts on the capsule of the liver (Fig. 4.17). No Cl.novyi type B organisms were isolated from the liver or other organs. Sections of the liver stained with haematoxylin and eosin showed heavy cellular infiltration around the fluke tracts (Fig. 4.18). The total number of flukes recovered from the liver is shown in Table 4.11 (above).

IV Sheep 23 and 43

These sheep were used as fluke controls in experiments 1 and 3 and were infected orally with 2,000 and 4,000 metacercariae of Fasciola hepatica respectively. These 2 animals were killed 10 weeks and 9 weeks respectively after infection with metacercariae of F.hepatica.
Liver of sheep 33 (fluke control) which died 7½ weeks following infection with 3,000 metacercariae of *F. hepatica*.

**Fig. 4.17.**

Section of the liver in Fig. 4.17 stained with haematoxylin and eosin, showing heavy cellular infiltration around the fluke tracts. X 80.
The animals were in good condition. In each animal the abdomen contained about 200ml straw-coloured fluid. The liver was slightly enlarged, friable and dark red in sheep 23 but hard, fibrous and reddish-yellow in sheep 43 (Fig. 4.19). Numerous fluke tracts were seen on the capsule of the liver in each case, and the bile ducts were hard and fibrous. A very small amount of fluid was found in the pericardial sac.

Bacteriological examination of all the organs including the liver did not reveal the presence of *Cl. novyi* type B. The number of flukes recovered from each of these livers is shown in Table 4.11 (above).

V Sheep, 24, 34, 44

These sheep were used as spore controls in experiments 1, 2 and 3 and were all infected orally with spores of *Cl. novyi* type B. They were all killed at the end of the experiments and examined.

At autopsy all three animals were in good condition. All the organs including the liver (Fig. 4.20) were grossly normal. No fluid was noticed in either the abdominal cavity or pericardial sac. *Cl. novyi* type B was isolated from the livers of
Liver of sheep 43 (fluke control) killed 9 weeks after infection with 4,000 metacercariae of *F. hepatica*.

Liver of sheep 24 (spore control) killed 8 weeks after infection with 100 million viable spores of *Cl. novyi* type B.
the 3 animals as well as from the spleens of sheep 24 and 44; no \textit{Cl. novyi} type B organisms were detected from other organs. No flukes were found in the livers of these sheep.

THE EFFECT OF PUTREFACATION ON THE ISOLATION OF \textit{CL. NOVYI}

I Guine-a-pig carcasses stored at 23°C

Two experiments were carried out for this investigation using a total of 65 guinea-pigs. In the first experiment 30 guinea-pigs were used; 6 groups of 4 guinea-pigs being infected orally with \textit{Cl. novyi} type B spores and the remaining 6 guinea-pigs kept as uninfected controls. They were all killed 24 hours later and the carcasses were incubated at 23°C for 6, 12, 48, 60 or 72 hours.

In the second experiment 35 guinea-pigs were used, 7 groups of 4 guinea-pigs being infected and the remaining 7 guinea-pigs being kept as uninfected controls. The periods of incubation of the carcasses were similar to those in the first experiment except that some carcasses were also incubated for 96 hours.
The number of fluorescing organisms in ten X50 fields from smears made from each organ were recorded and the means were calculated for each animal and each group of four infected guinea-pigs at each period of incubation in both experiments. Table 4.12 gives the details of the counts of fluorescing organs from all the infected guinea-pigs used in the 2 experiments.

The livers and spleens of the control guinea-pigs in the 2 experiments did not show any fluorescing organisms at any time.

Table 4.12 shows that fluorescing Cl. novyi organisms could only be found after incubation of the infected carcasses for at least 24 hours. The relationship between the mean number of fluorescing organisms counted and the time of incubation of the carcasses is shown in graph 4.10. It can be seen that the number of organisms increased in both the liver and spleen up to 48 hours after incubation, thereafter decreasing in the spleen but not reaching a peak in the liver until after 72 hours of incubation.
Table 4.12
Mean number of fluorescing *Cl. novyi* organisms in the livers and spleens of 52 carcasses of infected guinea-pigs incubated at 23°C

<table>
<thead>
<tr>
<th>Time of incubation of the carcass (hours)</th>
<th>Mean number of fluorescing organisms per X50 (fluorite oil-immersion) field with standard deviations for the means of the four carcasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>6</td>
<td>nil</td>
</tr>
<tr>
<td>12</td>
<td>nil</td>
</tr>
<tr>
<td>24</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>48</td>
<td>13.4 ± 1.0</td>
</tr>
<tr>
<td>60</td>
<td>22.3 ± 2.0</td>
</tr>
<tr>
<td>72</td>
<td>26.8 ± 4.0</td>
</tr>
<tr>
<td>96</td>
<td>15.9 ± 1.1</td>
</tr>
</tbody>
</table>
Graph 4.10.

The relationship between the mean number of fluorescing organisms in the liver and spleen and the time of incubation of the infected guinea-pig carcasses.
Temperature 23 °C

Mean number of Fluorescing Organisms

Incubation of Carcase (hours)
II The detection of alpha toxin of \textit{Cl. novyi} type B in incubated, infected livers

This experiment was carried out on the assumption that \textit{Cl. novyi} spores present in the liver in putrefied carcasses proliferate and consequently produce the alpha toxin; the presence of this toxin was used to detect the extent of multiplication of \textit{Cl. novyi} spores in infected guinea-pig and sheep livers, normal post-mortem putrefaction being simulated by incubation.

Guinea-pig livers

The livers of 21 guinea-pigs were used; 14 infected livers and 7 normal livers. These were incubated anaerobically in parallel at 23°C and 37°C for 6, 12, 18, 24, 60 and 72 hours. After each period of incubation, 4 mice were injected intravenously with 0.2ml of a liver tissue macerate (10g of liver in 10ml of sterile normal saline). The percentage of mice which died are shown in Table 4.13. Control mice which were injected with a mixture of the macerate and \textit{Cl. oedematiens} type B anti-serum (Burroughs Wellcome) did not die (see Methods).
Sheep livers

Infected livers from sheep used for the study of the behaviour of \textit{Cl.novyi} type B spores (see above) were used to study the effect of putrefaction. In addition normal, uninfected sheep livers were included as controls. The livers were incubated anaerobically at 23°C and 37°C for 6, 12, 18, 24, 60 and 72 hours. After each period of incubation, 4 mice were infected with a liver tissue macerate in sterile normal saline. The percentage of mice which died are shown in Table 4.14. All control mice did not die.

A comparison of the periods of incubation of infected guinea-pigs and sheep livers at 23°C and 37°C and the percentage of mice which died is shown in graph 4.11. This shows that after 60 hours of incubation of infected livers at 23°C or 37°C, the spores of \textit{Cl.novyi} type B present in the liver produce sufficient alpha toxin to kill all the mice but that the amount may drop thereafter. However, incubation of the infected livers at 37°C appeared to stimulate the organism to proliferate more quickly than incubation at 23°C.
Table 4.13

Alpha-toxin produced in incubated guinea-pig livers infected with Cl.novyi type B

<table>
<thead>
<tr>
<th>Time of incubation (hours)</th>
<th>Percentage of mice dying</th>
<th>Incubated at 37°C</th>
<th>Incubated at 23°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>75</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>75</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>
### Table 4.14

**Alpha-toxin produced in incubated sheep livers infected with Cl. novyi type B**

<table>
<thead>
<tr>
<th>Time of incubation (hours)</th>
<th>Percentage of mice dead</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubated at 37°C</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>75</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>72</td>
<td>75</td>
</tr>
</tbody>
</table>
Graph 4.11.

The relationship between the periods of incubation of infected guinea-pig and sheep livers at 23°C and 37°C and the percentage of mice which died following injection of a liver tissue macerate.
CHAPTER V

DISCUSSION

(I) The relevance of the present findings to the diagnosis of infectious necrotic hepatitis

The correct diagnosis of infectious necrotic hepatitis requires the use of reliable laboratory techniques to confirm a tentative clinical diagnosis. This laboratory confirmation of field diagnosis of infectious necrotic hepatitis has been hampered by the difficulties experienced when attempting to isolate *Cl. novyi* type B, as it is a fastidious demanding anaerobe. The need for practical and dependable techniques for the isolation of this organism has never been completely satisfied.

(a) The cultural approach. The isolation of *Cl. novyi* type B organisms in pure culture from the livers of sheep suspected to have died from infectious necrotic hepatitis requires the production of an abundant surface growth of the organism on solid media, from which single colonies can be easily picked out and identified. The task of growing *Cl. novyi* type B on solid media directly from infected tissues is usually
complicated by the fact that most of the livers to be examined are contaminated with other anaerobes, which overgrow the *Cl. novyi* type B. Hence there is a need for techniques to reduce such contamination from the infected materials. Heating infected, contaminated material at 100°C for 3-10 minutes, either directly or after inoculation into cooked-meat broth, reduces the number of contamination; *Cl. novyi* type B is unaffected by this temperature (Turner, 1930b; Williams, 1962; Roberts *et al.*, 1970b). The incorporation of inhibitory materials in the solid media further helps to reduce this contamination and the use of selective sheep blood agar as described by Rutter (1968) gives satisfactory results, the number of contaminants being reduced to a minimum.

Early workers on infectious necrotic hepatitis found great difficulty in obtaining a heavy surface growth of *Cl. novyi* type B on solid media (Edgar, 1928; Turner, 1930b; Jamieson, 1949; Williams, 1962). The failure of this anaerobe to grow readily on solid media may be due to its fastidious nutritional requirements and to its sensitivity to oxygen. Thus it has been found that a careful, invariable anaerobic
jar procedure and the use of suitable, freshly prepared, solid media are necessary for the growth of this organism. The anaerobic jar procedure used in this work gave good results and supported continuous growth of *Cl. novyi* type B. Sheep blood agar gives good growth of this organism on surface culture although human blood agar, if available, affords even better growth. The use of iron-filings on the solid media helps to give heavy and rapid surface growth of *Cl. novyi* type B. This effect also occurs with *Cl. novyi* types C and D, and its use as an aid to encourage rapid growth of *Cl. novyi* merits further investigation. The medium in which dithiothreitol is incorporated (Moore, 1968) gave reliable, heavy growth of *Cl. novyi* type B and modifications of this technique are suitable for the regular isolation of *Cl. novyi* types B and D (Collee and Watt, personal communication).

Typing the strains of *Cl. novyi* isolated from the livers of sheep dying from apparent infectious necrotic hepatitis is an important final step in the identification. Cooked-meat broth is a reliable routine fluid medium for the isolation of *Cl. novyi* type B, and the growth of the organisms in this medium is sufficient to allow the identification of the recognized soluble antigens for typing. In these
studies, neutralization tests for haemolysin (HL) and lecithovitellin (LV) activities gave useful results when used in parallel for routine typing, although some type B strains of Cl. novyi gave a negative LV reaction; this was also encountered by Keppie (1944), Jamieson (1949), and Rutter (1968). Rutter and Collee (1969) suggested that this occurs when the unconcentrated cooked meat broth cultures of the type B strains of Cl. novyi contain insufficient amounts of the beta-antigen, a haemolytic lecithinase, to give a positive LV reaction. The neutralization of the lethal toxin of type B strains of Cl. novyi in the supernatant fluid from growth in cooked-meat broth or directly in the peritoneal exudates from an animal dying of infectious necrotic hepatitis is another useful procedure for type identification.

(b) Immunofluorescence. Despite the controversy on the reliability of the fluorescent antibody technique as a sole criterion for the identification of Cl. novyi, the advantages of ease of application and the rapidity of the test have resulted in wide acceptance of the technique by many diagnostic laboratories. Although this technique does not differentiate between the types
of Cl. novyi, it presents a rapid tool for the identification of this fastidious anaerobe and is preferable to the time-consuming cultural techniques for preliminary identification of these organisms. The technique is particularly important in surveys, when large numbers of samples are to be examined for the presence of Cl. novyi. It has been used satisfactorily in this study for a survey of the incidence of Cl. novyi in the livers of sheep. Other workers have also used the technique for surveys of the incidence of this organism in ovine and bovine livers (Corbould, 1966; Batty et al., 1967; Thomson et al., 1968; Niilo et al., 1969; Roberts et al., 1970b). This technique was also utilized in the present work to survey the incidence of Cl. novyi in pasture soils, and its use in combination with the cultural techniques described by Nishida and Nakagawara (1964) for such surveys reduced the time needed for the preliminary identification of these organisms in soil samples.

There are however certain factors which should be considered when using the fluorescent antibody technique for the routine identification of these organisms. These are:

(i) Proper selection and preparation of the stained material.
It was clearly seen during these studies that the degree of fluorescence varies according to the source of material from which the smears were made. Organisms from smears made from animal tissues showed brighter fluorescence; this was previously observed by Batty and Walker (1964). Batty (quoted by Rutter, 1968) pointed out that the antiserum in the commercial fluorescent stain is usually prepared using a very "short-growth culture" in order to get a monospecific serum and stated that the degree of fluorescence seems to depend largely on the material that is used to prepare the smears.

The staining procedure used is also critical in getting a clear image. This requires proper fixation of the slides, the use of reliable, absorbed stains and the removal by washing of all untreated stain so as to cut down autofluorescence.

(ii) The use of reliable and simple optical equipment

An adequate intensity of the exciting beam, a broad field of view, good contrast and in addition simplicity of manipulation are desirable for the fluorescent microscope. The Vickers fluorescent
microscope used in this work proved satisfactory and in particular it is simple to set up the filter combination for blue or ultra-violet light.

The intensity and contrast of an absorbed image is always affected by the magnification used and it was found that the use of a X50 (Fluorite oil-immersion) lens - rather than a X100 lens - afforded the clearest image of the fluorescing organisms.

(iii) Interpretation of the results.

The significance of the detection of *Clostridium novyi* by the fluorescent antibody technique in smears from the livers of sheep suspected to have died from infectious necrotic hepatitis has been the subject of argument by many workers. A number of workers have interpreted the findings of *C. novyi* in fluorescent stained smears from post-mortem material as indicating the cause of death in sheep (Batty *et al.*, 1964; Hart, 1965) and pigs (Bourne and Kerry, 1965; Corbould and Munday, 1966). However others established that in most cases the incidence of fluorescent - positive smears from post-mortem material from cattle bore no relation to the cause of death (Thomson *et al.*, 1968; Niilo *et al.*, 1969).
The high incidence of *Cl. novyi* in post-mortem specimens prompted some workers to examine ovine livers from slaughtered animals using the fluorescent antibody technique, so as to demonstrate the incidence of these organisms (Corbould, 1966; Batty et al., 1967; Roberts et al., 1970b and c). Batty et al., (1967) concluded that a diagnosis of infectious necrotic hepatitis based on the use of the fluorescent antibody technique with smears from post-mortem material must be supported by other factors such as clinical history, post-mortem findings and the number of fluorescing organisms per microscopical field, being assessed in relationship to the state of decomposition of the carcass when the impression smear was taken.

(c) **History and post-mortem lesions**

When a carcass is examined soon after death, a diagnosis of infectious necrotic hepatitis based on history and post-mortem findings presents very little difficulty but when the carcass is decomposed, the characteristic lesions may no longer be apparent.

A history of sudden death has been considered by some workers as contributory evidence of the disease but in such circumstances other causes of sudden death
such as acute fascioliasis, enterotoxaemia, braxy, anthrax, hypomagnesaemia, plant or chemical poisoning should be considered and eliminated. All the liver samples received from the various Veterinary Investigation Departments during these studies for confirmation of infectious necrotic hepatitis, were from animals which had died suddenly. In addition death occurred suddenly in all the sheep which died during the experimental infections.

The post-mortem lesions and particularly the characteristic necrotic area in the liver have been considered to be pathognomonic by many workers (Albiston, 1927; Turner, 1930b; Jamieson et al., 1948; Williams, 1962). Others however did not notice any specific lesions in the liver in many of the livers they examined (Batty et al., 1964). When the liver lesion is small, when there is considerable liver damage by flukes or when the carcass is decomposed, the lesions may be difficult to detect. The size of the necrotic lesions and the intensity of the leucocyte response probably depends on the number of spores activated and on the potency of the toxin. In the sheep which were used for the induction of experimental infectious necrotic hepatitis, the liver lesions were
small, poorly demarcated and in some cases they were not visible on the surface of the liver (Figures 4.8, 4.9, 4.10 and 4.11). This appears to be a difference between the liver lesions in natural and experimental infectious necrotic hepatitis. Jamieson (1949) noticed the same difference in liver lesions in his experimental guinea-pigs which died from the disease. This difference may result from the activation in the experimental infections of a larger number of spores than are present in natural infections, with subsequent death of the animals before the defence mechanism has reached the stage usually seen in the natural disease in sheep. This may also be the explanation for some of the cases of sudden deaths in sheep encountered by Batty et al., (1964), in which they could not detect any specific lesions. It would therefore appear to be necessary to dissect the liver systematically while searching for these necrotic lesions.

The presence of a secondary pathogen is also necessary and this usually involves the presence of flukes and the parenchymal damage caused by these flukes. While the majority of workers have reported the presence of liver flukes in the livers from cases of infectious necrotic hepatitis, some workers (Batty et al., 1964) did not notice evidence of flukes in some
of the livers they examined. However in the absence of flukes other causes of hepatic injury should be considered as infectious necrotic hepatitis can be caused by other parasites and other agents capable of producing liver necrosis.

The amounts of fluid in the abdominal cavity and the pericardial sac and the colour of the subcutaneous tissues provide valuable contributory evidence for diagnosis of this disease, but the reliability on these factors depends on the state of decomposition of the carcass.

(d) The effect of putrefaction on the diagnosis of infectious necrotic hepatitis

The state of decomposition of the carcass has long been considered an important factor in the diagnosis of infectious necrotic hepatitis. The post-mortem lesions such as the necrotic liver lesions, the presence of flukes and the damage caused by these flukes may be more difficult to detect in a decomposed liver and in addition the liver appears to be a very favourable medium for the multiplication of Cl. novyi; the number of organisms increasing after death (Batty et al., 1967; Niilo et al., 1969).
The fact that \textit{Cl.novyi} organisms increase after death was investigated in the present work (Table 4.12 and Graph 4.10) in an attempt to correlate the increase in the number of organisms in the livers and spleens with the state of decomposition of infected guinea-pig carcasses kept at 23°C. Only small numbers of fluorescing organisms were found in impression smears taken from the liver and spleens of these carcasses after 24 hours incubation, but the number was about 8 times greater after 48 hours in both organs and increased to about 16 times greater in the liver after 72 hours.

In another experiment in which putrefaction was simulated by incubation of the liver at 23°C (Tables 4.13 and 4.14 and Graph 4.11) the findings suggest that the \textit{Cl.novyi} spores present in infected livers proliferate rapidly after death and produce the alpha toxin which is lethal to mice in about 24 hours, the amount of toxin present reaching a peak in about 60 hours.

There has been argument as to whether the appearance of \textit{Cl.novyi} in the liver represents post-mortem invasion by organisms originally present in the intestine or germination of dormant spores already in the liver.
The present findings agree with the latter most commonly accepted hypothesis, in that all carcasses or livers from infected animals showed evidence of proliferation of \textit{Cl. novyi}, whereas all the uninfected controls remained negative even after prolonged incubation.

There is no strict time limit for the validity of the post-mortem demonstration of the presence of \textit{Cl. novyi} in liver smears in the diagnosis of infectious necrotic hepatitis but the above findings tend to agree with the conclusion reached by Batty \textit{et al.}, (1967) that the examination of post-mortem material more than 48 hours after death is unreliable for confirmation of this disease.

(II) The epidemiology of infectious necrotic hepatitis

The spores of \textit{Cl. novyi} type B occur naturally in soil and sheep grazing on infected pastures are continually ingesting these spores. A number of workers have shown that healthy sheep carry these spores in their livers (Edgar, 1928; Jamieson, 1949; Williams, 1962) and that sheep from areas in which infectious necrotic hepatitis is prevalent carry more \textit{Cl. novyi} spores in their livers than those from other areas (Jamieson, 1949). However, Katitch \textit{et al.}, (1969a), who recently examined 500 liver samples from sheep in infectious necrotic hepatitis areas, could not detect \textit{Cl. novyi} spores in any of the livers. The
findings of Katitch et al. may be due to the size of the portions of the liver examined, as Edgar (1928) early pointed out that at least 50 grams of liver should be examined to obtain reliable findings from a sheep liver being examined for the presence of Cl. novyi spores.

The surveys undertaken during these studies confirmed the presence of Cl. novyi type B in soil from the pastures and in the livers of sheep and were in keeping with the findings of Jamieson (1949) in that there was a difference in the incidence of these organisms on farms with a history of infectious necrotic hepatitis compared with those with no such history (Tables 4.5 and 4.6). These surveys also revealed that the incidence of Cl. novyi type B in soils and sheep livers and the incidence of Fasciola hepatica in sheep livers were positively correlated both with each other and with the incidence of infectious necrotic hepatitis on the surveyed farms (Graphs 4.1, 4.2 and 4.3). From this it would seem possible that Fasciola hepatica, or more probably the intermediate host snail, Lymancea truncatula, and Cl. novyi spores may have a similar optimal soil environment.
In these surveys, *Cl.novyi* was first identified in infected livers or soil samples by the fluorescent antibody technique, the organisms were subjected to differential heating either directly or in fluid media and the identification later confirmed by typing. Most of the type B strains isolated produced relatively little toxin, i.e., were of low pathogenicity to mice. This phenomenon was previously noted by Corbould (1966) in his survey by the fluorescent antibody technique of the incidence of *Cl.novyi* in the livers of sheep and more recently by Roberts et al. (1970b), who found that of 4 *Cl.oedematiens* type B strains, isolated from the livers of normal sheep, 3 were fully toxogenic and indistinguishable from strains found in cases of infectious necrotic hepatitis, but one strain was only weakly toxogenic. Roberts et al. also found that only 9 per cent of the organisms found in the livers of normal sheep, which stained with fluorescent anti-*Clostridium oedematiens* anti-serum were *Cl.novyi* type B, a proportion which is rather smaller than that found in the survey in this study. This difference probably arises because two of the farms from which the samples came in the survey were known to have a history of infectious necrotic hepatitis. Roberts et al. pointed out that the low incidence of pathogenic strains of
Cl. novyi in livers from normal sheep suggests that infectious necrotic hepatitis may be only an occasional terminal syndrome of fascioliasis and that the Cl. novyi organisms found proliferating around infarcts may not necessarily be toxogenic type B.

An explanation of the low pathogenicity of type B strains found in the survey in this study is that the liver or soil samples were subjected to selective heating procedure before typing; so that the bacterial population would be derived from spores and Nishida and Nakagawara, (1965) have shown that such populations are frequently of low toxogenicity.

(III) The pathogenicity of infectious necrotic hepatitis

It was once thought that Cl. novyi spores reach the livers of sheep by being passively carried there by young flukes (Dodd, 1921). However later reports of the presence of Cl. novyi spores in the livers of healthy sheep (Edgar, 1928; Turner, 1930a and b; Jamieson, 1949) showed that the flukes have no essential role in carrying the spores to the liver. The present findings in guinea-pigs and sheep (Tables 4.3 and 4.4) confirm that spores of Cl. novyi reach the liver without the aid of flukes within 24 hours after being given orally to animals. Furthermore
the spores which led to the proliferation of Cl.novyi in the livers in the experimental cases could not have been carried there by the flukes, as they were administered two weeks after the metacercariae, and in no case has Cl.novyi been found in any of the experimental controls, which had not received a dose of spores.

The findings also throw some light on the pathway by which these spores reach the liver after ingestion. Thus the presence of the spores in the mesenteric lymph glands at an earlier time after ingestion than they are to be found in the liver and spleen, at first sight, supports Edgar's (1928) hypothesis that the spores may be carried by the lymphatics of the intestines (lacteals) and then by the general circulation to the liver. However, in this case one would expect to find spores in other organs such as the kidneys and also in the heart blood, yet none of these contained Cl.novyi in either the guinea-pigs or sheep. The only case in which organisms were found in the kidneys and heart blood was in a rabbit to which the spores had been administered intravenously. This suggests that the systemic circulation is not normally involved in the
dissemination of the spores. As the spores are to be found in the intestinal lymph glands, it seems probable that they may also pass from the intestine to the hepatic lymph glands, as these receive efferent vessels from the duodenal lymph glands (Sisson, 1965). They may then pass out directly into the liver from the gland, although this would involve movement against the lymph flow. On one occasion the spores were found in the peritoneal washings in a guinea-pig (Table 4.3) and all guinea-pigs (but not rabbits), which had been given the spores intraperitoneally for the production of the disease, died of infectious necrotic hepatitis (Table 4.7). This suggests that an alternative route for the spores may be directly across the peritoneal cavity to the liver. However the way in which spores can migrate in sites where they are not carried by a directional fluid flow is, as yet, unknown.

It was thought that the natural disease may involve unknown factors which initiate the growth of Cl.novyi spores already present in the liver, especially as it has been shown that a large proportion of animals carry both pathogens, - i.e. Cl.novyi type B and Fasciola hepatica - in their livers without developing infectious necrotic hepatitis. This possibility was studied by experimentally producing the disease in guinea-pigs, rabbits and sheep.
In guinea-pigs and rabbits, death from the disease occurred 4-6 weeks after infection with *Fasciola hepatica* (Tables 4.8 and 4.10). This is about the time of the critical period of severe liver damage caused by *F. hepatica* infection in mice, rats and rabbits (Urquhart, 1956; Sewell, 1961; Dawes, 1963b; Thorpe, 1965). The ability of *Cl.novyi* spores to elicit the disease in these hosts depended on the route by which they were administered. Spores given by the oral and intravenous routes were most likely to cause death in these animals although those given by the intraperitoneal route also resulted in death in guinea-pigs, but not in rabbits.

In the studies of experimental infectious necrotic hepatitis in sheep, emphasis was laid on the role of the flukes in the causation of the disease by using increasing doses of metacercariae of *Fasciola hepatica* and a constant dose of *Cl.novyi* type B spores. The results (Table 4.11) show that deaths from the disease may occur in sheep following a dose of 2000-4000 metacercariae and about 100 million viable spores of *Cl.novyi* type B. There appeared to be some relationship between the number of flukes present in the livers of the sheep and death from infectious necrotic hepatitis. All the sheep which had more than
1000 flukes in their livers and had received orally administered spores died from infectious necrotic hepatitis (sheep 32, 41 and 42, see Table 4.11), but only one of the three sheep which had received the dual infection but had less than 1000 flukes in its liver also died from the disease (sheep 22).

Two of the fluke controls (sheep 33 and 43) had more than 1000 flukes in their livers. Of these, sheep 43 did not show any symptoms of acute fascioliasis before being killed but sheep 33 died, apparently of the fluke infection. The death of this sheep was surprising in that the dose of metacercariae given was not expected to cause acute fascioliasis, and death from this disease usually occurs more than 7½ weeks after infection (Ross et al., 1967a; Pullen, 1968). The post-mortem lesions in this animal were more similar to those of subacute fascioliasis as regards the clinical symptoms and the small amount of fluid in the abdominal cavity, but death from sub-acute fascioliasis usually occurs about 12 weeks post infection and there was no evidence of haemorrhage into the bile ducts (Ross et al., 1967a; Pullan, 1968).
As might be expected the serum G.D. activities and absolute eosinophil counts of all the sheep with more than 1000 flukes (sheep 32, 33, 41, 42 and 43 - Group 1) tended to be higher than those in sheep which had less than 1000 flukes in their livers (sheep 21, 22, 23 and 31 - group 2) or those with no flukes in the livers (sheep 24, 34 and 44 - group 3), (Graphs 5.1 and 5.2).

Hence the degree of parenchymal damage to the liver by immature flukes as measured by the serum glutamic dehydrogenase (G.D.) activities and the host reaction to this infection, as measured by the absolute eosinophil counts (Appendix Table I) also appeared to be related to the probability of the animals dying from infectious necrotic hepatitis. Thus the serum G.D. levels from 2 weeks after infection and the absolute eosinophil counts between 2 and 5 weeks after infection tended to be raised more in the sheep which died of experimental infectious necrotic hepatitis than in those which survived the dual infection (Graphs 5.3 and 5.4).

All the sheep which developed infectious necrotic hepatitis (22, 32, 41 and 42) died within 6-8 weeks after infection with the metacercariae of *F. hepatica*.
Graph 5.1.

Serum glutamic dehydrogenase activities

Both graphs show a comparison between sheep infected with more than 1,000 flukes (Group I), those with less than 1,000 flukes (Group 2) and those not infected with flukes — the spore controls (Group 3).

Group I = Sheep numbers 32, 33, 41, 42 and 43.

Group 2 = Sheep numbers 21, 22, 23 and 31.

Group 3 = Sheep numbers 24, 34 and 44.

The dotted lines after 6 weeks post-infection refer to results from fewer animals.

Graph 5.2.

Absolute eosinophil counts
Both graphs show a comparison between sheep dying from infectious necrotic hepatitis after infection with both flukes and spores (Group 4), sheep which were also infected with both pathogens, but did not die (Group 5) and those which were not infected with flukes - the spore controls (Group 3).

- Group 4 = Sheep numbers 22, 32, 41 and 42.
- Group 5 = Sheep numbers 21 and 31.
- Group 3 = Sheep numbers 24, 34 and 44.

The dotted lines after 6 weeks post-infection refer to results from fewer animals.
All these sheep showed high serum G.D. levels during this period, which coincides with the period in the fluke's life cycle in which there is maximal parenchymal damage before the flukes enter the bile duct (Ross et al., 1967a). Thus, as with the guinea-pigs and rabbits, it is this period of maximal parenchymal damage which seems to be most suitable for the germination of the spores dormant in the liver.

When a large number of flukes and spores are present in the liver at this time, it is relatively probable that some spores will chance to be situated at a site where the parenchymal necrosis will render the environment suitable for multiplication even by a fastidious anaerobe such as \textit{Cl.novyi} type B, with subsequent toxin production and the onset of infectious necrotic hepatitis. If the number of flukes or spores are fewer, then the probability of this occurring becomes less and the sheep may survive, although both flukes and spores have been present in the liver. This occurred in two of the experimental cases, where there were fewer than 1,000 flukes in the liver, and will occur even more frequently in the field where infection levels are usually lower. This explanation thus readily accounts for the regularity with which both flukes and \textit{Cl.novyi} type B spores have been found in livers from healthy sheep during the surveys.
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### RESULTS FROM EXPERIMENTAL INFECTIOUS NECROTIC HEPATITIS IN SHEEP

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