

The University of Glasgow

JOHNE'S DISEASE

Experimental Infection in Mice with Studies  
on the Isolation and Cultivation of  
Mycobacterium Johnei

by

JOHN CAMERON, B.Sc.

(Medical Research Council Scholar)

Thesis submitted for the Degree of Ph.D.  
in the Faculty of Science

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Summary of thesis - "John's Disease. Experimental Infection in Mice with Studies on the Isolation and Cultivation of Mycobacterium Johnei" - submitted by John Cameron, B.Sc., for the degree of Ph.D. in the Faculty of Science.

The methods for the isolation of Myco. johnei from intestinal mucosa and media for its cultivation are discussed; 30 bovine and 2 pigmented ovine strains of Myco. johnei have been isolated. A new method for the isolation of the organism from faeces has been devised.

A vitamin-K compound was found to stimulate the growth of a strain of Myco. johnei not requiring the growth factor supplied by Myco. phlei. This compound did not support the growth of 3 typical strains of Myco. johnei requiring the growth factor. Other aspects of the metabolism of Myco. johnei were also investigated.

The main object of this study was to reproduce John's disease in mice. For this purpose, 6 strains of Myco. johnei were inoculated as cultures: of these, 4 were virulent, one was avirulent, and one was tested only with what may have been sub-minimal inocula. Five strains were inoculated as suspensions of infected viscera: of these, 3 were virulent, and the virulence of the other 2 is as yet unknown. The infection was produced by the intraperitoneal and intravenous, but not by the oral route.

John's/

Johne's disease in mice is characterised by a very long incubation period - 8 to 16 months, depending on the size of the inoculum - at the end of which Myco. johnei appears in the faeces and rapidly increases in numbers as the infection progresses. The disease is almost asymptomatic, yet in some experiments the death-rate was very high. Although it was possible to produce the infection in approximately 14 months with 0.5 mgm. dry weight of organisms, the use of larger doses shortened the incubation period. The shortest incubation period observed was 6 months.

Post-mortem examination of infected mice reveals thickening of the intestine and enlargement of the mesenteric lymph glands; microscopically, innumerable organisms are seen in smears of the viscera. The lesions, infiltrated by macrophages packed with acid-alcohol-fast bacilli, and sometimes by giant cells, were found in the intestinal mucosa and submucosa, and in the mesenteric lymph glands, liver, and spleen; the intestinal lesions are a replica of those seen in cattle, although generally more extensive.

Myco. johnei was re-isolated from the faeces and organs of experimentally infected mice, and the infection was reproduced by inoculation of the viscera of such animals.

Of a total of 205 mice inoculated with cultures, 51 received sub-minimal inocula, 53 received the avirulent strain/

strain, and 55 were killed for histological examination within 4 weeks of inoculation. Of the remaining 46, 27 were killed after 9 months; of these 27, 11 had Myco. johnei in the faeces and 5 of the 11 examined histologically had lesions. Another 12 of the 46 had Myco. johnei in the viscera 12 to 16 months after inoculation and 11 of them, examined histologically, had lesions. The remaining 7 of the 46, still alive, have large numbers of Myco. johnei in the faeces.

Of 54 mice inoculated with bovine intestinal mucosa, 7 died shortly after inoculation, 25 are in the incubation period, and 22 developed the infection. Of these 22, 12 are still alive and 10 are dead. of which 7, examined histologically, had lesions.

Of 74 inoculated with mouse viscera, 12 died shortly after inoculation, 25 still alive have large number of Myco. johnei in the faeces, 18 are in the incubation period, 4, inoculated 11 months ago, probably received a sub-minimal dose, and 15 developed the infection, Of the 15, 7 examined histologically had lesions.

Reasons are suggested for the failure of other workers to transmit Johne's disease to laboratory animals. The size of the inoculum, the length of the observation period, and differences in virulence of strains of Myco. johnei and in the susceptibility of animals, appear to be the main factors.

P R E F A C E

Where'er you touch there's  
interest without end.  
Goethe.

Johne's disease is a common, often fatal disease of cattle, affecting less frequently goats and sheep, and occasionally other animals; the causative agent, Myco. johnei, can be cultivated, but only with difficulty.

Over the last sixty years, a remarkable amount of work (of which this thesis mentions only a fraction) has been devoted to Johne's disease. In spite of that, there is no real knowledge either of the pathogenesis of the infection or of the toxins of the causative agent; and there is no successful chemotherapy. This is hardly surprising, because large-scale experiments on cattle are obviously impossible, and there is no small laboratory animal in which the disease can consistently be reproduced.

A/

A review of the literature shows that an intestinal infection resembling Johne's disease has, on a few occasions, been produced in laboratory animals (mice, rats, rabbits, and voles), the first report dating as far back as 1912. Such reports, however, have never been generally accepted and there is a widespread belief, finding expression in dogmatic statements in text-books of bacteriology, that the disease is not transmissible to animals other than the natural hosts. This contradiction between the text-book view and what has actually been accomplished in the field of transmission to laboratory animals, makes Johne's disease, as a topic for research, both intriguing and discouraging. It was felt that the contradictory results might be reconciled and the problem of the transmissibility of Johne's disease to other than the natural hosts solved, only with as full a knowledge as possible of the biology of Myco. johnei and of the natural disease. For these reasons, the introductory chapter to this thesis is not confined to the subject of the experimental infection in small laboratory animals.

The present work was undertaken with the object of reproducing Johne's disease in the mouse; it is felt that the aim has been achieved.

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## HISTORICAL

Pathological changes in the bovine intestine described by Hurltrel d'Arboval in 1826 (quoted by Stableforth, 1930) were probably due to Johne's disease; prolonged diarrhoea, possibly a symptom of Johne's disease, was earlier described in cattle by Lawrence in 1798, by White in 1815, and again by Cartwright in 1829 (all quoted by McGregor, 1934). The disease is now known throughout the countries of Europe, in North and South America, Barbados, Jamaica, Trinidad, Kenya, India, Ceylon, Sumatra, Australia and New Zealand.

Most reports on Johne's disease describe the infection in cattle, but there are several reports of the disease in sheep (Stockman, 1911; Twort & Ingram, 1912a; Howarth, 1932; Dunkin & Balfour Jones, 1935; Thiery & Getas, 1935; McEwen, 1939; and Wilson Taylor, 1945, 1951a;). It has also been found in goats and swine (Eveleth & Gifford, 1943), in deer (McFadyean, 1907; Bourgeois, 1940, 1944;), and in Indian zebu, hybrid zebu, and antelope (Dorofeev & Kalachev, 1949).

Johne and Frothingham in 1895 demonstrated an acid-alcohol-fast bacillus in an atypical tuberculous bovine intestine, atypical insofar as it revealed, in addition to tuberculous lesions, lesions which today would be recognised as characteristic of Johne's disease. Until the experiments of Bang in 1906, the disease was considered to be a form of intestinal/

intestinal tuberculosis; McFadyean (1907) drew attention to the undesirability of any name implying a relationship to tuberculosis and to emphasise the dissimilarity he suggested the name Johne's disease, but the connotation with tuberculosis still exists in such synonyms as chronic paratuberculous enteritis of cattle, pseudo-tuberculous enteritis of cattle, l'entérite paratuberculeuse des bovidés, and chronische Paratuberkulose Darmentzündung beim Rinde.

It is probably fair to say that a careful investigation before 1906 would have revealed that, apart from being caused by an acid-alcohol-fast bacillus, Johne's disease has little in common with tuberculosis. However, confusion between them may have arisen from the difficulty in producing Johne's disease in adult animals and from the remarkably long incubation period in Johne's disease; in calves, which are much more susceptible to Johne's disease than adult beasts, the incubation period may be 18 months or longer. The transmission experiments of Bang (1906) showed that Johne's disease was an entity, and 5 years later Twort & Ingram (1911-1912), for the first time, cultivated the causative agent, now known as Myco. paratuberculosis (Breed, Murray, & Parker Hitchens, 1948), Myco. johnei, or Johne's bacillus. These workers found that with the addition of one per cent of Myco. phlei, a good egg medium supported the growth of Myco. johnei/

Myco. johnei; since then, many media have been used, the feature common to them all being the presence of Myco. phlei, which provides a growth factor (or factors) as yet unidentified.

### THE DISEASE

Description of Infected Animals. The commonest symptoms of Johne's disease are diarrhoea and emaciation, the latter to a large extent due to atrophy of the dorsal muscles. These, of course, are late symptoms, and occur when the disease is well established; at this stage, too, animals are unthrifty, have a low milk yield, a ravenous appetite, and polydypsia. Diarrhoea per se is not necessarily characteristic of Johne's disease, and as Meyer (1913-1914) pointed out, when it occurs the possibility of chronic tuberculosis or of parasitic enteritis should be considered. For this reason, colloquial names for Johne's disease, such as "scrapy", "scours", "scouring rot", "scantering", "shooting", and "wet scanter", which emphasise the ever-present diarrhoea, can be misleading. There may be remissions in the diarrhoea, and in some animals (Bang, 1910; Taylor, 1932) motions are normal or almost so. In such cases the other symptoms, emaciation and low milk yield in spite of great appetite, <sup>may be present.</sup> ~~become the ones to be guarded~~ against. Nevertheless, in the course of the disease there is always/

always, at some period, serious diarrhoea.

Several other symptoms, not generally mentioned in the description of the disease, have been observed from time to time. In one of the earliest and most discriminating reports on Johne's disease, Bang (1906) remarked that some animals were nervous, had a poor appetite (as opposed to the usual ravenous appetite), and no temperature, and revealed at post-mortem a lack of fat tissue. Bongert (1910) considered anaemia a characteristic of the infection, becoming more pronounced as the disease progressed. Anaemia was later observed by Dryerre (1934) in infected sheep; he reported a 30 to 60 per cent haemoglobin deficiency in an infected flock compared with a 10 to 20 per cent deficiency in uninfected animals. A marked depression of the blood level of calcium and magnesium, particularly of magnesium, was noted by Stewart, McCallum, & Wilson Taylor (1945); this finding, although generally accepted, was not confirmed by Ford (1952).

Description of Lesions. There is no more accurate or complete a description of the macroscopic and microscopic changes in an infected intestine than that given by Wilson & Miles (1946, page 1364).

"Pathologically, the main lesions are situated in the last 50 feet of the small intestine, and in the neighbourhood of/  
of/

of the ileocaecal junction, but sometimes the whole gut is affected. The intestine is thick and rigid, looking not unlike a hose pipe; the mucosa is greatly thickened and is thrown into regular corrugations, resembling the convolutions of the cerebrum. The mucosa is smooth and pale or pink in colour, and covered with slimy material; on the surface of the corrugations it is dotted with red spots or haemorrhages: between the folds it often has a warty appearance. Occasionally small nodules are seen, due to enlargement of solitary lymphoid follicles. There is no ulceration of the mucosa, and the peritoneal surface appears normal. The mesenteric lymph glands are usually enlarged, oedematous and pigmented". It should be added that normal bovine intestinal mucosa has corrugations which disappear when the intestine is gently stretched; the corrugations due to Johne's disease do not disappear when the intestine is stretched.

Histologically "the mucosa, and to a less extent the submucosa, are infiltrated with lymphoid and epithelioid cells, which are responsible for the thickening. Near the surface of the gut the mucosa is absolutely structureless; all traces of nuclei and cell outlines have disappeared. Giant cells are rare; there is no caseation, ulceration, fibrosis, or calcification. Short acid-fast bacilli, often in enormous numbers, are found in the mucosa lying between the glands and in the lymphoid tissue of the solitary follicles; they/



they may invade the submucosa, and very occasionally the underlying muscle layer. The bacilli are arranged in dense clumps, and may be intra- or extracellular. Johne and Frothingham were of the opinion that they were primarily intracellular in position, but that owing to the subsequent disintegration of the cell, they were set free in the tissue. The cellular reaction around the bacilli is diffuse, not localised as in tuberculosis. Bacilli are generally found in the mesenteric glands and sometimes in the sublingual and submaxillary glands". More extensive descriptions are given by McFadyean (1918) and Hallman & Witter (1933).

A suggestion that Myco. johnei could be found in sites other than the intestine was made in 1929 by Alexejeff-Goloff, who described in one cow necrotic centres in the liver, and thickened mucous membrane in the gall bladder and urinary bladder. In this and in other 3 cows, acid-alcohol-fast bacilli were present in the heart and peripheral blood, lymphatic glands, heart muscle, lungs, liver, spleen, suprarenals, kidneys, gall bladder, urinary bladder, ovaries, mesenteric lymph glands, and mucous membrane of the intestine. They were also present in "foetal envelopes" and fluids, a pure culture being obtained from the foetus. The author concluded that Johne's disease is a bacteraemia causing intra-uterine infection. McFadyean (1935) thoroughly disagreed with this conclusion and with the findings in general.

Two cases were reported by Mathews in 1930, those of an 11 year old Holstein cow and of a 7 year old Jersey bull, in both of which lesions were observed in the liver as well as in the intestine.

Houthuis (1932) isolated Myco. johnei from the liver, lungs, and portal glands of infected cattle, remarking at the same time that these cases were rare and unusual. He suggested that lymphatic spread might have been responsible for this effect, but did not rule out the possibility that the blood might also be involved.

Later, Brodenok (1941) observed in 2 cows a similar, but not quite so extensive, distribution of Myco. johnei. Besides the usual intestinal localisation, he reported the presence of organisms in the gall bladder, portal lymph nodes, and spleen: in one animal the changes in the gall bladder were the same as those in the intestine in advanced cases of Johne's disease, and in the other they were more acute, showing enlargement, thickening, hyperaemia and petechial haemorrhages.

Far from considering extra-intestinal localisation rare and unusual, Wilson Taylor (1953) remarked that Myco. johnei could be regularly isolated from "almost any of the lymph nodes or abdominal or thoracic viscera of cattle showing clinical signs of the disease". It would thus appear that, while the normal site of infection is the intestine in the region/

region of the ileo-caecal valve, extra-intestinal localisation of Myco. johnei does occur.

Experimental Infection in Natural Hosts. Five years before Myco. johnei was first isolated, Bang (1906) reproduced the disease in calves by feeding and by inoculating intravenously or intraperitoneally a mixture of infected bovine intestinal mucosa and infected lymph-node tissue. These results were confirmed 4 years later by Miessner & Trapp (quoted by Twort & Ingram, 1911-12). Twort & Ingram (1912) successfully infected cattle by both the intraperitoneal and intravenous inoculation of cultures of Myco. johnei; a calf inoculated subcutaneously with a culture also developed the disease, but in this instance the possibility of an earlier natural infection was not excluded.

McFadyean & Sheather (1916), on the other hand, failed to infect the majority of their animals - cattle, donkeys, sheep, and a pony - as judged by the symptoms exhibited during life and by the results of post-mortem examination. In the light of what is now known, their results can be ascribed to the use of adult animals; such animals are thought to be more refractory than young ones. Subsequent work by Hagan & Zeissig (1933a), and Hagan (1935, 1938) confirmed the original findings of Bang (1906).

Meyer/

Meyer (1913-14) was the first to realise that the age of animals might play a role in experimental infection with Myco. johnei; his observation is now referred to as the age-immunity phenomenon. A cow and 2 of its calves, a 2 year old heifer, and a 10 month old bull, were exposed by Meyer for 2 years to contact with diseased cattle in a stable and exercise-yard heavily infected with Myco. johnei: the organisms were demonstrable in the manure in the yard. Calving and infectious abortion, both of which Meyer thought would hasten the onset of clinical symptoms, were without effect on the heifer; at post-mortem the animals were found not to have contracted Johne's disease. From this experiment Meyer concluded that infection takes place during the first 3 or 4 months of life. The failure of McFadyean & Sheather (1916) to infect the majority of their animals, which were adult, indirectly confirmed Meyer's hypothesis.

Working with a mixed herd exposed to infection for 4 years, Hagan reported in 1938 that of 16 animals dying in the herd, 15 were born therein and exposed to infection from birth. No cases developed in animals exposed to infection from 4 months. Experimental infection by feeding massive doses of infected intestinal mucosa proved possible in animals less than 2 years old. It would appear from these carefully controlled experiments that infection occurs in the calf very early in life.

Even/

Even when infection becomes apparent late in adult life, it does not follow that it was contracted in adult life (Doyle, 1953). A very interesting case was described by Dunkin (1935) in which a calf from an infected mother was caught in a canvas bag at birth and throughout life was carefully isolated from any possible contact with Myco. johnei. Three and a half years later, although appearing until then in excellent condition, it began to scour; acid-alcohol-fast bacilli were found in the faeces; and at post-mortem the characteristic lesions of Johne's disease were observed. Since it is unlikely that infection took place in adult life, it probably occurred either in utero, or during the brief period of birth, in which case this particular calf represents an instance of an unduly long incubation period. Moreover, as Doyle (1953) remarked, if the infection indeed occurs in the first few months of life, the incubation period must, in many cases, be longer than the generally postulated 18 to 24 months.

Incidence, Mode of Spread, Control, and Treatment. The incidence of Johne's disease is not really known since it is not a notifiable disease and since it can be diagnosed with certainty only at a post-mortem examination. In the United States, Beach & Hastings (1922) estimated the annual loss from this/

this disease to be between 2 and 12 per cent in selected herds; they quote Bang as saying "the future of a certain breed of dairy cattle in Denmark depends on the ability to eliminate Johne's disease". Houthuis (1932) reported an annual loss in Holland of 12 to 15 per cent in herds of about 40 animals. In Assam, where the incidence is high, Pande (1940) examined 9 breeds of cattle, in 8 of which the incidence was about 25 per cent, and in the 9th, Bihari crosses, only 16 per cent. Much of the disease in India was traced to Government-owned bulls which were hired to villages for breeding. Infection among these bulls ensured a thorough spread of the disease. In a heavily infected flock of 55 sheep, Sigurdsson, Vigfússon, & Theodors (1945) reported from Iceland that serological diagnosis, confirmed by post-mortem examination, revealed an incidence of 54 per cent.

The results of an investigation by Wilson Taylor (1949, 1952a) must cast doubt on the reliability of any figure quoted as the incidence of Johne's disease, since the diagnosis depends so largely on the finding of the organism. In a survey of apparently healthy cattle delivered to the Reading abattoir Myco. johnei was cultured from the mesenteric lymph glands of 15 per cent of the animals.

Investigating the susceptibility to Johne's disease in relation to age, Doyle (1953) remarked, of animals aged 7 to/

to 13 years and suffering from Johne's disease, "it is impossible to say when these animals became infected; they may have contracted the disease during early life and remained carriers until some adverse condition reactivated it, or they may have picked up infection during adult life. It is probable that many young animals, and perhaps more adult animals, contract infection and recover or remain permanent carriers without exhibiting clinical disease". Sheather (1933) seems to have been one of the first to realise this possibility; two other authors, Smythe (1950) and Wilson Taylor (1949, 1951b), express the same thought in a different manner, inclining rather to the view that Myco. johnei may be a commensal in the bovine intestine, becoming pathogenic as the result of some unknown "trigger mechanism".

The presence of Myco. johnei in cattle not clinical cases of Johne's disease suggests that the present "incidence" is, in effect, the mortality rate, and that the actual incidence is considerably higher. The existence of non-clinical cases may also explain some of the positive reactions in healthy animals to johnin skin tests (see page 24).

Johne's disease is not easily diagnosed in its early stages, and a farmer may unwittingly sell an infected animal thereby possibly establishing a new focus of infection. As mentioned/

mentioned earlier, much of the disease in India was at one time spread in a similar fashion.

There are usually large numbers of organisms in the faeces of a clinical case of Johne's disease, and as a result, bedding, drinking water, and pasture become sources of infection. Since infection probably enters by the alimentary canal, it is easy to imagine how the disease spreads. Bedding in good farms is changed once, sometimes twice, daily, so that the greatest danger probably arises from contaminated drinking water and pasture. The reports of Vishnevskii, Mamatsev, Chernyshev, & Chernyshev (1940) and of Lovell, Levi, & Francis (1944) show quite clearly that the danger from such sources can hardly be overstated. The former workers found that Myco. johnei remained viable in cattle manure and in black soil for more than 11 months. Lovell et al found that a culture of Myco. johnei suspended in river water was sterile after 30 days, while a culture suspended in sterile pond water containing mud survived for 9 months; in infected faeces exposed to the weather, Myco. johnei survived for 246 days and for 163 days when intestinal scrapings from diseased animals were suspended in unsterilised river water.

Wilkinson (1934) is of the opinion that careful attention to hygiene, in the broadest sense, can do much, by lessening the chances of exposure, to prevent the infection of/



of young cattle. He suggested draining stockyards and surfacing them with a deep layer of well-rolled ballast, flooring surface-drained young stock boxes with concrete, and storing manure away from sheds. Fields were to be dressed with lime, also recommended by Houthuis (1932), and left exposed all summer before receiving a fresh stock of calves; calf pens were to be thoroughly disinfected and permanently-fixed batten-floors removed and replaced by floors easily removable for disinfection. Provision was also to be made for the immediate weaning of calves at birth to prevent infection from an infected parent, a measure also suggested by Miessner (1914). Most important of all, drinking ponds were to be railed off to prevent contamination with faeces. Similar measures were suggested by Minett (1933) and were successfully adopted by Downham (1951a).

Since the disease is not notifiable another problem is the disposal of infected animals. In the case of valuable stock, they may be segregated (Rinjard, 1934) - Edwards (1947) reported the elimination of Johne's disease at the Agricultural Research Council Field Station, Compton, by such a policy - but segregation is not always practicable. Four workers, Bang (1906), Houthuis (1932), Dunkin (1934), and Rinjard (1934), are of the opinion that the wisest procedure is to kill them, since the first loss is generally the smallest: /

smallest: if they are in good condition they will fetch a price at the abattoir.

There is no successful treatment for Johne's disease. The main object of the earliest treatment recorded in the literature appears to have been to control the diarrhoea with such mild laxatives as castor oil and kreolin, followed by styptics, bismuth subnitrate, tincture of opium, catechu, krameria, and zinc sulphocarbulates (Meyer, 1913-14). Ferrous sulphate, copper sulphate, and sulphuric acid, interspersed with opium, tannoform, and astringent powders of nux vomica, catechu, and chalk, were used by McFadyean, Sheather, & Edwards (1915); there was occasional control of diarrhoea, mainly by the ferrous sulphate. In the course of treatment some animals put on weight, only to lose it again: the most important point was that treated animals, when killed, still harboured enormous numbers of Myco. johnei in the intestine.

Sheather (1927), following a claim by Tutt (1926), injected some infected animals intravenously with formaldehyde, and the initial results were very discouraging; the condition of the treated animals improved and the diarrhoea was arrested. As with previous treatments, however, this was followed by a relapse, after which the animals were refractory to further treatment.

The/

The earliest treatment directed against the causal agent was the use of chaulmoogra oil by Williams (1928), who claimed some success; later Downham (1933) and Gunning (1933) found it ineffective in small scale experiments. Chaulmoogra oil was again used by Downham (1951b) who, on this occasion, reported that one of 2 animals which were treated for 6 months showed great improvement: unfortunately, it died of an oil embolism.

Of the newer anti-tuberculous compounds 4:4' diamino diphenylsulphone, iso-nicotinic acid hydrazide, 4-amino salicylic acid, streptomycin, and 4-acetamido-benzaldehyde thiosemicarbazone, have been used in vivo with discouraging results (Larsen, Vardaman, & Groth, 1950; Cauchy, Goret, Merieux, & Verge, 1951; Oostenbrug & Talsma, 1951; Verge, Goret, & Cauchy, 1951; Ford, 1952; Larsen & Vardaman, 1952, 1953; Deans Rankin, 1953). In some animals there was a temporary weight gain and cessation of diarrhoea, but all treated animals finally died or were killed because of Johne's disease.

Several attempts have been made to correlate the mineral content of the diet and of the pasture with the incidence of the disease (Spicer, 1932, 1933, 1934, 1936; Haddow, 1934; Wilkinson, 1934; Sen, 1935; Smythe, 1935; Pande, 1940; Downham, 1951a). Spicer (1932), first reported that a high/

high mineral content afforded a dietary means of controlling Johne's disease. A complementary report followed (Haddow, 1934) suggesting that animals with no minerals in their diet were more susceptible to Johne's disease: a further contribution (Pande, 1940) again stressed the value of mineral supplements in reducing the incidence of the disease. Much correspondence has appeared in the columns of veterinary journals on this subject. No single mineral has, with certainty, been implicated - calcium has been suggested - but there is no doubt that diseased cattle, other than hopeless cases, improve with improved feeding. Johne's disease today certainly appears to be more frequent in farms where the general level of nutrition is low (McIntyre, 1953).

A liberal daily supply of iodised bone-flour, especially to milch cows, was recommended by Wilkinson (1934), and his results speak for themselves: in a herd, more or less given up for lost because of the inroads made by Johne's disease, the calves, born and reared in the year these measures were introduced, were the best ever and the milk yield was increased.

Diagnostic Methods. Before the onset of clinical symptoms there is no means of diagnosing Johne's disease with any accuracy. With experience, observant stockbreeders can/

can pick it out, an early pre-clinical sign being an oedematous swelling between the rami of the lower jaw. This swelling may on occasion be noted 2 years before diarrhoea, although it is more generally observed only weeks or months before its onset. Both Cartwright (1829, quoted by McGregor, 1934) and Motton (in a personal communication quoted by Dunkin, 1934) observed this oedematous swelling in cattle; Johnston (1933) and Mudaliar (1944) reported it in sheep; in Cornwall the local name for Johne's disease is "chuck felon" (a swelling of the intermaxillary space) and according to Smythe (1935), "the least degree of fulness or swelling in this region in an apparently healthy or otherwise valuable animal, today completely mars the sale in the open market".

Acid-alcohol-fast bacilli in densely packed clumps, an arrangement characteristic of Myco. johnei in faeces, may be found in smears of faeces and of rectal mucous membrane from clinical cases. Markus (1904) suggested the examination of such smears as a means of diagnosing Johne's disease; such an examination is seldom carried out as a first step in diagnosis but is generally used to confirm a clinical diagnosis: a negative finding does not exclude Johne's disease. Houthuis (1932) stated that in his experience only 10 per cent of cases confirmed at post-mortem examination could be diagnosed by the examination of faeces.

The serological methods of diagnosis, agglutination and complement-fixation, are of little value in mycobacterial infections because the existence of antibodies to mycobacteria is not easily shown in vitro and because cross-reactions among mycobacteria are very common. Neither method has so far been adequately developed for general use although Hagan & Zeissig (1933b) considered the complement-fixation test to be very useful.

In his agglutination experiments Twort (1912) used as antigen a saline suspension of Myco. johnei and found that it was of limited specificity when compared with similar suspensions of Myco. phlei and of the avian, bovine, and human tubercle bacilli. A serum agglutinating its homologous organism was also positive, to a lesser degree, with suspensions of the other organisms. When testing sera the antigen itself had to be watched because of the possibility of spontaneous agglutination. Sera from naturally and experimentally infected cattle were titrated, the most strongly positive giving a titre of 1 in 15. The sera of sheep and goats inoculated with Myco. johnei were negative and those of rabbits gave poor results. The sera from fowls after 2 inoculations were strongly positive, some giving complete agglutinations at 1 in 25. On the other hand, the serum of an uninoculated fowl was also strongly positive and the/

the serum of another fowl agglutinated with suspensions of avian, bovine, and human tubercle bacilli at 1 in 5.

Many antigens have been used for the complement-fixation test in the diagnosis of Johne's disease. Twort & Ingram (1913a) used cultures of Myco. johnei, but later obtained better results with Myco. tuberculosis. Other antigens which have been used are avian tuberculin (Bang & Andersen, 1913), an extract of infected mucous membrane (Miessner & Kohlstock, 1912), and a methyl-alcohol extract of Myco. johnei previously defatted with acetone (Boquet, 1927a). Unfortunately, although complement is fixed in the presence of these antigens and infected serum, it is also fixed in the presence of anti-tuberculous sera. Furthermore, similar extracts of Myco. tuberculosis give positive complement-fixation with sera from cattle suffering from Johne's disease (Boquet, 1927a).

More recently Sigurdsson (1945, 1946, 1947a, & b), reported the existence of a specific antigen in the intestinal mucosa of infected sheep. According to this worker, it is recoverable in a "masked" or inactive form by saline extraction at 100°C of intestine previously dried at low temperature and under reduced pressure. The antigen is "unmasked" by grinding the dried intestinal mucosa in a ball mill. Sera from infected sheep, in the presence of this antigen, fix complement to a high titre; some uninfected sheep also fix complement, but only to a very low titre (Sigurdsson et al, 1945).

A slightly modified haemolytic complement-fixation test now in use in this country (Hole, 1952a, & b, 1953;) has so far proved useful in determining whether or not, in an attested herd of cattle, a clinically suspect animal has Johne's disease; animals reacting to avian tuberculin have not been excluded (see page 31). The standard dose of antigen, a washed, saline suspension of heat-killed Myco. johnei, is left in contact for an hour at room temperature with dilutions of the test serum, previously held at 56°C for half-an-hour to reduce its anti-complementary activity; a higher temperature is avoided because Hole believes that the units comprising the antibody are somewhat thermolabile. The mixing of the serum and antigen allows adsorption of antibody from the serum; after an hour the suspension is centrifuged, the supernatant discarded, and the organisms resuspended in fresh saline. The dose of complement, estimated in the presence of the antigen, is added to this suspension, and the whole left for half-an-hour at room temperature for fixation of complement to take place. The haemolytic system is then added, the suspension incubated at 37°C for half-an-hour, the tubes centrifuged, and the results read against an illuminated screen; the use of a positive and of a negative serum of known titre as controls is advised. Fixation of complement at a serum dilution of 1 in 5 is considered positive; a strongly/



strongly positive serum may have a titre of 1 in 80, and occasionally 1 in 160; titres above this have not been encountered.

The suspensions of Myco. johnei used as antigen did not always give satisfactory results, even when prepared from the same strain of organism; this was thought to be due to the presence of rough and smooth variants in the culture, and indeed, not only did the smooth variant give better suspensions and the rough variant tend to auto-agglutination, but later experiments showed that, with regard to antigenic properties, the rough variant was inactive.

At the post-mortem examination of 330 clinically suspect animals, 285 had Johne's disease, 13 were "suspicious", and 32 negative. Of the 285 proven cases, 267 were positive by the test, 12 doubtful, and 6 negative; of the 13 "suspicious", 9 were positive by the test, 2 doubtful, and 2 negative, and of the 32 negative at post-mortem examination, 3 were positive by the test, 12 doubtful, and 17 negative. The post-mortem examination of 121 animals not clinically suspect revealed that of 49 positive post mortem, 41 were positive by the test and 8 doubtful. Of 17 "suspicious" at post-mortem examination, 13 were positive by the test, 2 doubtful, and 2 negative, and of 55 negative at post-mortem examination, 19 were positive by the test, 17 doubtful, and 19 negative.

Johnin/

Johnin culture filtrate, intradermal johnin, and johnin P.P.D. were used as antigens by Larsen, Porter, & Vardaman (1953), in a modified Middlebrook-Dubos haemagglutination test (Middlebrook & Dubos, 1948) for the diagnosis of Johne's disease in cattle. In preliminary experiments, sheep erythrocytes sensitised with either of the first 2 antigens gave unreliable results with known positive sera; these antigens were discarded in favour of johnin P.P.D.

Of 55 animals with no history of either Johne's disease or of tuberculosis the serum of 50 did not agglutinate at a dilution of 1 in 4, that of 5 agglutinated at 1 in 4, and none at 1 in 32. Consequently, agglutination at 1 in 4 to 1 in 16 was considered the normal range and 1 in 32 or more was taken to indicate an infected serum. Thus, of 67 animals from herds infected with Johne's disease, 10 were reactors; of 13 animals not reacting to tuberculin and from a herd infected with tuberculosis, 2 were reactors. In animals sensitised to Myco. johnei by the intraperitoneal inoculation of a mineral oil suspension of Myco. johnei, 27 of 28 were reactors as were 16 of 18 similarly sensitised to the bovine tubercle bacillus.

Comparing the results on the 67 animals from infected herds with the results of the intradermal johnin test, 28 animals with sera agglutinating at 1 in 16 or less were negative/

negative to johnin, and 10 at 1 in 32 or more were positive. However, 14 at 1 in 16 or less were positive and 15 at 1 in 32 or more were negative.

Allergic reactions, because of their success in the diagnosis of tuberculosis, have been widely applied to the diagnosis of Johne's disease but without the same success; of the many contributors in this field the following is an incomplete list: Bang (1909), Twort & Ingram (1913a), Meyer (1913-14), McFadyean, Sheather, & Edwards (1916), Beach & Hastings (1922), Boquet (1927a), Dunkin (1928, 1933), Turner (1928), Wright (1928), Hagan & Zeissig (1928-29), Broerman & Fogle (1932), Rinjard (1934), Minett (1935), Rinjard & Vallée (1936), McIntosh (1939), Glover (1941a & b), Johnson, Milligan, & Cox (1941), Johnson & Cox (1942), Konst & Watson (1943), Johnson (1944a & b), Johnson & Pratt (1944), Johnson, Larsen, Henley, & Groth (1949), Larsen & Johnson (1949), Baisden, Johnson, & Larsen (1950), Sikes & Groth (1950), Smythe (1950, 1951), Sikes, Johnson, & Oglesby (1951), Jones, Larsen, Vardaman, & Baisden (1953), Sikes (1953).

The first attempt to detect Johne's disease by an allergic skin reaction was made by B. Bang (quoted by O. Bang, 1909): he used an aqueous, glycerol extract of infected intestinal mucosa as the diagnostic agent and the results were inconclusive.

The/

The early confusion between Johne's disease and avian tuberculosis probably accounted for the use of avian tuberculin by early workers. Thus Bang (1909) reported that 20 cattle, clinical cases of Johne's disease, reacted to avian tuberculin and not to mammalian tuberculin. He recommended avian tuberculin as a diagnostic agent, but subsequent reports have shown that considerable numbers of tuberculous cattle also react to avian tuberculin.

Twort & Ingram (1913a) were the first to prepare a product analogous to tuberculin for the diagnosis of Johne's disease. As in tuberculin production, a profuse growth of Myco. johnei on the surface of a fluid medium was steamed, the dead organisms removed by filtration through paper, and the filtrate concentrated to a small volume by heat. These johnins, as the preparations are called, can be used intradermally, intravenously, or subcutaneously, and the reaction is gauged either by measuring the local swelling after intradermal or subcutaneous injection, or by the rise in rectal temperature after intravenous injection. The ophthalmic route, where the response is judged by inflammation of the conjunctiva, is now seldom used.

A johnin prepared by Rinjard (1934) by the extraction of Myco. johnei with cold distilled water was used with excellent results in cattle vaccinated by the method of Vallée, /

Vallée, Rinjard, & Vallée (1934a). The results on naturally infected animals were less encouraging. It must be pointed out that in 1929 Hagan & Zeissig had used a similarly prepared johnin, and in calves sensitised by a subcutaneous inoculation of a mineral oil suspension of Myco. johnei, they were unable to demonstrate that their johnin had any advantage over avian tuberculin.

A subsequent report from France indicated that even an efficient johnin can have its drawbacks. Rinjard's preparation had to be withdrawn because some farmers were selling their positive reactors, thereby establishing new foci of infection (Rinjard, 1934).

Reviewing the value of johnins, a committee of the Agricultural Research Council (Report, 1941) considered that further research was required to improve their specificity and to estimate their potency. The committee also suggested that methods for the detection of avian tuberculosis in cattle should be improved and that enquiries should be made into the incidence of positive reactions to johnin in animals infected with bovine or avian tuberculosis. It should perhaps be remarked that, unlike tuberculin, there is as yet no standard johnin preparation.

It is interesting to note that a strain of Myco. johnei used in this country for the preparation of johnins, strain Teps,/

Teps, grows without the Myco. phlei growth factor; such strains are discussed later (see page 43). Wilson Taylor (1951a), commenting on the use of such strains for the preparation of johnin, wondered if the johnins prepared from them were the same as would be johnins prepared from freshly isolated, typical strains of Myco. johnei requiring the growth factor.

Johnin purified protein derivatives - johnin P.P.Ds. - were prepared by Glover (1941b) from heat-concentrated culture filtrates of strain Teps by precipitation with ammonium sulphate or trichloroacetic acid and by adsorption on benzoic acid. The yield varied from one preparation to another by as much as 100 per cent - from 10.25 to 22.07 mgm. of johnin P.P.D. per 100 ml. of original culture medium. However, regardless of the method of preparation or of the yield 0.0003 to 0.0004 gm. of johnin P.P.D. was equivalent to 0.1 gm. of heat-concentrated johnin. A solution of johnin P.P.D. containing 5 mgm. per ml. retained its activity for 175 days.

Comparing johnin P.P.Ds. with a heat-concentrated johnin by the double intradermal method in a small number of infected cattle, Glover found that both gave equally extensive, oedematous swellings; in uninfected cattle the injection of the same concentration of P.P.D. - 2 doses of 0.1 ml. of a solution containing not more than 3 mgm. P.P.D. per ml. - was/

was followed by negative reactions. It was later shown by Baisden et al (1950) that when the American Bureau of Animal Industry johnin (B.A.I. johnin) was treated with trichloroacetic acid, silicotungstic acid, acetone, or ethanol, a johnin protein was precipitated. When this was redissolved in an appropriate solvent the solution was of greater specificity than the B.A.I. johnin. Another johnin protein derivative, also of greater specificity than the B.A.I. johnin, was prepared by Jones et al (1953). Their method consisted of concentrating a dilute culture filtrate of Myco. johnei in a vacuum desiccator, dialysing, extracting with phenol, and precipitating the phenol extract with ethanol; the fraction precipitated with 50 to 80 per cent ethanol was the most active.

The practical value of johnins is difficult to assess: in experimentally infected cattle some johnins give excellent results (Rinjard, 1934), but they are not so successful as tuberculin in field work; on the other hand, they have not been so extensively investigated. Positive reactions to johnin must be interpreted in the light of clinical symptoms; the possibility of avian or bovine tuberculosis must also be borne in mind. Negative reactions must be regarded with caution since the initial and terminal stages of Johne's disease are characterised by an anergic condition.

The problem of allergic reactions in small laboratory animals/

animals was investigated by Boquet (1927a). Rabbits, sensitised by the intravenous inoculation of 60 to 80 mgm. of Myco. johnei, reacted, with a rise in temperature of 1°C by the 5th hour, to the injection of 0.1 to 0.5 ml. of johnin - a glycerol extract of Myco. johnei - or of tuberculin. On the other hand, rabbits infected for 2 months with Myco. tuberculosis reacted to the intravenous injection of 0.5 to 0.7 ml. of johnin with a rise in temperature of 0.5 to 0.7°C, the rise with 0.7 ml. reaching 1.7°C in the 5th hour. Although tuberculous rabbits were found to be sensitised to johnin, they withstood, without great discomfort, as much as 1 ml. of johnin intravenously.

Guinea-pigs inoculated intraperitoneally with 100 to 150 mgm. of Myco. johnei reacted to the intradermal injection of 0.01 ml. of johnin and of tuberculin; they tolerated, subcutaneously, as much as 1.5 ml. of johnin and 1.0 ml. of tuberculin. Tuberculous guinea-pigs gave a typical, papular reaction after the intradermal injection of 0.02 ml. of johnin; like rabbits they were found to be sensitised to johnin yet withstood 1.5 ml. of johnin subcutaneously.

In guinea-pigs 20 days after the intraperitoneal inoculation of 100 mgm. of Myco. johnei, skin reactions followed the intradermal inoculation of 5 mgm. of Myco. johnei, Myco. phlei, and Myco. tuberculosis, the reaction with Myco. tuberculosis being most marked. The local reaction which followed/



followed the intracutaneous inoculation of 10 to 20 mgm. of Myco. johnei into tuberculous guinea-pigs was very feeble and less necrotic than that following the intracutaneous inoculation of Myco. tuberculosis.

Twenty to 60 days after inoculation with Myco. tuberculosis, or with 20 to 30 mgm. of Myco. johnei, intraperitoneally or subcutaneously, guinea-pigs withstood the intraperitoneal inoculation of 100 to 150 mgm. of Myco. johnei; death, in 8 to 15 hours, followed the intraperitoneal inoculation of 60 to 80 mgm. of heat-killed tubercle bacilli. In spite of the allergic cross reactions between Myco. johnei and Myco. tuberculosis, inoculation with Myco. johnei did not confer immunity against tuberculosis.

Glover (1941a) experienced difficulty in sensitising guinea-pigs for comparing and standardising his johnins. The animals were best sensitised by the intraperitoneal inoculation of 1 ml. of a suspension in liquid paraffin of 30 mgm. per ml. of Myco. johnei, strain Teps. Such sensitised guinea-pigs reacted to 0.00012 to 0.000074 gm. of johnin P.P.D., compared with 0.0001 gm. in non-sensitised control animals.

Guinea-pigs were poorly sensitised by the intramuscular, intraperitoneal, intravenous, or subcutaneous, inoculation of aqueous suspensions of strain Teps. Occasionally a group of animals became sufficiently sensitised for experimental purposes, /

purposes, but reproduction of this sensitivity in successive groups was not possible. It should, of course, be remembered that guinea-pigs have not so far been shown to be susceptible to infection with Myco. johnei.

Opinion is divided whether johnin or avian tuberculin is more reliable for detecting Johne's disease (Hagan & Zeissig, 1928-29). It is true that some infected animals react well to avian tuberculin, yet no relationship has been shown to exist between Myco. johnei and Myco. tuberculosis var. avium other than that both are mycobacteria. However, when investigating the specificity relationships of mycobacterial P.P.Ds., Green (1946) found that there is a fairly close relationship, as judged by reactions in the guinea-pig, between avian tuberculin P.P.D. and johnin P.P.D. He defined specificity factor as "the number of weight units of heterologous P.P.D. required to elicit the same intensity of skin reaction as one unit of homologous P.P.D.", and showed that, in guinea-pigs sensitised with a suspension of Myco. johnei in oil, one unit of johnin P.P.D. was equivalent to 3 units of avian tuberculin P.P.D. and to 10 of human and bovine tuberculin P.P.Ds. In guinea-pigs sensitised with Myco. tuberculosis var. avium, one unit of avian tuberculin P.P.D. was equivalent to 3 units of johnin P.P.D., and to 20 of human and 40 of bovine tuberculin P.P.Ds. These figures at least provide one explanation for the value of/  
of/

of avian tuberculin in the diagnosis of Johne's disease.

Vaccination. The method of vaccination against Johne's disease proposed by Vallée & Rinjard (1926) and Vallée et al (1934a) stems from the basic conception of Calmette that in mycobacterial diseases only live vaccines can afford immunity. It is based on a method originally recommended by Vallée (1924) for vaccination against tuberculosis, and consists of the subcutaneous inoculation into unweaned calves of 5 to 10 mgm. of live, dried Myco. johnei suspended in a non-resorbable excipient - 1 ml. of olive oil, 1 ml. of vaseline oil, and 10 to 20 mgm. of inert powder (pumice preferably, or sandstone). At a later date (Vallée et al, 1941) 2 vaccines were used; in one the organisms were resuspended in their culture fluid and thus incorporated into the excipient, and in the other they were incorporated into the excipient together with the vacuum-dried synthetic culture fluid in which they had been grown to exhaustion. The vaccine is inoculated subcutaneously into the neck and produces an inflammatory reaction which is gradually replaced by a cold, sclerotic lesion. Post mortem, the lesion, adhering to the skin and adjacent tissues, is sclerotic at the periphery, caseous internally, and resembles a tuberculous focus; it weighs 150 to 200 gm. The oily mass of the inoculum is in the centre and at the periphery there are numerous caseous/

caseous tubercles, about 0.5 cm. in diameter and rich in organisms. The whole is enclosed in a sclerotic network which isolates it from the surrounding tissue.

The harmless nature of the vaccine was demonstrated before field trials were undertaken (Vallée et al, 1934b): 16 calves not reacting to johnin were inoculated and remained well. After a year's field trial, in which 277 cattle were vaccinated and 90 were left unvaccinated as controls, only one vaccinated animal had to be killed because of Johne's disease; several control animals contracted the infection.

Immunity was thought to last as long as the vaccination lesion persisted but Vallée et al, (1941) later considered that immunity survives the local lesion, as judged by results in endemic areas: they believed that this was due to the dissemination, throughout the host, of small particles of the original inoculum, in which the organisms are protected by oil. They agreed also with the opinion of Laporte (1940) that the oil component of their vaccine enhanced the antibody response to Myco. johnei.

Vaccination is said to afford protection for 8 to 12 months, occasionally 24 months, and in exceptional cases 72 months; throughout this period vaccinated animals react to johnin, but to neither human nor bovine tuberculin. By 1941, 250,000 cattle had been vaccinated.

In the United States, Hagan (1935) repeated the experiments/

experiments of the French workers. His vaccine, originally 50 mgm. of Myco. johnei suspended in 1 ml. of liquid paraffin, was injected subcutaneously into the neck where it produced large tumours. In subsequent experiments, the same mass of organisms was suspended in 5 ml. of saline and the calves were vaccinated at intervals of 6 months. The results were not so encouraging as those reported from France, but Hagan concluded that the method was probably useful. The French vaccine was recently studied by Larsen (1950) who confirmed both its efficacy and the duration of protection it gave.

In this country Doyle (1945) used a non-exacting strain of Myco. johnei (see page 43); in cattle and goats inoculated subcutaneously with 30 mgm. of organisms suspended in liquid paraffin, there was no trace of Johne's disease 32 months later at post mortem. He considered that the method merited a trial and suggested vaccination 3 or 4 times at intervals of 12 to 15 months. Unlike the French workers, he found that vaccination interfered with the tuberculin reaction and suggested that this was possibly because of the increase in potency of tuberculins since 1926. Vaccination against Johne's disease (Report, 1946) was initiated jointly by the Agricultural Research Council and by the Ministry of Agriculture and Fisheries. The vaccine, "a suspension of living Myco. johnei in pumice and oil", was to be inoculated subcutaneously in 2 ml. amounts into calves less than one month old and the animals were to be vaccinated 12 to 18 months later. Herds were accepted for the experiment if/

if Johne's disease was well established therein and if recruitment was from calves born and reared in the herd; comparative tuberculin tests were to be done before vaccination. The results have not yet been evaluated.

For the vaccination of sheep Sigurdsson & Tryggvadóttir (1949, 1950) prepared a vaccine consisting of heat-killed Myco. johnei suspended in mineral oil. Two strains of organisms were used; one of them, Teps, although not requiring the growth factor (see page 47), was grown in its presence. Each vaccinated lamb received 2 ml. of mineral oil containing 0.3 per cent phenol and 50 mgm. dry weight of organisms, and 2 years later, 1 ml. containing 5 mgm. of organisms. The experiment lasted 3½ years, 289 lambs were vaccinated, 266 were left unvaccinated as controls, and all animals were autopsied.

During the experiment 16 unvaccinated animals and no vaccinated animals died of Johne's disease; at post-mortem examinations on the survivors, lesions of Johne's disease were found in 21 unvaccinated animals and in 4 vaccinated animals. According to Sigurdsson (1952), "both these differences are statistically highly significant".

#### MYCOBACTERIUM JOHNEI

Description. According to Wilson & Miles (1946, page 441), Myco. johnei is a "short, thick acid-alcohol-fast bacillus,/"

bacillus, 1 to 2  $\mu$  long, generally straight, with rounded ends and parallel or slightly bulging sides. It is non-motile, Gram-positive, and generally stains uniformly but may occasionally be granular."

"On Dorset's egg medium, containing 4 per cent of glycerol <sup>and 1 per cent of dead ground Myco. johnei</sup> primary cultures of Johne's bacillus consist of tiny, dull-white colonies, rarely visible to the naked eye in less than 4 weeks; they are more or less circular in shape and may be discrete or confluent. As they grow older, they increase in size, become more elevated, and turn a dull yellowish-white colour; the edges remain thin and from them numerous irregular striations rise towards the central peak. In subcultures the growth is more copious, more confluent, and may be slightly wrinkled".

Isolation. Myco. johnei can generally be isolated from the faeces, rectal mucous membrane, intestinal mucosa, and mesenteric lymph nodes of infected cattle. In advanced cases, the mucosal layers and lymph nodes, the commonest sources of Myco. johnei, may contain innumerable organisms. Their isolation, on occasion quite simple, presents a problem in many respects similar to that involved in isolating Myco. tuberculosis.

The earliest method of isolation, that of Twort & Ingram (1911-12), involved washing the surface of an infected lymph/

lymph gland or section of intestine with running water, searing it with a hot spatula, and removing from beneath the seared surface, with sterile scissors, a piece of tissue, which is smeared over the surface of media. More generally, infected intestinal mucosa is exposed to a disinfectant which is lethal for contaminating organisms, and which usually destroys the tissue where the mycobacteria are located: antiformin, sodium hydroxide, sulphuric acid, and oxalic acid, are used for this purpose, the last named having the advantage that, after treatment, infected material can be sown straight on to media. A reagent not now in use, ericolin (Twort, 1909), was used by Twort & Ingram.

Minett (1942) studied the effect of antiformin, sodium hydroxide, and sulphuric acid, on normal, infected, and mixtures of normal and infected, intestinal mucosa; he also exposed infected intestinal mucosa to papain or to trypsin, then to antiformin. Since these appear to be the only experiments designed to determine the best disinfectant for sterilising normal and infected intestinal mucosa, and to observe its action on Myco. johnei, they may, with advantage, be described in detail.

A saline suspension of normal, lightly washed, bovine intestinal mucosa was sterilised, in 5 to 30 minutes at room temperature, by exposure to either 10 or 20 per cent antiformin; 0.5/



0.5 to 1.5 N-sodium hydroxide was unreliable in its action, even after 3 hours, and 1.2 N-sulphuric acid was ineffective in under 2 hours.

In a second experiment, serial tenfold dilutions, to  $10^{-4}$ , were made in saline of a saline suspension of well-washed, heavily infected, bovine intestinal mucosa; to one volume of each dilution were added 9 volumes of a saline suspension of normal bovine intestinal mucosa. The mixtures were exposed to 10 per cent antiformin for 20 minutes at room temperature, and to 1.0 N-sulphuric acid for 3 hours at  $37^{\circ}\text{C}$ . Myco. johnei was cultured from all dilutions; a few tubes were contaminated. Exposure to 1.0 N-sodium hydroxide, for 3 hours at  $37^{\circ}\text{C}$ , did not eliminate contaminants; in a later experiment, exposure for 18 hours at  $37^{\circ}\text{C}$  killed all contaminants and allowed growth of Myco. johnei; the same treatment sterilised a suspension of Myco. johnei in serum.

In one experiment, Minett found that in infected tissue Myco. johnei withstood exposure to 1.0 N-sodium hydroxide for 42 hours at  $37^{\circ}\text{C}$ , but not to 1.0 N-sulphuric acid for more than one hour at  $37^{\circ}\text{C}$  (but see above). He concluded from this that sulphuric acid is more lethal for Myco. johnei in infected tissue than is sodium hydroxide.

When lightly infected intestinal mucosa was exposed overnight/

overnight at 37°C, either to papain powder (5 per cent), or to trypsin (Allen & Hanbury, 10 per cent, or trypsin powder 0.15 per cent) and then to 15 per cent or 20 per cent antiformin, no growth of Myco. johnei was obtained. In controls exposed only to antiformin, growth of Myco. johnei was obtained.

Oxalic acid, introduced for the isolation of Myco. tuberculosis by Corper & Uyei (1929-30), is considered by Wilson Taylor (1950) to be the most satisfactory reagent for the isolation of Myco. johnei from infected intestinal mucosa. In his method for the isolation of Myco. johnei, a mixture of about 1 gm. of infected mucosa and 10 ml. of 5 per cent oxalic acid is ground in a mortar with sterile sand, the resulting suspension allowed to stand for 10 minutes, and the supernatant filtered through 2 thicknesses of cheese-cloth into a sterile container which is placed for 30 minutes in a water-bath at 37°C. The container is then centrifuged, the supernatant discarded, and the sediment sown on to culture medium.

From the clinical point of view, as an aid to diagnosis, the isolation of Myco. johnei from faeces would be very useful. However, the problem is rather complex, and in a review of the existing methods Cauchemez & Sargent (1934) concluded that, apart from the initial difficulty of concentrating Myco. johnei in faeces, there is no generally acceptable/

acceptable method for its isolation therefrom. The difficulty is that disinfectants such as antiformin, oxalic acid, sodium hydroxide, and sulphuric acid, will kill in a short time all bacteria in faeces other than acid-alcohol-fast bacilli, spores, and certain fungi. On the other hand, if the exposure is lengthened to kill the spores and fungi, the acid-alcohol-fast bacilli may also be killed.

Antiformin was used by Levi (1948), who isolated Myco. johnei from 81 of 91 specimens of faeces. He suspended 0.5 to 1.0 gm. of faeces in distilled water, filtered the suspension through muslin, and allowed the filtrate to sediment for 10 to 15 minutes; 1.5 to 2.0 ml. aliquots of the supernatant were then exposed to 10, 15, or 20 per cent antiformin, at room temperature for 20 to 30 minutes. Thereafter the mixtures were diluted with sterile distilled water, centrifuged at 2000 r.p.m. for 15 minutes, and the sediments cultured on Dunkin's medium (Minett, 1942).

Wilson Taylor (1950) considered treatment with antiformin to be "the method of choice", at the same time remarking that "there is at present no completely reliable method of obtaining Myco. johnei in culture from infected bovine faeces, particularly from samples in which they may be present in very small numbers". However, Myco. johnei has been isolated from infected faeces both by Glover (1952a), who/

who did not specify his method, and by Wilson Taylor (1953) who used antiformin.

Cultivation and Biochemical Characters. Two

mycobacteria, the avian tubercle bacillus and Myco. phlei, suggested themselves to Twort & Ingram (1911-12) as sources of growth factor, the former because of the reactions to avian tuberculin in cattle with Johne's disease and the latter because they felt that "it may be the wild ancestor and originator of Johne's bacillus". Myco. phlei proved to be a better source of growth factor than the human tubercle bacillus, but the avian tubercle bacillus was a poor one. These workers later showed that Grassberger's butter bacillus, Karlinski's Nasenschleim bacillus, Marpmann's urine bacillus, and the Pseudoperlsucht and smegma bacilli of Moeller, all support the growth of Myco. johnei. They thought that the avian and bovine tubercle bacilli were of no practical value but their opinion was challenged by McFadyean, Sheather, & Edwards (1912), who suggested that, if anything, the avian tubercle bacillus was the best source of growth factor and the bovine a good one.

Growth-factor activity, freed from bacterial cells, is required in media for the preparation of johnins and is conveniently prepared by the method of Boquet (1928). This consists/

consists of suspending, in 300 ml. of saline containing 20 per cent of glycerol, 10 gm. of dry Myco. phlei from a 2-week-old 4 per cent glycerol-broth culture, and autoclaving the suspension for an hour at 120°C. The suspension is then allowed to stand for several hours and the supernatant used at a final concentration of 10 per cent. The supernatant can be further freed from bacillary bodies by centrifuging; there is still some activity in the bacterial residue (Twort & Ingram, 1911-12). Glover (1952b), without specifying the mass of organisms extracted, used the supernatant from a 20 per cent glycerol-saline extract of Myco. phlei.

Numerous media, some very complex, have been devised for the growth of Myco. johnei; they generally contain a large proportion of egg and for further information the paper of Minett (1942) should be consulted. The feature common to them all is the presence of the growth factor - Myco. phlei, or an extract prepared from it - and provided this is present, even simpler media give excellent results (Boquet, 1927b, 1928). The optimum amounts of egg-yolk - 37 per cent - and of glycerol - 4 per cent - were discussed in a recent short paper by Wilson Taylor (1950). Diffuse growth of Myco. johnei was obtained by Glover (1952b, c) and by Williams Smith (1953) in fluid media of the Dubos type (Dubos, 1945, 1946; Dubos & Davis, 1946; Davis & Dubos, 1946, 1947; Davis, 1947; Davis & Dubos, 1948; Dubos & Middlebrook, 1948;).

The/

The cultivation of Myco. johnei on the surface of a fluid medium is not easy. Bang (1914) gave up trying to obtain surface cultures because they no sooner became the size of a penny than they sank. Boquet (1928) grew his strains on potato slopes partly immersed in Sauton fluid (1912) containing the growth factor; when a thin surface film had formed over the potato and the fluid in which it was resting, the film was used to inoculate flasks of the same fluid medium. He recounted no difficulties in obtaining surface films. Strain Teps, which requires no growth factor (see below and Dunkin, 1928, 1933;) and which has been used in this country since 1933 for the preparation of johnin, grows readily on the surface of a chemically defined fluid medium. Some strains of Myco. johnei grow as a surface film more easily than others, and it is noteworthy that the former generally do not require the growth factor.

While it is true that the classical or typical Myco. johnei requires the growth factor, there are many reports of strains of Myco. johnei capable of growing without the addition of the growth factor (Twort & Ingram, 1912b; Twort, 1914; Dunkin, 1933; Knight, 1936; McIntosh, 1939; Boquet, 1939; Johnson, 1944b; Doyle, 1945; Allen, 1952a; Spears, 1953;). Such strains can be grown on chemically defined media and are described as non-exacting. They are used for the production of johnin (Allen, 1953), to sensitise small animals for skin tests/

tests (Glover, 1941b; Johnson & Cox, 1942;), and in different methods of vaccination (Doyle, 1945; Sigurdsson & Tryggvadóttir, 1949, 1950;). Although it is not known whether they are pathogenic for cattle, Twort (1914) infected rabbits, rats, and mice, with a non-exacting strain; in our experience of 2 of these strains one, M.143 (Allen, 1952a) is capable of infecting the mouse, and the other, Teps, appears to be non-pathogenic for the mouse.

Some workers believe that on prolonged culture, strains of Myco. johnei become non-exacting. In a chemically defined fluid medium without growth factor Allen (1952b) observed growth of one strain of Myco. johnei which in the previous passage was growing on full medium.

The review by Boquet (1928) contains practically all the available information on the biochemical properties of Myco. johnei. It is strictly aerobic with an optimum temperature of 39 to 40°C; for abundant growth it is necessary to subculture at intervals of 4 to 5 weeks, growth reaching its maximum by the 5th or 6th passage, and not increasing with further passages. Unlike other mycobacteria, it can survive 6 months in the incubator without subculture; Hagan & Zeissig (1933b) extended this period to more than 12 months, and our own observations show that some strains can be subcultured after 29 months.

The/

The optimum pH is not known: Boquet found that in Sauton fluid the reaction declined from about pH 7.0 to pH 4.9 over 8 to 10 weeks; in egg media, Minett (1942) found that pH 6.3 was the optimum for the commencement of growth, and that subsequently alkalinity was produced. Presumably both are correct, depending on the strain of Myco. johnei.

Asparagine, which is usually the main source of nitrogen for mycobacteria grown in chemically defined media, can be replaced by leucine, but not by alanine or aspartic acid; inorganic nitrogen cannot be utilised. Boquet considered the mineral ingredients, magnesium sulphate and dipotassium phosphate, to be indispensable as well as a small amount of ferric ammonium citrate or ferric or manganese lactate.

Glycerol, as with other mycobacteria, enhances the growth of Myco. johnei: Boquet and Wilson Taylor (1950) both agree that the optimum concentration is 4 per cent, the value found also by Johnson (1944b) for non-exacting strains. Glucose at the same concentration cannot substitute for glycerol.

The viability of Myco. johnei under various conditions was investigated by Vishnevski, Mamatsev, Chernyshev, & Chernyshev (1940). An emulsion of 3 mgm. per ml. of Myco. johnei and strips of filter paper soaked in the emulsion were both/



both placed in sealed, glass containers and exposed to direct and indirect sunlight; 10 months later, Myco. johnei was recovered from both containers. A saline suspension in 10 to 50 per cent glycerol also contained viable organisms after 10 months.

The same authors found that a 10 to 20 per cent solution of chlorinated lime destroyed Myco. johnei in 10 minutes, 5 per cent lysol in 5 to 10 minutes, 0.2 per cent corrosive sublimate in 10 minutes, 5 per cent creolin in 2 hours, and 5 per cent sodium or potassium hydroxide in 24 hours. Rivanol and potassium permanganate from 0.05 to 0.8 per cent were ineffective.

Several antibiotics active against Myco. tuberculosis have been tested in vitro against Myco. johnei. Hauduroy & Rosset (1948) found that, in a Dubos type medium, 46 of 58 strains of Myco. johnei were sensitive to 10  $\mu\text{gm.}$  per ml. of streptomycin. Three strains were resistant to 1000  $\mu\text{gm.}$  per ml. but were sensitive to 10,000  $\mu\text{gm.}$  per ml. and mutants of 2 of these strains were less sensitive than the parent strains. Streptomycin, promin, 4-amino salicylic acid, stilbamidine di-isethionate, and pentamidine isethionate, were examined by Larsen et al (1950), who concluded that in low concentrations - about 1  $\mu\text{gm.}$  per ml. - only streptomycin was inhibitory to Myco. johnei. Later, Larsen & Vardaman (1952) found viomycin to be inhibitory at 8  $\mu\text{gm.}$  per ml.; 4 thiosemicarbazones/

thiosemicarbazones, 4-hydroxy benzaldehyde thiosemicarbazone, 4-isobutoxy benzaldehyde thiosemicarbazone, 4-formyl acetanilide, 4-isobutyl 3-thiosemicarbazone, and 4-formyl acetanilide thiosemicarbazone, were inactive below 150  $\mu$ gm. per ml. Iso-nicotinic acid hydrazide was shown by Larsen & Vardaman (1953) to inhibit Myco. johnei at 25  $\mu$ gm. per ml., but, according to Deans Rankin (1953), not at 10  $\mu$ gm. per ml.

The Growth Factor. It seems strange, 43 years after the demonstration of the growth-promoting property of Myco. phlei and of other mycobacteria for Myco. johnei, that the growth factor is still unidentified. In this time the existence and identity of various growth factors has been ascertained; yet, although several properties of this particular growth factor are known, only recently (Francis, Madinaveitia, Macturk, & Snow, 1949; Francis, Macturk, Madinaveitia, & Snow, 1953;) has any attempt been made to identify it and none to determine its role in the metabolism of Myco. johnei.

In addition to mycobacteria, human hair, rat skin and hair, honey, tree barks, seaweeds, wood shavings, flowers, fruits, roots, vegetables, seeds, spices, streptomycetaceae, yeasts, fungi, and wines, were investigated by Twort & Ingram (1914) for growth-factor activity; glycerinic (glyceric?) acid, of numerous chemicals examined (Twort & Ingram, /

Ingram, 1913a), and extracts of such diverse materials as linseed, barley, wheat, maize, oats, dairi, culinary peas, physostigma beans, fig seeds, tamarinds, dried currants (grapes), figs, muscatels, and a mushroom, Cantharellus aurantiacus, all showed a small degree of activity but none was so active as Myco. phlei or its extracts.

Twort & Ingram (1911-12) also found that the growth-factor activity of Myco. phlei depends on the conditions under which it is grown: organisms grown for 2 weeks on glycerol-broth or on glycerol-agar had good growth promoting activity, whereas organisms grown for 4 weeks on glycerol-liver-broth had no activity; when grown on Dorset's egg medium, Myco. phlei had only moderate activity. It was found by Woolley & McCarter (1940) that Myco. phlei grown on a chemically defined fluid medium supported the growth of Myco. johnnei, a finding which Francis et al (1953) failed to confirm.

All the growth-promoting activity, after extracting Myco. phlei in a Soxhlet apparatus for 3 hours with ethanol, is contained in the extract (Twort & Ingram, 1913b); the white precipitate which forms on cooling contains a little activity, but the activity is mainly found in the clear supernatant which, on evaporation, yields a tacky residue partly soluble in chloroform. Most of the activity is contained in the chloroform-insoluble fraction; this fraction is soluble in water and a little activity contained in the chloroform-soluble fraction/

fraction can be extracted therefrom with water. The growth factor can also be extracted from mycobacteria with chloroform and methanol (Twort & Ingram, 1914). According to Boquet (1928) acetone at 37°C can extract some activity from both Myco. phlei and Myco. tuberculosis, the latter extract having more activity; in both cases there is activity in the bacterial residue.

Although the growth factor appears to be water-soluble, there is doubt whether it can be extracted directly from organisms with water. Twort & Ingram (1911-12) found that extraction with water for 30 minutes at 120°C did not remove any activity from the cells, whereas Woolley & McCarter (1940) found that activity could be extracted with boiling water.

For the preparation of media, most workers either extract the growth factor at 120°C with glycerol-saline mixtures or incorporate bacterial bodies previously steamed in their culture fluid. The high temperature and the use of glycerol as solvent are probably responsible for the extraction of the growth factor.

The growth of a non-exacting strain of Myco. johnei (see page 43) was found by Woolley & McCarter (1940) to be stimulated by the 2 anti-haemorrhagic compounds of the vitamin-K group, phthiocol and 2-methyl naphthaquinone.

Phthiocol/

Phthiocol, at a concentration of 1  $\mu$ gm. per ml. of medium, was almost as active as 0.4 per cent Myco. phlei in promoting growth. It should perhaps be mentioned that Twort & Ingram (1913b) sometimes found that the addition to media of ethanolic extracts of Myco. phlei, equivalent to 0.25 to 0.5 per cent of Myco. phlei, did not support the growth of Myco. johnei; however, they found that if the level of Myco. phlei, as represented by the ethanolic extract, was raised, growth was obtained, whereas Woolley & McCarter found that a higher concentration of phthiocol was inhibitory. It may also be relevant, because for their experiments Woolley & McCarter grew Myco. phlei on a chemically defined medium, that Myco. phlei grown on such a medium was found by Francis et al (1953) to be devoid of growth-factor activity.

In another experiment with the same non-exacting strain Woolley & McCarter found that 2 methyl-naphthaquinone at a concentration of 0.1  $\mu$ gm. per ml. was more active than a 30-times concentrated Myco. phlei culture fluid at a concentration of 0.05 ml. per ml. Francis et al (1949, 1953) were unable to obtain growth of Myco. johnei either with 0.05 to 50  $\mu$ gm. per ml. of phthiocol or with 1 to 100  $\mu$ gm. per ml. of 2 methyl naphthaquinone, whereas an acetone extract of Myco. phlei allowed full growth. They also isolated from Myco. tuberculosis a substance resembling the vitamins K:/

vitamins K: it failed to support the growth of Myco. johnei on egg medium.

Woolley & McCarter themselves admitted that growth was better with an extract of Myco. phlei than with the 2 anti-haemorrhagic compounds and they suggested that Myco. phlei probably supplies some additional factor. It must be emphasised that since their work was done with a non-exacting strain, no proof was brought of the identity of vitamin K and the growth factor; nevertheless, it is from this paper that their alleged identity arose.

A compound, capable of promoting vigorous growth of Myco. johnei and, in the opinion of its discoverers, quite unrelated to the naphthaquinones, was isolated from Myco. phlei by Francis et al (1949, 1953): they propose to call it mycobactin. It was obtained as a colourless, crystalline, aluminium derivative, m.p. 216.5 to 217°C, and as an aluminium-free, amorphous powder, m.p. 165 to 166.5°C, of the empirical formula  $C_{47}H_{75}O_{10}N_5$ . Mycobactin has an absorption spectrum in methanol with maxima at 250 and 310 m $\mu$ . and a characteristic shoulder at 258 m $\mu$ . It is stable to heat up to 150°C and is not oxidised in air; it dissolves in solutions of caustic alkalis but not of sodium bicarbonate. Alkaline solutions rapidly decompose, even at 0°C, with loss of growth promoting activity. It does not dissolve in dilute mineral/

mineral acids but dissolves in concentrated sulphuric acid without immediate decomposition, and has a slight solubility in concentrated hydrochloric acid; warming with mineral acids causes inactivation. It is weakly basic and has a tendency to chelation with metals; complexes with aluminium and with copper have been isolated. Since the methods of isolation are rather complex, and since the data on properties and derivatives are of a specialised nature, they will not be further discussed. Mycobactin was estimated to be present in Myco. phlei at a concentration of 3 mgm. per gm. of wet weight; the optimum concentration for the growth of Myco. johnei was estimated to be 40 to 80  $\mu$ gm. of mycobactin per ml. of medium.

As will be realised, there is much conflicting information on the properties of the growth factor. Thus, according to Twort & Ingram (1911-12), the growth factor cannot be extracted directly from Myco. phlei with water at 120°C; according to Woolley & McCarter (1940) it can. All workers agree that it can be extracted by autoclaving Myco. phlei with glycerol-saline mixtures; it is also agreed that it can be partly, or wholly, extracted with acetone, ethanol, and methanol (Boquet, 1928; Woolley & McCarter, 1940; Francis et al., 1953; Williams Smith, 1953;). On the other hand, when these solvents were removed - by distillation - the growth factor in the residue was found by Twort/

Twort & Ingram (1911-12) to be only partly soluble in chloroform and by Woolley & McCarter (1940) to be soluble in ether; in contrast to these findings, Twort & Ingram (1914) found that it could be extracted from Myco. phlei with chloroform, and according to Williams Smith (1953) not with ether. The latter was also unable to extract it from cells with chloroform. Other solvents in which the growth factor was found to be soluble are water (Twort & Ingram, 1911-12; Boquet, 1928;), petroleum ether (Boquet, 1928), glacial acetic acid, light petroleum, pyridine, and xylene (Francis et al, 1953). In one of their methods for isolating the growth factor, Francis et al (1953) found that the addition of light petroleum to the concentrated acetone extracts of Myco. phlei gave a precipitate which contained all the activity; water, according to Twort & Ingram (1911-12) and Boquet (1928) a solvent for the growth factor, was used to remove "further inactive materials" from the light petroleum precipitate. The empirical formula,  $C_{47}H_{75}O_{10}N_5$ , which Francis and his co-workers assigned to the growth factor, hardly suggests a water soluble compound. What information there is seems, in fact, to suggest that there may be more than one substance in Myco. phlei capable of supporting the growth of Myco. johnei.

EXPERIMENTAL/



EXPERIMENTAL INFECTION OF LABORATORY ANIMALS

The earliest attempts to produce Johne's disease in laboratory animals were bedevilled by the inability to cultivate Myco. johnei and by the confusion between Johne's disease and tuberculosis, arising from the original publication of Johne & Frothingham (1895).

There are many reports in the literature of the inability of Myco. johnei to produce an infection in small laboratory animals; among the animals used were cats, chickens, fowls, guinea-pigs, hamsters, pigeons, rabbits, and rats. On the other hand, several authors were successful in producing one or other of 2 types of infection in laboratory animals, one having little in common with Johne's disease, and the other resembling in many respects the natural infection. The latter group of reports has not been generally accepted, and there is a general belief, fostered by statements in text-books (Wilson & Miles, 1946; Breed, Murray, & Parker Hitchens, 1948; Browning & Mackie, 1948; Mackie & McCartney, 1953;), that Johne's disease cannot be reproduced in laboratory animals.

The first to kill, if not to infect, laboratory animals with Myco. johnei, appear to have been Twort & Craig (1913). Their results are difficult to interpret and the death of the animals was possibly an allergic phenomenon.

They/

They inoculated rabbits of unstated age intravenously with 30 to 120 mgm. moist weight of bacilli at intervals of 3 to 5 days, with sometimes a 3rd dose 4 to 6 weeks later, or the 2nd or 3rd dose 15 to 30 days later. When 2 doses were given within 5 days, no ill effects were observed, but when doses were separated by more than 15 days, the animals became emaciated and died; at post mortem, the authors found masses of bacilli, intracellularly and extracellularly in the lungs. They found that Myco. johnei disappeared from the lungs 15 days after the last inoculation and persisted intracellularly in the spleen for 30 days, while in the intestine there were neither acid-alcohol-fast bacilli nor pathological changes at any time. The authors considered that since the organisms are rapidly absorbed by the liver, and only a small number are excreted by the kidney, it was possible that many might pass through the bile ducts into the intestine and be excreted with the faeces: organisms were indeed cultivated from the bile.

Intraperitoneal inoculation of 100 to 120 mgm. of bacilli produced no ill effects, regardless of the interval between inoculations. Several weeks after inoculation well stained organisms could be seen inside the phagocytes, and 2 or 3 months later, nodule formation was observed in the peritoneal cavity. These nodules, similar to tuberculous lesions/

lesions, varied in size "from a match head to a bean", Abdominal lymphatic glands showed necrotic changes similar to those caused by members of the acid-alcohol-fast group of organisms.

Subcutaneous inoculation produced caseous abscesses persisting for "a great length of time", the most noticeable feature being the resistance of the bacilli to destruction. Dead bacilli were equally resistant to destruction.

Comparing the effects of Myco. johnei with those of 12 other acid-alcohol-fast bacilli, the authors concluded that the commonest pathological condition caused by live or dead acid-alcohol-fast bacilli is the presence, in the peritoneal cavity, of caseous masses which apparently cause no inconvenience.

Boquet (1925) found that in rats a single intraperitoneal inoculation of 5 to 10 mgm. of Myco. johnei produced an infection involving the peritoneal cavity and the tracheo-bronchial glands. Greyish, pin-head nodules in the peritoneal cavity and on the omentum contained pus, very rich in bacilli; the mesenteric ganglions were hypertrophied. The other abdominal organs showed no macroscopic lesions, although the spleen and liver contained large numbers of organisms; the tracheo-bronchial glands, as large as a grain of wheat, were hard and sclerotic and smears showed large numbers of bacilli in/

in typical groups and masses; there were no organisms in the lungs.

Repeated intraperitoneal inoculations of 10 to 30 mgm. of Myco. johnei at intervals of 15 to 20 days gave rise to disseminated, peritoneal tubercles and nodules on the stomach wall, small intestine, liver, and diaphragm. Hypertrophy of the tracheo-bronchial glands was more marked, smears again showing large numbers of bacilli. The liver and spleen, apparently normal, also contained large numbers of bacilli; there were fewer in the mesenteric ganglion. The kidneys and lungs were not involved and the small intestine appeared undamaged, but organisms were present both in the mucosa and in the intestinal wall.

Following intra-testicular inoculation of 5 to 10 mgm., there was an inflammatory swelling of the organ and a small abscess formed in the paracenchyma, where the organisms multiplied. The infection then spread to the peritoneum, with nodule formation, and to the liver, spleen, and tracheo-bronchial glands.

The histological changes, described by Morin (1925), consisted of an epithelioid-cell proliferation in the lymphatic tissue, and central caseation with few giant cells in the sub-peritoneal tissue, the lesions on the whole resembling those of leprosy.

tissue/

Tissue containing bacilli produced the same lesions as suspensions of organisms. Nothing was observed after either subcutaneous or intradermal inoculation, or after the feeding of Myco. johnei, with or without Myco. phlei, 4 to 5 times a day every 2 or 3 days.

Boquet compared the pathogenicity of Myco. johnei for the rat to that of a low-virulence tubercle bacillus, the former being characterised by its greater affinity for the lymphatic glands.

The white mouse he found less sensitive than the rat; 5 to 10 mgm. Myco. johnei intraperitoneally gave rise to nodules on the serous coat and hypertrophy of the tracheo-bronchial glands; the liver and spleen were unaffected.

Hagan & Mansfield (1930) inoculated guinea-pigs with large doses of cultures and of infected intestinal mucosa, and killed them 3 weeks to 6 months later. After intraperitoneal inoculation, lesions were found in the peritoneal cavity, mainly near the free end of the longer omentum; in some of the male animals there were lesions in the scrotal sac. Occasionally there were small lesions on the diaphragmatic surface of the liver, on the surface of the spleen, and of the intestine. In the omentum there were either nodules or diffuse thickenings, the nodules ranging in size from pin-point to 6 to 8 mm: the diffuse thickenings were several centimetres long and nearly 1 cm. thick. With smaller doses/

doses the lesions were similar but there was less pus and less necrosis. Intramuscular inoculation was without effect; subcutaneous inoculation occasionally produced the same type of lesions as intraperitoneal inoculation: this was thought to have arisen because the needle, in the course of the subcutaneous inoculation, entered the peritoneal cavity.

Myco. johnei was not recovered from the lesions. Since similar lesions were obtained with heat-killed Myco. johnei, heat-killed Myco. tuberculosis, and non-pathogenic mycobacteria (grass bacilli), the authors considered that they were formed without multiplication of the organisms. It was their opinion that "the lesions are undoubtedly identical to those which were seen by Twort in rabbits and Boquet in white mice and rats, injected intraperitoneally with heavy doses of Myco. johnei".

An altogether different approach was that of Mohler (1939), who inoculated guinea-pigs, rabbits, rats, and mice, with Myco. johnei suspended in liquid paraffin, the inoculation killing the animals with unfailling regularity. The animals developed the clinical symptoms of Johne's disease, diarrhoea and emaciation, and lesions of unspecified nature were found in the intestine. Infected goat faeces, suspended in liquid paraffin, produced a similar result in guinea-pigs. Successful passages, requiring no liquid paraffin as excipient/

excipient, were made with the organs of infected animals. An instance of intra-uterine infection in guinea-pigs was also reported. Mohler's findings were weakened by the fact that he described small foci and nodules of various sizes on the liver surface, spleen, lungs, and diaphragm, and enlargement of some lymph glands as "typical lesions of Johne's disease". These by no means conform to the lesions of Johne's disease in cattle (see page 4).

That mycobacteria, dead or alive, suspended in an oil or fat menstruum, can produce lesions in the peritoneal cavity and lungs very similar to those observed by Mohler was first shown by Rabinowitsch (1897, 1900): the vehicle in this case was butter. Hagan & Levene (1932), and Laporte (1940), showed that liquid paraffin acts in a similar manner.

Confirmation of Mohler's results was not forthcoming; Wilson Taylor (1950) conceded that suspension of Myco.johneii in liquid paraffin could be fatal for guinea-pigs, while Sahai (1940, 1941) and Levi (1941), confirming the existence of peritoneal lesions, pointed out, as Rabinowitsch had done previously, that similar lesions can be produced by live and dead mycobacteria.

From their vaccination studies on Johne's disease, Vallée et al (1941) concluded that their strain of Myco. johnei suspended in liquid paraffin was harmless when inoculated intraperitoneally into guinea-pigs.

It seems fairly certain that the inoculation of Myco. johnei suspended in liquid paraffin can give rise to extensive lesions in the peritoneal cavity and even in more distant organs. Glover (1941a, b) used suspensions of Myco. johnei in liquid paraffin for sensitising guinea-pigs which were then used in the standardisation of johnins. After repeated skin tests, some of these animals died, and at post mortem the peritoneal cavity frequently contained a large amount of a clear, ascitic fluid. The mesentery was thickened and loops of small intestine, caught up in the intense inflammatory reaction, were adhering either to one another or to the abdominal wall. The liver and spleen were slightly enlarged and showed on their surface irregular fibrous bands or foci. The gastro-splenic omentum was always thickened, and beset with firm, yellow or white nodules, containing a thick caseo-pus very rich in acid-alcohol-fast bacilli, while firm, well circumscribed foci were seen in the liver and in the spleen. Occasionally the tracheo-bronchial glands were enlarged and haemorrhagic lesions were present in the lungs. Guinea-pigs inoculated with Myco. johnei suspended in liquid paraffin, but not subjected to skin tests, showed similar but less extensive lesions. Were a single injection of Myco. johnei in liquid paraffin to cause a progressive fatal disease, Glover's method could hardly be of value for sensitising animals.

In/



In the United States, Glover's findings were confirmed by Johnson & Cox (1942) who used "a mineral oil" as excipient. Their doses ranged from 200 mgm. moist weight of Myco. johnei in 2 ml. of mineral oil for rabbits, to 50 mgm. per 0.5 ml. for guinea-pigs; here again, the only deaths reported were in guinea-pigs and were from shock following the intradermal injection of 0.1 ml. of johnin.

It would appear that on the whole, when liquid paraffin is used as an excipient, Myco. johnei may cause lesions in experimental animals, which are neither commonly fatal nor specific. One point should certainly be borne in mind - namely, that liquid paraffins and mineral oils are not chemically defined substances and that brands may differ both in composition and in effect.

Alikaeva (1941) found that rabbits which received Myco. johnei orally or intravenously developed multiple caseous abscesses of joints and pleura and contracted kidneys; gastro-intestinal lesions were rare. Organisms were cultured from kidneys, liver, heart, and spleen.

An instance of presumably completely non-specific reaction was described by Meyn & Weiske (1943) who inoculated hens intramuscularly with Myco. johnei suspended in a mixture of kieselguhr, trypan blue, potassium iodide, and saline. There was a local reaction from which Myco. johnei was recovered, /

recovered, resuspended in the same mixture, and reinoculated into more hens. On the basis of these results the authors reported that the hen could be used as an experimental animal in studies on Johne's disease.

Cats and splenectomised hamsters were used by Johnson & Pratt (1944). Fourteen cats were fed with cultures of Myco. johnei and with infected intestinal mucosa and were killed 2 weeks later, which seems unusually early in view of the lengthy incubation period of the natural infection. The post-mortem examinations, however, revealed a thickening of the intestine, and, microscopically, a few epithelioid cells containing acid-alcohol-fast bacilli, and no giant cells. The hamsters were refractory. It is unfortunate that the cats were not kept alive for a longer period, a thought also expressed indirectly by the authors.

A comparison between groups of guinea-pigs, inoculated intraperitoneally or subcutaneously in the thigh with 5 mgm. of Myco. johnei suspended in liquid paraffin or saline, was made by Verlinde & Bekker (1945). The animals were killed up to 90 days after inoculation and in those inoculated with the saline suspension the commonest lesions were nodules (tubercles) in the liver, composed of epithelioid cells surrounded by a zone of lymphocytes. The animals had nodules in the spleen consisting of epithelioid tissue and giant cells.

After/

After subcutaneous inoculation of organisms suspended in liquid paraffin, half of the animals had liver nodules and one had nodules in the lungs; all showed inguinal lymph-node involvement, the granulation tissue consisting of epithelioid cells, giant cells, lymphocytes, and fibroblasts. Of 11 animals inoculated intraperitoneally with the suspension in oil, 4 died; 3 had nodules in the spleen, 8 had nodules in the liver, and 5, nodules in the lungs. There were no lesions suggestive of Johne's disease in any animal, nor could the lesions evoked by the subcutaneous or intraperitoneal inoculation of Myco. johnei in liquid paraffin be differentiated from those induced by Myco. bekkeri or by the bacillus Calmette-Guérin, similiarly inoculated.

The most recent report in the group of non-specific infections with Myco. johnei is that of Stavitsky and Beck (1946), who used 11-day-old developing chick embryos as experimental animals. Nine strains of Myco. johnei were used, all but one requiring the growth factor; they were inoculated, as saline suspensions of 0.05 to 0.5 mgm. per ml., on to the chorioallantoic membrane, or into the allantoic cavity, the yolk sac, or the allantoic venule; there were 2 control groups, opened uninoculated embryos and embryos inoculated with sterile saline.

The changes on the chorioallantoic membrane after the deposition of Myco. johnei were mainly proliferative. After incubation/

incubation, for 9 to 10 days, the embryos which received the typical strains of Myco. johnei were opened and the yolk sac, chorioallantoic membrane, liver, spleen, glandular stomach and small intestine examined histologically. There were no acid-alcohol-fast bacilli to be seen, nor were there microscopic changes in any tissue other than the liver, which was fatty. The non-exacting strain of Myco. johnei produced more intensive and extensive proliferative changes on the chorioallantois, reminiscent of the changes seen in the natural disease; the authors observed multiplication, and cultures of Myco. johnei were obtained. The lesions in sites other than the chorioallantois were indistinguishable from those produced by the typical strains of Myco. johnei; there was no multiplication of organisms.

As already mentioned, there are several reports of an infection in small laboratory animals, resembling in many respects the natural infection. The first is that of Twort & Ingram (1913a) who, in an addendum to their monograph, reported an intestinal infection in a rabbit which had received 2 intravenous inoculations of a non-exacting strain of Myco. johnei; the intestinal changes in the rabbit were similar to those found in cattle and sheep in the early stages of the disease.

Twort/

Twort (1913, 1914), also using a non-exacting strain, (presumably the same one) successfully infected rabbits, rats, and mice; because of their importance his results are given in detail.

<u>Route of Inoculation</u>	<u>Number of Animals</u>	<u>Number Infected</u>
Intravenous	11 Rabbits	5
Intraperitoneal	8 Rabbits	0
Intraperitoneal	28 Mice	5
Intraperitoneal	12 Rats	3
Intraperitoneal	6 Guinea-pigs	0
<u>Per os</u>	4 Rabbits	0
<u>Per os</u>	12 Mice	1

In rabbits the disease was limited to the ileo-caecal valve and about 30 cm. of the ileum; in some cases there were bacilli in the appendix and in the duodenum. In one animal the small intestine appeared to be thickened throughout its entire length; Myco. johnei was recovered from the intestine and from the mesenteric glands. There were numerous bacilli in the liver and spleen and considerable, disseminated, cellular reaction, consisting of small agglomerations of lymphocytes; the bacilli were intracellular and extracellular. The authors thought that possibly all their animals were infected after 2 to 3 months, but that there was a spontaneous regression of the disease. Later, when no lesions were/

were seen in the intestine, organisms were cultured from the mesenteric and thoracic glands for as long as 157 days after inoculation; this was also true of feeding experiments. Throughout the course of the experiments, the rabbits ate well and lost no weight, but had a shaggy coat and were listless: there was no diarrhoea.

In mice at post mortem the alimentary tract was found to be infected from the stomach to the rectum, the largest numbers of bacilli being found in the duodenum and jejunum; there were only a few in the ileum. The bacilli were found mainly in the apices of the villi, the submucous tissue and muscle layers also being affected. Large numbers of bacilli were found in the abdominal and thoracic lymph glands, liver, spleen, and faeces. One mouse, which developed an infection throughout the length of its small intestine, also had organisms in the abdominal lymph glands, but none in the thoracic lymph glands, liver, or spleen. Myco. johnei was recovered from the mesenteric lymph glands of mice, even from those thought not to be diseased; there was neither diarrhoea nor emaciation.

The infection in rats, not discussed at length, affected the ileum in 2 cases and the duodenum in a third case, 4 to 5 months after inoculation.

Guinea-pigs were found to be refractory, although organisms/

organisms were recovered from mesenteric and thoracic glands 157 days after inoculation; caseous masses in the abdomen were found to contain dead bacilli.

In 1914, when trying to produce Johne's disease in goats with suspensions of infected bovine intestinal mucosa, Andersen inoculated a rabbit, apparently as a control animal; he recounted his surprise 12 months later when he found a well established intestinal infection in the rabbit, which appeared to be in excellent condition. This was probably the stimulus for further experiments, and in 1922 he reported that 2 of 9 rabbits, inoculated subcutaneously, were infected 11 and 18 months later respectively, and 4 of 7, inoculated intravenously, 3 to 15 months later. These results were obtained with infected, bovine, intestinal mucosa, and with cultures and infected mesenteric glands from a goat, which had been infected with the bovine strain of Myco. johnei.

The intestinal lesions closely resembled those in cattle. The small intestine was thickened and the mucosa in folds; between the large folds there were small wart-like protrusions and small haemorrhages; the author considered that it was an exact replica of an infected bovine intestine. Histologically, the villi were larger and thicker, forming club-like swellings. The epithelium was normal, but the villi were packed with epithelioid cells containing innumerable/

innumerable acid-alcohol-fast bacilli; on low magnification the villi appeared entirely red. The deeper layers of the mucosa were also affected. Numerous bacilli were present both intracellularly and extracellularly. The mesenteric glands, the caecum, and the large intestine contained many bacilli, but the histological changes in the colon were not so marked as in the small intestine. The lungs, liver, spleen, and kidneys were normal, and no bacilli were seen; they were, however, cultured from mesenteric lymph glands. The rabbits were very fat and there was no diarrhoea. Passage experiments, rather surprisingly, were unsuccessful. Experiments with guinea-pigs and a fowl were also unsuccessful.

Andersen's overall results of 6 animals successfully infected of 22 inoculated hardly do him justice; closer examination of his results shows that 4 out of 7 rabbits inoculated intravenously became infected. In view of the many reports that Myco. johnei is harmless when inoculated subcutaneously, Andersen's infection of even 2 animals by this route is a striking result.

Intestinal lesions of unspecified character were found in mice by Popp (1940) following the intraperitoneal or intravenous inoculation of Myco. johnei suspended in liquid paraffin. There was no indication whether the organisms were present in the lesions. If so, they were not recovered, nor were passage experiments successful.

Popp's/



Popp's results seem to be much in line with those of Twort & Craig (1913), and Hagan & Mansfield (1930); in no case was it possible to isolate the organisms from the lesions. It is, of course, not always easy to culture mycobacteria from caseous lesions (Dubos, 1952, 1953). On the other hand, lesions closely resembling those obtained with live organisms have been obtained in experimental animals inoculated with suspensions of heat-killed mycobacteria, or even with bacterial extracts (Ungar, Coulthard, & Dickinson, 1948). The results of Twort & Craig, Hagan & Mansfield, and Popp, may be taken at their face value, and the lesions produced in their experimental animals ascribed to organisms which died, either before inoculation, or in situ.

The most striking results are those of Francis (1943) who produced Johne's disease in very young mice with both cultures of Myco. johnei and infected mouse liver; most of the mice were inoculated intraperitoneally and generally received 2 inoculations, the second 7 days after the first.

Mice, 15 days old, were inoculated with 2 doses of Myco. johnei, each of 2.5 mgm.; 24 to 34 weeks later 2 of the 4 surviving mice had intestinal lesions and Myco. johnei was isolated from the liver and spleen of one of them and from the spleen of one of the mice which had not developed intestinal/

intestinal lesions. Four further groups of mice were inoculated from this original group. Seven mice, 7 days old, received 2 doses of a culture from spleen; 5 received 1 mgm. followed by 0.4 mgm., and 2, 0.5 mgm. followed by 0.75 mgm. One died shortly after inoculation, 3, killed 12 to 19 weeks later, were infected, 2, killed 20 weeks later, were either refractory or the infection had regressed, and in the last, killed after 24 weeks, the liver and spleen were heavily infected and there were small numbers of organisms in the intestine. In the second group inoculated from the original group, 4 mice, 10 days old, received 0.1 ml. of a liver suspension, and another 4, also 10 days old, 0.1 ml. of a spleen suspension; all mice received a second inoculation of 0.1 ml. of a liver suspension from another mouse in the original group: 2 died shortly after inoculation and the other 5, killed 17 to 27 weeks later, were all negative. The third series infected from the original group consisted of 9 mice, 10 days old. They were fed 0.02 ml. of a 1 in 4 saline suspension of a heavily infected intestinal tract; 3 days later they received 0.4 ml. of the suspension and 4 days later 0.08 ml. The death of 6 of these mice was recorded and was due in each case to salmonella infection; no acid-alcohol-fast bacilli were seen in smears from any of them other than in one which died 10 weeks after inoculation and in/

in which 2 large clumps of acid-alcohol-fast bacilli were seen in one intestinal smear. The fourth group inoculated from the original consisted of 7 mice, 13 days old, which were given 0.1 to 0.2 ml. of a 1 in 4 saline suspension of an infected liver; some of the mice received a second inoculation 7 days later. One died soon after inoculation, 3 developed intestinal lesions, and 3 were probably in the early stages of infection. Liver tissue from one of the mice was treated with antiformin and 0.05 gm. of this material was inoculated into 15 mice, 14 days old. All but one died within 5 weeks of inoculation and in those dying the infection was developing in the liver, spleen, and intestine; the survivor died after 8 months and no lesions of Johne's disease were found.

In all these experiments, of 50 mice inoculated with Myco. johnei or infected material, or fed infected material, 8, possibly 12, developed Johne's disease.

Histologically, the small intestine was extensively invaded, the villi thickened, and the mucosa and submucosa packed with bacilli, which were also fairly numerous in the longitudinal and circular muscle layers and in the mucosa. The histology of the intestinal lesions was not fully described, but the author considered them to be comparable to the lesions found in cattle. In the liver, the lesions were/

were proliferative in type and were found in all parts of the lobules, even adjacent to the central lobular vein, the largest being in the portal tract. When well developed, the lesions consisted of masses of epithelioid cells, packed with bacilli, surrounding the blood vessels and the bile duct. The circular aggregations of epithelioid cells within the liver parenchyma sometimes appeared to be surrounded by a thin layer of true endothelial cells; no central necrosis was observed, nor any connective tissue separating the lesions from normal liver cells.

There is little doubt that an intestinal infection closely resembling the natural infection was established in these animals.

Hamsters were somewhat refractory although passage and multiplication of organisms was obtained; rabbits and 2-day-old guinea-pigs were refractory.

In voles about 6 weeks old Levi (1950) found that the intraperitoneal inoculation of 0.0001 to 2.0 mgm. moist weight of Myco. johnei in 0.5 ml. of saline produced an intestinal infection in 44 to 450 days in 17 of 21 animals; 10 to 13 mgm. per os produced an infection in 42 to 500 days in 5 of 8 animals. Nine recently isolated strains of Myco. johnei were used, 7 in the feeding experiments and 9 in the others.

At/

At post-mortem the only macroscopic lesions, caseous nodules in the abdominal cavity and a thickening of the omentum, were also seen in voles inoculated intraperitoneally either with heat-killed Myco. johnei or with Myco. phlei (see also page 70).

The lymphoid follicles or Peyer's patches in the intestine seemed to be the sites of predilection, the submucosa and villi becoming progressively involved. Massive numbers of organisms seemed to have little effect on the animals and death appeared to be due to intercurrent infection; in voles other than those dying of intercurrent infection no macroscopic lesions were seen at post mortem. Only in long-established infections was the cellular reaction marked, and it appeared insignificant in relation to the large number of organisms which, at some levels of the intestine, were found in the villi, submucosa, muscle layers, and on the peritoneal surface. The extent of the lesions and the number of organisms appeared to depend on the time since inoculation rather than on the size of the inoculum. The small intestine was more frequently involved than the large intestine and 6 months or more after inoculation large numbers of Myco. johnei were found in the faeces of some animals.

The histological changes in the mesenteric lymph nodes, spleen, and less frequently, in the lungs, appeared to consist of/

of granulomatous foci of epithelioid cells with occasional lymphocytes or small mononuclear cells: giant cells were not seen. There was no necrosis, caseation, or calcification, and little or no connective-tissue reaction. In the liver there were accumulations of Kupfer-like cells and occasional epithelioid cells in the portal tracts and sometimes near the centres of the lobules.

Intestinal localisation of Myco. johnei followed both inoculation and feeding. Shortly after inoculation there were more organisms in the liver, spleen, and lungs, than in the intestine, but as the infection progressed there were more in the intestine than in the other organs; in animals infected orally smears of the intestinal mucosa were richest in organisms. In control experiments heat-killed Myco. johnei and Myco. phlei failed to produce the infection.

In 2 of 3 voles inoculated intraperitoneally with the avian tubercle bacillus there were numerous organisms and foci of epithelioid cells in the intestine and in other organs. The author was of the opinion that the reaction of the vole to the avian tubercle bacillus seemed to be similar to the reaction to Myco. johnei; he also likened the lesions caused by Myco. johnei to those following the inoculation of small doses of the human tubercle bacillus.

It/

It is certainly true that the successful inoculation of small laboratory animals with Myco. johnei has been accomplished only at infrequent intervals; nevertheless, it appears to be possible to reproduce Johne's disease in laboratory animals. Since opinion seems to be against the possibility of infecting laboratory animals, the choice of animal is open. However, the experimental infection has twice been produced in the mouse and this, together with the fact that large numbers of mice can be easily accommodated, suggested the mouse as a suitable experimental animal. If, as is possible, individual animals differ in their susceptibility, or individual strains of Myco. johnei in their virulence, or even if additional factors, such as age, have an influence on the infection, only large-scale experiments will provide a statistically significant answer; the mouse appears to be well suited for such investigations.

## EXPERIMENTAL

An account of my own experimental work, under the headings "In-vitro Experiments" and "In-vivo Experiments", now follows.



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IN-VITRO EXPERIMENTS

Media for the Cultivation of Myco. johnei. The medium which I used at first for the isolation of strains was that of Wilson Taylor (1950); it has the following composition:-

Egg-yolk	60 ml.
Egg-white	15 ml.
Glycerol	4 ml.
Congo red solution (1 per cent)	1 ml.
Heat-killed <u>Myco. phlei</u>	1 gm.
Normal saline	19 ml.

The heat-killed Myco. phlei is ground in a mortar with the glycerol-saline solution to give an even suspension which is autoclaved at 120°C for 20 minutes. When cool, the suspension is thoroughly mixed with the egg-congo red mixture and the medium dispensed in sterile, universal containers. The containers are sloped, inspissated at 90°C for 2 hours, and on 2 further successive days are held at 80°C for 2 hours in the Koch steamer. They are then incubated at 37°C for a week and contaminated tubes are discarded.

This medium proved excellent for passaging Myco. johnei. However, when used for the isolation of Myco. johnei from intestinal mucosa and from faeces the results were disappointing; the growth of organisms, other than Myco. johnei, which survived exposure to oxalic acid was not always suppressed. This may be because the dye in the medium, congo red, is poorly bacteriostatic. Malachite green, on the other hand, is very effective in greatly reducing contamination in  
Lowenstein-Jensen/

Lowenstein-Jensen medium, and I decided to use it in place of congo red in Wilson Taylor's medium. Minett (1942) had already observed that 0.06 per cent of malachite green is slightly inhibitory when the inoculum of Myco. johnei is very small, but not 0.04 per cent. Accordingly, a batch of Wilson Taylor's medium was prepared containing 0.04 per cent malachite green, and this medium proved excellent both for the isolation and passaging of Myco. johnei. Early growth is indicated by the colour of the dye changing to a creamy yellow: this was previously observed and commented upon by Dunkin (1928).

Other media were tried in parallel with that of Wilson Taylor: as reported by Boquet (1939), excellent results were obtained on Lowenstein-Jensen whole-egg medium containing 1 per cent of heat-killed Myco. phlei.

A medium devised in the course of the present work allowed good growth of some strains of Myco. johnei but not of others. Its base is fresh bovine plasma and the composition is as follows:

Bovine plasma	450 ml.
Asparagine	2 gm.
Sodium citrate	1 gm.
Dipotassium phosphate	0.25 gm.
Magnesium sulphate	0.25 gm.
Ferric citrate	0.03 gm.
Congo red, 1 per cent solution	5 ml.
<u>Myco. phlei</u>	5 gm.
Distilled water	45 ml.
Glucose	5 gm.
Glycerol	20 ml.

The/

The salts and glucose are dissolved in the distilled water and glycerol, the pH of the resulting solution, about pH 7.4, being left unadjusted. The Myco. phlei is ground in a mortar with this solution to form an even suspension to which the plasma-congo red mixture is added before inspissation. Glucose at 2 per cent allowed good growth and glycerol at 4 per cent did not; the combination of the 2 gave better growth than glucose alone.

Following the work of Dubos and his associates on the diffuse growth of Myco. tuberculosis, Glover (1952b) showed that bovine serum treated with Liquoid (sodium polyanethol sulphate, Roche Products Limited) can be substituted for fraction V albumin in the Dubos albumin-Tween 80 medium, and that when a suitable extract of Myco. phlei is added, the medium allows diffuse growth of Myco. johnei. In my experiments Glover's medium was used in a slightly modified form which has the following composition:-

Disodium phosphate	0.63 gm.
Potassium dihydrogen phosphate	0.10 gm.
Sodium citrate	0.15 gm.
Magnesium sulphate	0.06 gm.
Ammonium succinate	0.8 gm.
Succinic acid	1.2 gm.
Ferric ammonium citrate	0.002 gm.
Copper sulphate, 0.01 per cent solution	3.0 ml.
Zinc sulphate, 0.01 per cent solution	3.0 ml.
Glycerol	0.2 ml.
Distilled water to	100 ml.

To the salt base, the pH of which is 7.0 to 7.2, is added 4 per cent of Liquoid-treated serum containing 2.5 per cent/

cent of Tween 80 and 4 per cent of Boquet's (1928) glycerol-saline extract of Myco. phlei. Diffuse growth of Myco. johnei was obtained; no attempt was made to isolate Myco. johnei in this medium.

Isolation of Myco. johnei from Faeces. Methods for the isolation of Myco. johnei from faeces (see page 39) are unreliable and the results discouraging because of the presence of many moulds and sporing organisms in the faeces, and because of the difficulty of inhibiting their growth without interfering with that of Myco. johnei. Preliminary experiments, an account of which now follows, were carried out with a view to overcoming these difficulties.

An attempt was first made to induce germination of spores, and then to expose the vegetative forms to antiformin, oxalic acid, or sulphuric acid. Faeces containing Myco. johnei were ground with sand and saline in a mortar and the suspension allowed to sediment for 15 minutes. The supernatant was then drawn off, mixed with an equal volume of meat-extract broth in a universal container, and the container placed in a water-bath at 37°C. At hourly intervals, for 7 hours, 3 aliquots were drawn off, mixed with equal volumes of 20 per cent antiformin, 10 per cent oxalic acid, or 10 per cent sulphuric acid, and all 3 suspensions returned to the water-bath at 37°C. From these suspensions, further aliquots/

aliquots were drawn off at hourly intervals, again for 7 hours, centrifuged, the supernatants poured off, and the sediments washed twice with saline. These were then sown on to meat-extract agar slopes and into tubes of meat-extract broth and incubated at 37°C for 7 days.

Sterilisation of faeces was achieved, but Myco. johnei did not survive the treatment, so that the method is of no practical value.

A method was next devised with oxalic acid and malachite green. About 1 gm. of faeces, containing large numbers of Myco. johnei, was suspended in 10 ml. of distilled water and the suspension filtered through 2 thicknesses of cheese-cloth to remove debris. The filtrate was then centrifuged at 3500 to 4000 r.p.m. for 30 minutes, the supernatant discarded, and the sediment transferred to an all-glass tissue grinder where it was resuspended with grinding, in 10 ml. of distilled water. The suspension was allowed to stand for 15 minutes, then equal volumes of the supernatant and of 10 per cent oxalic acid, containing 0.02 per cent malachite green, were mixed and placed for 30 minutes in a water-bath at 37°C. This suspension was centrifuged at 3500 to 4000 r.p.m., the supernatant discarded, and the sediment tested, as previously, for sterility. After 7 days at 37°C there was no growth. So encouraging were the results that/

that in the next experiment the sediment was sown on to Lowenstein-Jensen medium containing one per cent of Myco. phlei and on to Wilson Taylor's medium (see page 79) containing malachite green or congo red; in all cases there was no growth of organisms other than Myco. johnei. Since the congo red medium itself is only weakly bacteriostatic, the success of the method must be ascribed to the joint bactericidal action of oxalic acid and malachite green. Myco. johnei has now been isolated by this method from 4 of 4 specimens of bovine faeces and from 5 of 5 specimens of mouse faeces.

An alternative procedure is to expose faeces, first to the action of oxalic acid, then to the action of malachite green, instead of to a solution containing both oxalic acid and malachite green. After the preliminary treatment described on page 83, the faecal suspension from the tissue grinder was allowed to stand for 15 minutes, then equal volumes of the supernatant, and of 10 per cent oxalic acid, were pipetted into a universal container which was placed for 30 minutes in a water-bath at 37°C; the suspension was then centrifuged, the supernatant discarded, and the sediment transferred to a sterile tissue grinder, where it was suspended in 0.01 per cent of malachite green. This suspension was centrifuged for 30 minutes at 3500 to 4000 r.p.m., the supernatant/

supernatant discarded, and the sediment sown on to Lowenstein-Jensen medium containing one per cent of Myco. phlei. This method also proved successful for the isolation of Myco. johnei from 3 of 3 specimens of faeces of mice; it was not so successful with bovine faeces, possibly due to qualitative differences in the bacterial flora: 2 of 4 specimens of faeces yielded cultures of Myco. johnei.

When solutions of antiformin and malachite green are mixed a precipitate forms. It was decided, therefore, not to expose faeces to such a mixture, but to expose them, as in the previous method, first to the one reagent then to the other. The procedure was the same as that described on page 84, except that instead of being exposed to oxalic acid the faecal suspension was exposed at 37°C for 15 minutes to a final concentration of 20 per cent antiformin. It was then centrifuged at 3500 to 4000 r.p.m. for 30 minutes, the sediment washed carefully twice in distilled water, and resuspended in 0.01 per cent malachite green. This suspension was centrifuged and the deposit sown on to media as before. Myco. johnei was isolated from 3 of 3 specimens of faeces of mice by this method.

#### Isolation of Myco. johnei from Intestinal Mucosa.

Altogether 30 bovine strains of Myco. johnei and 2 pigmented ovine strains of Myco. johnei were isolated from intestinal mucosa; /



mucosa; some of the specimens of infected intestine contained enormous numbers of acid-alcohol-fast bacilli, others relatively few. The most successful method for the isolation of Myco. johnei from these specimens was Wilson Taylor's modification of the oxalic acid method of Corper & Uyei (1929-30) (see page 39), with a minor modification of my own.

Mucosal tissue, about 2 gm., instead of being ground with sand as in Wilson Taylor's method, is ground in a sterile tissue grinder with 10 per cent oxalic acid. The advantages are a more uniform particle size and less chance of contamination with airborne organisms. There is sufficient material to seed 12 tubes of media.

The ease with which Myco. johnei can be isolated from intestinal mucosa, as opposed to the difficulty with faeces even very rich in Myco. johnei, prompted a brief investigation of the bacterial flora of infected intestinal mucosa around the ileo-caecal valve. Only the aerobic flora was investigated since it is the presence of aerobic moulds and spores in faeces which makes the isolation of Myco. johnei from this source so difficult.

The population was found to consist mainly of Gram-negative organisms (Escherichia, paracolon types, Aerobacter, and Proteus), with an occasional Streptococcus and Sarcina.  
The/

The result of such an examination does not exclude the presence of sporing organisms; it suggests, however, that very few are to be found around the ileo-caecal valve. It is tempting to assume that this is the reason for the relative ease with which Myco. johnei can be isolated from intestinal mucosa.

The Growth Factor and Non-exacting Strains. As already mentioned, media for the isolation of Myco. johnei must contain either one per cent of heat-killed Myco. phlei or an active extract of Myco. phlei, the organisms or the extract supplying the growth factor; the properties of the growth factor have already been discussed (see page 47). Acid-alcohol-fast bacilli not requiring this growth factor on isolation cannot at present be termed Myco. johnei; our own strains all require the growth factor and are, by this criterion, strains of Myco. johnei.

The impression conveyed by the literature is that strains of Myco. johnei become non-exacting after many passages on laboratory media; Glover (1952a) and Wilson Taylor (1951b, c, 1952b) are of this opinion. Dunkin (1933) was the first to report the possibility of training strains of Myco. johnei to dispense with the growth factor and ultimately to grow on a chemically defined medium; such strains are known as non-exacting strains of Myco. johnei (see/

(see page 43).

The nature of the growth factor is unknown; Woolley & McCarter (1940), however, showed that substances with vitamin K activity stimulated the growth of one non-exacting strain of Myco. johnei, and this raises the question whether vitamin K can also act as the growth factor for freshly isolated, i.e. fully exacting strains of Myco. johnei.

In an attempt to answer this question I carried out the following experiment: an alcoholic extract of a mycobacterium known to support the growth of freshly isolated strains of Myco. johnei was examined in ultraviolet light. It is well known that the vitamins K and structurally related compounds have a specific absorption peak in the ultraviolet range at about  $2490\text{\AA}^{\circ}$ . No spectrum of any vitamin K-like substance was found in the extract. Conversely, vitamin K (Synkavit, the calcium salt of the diphosphoric ester of 2 methyl 1:4 naphthahydroquinone, Roche Products Limited) incorporated in egg medium did not support the growth of a freshly isolated strain of Myco. johnei, though in agreement with Woolley & McCarter (1940), it stimulated the growth of a non-exacting strain, Teps, on the surface of Sauton fluid.

Before further discussion of the growth factor, it seems necessary to describe experiments carried out with strain Teps. A covering note, received with the culture from H.R. Allen, Royal Veterinary College, Streatley, Berkshire, stated/

stated that "with plenty of inoculum - and patience - a surface film can be obtained on a fluid medium". The strain in the first experiments was inoculated on to the surface of 3 chemically defined fluid media, those of Henley, of Long and Seibert (Report, 1939), and of Sauton, but there was no observable growth after 6 months' incubation at 37°C; Johnson (1944b) noted that some of his non-exacting strains also showed a preference for a particular medium.

A second culture of strain Teps was obtained, together with the formula of the medium on which it is grown. The composition of this medium differs but slightly from those mentioned before, the differences being 0.1 per cent dipotassium hydrogen phosphate as against 0.3 per cent in the others; 0.006 per cent ferric citrate as against 0.005 per cent ferric ammonium citrate and 0.05 per cent sodium citrate which is not present in the others. On this medium surface growth was obtained; on the media of Henley, of Long and Seibert, and of Sauton, surface growth was also obtained, but only in the presence of an extract of Myco. phlei. It seems that strain Teps may represent a type of strain of Myco. johnei capable of dispensing with the growth factor only under strictly specified conditions. Even in Allen's medium, better and more rapid growth is obtained in the presence of the growth factor.

The/

The rather confusing facts emerging both from my own experiments and from those of others, can be summed up thus:

- (a) extracts of Myco. phlei support the growth of exacting strains and stimulate the growth of one non-exacting strain of Myco. johnei.
- (b) the anti-haemorrhagic compounds do not support the growth of exacting strains of Myco. johnei; they stimulate the growth of a non-exacting strain, but even in this respect are inferior to Myco. phlei and its extracts.
- (c) extracts of a mycobacterium giving no vitamin K absorption spectrum support the growth of exacting strains and stimulate the growth of a non-exacting strain of Myco. johnei.

It would thus appear in agreement with Francis et al (1949, 1953) that the growth factor is not identical with vitamin K, which nevertheless stimulates the growth of a strain of Myco. johnei not requiring the growth factor. Moreover, since extracts giving no vitamin K spectrum also stimulate the growth of a non-exacting strain, it may be concluded that the growth of such strains can be stimulated by a variety of compounds.

The observation that strain Teps grows better in the presence of the growth factor, or of vitamin K, although capable of growing in their absence, suggested a simple method for the detection of growth stimulation. In the following/

following experiments various substances were incorporated into a chemically defined fluid medium without growth factor and the medium was inoculated from a growing surface film of strain Teps. Growth stimulation was indicated by better growth in the presence of the substance under investigation than in the control. The following substances were investigated, with the results noted, no attempt being made to assess the degree of stimulation:-

<u>Substance</u>	<u>Stimulation</u>
Precipitate from a 2-week-old <u>Myco. phlei</u> culture fluid (4 per cent glycerol meat-extract broth) precipitated with	
a. Acetic Acid	+
b. Hydrochloric Acid	+
Bovine plasma, 1 per cent	-
Bovine serum, dialysed, 1 per cent	-
Sheep serum, dialysed, 1 per cent	-
Bovine intestinal mucosa, freeze-dried, 0.01 per cent	-
Creatine, 1 per cent	-
Creatinine, 1 per cent	-
Vitamin E, concentrations from 0.01 to 10.0 µgm. per ml.	-
Vitamin K, 0.01 µgm. per ml.	-
0.10 µgm. per ml.	+
1.00 µgm. per ml.	+
10.00 µgm. per ml.	+
Vitamins E & K combined	
0.01 µgm. per ml. K + 10.00 µgm. per ml. E	-
0.10 µgm. per ml. K + 1.00 µgm. per ml. E	-
1.00 µgm. per ml. K + 0.10 µgm. per ml. E	+
10.00 µgm. per ml. K + 0.01 µgm. per ml. E	+

+ indicates growth stimulation

- indicates no growth stimulation

Similarly, inhibitors to Myco. johnei can be detected by incorporating the material to be investigated in a chemically/

chemically defined fluid medium containing growth factor and noting the resulting inhibition of strain Teps.

Three types of broth were investigated, meat-extract or infusion broth, digest broth, and digest broth containing 0.5 units per ml. 'Liquemin' (Heparin 'Roche' solution, Roche Products Limited); the last broth was chosen simply because of its availability.

The following results were obtained: after one month, digest broth at all concentrations appears inhibitory to strain Teps; after 3 months, growth in cultures with amounts of all broths up to 20 per cent is superior to that in the control (no broth); higher concentrations of broth are inhibitory throughout. Tentatively I suggest that a substance, or substances, inhibiting the metabolism of Myco. johnei, is present in broth. The organism can become adapted to this substance, and the ensuing growth is more abundant than in control media without broth; concentrations of broth above 50 per cent inhibit adaptation; 'Liquemin' appears to lift the inhibition due to low concentrations of digest broth. Boquet (1939) and Hagan (1952) suggested that various brands of broth prepared in laboratories probably contain inhibitory substances which, even in the presence of the growth factor, prevent the growth of Myco. johnei. My experiments also show that the brands of broth examined provide growth stimulation for strain Teps in addition to that provided by the growth factor.

The/

The growth factors of Myco. johnei seem to be a matter of great complexity, and the results so far obtained suggest that several factors are involved. The terms "growth factor" and "growth stimulating factor" have to be used with caution; thus it is probably incorrect to equate the stimulation of strain Teps by low concentrations of broth to that by the growth factor. It should be remembered that Myco. tuberculosis var. hominis grows well on chemically defined media, but grows better when serum is added to the media; the effect of low concentrations of broth on strain Teps may be similar. It is my opinion that until the Myco. phlei growth factor is identified the investigation of any substance for this activity should be carried out in a very rich medium such as that of Lowenstein-Jensen or of Wilson Taylor. These are probably as fully nutrient as media can be, and enhanced growth observed in them will most probably be attributable to the growth factor and not to an enrichment of the medium.



IN-VIVO EXPERIMENTS

Strains of Myco. johnei used. The experiments were carried out with 6 strains of Myco. johnei inoculated as culture suspensions, and with 5 strains inoculated as suspensions of intestinal mucosa. Where mucosa was used Myco. johnei was subsequently cultured from the material. Suspensions of liver, spleen, and intestine from infected mice were also inoculated.

The origin of the strains of Myco. johnei is as follows:-

Strain C. Isolated by the method of Wilson Taylor (1950) from bovine intestine received from J.T. Stamp, Edinburgh and East of Scotland College of Agriculture: inoculated as culture suspension.

Strain E. Isolated by the modification (see page 86) of the method of Wilson Taylor (1950) from bovine intestine, received from W.G. Johnston, West of Scotland Agricultural College, Veterinary Laboratory, Auchincruive: inoculated as suspension of intestinal mucosa.

Strain F. Isolated by the modification (see page 86) of the method of Wilson Taylor (1950) from bovine intestine, received from W.G.

Johnston/

Johnston, West of Scotland Agricultural College, Veterinary Laboratory, Auchincruive: inoculated as suspension of intestinal mucosa.

Strain Fass. Culture supplied by A.W. Stableforth, Ministry of Agriculture and Fisheries Laboratory, Weybridge, Surrey. The culture was labelled .... "Fass. Taylor Strain. 2nd Sub-culture"; inoculated as culture suspension.

Strain K. Isolated by the modification (see page 86) of the method of Wilson Taylor (1950) from bovine intestine, received from W.G. Johnston, West of Scotland Agricultural College, Veterinary Laboratory, Auchincruive: inoculated as intestinal mucosa.

Strain L. Isolated by the method of Wilson Taylor (1950) from bovine intestine, received from J.T. Stamp, Edinburgh and East of Scotland College of Agriculture: inoculated as culture suspension.

Strain M. Isolated by the method of Wilson Taylor (1950) from bovine intestine, received from J.T. Stamp, Edinburgh and East of Scotland College of Agriculture: inoculated as culture suspension, suspension of bovine intestinal mucosa, and suspension of mouse viscera.

Strain M.143/

Strain M.143. Culture supplied by H.R. Allen, Royal Veterinary College, Streatley, Berkshire. Requires no Myco. phlei growth factor: inoculated as culture suspension and suspension of mouse viscera.

Strain R. Isolated by the modification (see page 86) of the method of Wilson Taylor (1950) from bovine intestine, received from I.M. McIntyre, University of Glasgow Veterinary School, Bearsden, Glasgow: inoculated as suspension of intestinal mucosa.

Strain Teps. Culture supplied by H.R. Allen, Royal Veterinary College, Streatley, Berkshire. Originally isolated by Dunkin (1928, 1933); does not require Myco. phlei growth factor: inoculated as culture suspension.

Strains C and M were grown on Wilson Taylor's medium, strain Fass on Lowenstein-Jensen medium containing 1 per cent of heat-killed Myco. phlei, and strain L in the modified Glover's medium. Strain M.143 was grown on an egg-yolk agar, and strain Teps on potato slants flooded with Sauton fluid containing 10 per cent Myco. phlei extract (Boquet, 1928). Strains E, F, K, M (in some experiments), and strain R, were inoculated as a suspension of intestinal mucosa. Suspensions were/

were prepared by grinding about 2 gm. of well-washed, infected, intestinal mucosa with 10 per cent oxalic acid in a tissue grinder; the suspension in oxalic acid was then exposed for 30 minutes in a water-bath at 37°C, centrifuged, and the supernatant discarded. The sediment was transferred to a sterile tissue grinder and twice washed by grinding in sterile saline. It was then made up to a volume of 10 ml. with sterile saline and used for inoculations. Material thus treated is later described as "oxalated".

Agents for suspending Myco. johnei. The cultures of Myco. johnei were suspended in the following:-

- (i). Distilled water.
- (ii). Normal saline (0.85 per cent sodium chloride in distilled water).
- (iii). Meat-extract broth containing 4 per cent by volume of glycerol.
- (iv). Fresh bovine plasma.
- (v). An extract of bovine intestine. This was prepared by extracting 100 gm. of fresh, normal, bovine intestinal mucosa with 100 ml. of 20 per cent glycerol-saline for 20 minutes at 120°C. The resulting suspension was centrifuged for 30 minutes at 3500 to 4000 r.p.m. and the supernatant fluid/

fluid used as the extract of bovine intestine, intravenous inoculation of this material killed mice almost instantaneously; intraperitoneally, it was without effect.

It was thought that suspending agents (iv) and (v) might supply a factor or factors determining the susceptibility of the animals (cf. the role of activator in experimental staphylococcal infections (Smith & Hale, 1944)).

(vi). T.K.L. medium. This is the Sauton medium used by Boquet (1928) for the growth of Myco. johnei, with the following additions:-

Liver extract	1	per cent
Yeast extract	1	per cent
Lecithin	0.16	per cent
Choline	0.16	per cent

These additions were made to favour the multiplication and survival of Myco. johnei.

(vii) Liquid Paraffin B.P.

(viii) Milk.

(ix) Mucin (a 1.75 per cent solution of Wilson's hog gastric mucin (Smith, 1950)).

Agents (vii), (viii), and (ix) were used because of their known virulence-enhancing and anti-phagocytic action.

(x) Liqueid-treated serum. Liqueid (sodium polyanethol-sulphonate, Roche Products Limited), an anti-coagulant, has been shown to allow better growth of/  
of/

of a number of organisms than control media, by depressing the normal bactericidal properties of fresh blood. It has also been shown by Glover (1952b, c) that fresh bovine serum treated with Liquoid and calcium chloride can be substituted for fraction V albumin in a Dubos-type medium. It was thought that a combination of these 2 properties in a single suspending agent might offer advantages over simpler fluids.

Preparation of Suspensions of Myco. johnei. A

homogeneous suspension of mycobacteria, as is well known, cannot be obtained simply by transferring the organisms to the suspending agent and shaking in a stoppered container. A smooth suspension can be obtained by grinding the organisms in a small mortar with the agent chosen, but this method is clumsy.

In the present experiments suspensions of Myco. johnei were obtained by using a glass tissue-grinder. The organisms were removed from the surface of solid medium by a platinum spatula and transferred to a small glass tube, 0.5 cm. deep, 1 cm. in diameter, and sealed at one end. This was placed in a sterile Petri dish which in turn was placed, with the lid ajar, /

ajar, in the incubator at 37°C for at least 24 hours. The glass tube was then removed from the Petri dish, a small piece of sterile filter paper placed over the open end and the tube and cover weighed; the organisms were then transferred to a sterile tissue-grinder, the tube and filter paper again weighed, and the mass of organisms, in the inoculum, calculated by difference. The suspending agent was then added to the organisms and several turns of the plunger of the tissue-grinder gave a homogeneous suspension.

### Mice

Strain. W-Swiss mice (Howie, 1949) were used throughout the experiments; the stock was bred free of salmonella and resistant to Tyzzer's disease by Professor J.W. Howie at the Rowett Research Institute, and appears to be resistant to trauma, to secondary infection, and to the toxic action of suspending agents. Francis (1943) was unfortunate to lose many of his mice from salmonella infection in the course of a passage experiment; in our experiments losses from all extraneous causes were negligible. In only one experiment was there an unduly high death-rate, a complete litter dying of a diphtheroid infection. The inoculum was possibly infected, but since the infection developed at the beginning of the experiment, long-term results were not affected, and the animals were easily replaced./

replaced.

Age. At first, adult mice, generally 25 to 35 gm. were used. Later, as experience in giving intra-peritoneal inoculations was gained, animals 7 days' old were used.

Diet. The diets used did not seem to influence the experiments. Breeding and weaning mice were fed on water and cubes of the Rowett Research Institute's diet No.86, a fully nutrient diet of natural foods designed and described by Howie (1951). The diet was changed to cubes of white bread, moistened with milk, when the mice were four weeks old.

In some experiments the animals, after 6 months on bread and milk, were restored to the cube and water diet, and after a further 6 months, returned to bread and milk.

In other experiments, mice were fed on cube and water for 6 months, then transferred to bread and milk for the remainder of the experiment.

In the latest series of experiments the mice were maintained throughout on the cube and water diet.

### Criteria used in assessing Results.

Death of Experimental Animals. Only a small number of mice were allowed to die, the majority being killed at various stages in the course of the infection. In some experiments/



experiments a number of mice died when aged 12 to 16 months which is within the life, 2 to 3 years, of healthy W-Swiss mice. Heart-blood cultures were sterile and there was no evidence of secondary infection at post mortem; in view of bacteriological and histological evidence, I feel justified in ascribing death to the infection with Myco. johnei, although the immediate cause of death is not known.

Presence of Myco. johnei in Faeces. The examination of bovine faeces, intra vitam, for acid-alcohol-fast bacilli is used in veterinary practice to confirm a clinical diagnosis of Johne's disease (see plate 14, page 125). Acid-alcohol-fast bacilli were found in the faeces of mice which, on subsequent histological examination, were found to have been suffering from Johne's disease. To gauge the progress of the infection, therefore, I examined the faeces of mice, intra vitam, for acid-alcohol-fast bacilli. The count was made at monthly intervals over 50 fields under the oil-immersion lens, and the number of organisms noted, together with any clumps seen. A rising count was taken to indicate a progressing infection. When the count exceeded 1000 organisms per 50 fields, the mouse was considered "positive"; further counts were made from time to time on such mice to ensure that there was no regression. The number of organisms counted in this manner is indeed an indication of/

of the extent of the invasion of the intestine, a point which is discussed on page 137..

Appearance of Mice and Macroscopic Lesions.

During the experiments, a hollowing of the perineum was often observed in mice suffering from Johne's disease. Discussing the emaciation caused by Johne's disease in cattle, Lominet (1936) described "un enfoncement, le coup de poing, entre la mamelle, les masses musculaires crurales et la vulve, dépression d'autant plus accusée que l'amaigrissement est plus avancé"; this seems to be very similar indeed to what was observed in heavily infected mice in the present experiments.

Infected mice were much less active than healthy mice, and often showed little interest in food. Some animals, too, had a tendency to stand high on their legs, the abdomen, which was tumid, being held clear of the ground in an exaggerated manner; the coat was generally staring, lacking the usual lustre seen in the W-Swiss mice. The tail and pads were cool to the touch, and when handling heavily infected animals I was struck by their general lack of tone.

Following the intraperitoneal inoculation of cultures or of infected viscera, a local, subcutaneous nodule was observed in several mice at the site of inoculation. In some/

some, the lesion ruptured when a second inoculation was made in the vicinity, and the exuding pus was very rich in acid-alcohol-fast bacilli. These inoculation lesions gradually regressed and no trace of them could be found several months later (see Meyn & Weiske (1943), and page 62).

In animals dying a week to 4 months after inoculation, post-mortem examination very often revealed small white nodules, 1 to 2 mm. in diameter, on the diaphragm, liver and spleen. These probably corresponded to the nodules described by Boquet (1925) and were regarded as not necessarily specific for Myco. johnei.

In more advanced stages of the infection there might be small white lesions, about 1 mm. in diameter, on the surface of the liver lobes, and similar lesions were often present on the anterior, subcutaneous aspect of the abdomen; these lesions all contained large numbers of acid-alcohol-fast bacilli.

At an even later stage - 12 to 16 months after inoculation - there was complete absence of tone in the small intestine, the diameter of which was greatly increased throughout its length (see plates 15 and 16, pages 128 and 129); due to the enlargement of the intestine, the liver might be displaced; the axillary, inguinal, and mesenteric lymph glands were also enlarged; the lungs had a greenish appearance/

appearance not seen in normal mice, the cause of which was not ascertained. Other organs appeared normal, although the kidneys might be pale brown, not unlike the colour of milk chocolate; there was an absence of fat in the lower abdomen; this was observed by Bang (1906) in cattle, and it may well be related to the hollowing of the perineum previously described.

Before death, some animals were obviously ill, whereas others died although they seemed to be in perfect health. However, in spite of a lack of clinical symptoms which, as in cattle, might not appear until the terminal stages of the infection, extensive histological lesions were always found in infected mice.

Examination of Smears. When an animal died or was killed, smears were made from the colon (2 levels), small intestine (6 levels), kidney, liver, lungs, spleen, and pancreas. The numbers of acid-alcohol-fast bacilli were noted.

Histology. From each mouse sections of colon (2 levels), small intestine (6 levels) and associated lymph glands, kidney, liver, lung, pancreas, and spleen, were examined. In some cases sections of brain, inguinal lymph glands, and stomach, were also examined. The type of tissue reaction and the distribution of acid-alcohol-fast bacilli were noted.

## RESULTS

<u>JOHNE'S DISEASE IN MICE</u>	107
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JOHNE'S DISEASE IN MICE

In the first group of experiments, involving equal numbers of males and females, 82 mice were inoculated; the dose of organisms, 1 mgm., and the volume of the inoculum, 0.5 ml., were kept constant, but the age of the mice, the strain of organism, the suspending agent, and the route of inoculation were varied. Within a month of inoculation 55 of the 82 mice had been killed: in these no evidence of infection was found.

It was thought that since the natural infection in cattle requires 18 to 24 months to develop, it might also take a long time to appear in mice; accordingly, the remaining 27 animals in the group (table 1, page 108) were kept for a further 8 months and were then killed.

The first evidence of infection with Myco. johnei was found in these animals. There were acid-alcohol-fast bacilli, singly and in clumps, in smears made post mortem from the faeces, and from various organs of some of the mice, but only the presence or absence of clumps was noted. No estimate was made of the number of organisms, which were numerous in the faeces, intestinal mucosa, liver, pancreas, and spleen, and scattered in the kidneys and lungs. Thus, table 2 (see page 109), which summarises these findings, gives the impression of a more uniform distribution of organisms than was indeed the/

TABLE 1

Details of inoculation of 27 mice

Mouse* Number	Sex	Age or Weight	Route of Inocula- tion	Strain of <u>Myco.</u> <u>johne</u>	Suspending agent
1	M	25 to 35 gm.	I.P.	M	Saline
2	F	25 to 35 gm.	I.P.	M	Mucin
3	M	25 to 35 gm.	I.P.	M	B.I.M. **
4	M	25 to 35 gm.	I.P.	Fass	Saline
5	M	25 to 35 gm.	I.P.	Fass	T.K.L. ***
6 & 7	F	25 to 35 gm.	I.P.	Fass	Plasma
8	F	25 to 35 gm.	I.P.	Fass	B.I.M. **
9 to 11	F	25 to 35 gm.	I.V.	M	Plasma
12 to 14	M	25 to 35 gm.	I.V.	M	T.K.L. ***
15 & 16	M	25 to 35 gm.	I.V.	M	Saline
17	M	25 to 35 gm.	I.V.	C	Plasma
18	M	25 to 35 gm.	I.V.	C	Saline
19 to 22	F	25 to 35 gm.	I.V.	C	Mucin
23 & 24	M	21 days	I.P.	M	Plasma
25	F	21 days	I.P.	M	Milk
26	F	21 days	I.P.	M	Mucin
27	F	21 days	I.P.	M	T.K.L. ***

\* All mice received 1.0 mgm. of organisms per 0.5 ml. of inoculum.

\*\* Extract of bovine intestinal mucosa.

\*\*\* Medium described on page 98.

TABLE 2

Results of bacteriological examination of organs  
and faeces of mice detailed in Table 1 and killed  
9 months after inoculation

Mouse No.	Presence of acid-alcohol-fast bacilli.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Faeces	-	-	-	-	+	-	-	-	-	-	+	-	+	+
Intestine	-	-	+	-	+	-	-	-	-	-	+	-	+	+
Kidney	-	+	-	-	-	-	-	-	-	-	+	-	+	+
Liver	-	-	-	-	+	+	+	-	+	-	+	-	+	+
Lung	-	-	-	-	-	-	-	-	+	+	+	-	+	-
Pancreas	+	-	-	-	+	+	+	-	+	-	+	-	+	+
Spleen	-	+	+	-	+	+	+	-	+	+	+	+	+	+

Mouse No.	15	16	17	18	19	20	21	22	23	24	25	26	27
Faeces	+	-	+	-	+	+	-	-	-	-	+	+	+
Intestine	+	-	+	-	+	+	+	-	-	-	+	+	+
Kidney	+	-	+	-	+	+	-	-	-	+	+	+	+
Liver	+	-	-	+	+	+	-	-	+	+	+	+	-
Lung	+	+	+	-	+	+	-	-	+	-	+	-	+
Pancreas	+	-	+	-	+	+	-	-	-	-	+	+	+
Spleen	+	-	-	-	+	+	-	-	+	+	+	+	+

+ indicates the presence of clumps of acid-alcohol-fast bacilli.  
- indicates the absence of clumps; isolated organisms were disregarded.



the case. It was subsequently learned from the results of histological examination that lesions were present only in the abdominal viscera: there were no lesions in the kidneys and lungs.

In some mice, at post mortem, the small intestine was enlarged and the intestinal vessels congested. Because of this, and because of the bacteriological findings, 5 mice, 2 of which had been inoculated intraperitoneally and 3 intravenously, were examined histologically.

In the 3 mice inoculated intravenously, lesions were found in the intestine, liver, mesenteric lymph nodes, and spleen (table 3, page 111).

Microscopically, the enlargement of the small intestine appears due to a collection of large pale cells in the mucosa and submucosa. The villi are swollen and contain groups of these pale cells which have round nuclei and abundant, slightly granular acidophilic cytoplasm; often multinucleate, they form small giant cells, and appear to be derived from the reticulo endothelial system (see plate 1 page 112). Similar groups of cells are present in the deepest layers of the submucosa close to the muscle coat, (see plate 3, page 113), but lesions are not seen in the muscle coat itself; or on the peritoneal surface of the bowel. Sections stained by the Ziehl-Neelsen method show that these cells contain numerous/

TABLE 3

Presence of lesions in five mice  
examined histologically. The mice  
form part of Tables 1 and 2.

Organ	Presence of Histological Lesions in Mice				
	No. 5	No.15	No.19	No.20	No.25
Intestine	+	+	+	+	+
Kidney	-	-	-	-	-
Liver	+	+	+	+	+
Lungs	-	-	-	-	-
Pancreas	+	+	+	+	+
Spleen	+	+	+	+	+

+ indicates presence of lesions.

- indicates absence of lesions.

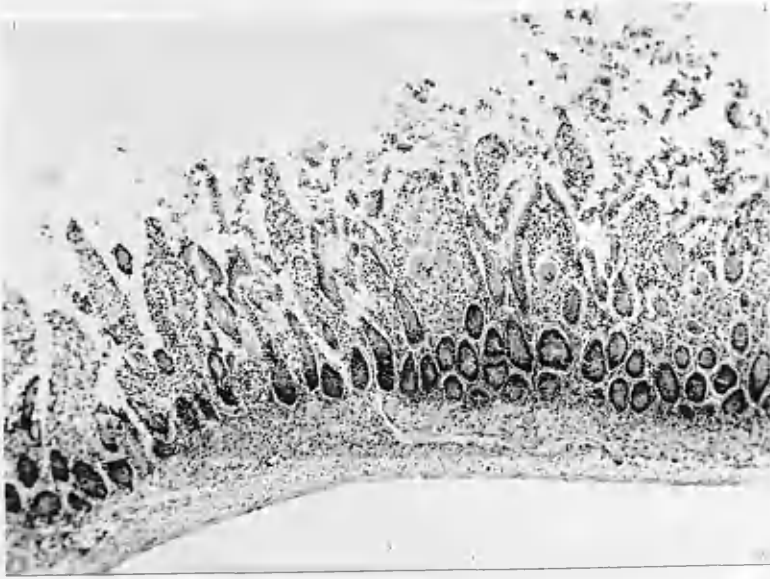


Plate 1. Section of intestine of mouse killed 9 months after the intravenous inoculation of 1 mgm. of Myco. johnei. Note macrophages in the villi and in the submucosa. Stained by haematoxylin-eosin. Magnification x25.

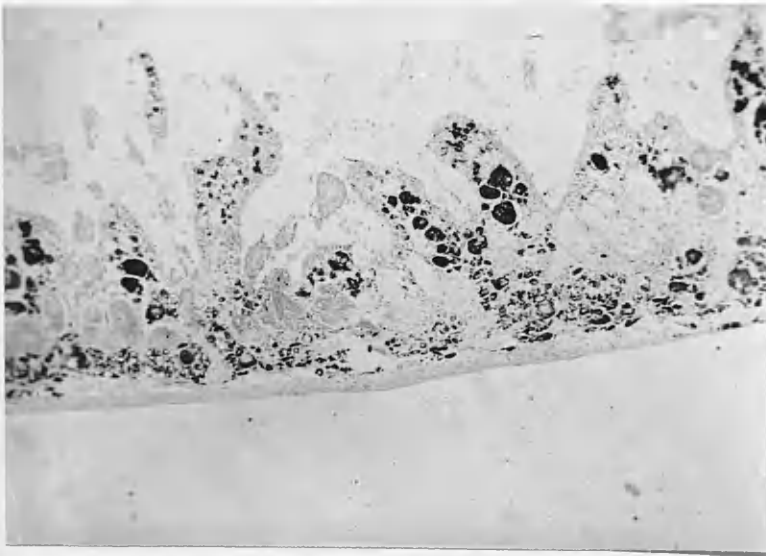


Plate 2. As Plate 1. Note large masses of acid-alcohol-fast bacilli in the villi and in the submucosa. Stained by Ziehl-Neelsen. Magnification x25.

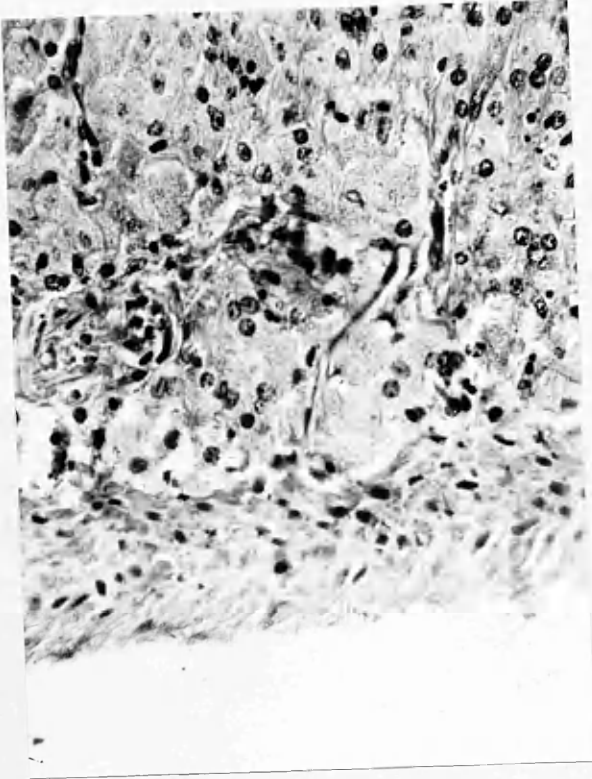


Plate 3. Section of intestine of mouse killed 9 months after the intravenous inoculation of 1 mgm. of Myco. johnei. Note macrophages in the submucosa. Stained by haematoxylin-eosin. Magnification x500.

numerous, small, acid-alcohol-fast bacilli (see plate 2, page 112). There is no surrounding tissue reaction, no cellular exudate or fibrosis, and no necrosis or caseation.

The local mesenteric lymph nodes show disorganisation of their structure, and contain large groups of pale, swollen, phagocytic cells similar to those seen in the bowel wall; these cells also are full of acid-alcohol-fast bacilli (see plates 4 & 5, page 115).

The spleen, with scattered groups of phagocytic cells containing numerous organisms, shows changes similar to those in the lymph nodes (see plates 6 & 7, page 116).

Groups of large, pale, phagocytic cells are present throughout the liver, mainly along the portal tracts (see plates 8, 9, & 10, pages 117 & 118). These groups are often surrounded by smaller cells with dark nuclei, cells also probably derived from the reticulo-endothelial system. There is some disturbance in the arrangement and staining of the hepatic cells; again the phagocytic cells are full of bacilli.

In the pancreas, small aggregations of acid-alcohol-fast bacilli are present in the interstitial tissue between the lobules; the organisms, lying in phagocytic cells, often elicit a macrophage reaction.

The kidneys show no gross abnormality; in sections stained by the Ziehl-Neelsen method small groups of acid-alcohol-fast/

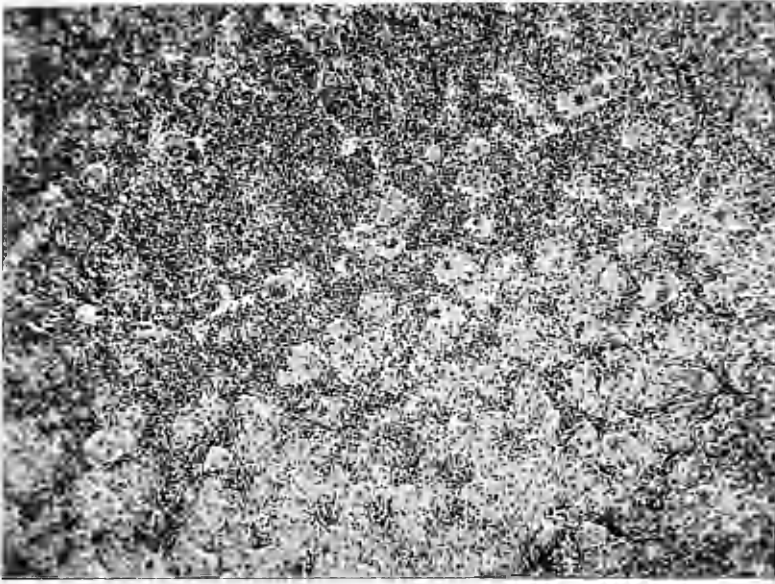


Plate 4. Section of mesenteric lymph node of mouse killed 9 months after the intravenous inoculation of 1 mgm. of Myco. johnei. The normal structure of the node is lost and large aggregates of macrophages are seen. Stained by haematoxylin-eosin. Magnification x150.

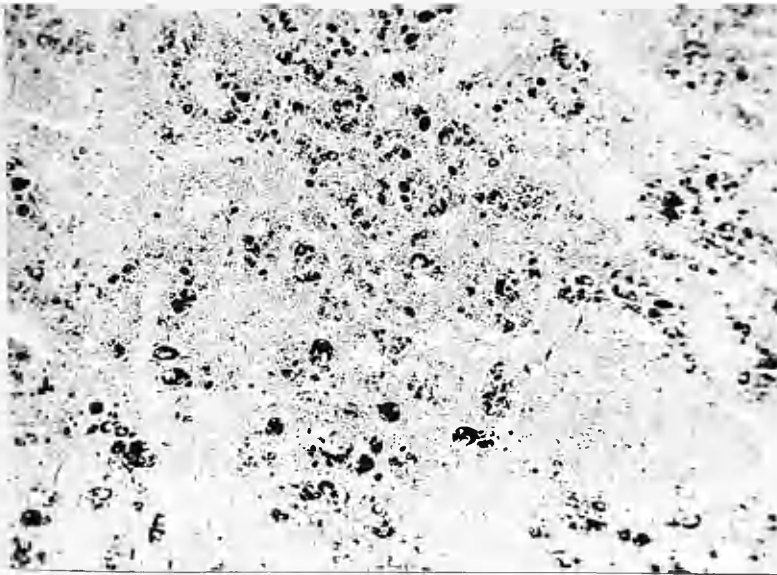


Plate 5. As Plate 4, showing large masses of acid-alcohol-fast bacilli within the macrophages. Stained by Ziehl-Neelsen. Magnification x150.

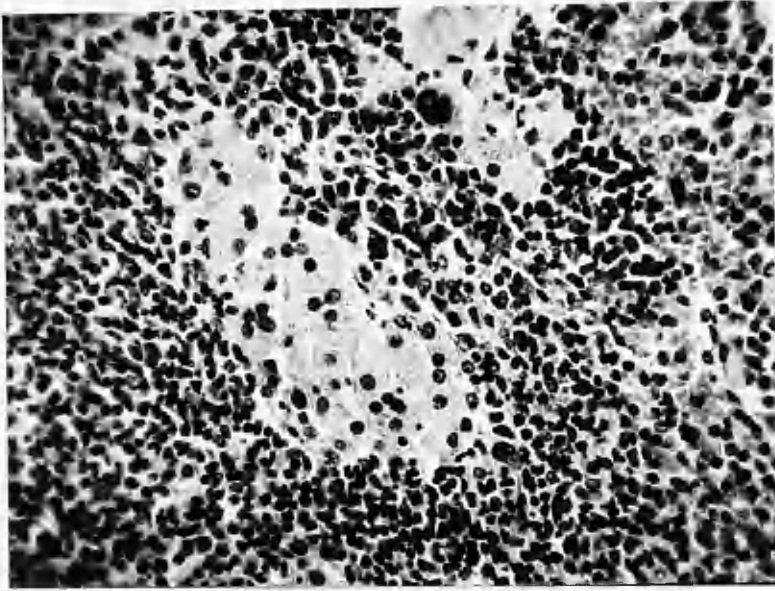


Plate 6. Section of spleen of mouse killed 9 months after the intravenous inoculation of 1 mgm. of Myco. johnei. Note large, pale macrophages. Stained by haematoxylin-eosin. Magnification x500.

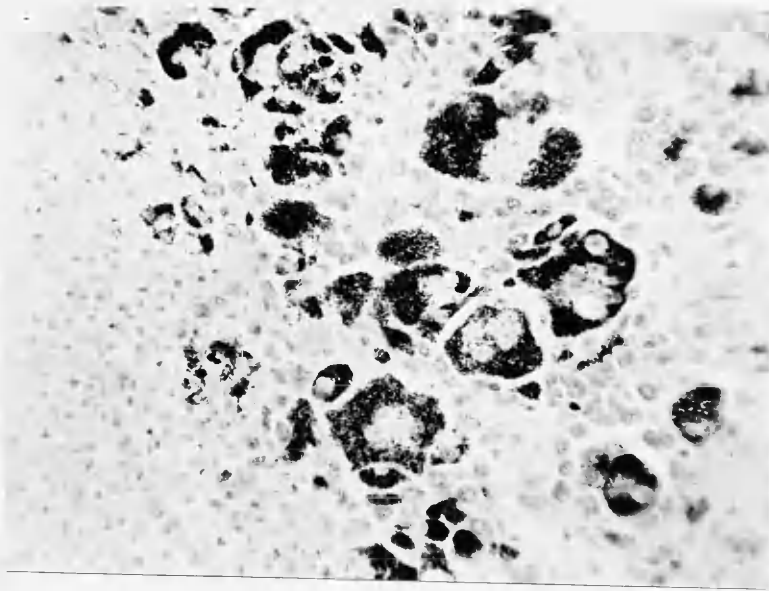


Plate 7. As Plate 6, stained to show masses of acid-alcohol-fast bacilli within the macrophages. Stained by Ziehl-Neelsen. Magnification x500.

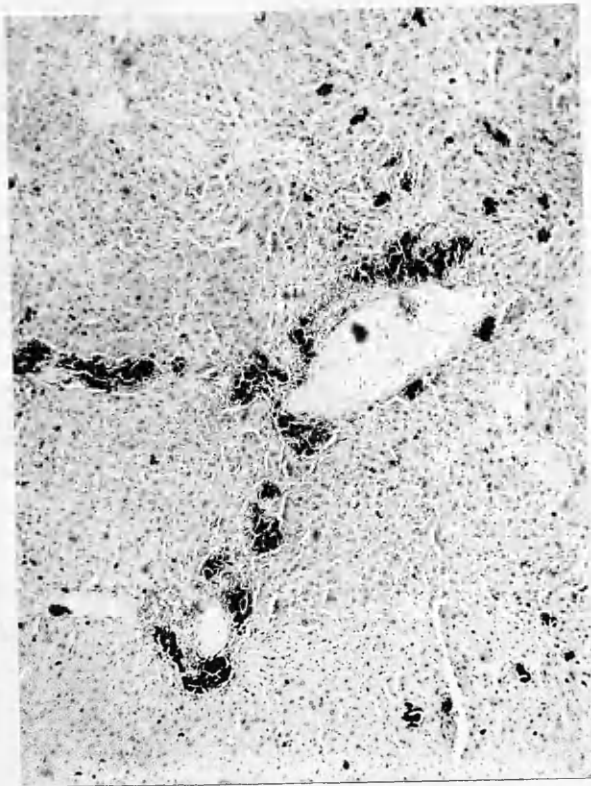


Plate 8. Section of liver of mouse killed 9 months after the intravenous inoculation of 1 mgm. of Myco. johnei. Note masses of acid-alcohol-fast bacilli around portal tracts. Stained by Ziehl-Neelsen. Magnification x50.



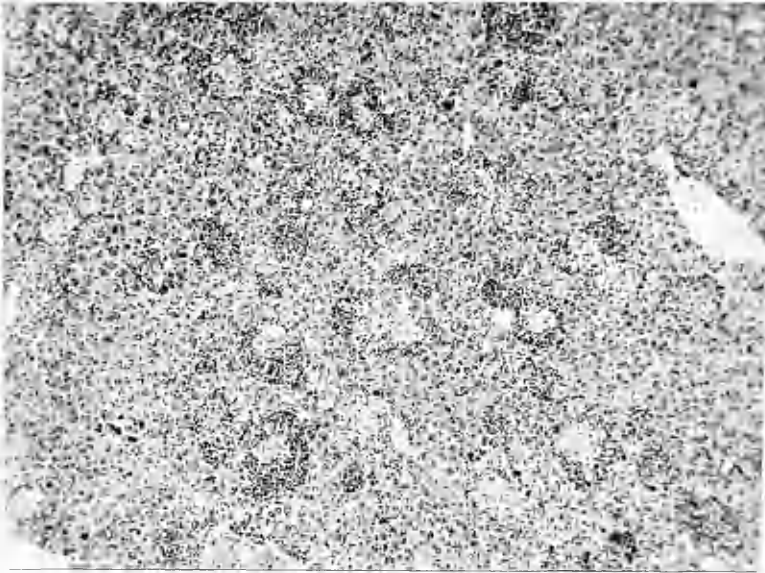


Plate 9. Section of liver of mouse more heavily infected than that shown in Plate 8. Note, scattered throughout the parenchyma, groups of pale macrophages surrounded by small dark cells. Stained by haematoxylin-eosin. Magnification x150.

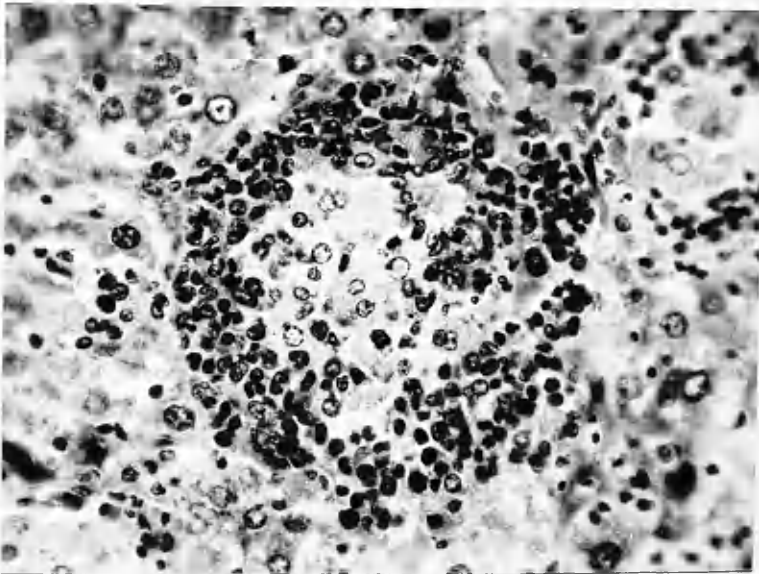


Plate 10. As Plate 9, showing a small granuloma composed of a group of macrophages surrounded by lymphocytes. Stained by haematoxylin-eosin. Magnification x500.

alcohol-fast bacilli are seen, mainly in the interstitial tissue between the tubules and in the junctional zone between the cortex and the medulla: cellular reaction is not seen. Similarly, in the lungs there are no gross histological lesions (see plate 11, page 120) but small groups of organisms are seen, particularly in the peribronchial tissue; there is no cellular reaction around these organisms, nor is it possible to determine whether they are intra- or extracellular.

Heart and brain appear normal; the stomach, oesophagus, and lower portion of the large bowel also appear normal and do not contain organisms (compare with findings in more advanced cases, page 130).

After intraperitoneal inoculation, the histological changes in the intestine, liver, and spleen are very similar, if not identical, to those seen after intravenous inoculation. In addition, small nodules, composed of dense fibrous tissue and swollen, phagocytic cells containing masses of organisms, are found on the peritoneal surfaces of the viscera.

In this experiment most of the factors later shown to be of importance in the study of Johne's disease in mice were set forth - the long incubation period, the intestinal localisation of Myco. johnei independent of both the suspending agent and the route of inoculation, and the co-existence/

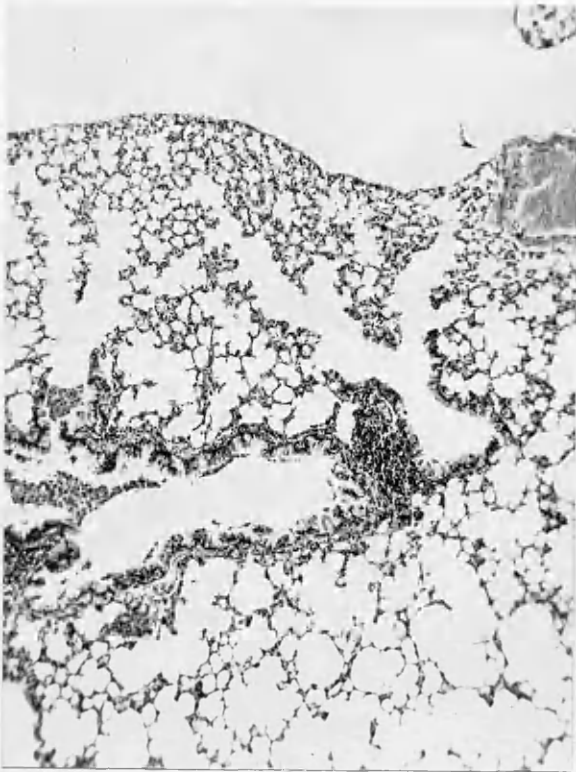


Plate 11. Section of lung of mouse killed 9 months after the intravenous inoculation of 1 mgm. of Myco. johnei. Note absence of cellular reaction. Appropriate staining shows only scant acid-alcohol-fast bacilli around the bronchi. Stained by haematoxylin-eosin. Magnification x25.

co-existence of intestinal lesions and acid-alcohol-fast bacilli in the faeces.

In the next experiment it was decided to try to obtain further information on the progress of the infection by counting, at monthly intervals throughout the experiment, the numbers of single acid-alcohol-fast bacilli - as opposed to clumps of acid-alcohol-fast bacilli - in the faeces. Since the incubation period in mice is very long, it was thought that inoculation at a very early age might ensure the development of the infection within the life of the mouse, hence the use of very young mice. It was thought that with adult mice there would be greater likelihood of the animals dying of old-age before the infection was established. Moreover, by comparing the results with those obtained on adult mice in the previous experiment it was thought that it might be possible to demonstrate the age-immunity phenomenon (see page 9).

The mice, 16 in number and detailed in table 4 (see page 122), were 7 to 12 days old, and were inoculated twice, the second inoculation 7 to 10 days after the first; 13 mice each received 1.96 to 2.64 mgm. of Myco. johnei, strain M, suspended in saline; 3 received a saline suspension of the washed, bovine, intestinal mucosa from which strain M was isolated.

For/

TABLE 4

Details of 16 young mice receiving 2 intraperitoneal inoculations of either a saline suspension of Myco. johnei, strain M, or the bovine intestinal mucosa from which strain M was isolated

	Mouse numbers	1 to 4	5 to 13	14 to 16
1st inoculation	Age in days when inoculated	7	9	12
	Weight of organisms in mgm.	1.32	0.56	M
	Volume of inoculum in ml.	0.25	0.1	0.2
2nd inoculation	Age in days when inoculated	14	19	19
	Weight of organisms in mgm.	1.32	1.4	M
	Volume of inoculum in ml.	0.25	0.25	0.25

M inoculated with bovine intestinal mucosa.

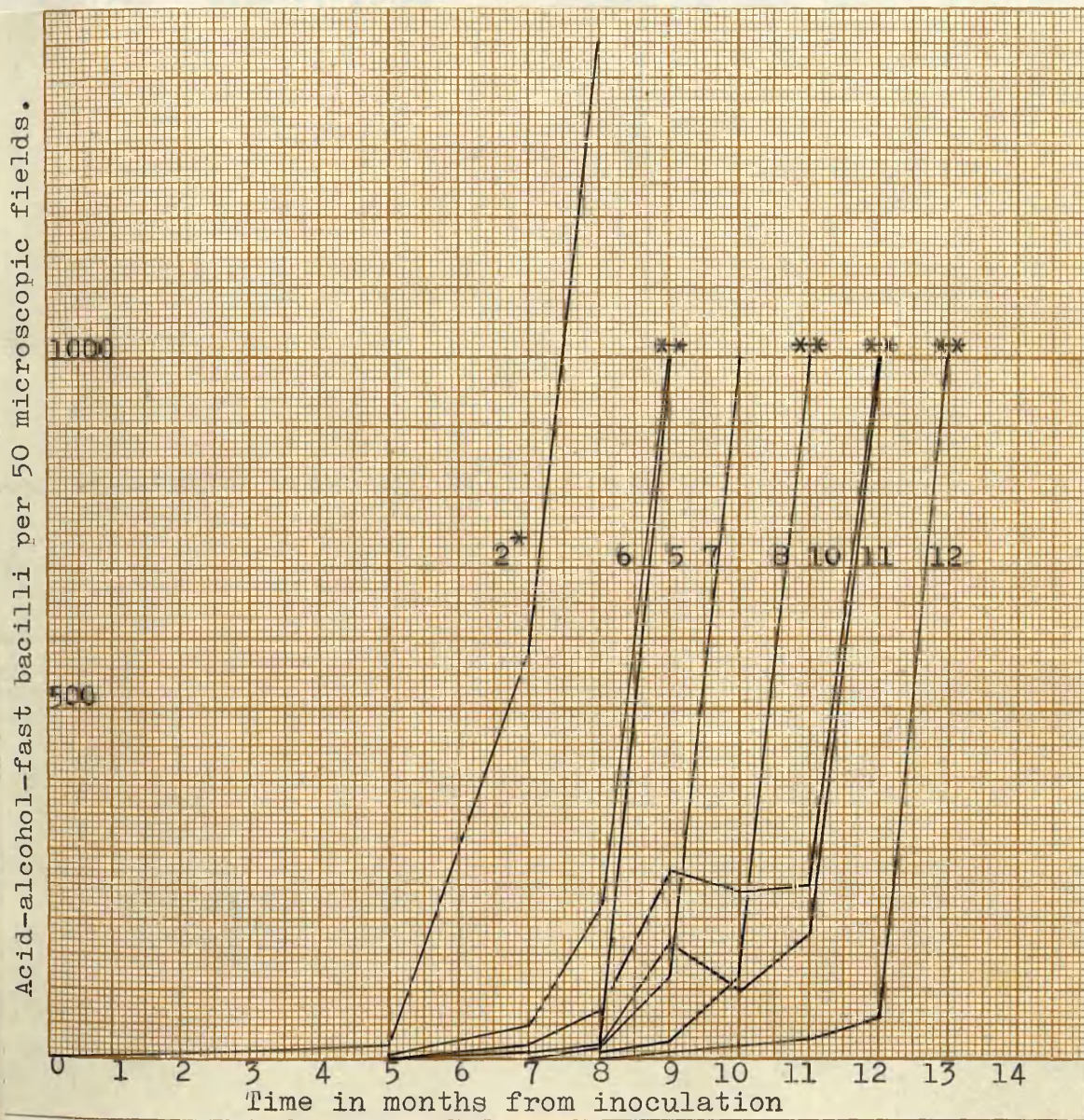
For the first few weeks there were single acid-alcohol-fast bacilli in the faeces; thereafter they became very scarce until 3 or 4 months after inoculation when they began to increase in number, although by the 5th month the numbers did not exceed 10 organisms per 50 microscopic fields, except in one case where there were 18 organisms. The results of these monthly counts are shown as graphs in figure 1 (see page 124).

Five months or more after inoculation there was a remarkably sudden increase in the number of clumps and of single organisms in the faeces, the latter rapidly exceeding 1000 per 50 fields (see plate 12, page 125). Counting then became so inaccurate and time-consuming that, with the exception of one mouse, counts of more than 1000 organisms were not made. It should be noted that only single acid-alcohol-fast bacilli were counted - no attempt was made to count the organisms in the clumps. The faeces were subsequently examined from time to time to ensure that there was no decrease in numbers; not only was there no decrease, there was an ante-mortem increase, enormous numbers of acid-alcohol-fast bacilli appearing in the faeces, the appearance being similar to that observed post mortem (see plate 13, page 125). In figure 1 the results of only 8 mice are detailed; the other 8 in the group died or were killed when the number of organisms in the faeces did not exceed 50 per 50 fields.

Of/

FIGURE 1

Numbers of single acid-alcohol-fast bacilli in the faeces of 8 mice observed over 13 months



\* Mouse number (see Tables 4 and 5).

\*\* This number was exceeded, but accurate counts were not carried out beyond 1000 organisms.

Plate 12, right. Myco. johnei in the faeces of a mouse. Stained by Ziehl-Neelsen. Magnification x2000.

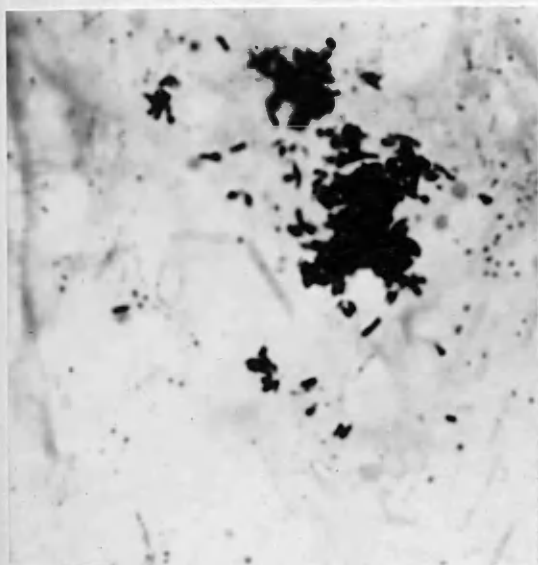
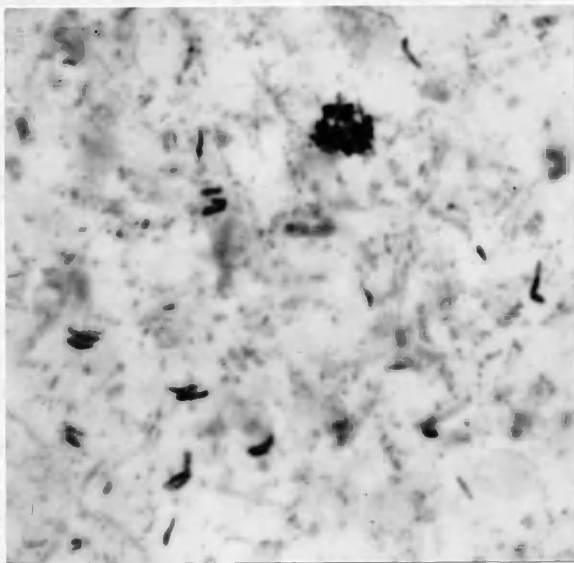
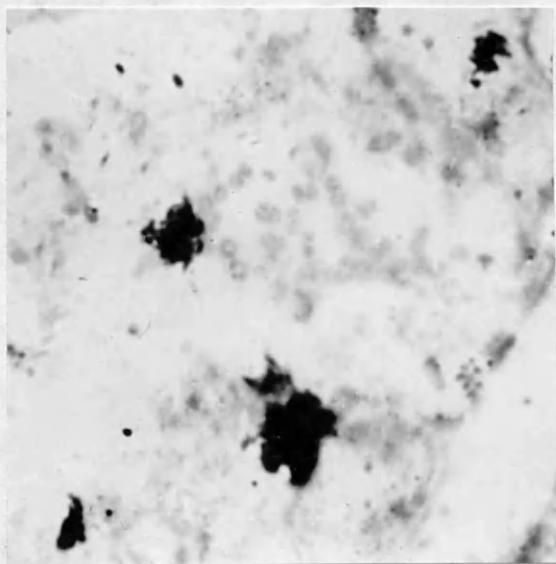


Plate 13, left. Myco. johnei in the faeces, post-mortem, of a mouse which died of Johne's disease. Stained by Ziehl-Neelsen. Magnification x2000.

Plate 14, right. Myco. johnei in bovine faeces. Compare with Plates 12 and 13. Stained by Ziehl-Neelsen. Magnification x2000.





Of the 16 mice in this experiment (see table 5, page 127) 2 died of an intercurrent infection, No.13 at 2 months, and No.14 at 4 months after inoculation; in both there were acid-alcohol-fast bacilli in all the organs, and in the intestine, but in such small numbers that negative histological findings were only to be expected. Three others, Nos. 4, 15, & 16, died after 4, 6, & 10 months respectively, and had heavily infected faeces during life; smears from all organs were heavily positive. The death of these animals was not immediately detected, and they could not be examined histologically because of putrefraction. Another 4 mice died with fairly extensive intestinal lesions, Nos. 1 & 9 after 6 months, No.3 after 7 months, and No.7 after 10 months; despite well established Johne's disease, the death of these 4 mice could not, with certainty, be attributed to Johne's disease because intercurrent infection was not ruled out.

Of the remaining 7 mice, Nos.2, 5, 6, & 8 died and Nos. 10, 11, & 12 were killed 12 to 16 months after inoculation. At post mortem they showed a characteristic appearance (see plates 15 & 16, pages 128 & 129). The small intestine was considerably thickened, and to some extent displaced the other abdominal viscera; the axillary, inguinal, and mesenteric lymph glands were markedly enlarged, and there was no fat in the lower abdomen. Heart-blood cultures/

TABLE 5

Correlation between the numbers of acid-alcohol-fast bacilli (A.A.F.B.) in the faeces of mice (detailed in Table 4) and the extent of lesions in various organs

Mouse Number		1	2	3	4	5	6	7	8
Months after inoculation when animal died or was killed		6D	12D	7D	4D	13D	13D	10D	14D
A.A.F.B. per 50 oil immersion fields at death		120	>10 <sup>3</sup>	130	10	>10 <sup>3</sup>	>10 <sup>3</sup>	>10 <sup>3</sup>	>10 <sup>3</sup>
Degree of histological lesions in	Intestine	++	+++	++	**	+++	+++	+++	+++
	Liver	++	+++	++	**	+++	+++	+++	+++
	Lung	+	+	+	**	+	+	+	+
	Kidney	+	+	+	**	+	+	+	+
	Spleen	++	+++	++	**	+++	+++	+++	+++

Mouse Number		9	10	11	12	13	14	15	16
Months after inoculation when animal died or was killed		6D	12K	16K	16K	2D	4D	6D	10D
A.A.F.B. per 50 oil immersion fields at death		30	>10 <sup>3</sup>	>10 <sup>3</sup>	>10 <sup>3</sup>	nil	5	50	250
Degree of histological lesions in	Intestine	++	+++	+++	+++	+	+	**	**
	Liver	++	+++	+++	+++	+	+	**	**
	Lung	+	+	+	+	+	+	**	**
	Kidney	+	+	+	+	+	+	**	**
	Spleen	++	+++	+++	+++	+	+	**	**

D indicates that animal died

K indicates that animal was killed

\*\* indicates that animal was not examined histologically

+ no tissue reaction; scanty acid-alcohol-fast bacilli

++ slight tissue reaction; moderate number of A.A.F.B.

+++ extensive lesions; abundant acid-alcohol-fast bacilli.

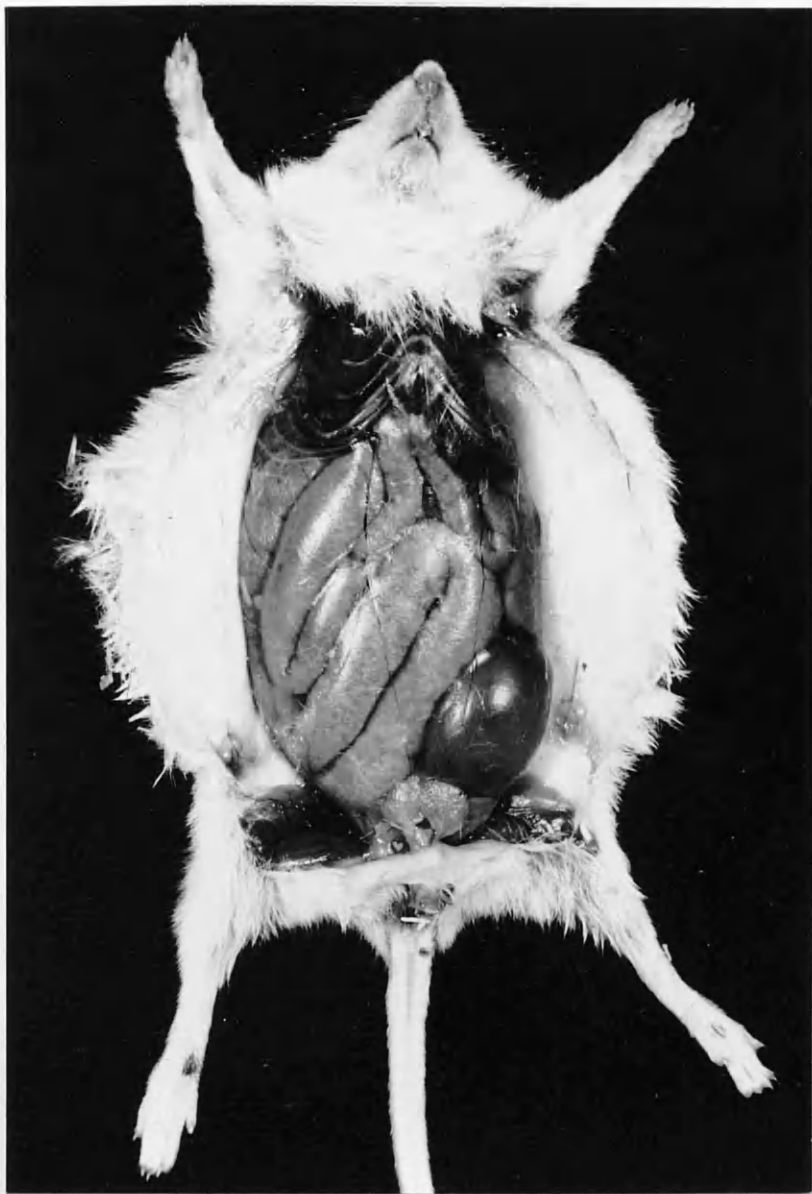


Plate 15. Appearance of mouse which died of Johne's disease 18 months after the intraperitoneal inoculation of a suspension of infected mouse viscera. Note enlargement of small intestine.



Plate 16. Appearance of mouse which died of Johne's disease 14 months after the intraperitoneal inoculation of 1.96 mgm. of Myco. johnei. Note small intestine and inguinal lymph glands.

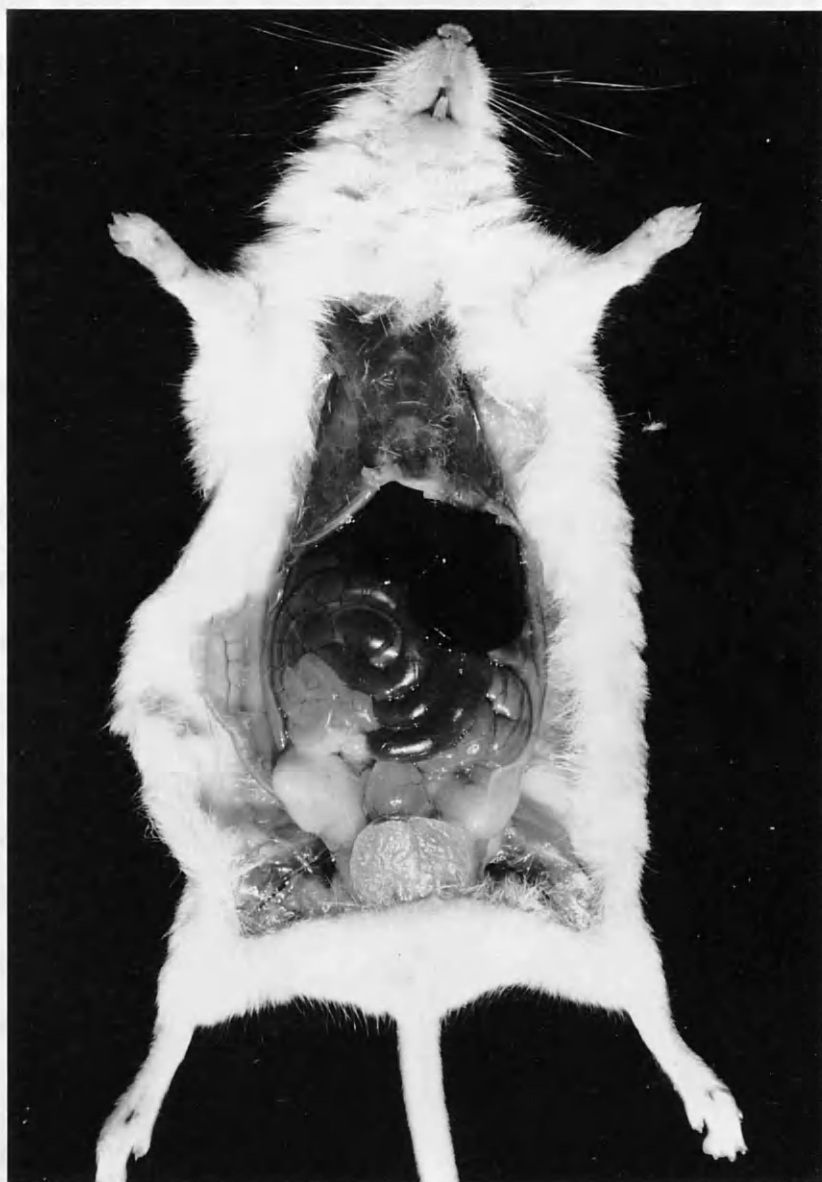


Plate 17. Normal mouse, 14 months old. Compare with Plates 15 and 16.

cultures from the animals which died were negative, and organisms other than acid-alcohol-fast bacilli were not found in the peritoneal cavity. Smears of the intestinal mucosa revealed organisms so numerous as to give the impression of looking at badly prepared smears from a culture (see plate 18, page 132).

The histological changes in these mice were similar to but more extensive than those described earlier. In the bowel the infection involved not only the whole of the small intestine, but also the colon and the stomach. The villi were greatly distended forming almost spherical projections into the lumen of the bowel, and there were large aggregations of acid-alcohol-fast bacilli both in the submucosa and subserosa (see plates 19 & 20, page 133); the surface epithelium was desquamated, but only slight evidence of polynuclear reaction was seen in the underlying tissues.

Other organs showed changes similar to those described previously. The results of this experiment are summed up briefly in table 5 (page 127).

A comparison of the histological lesions in the intestinal mucosa in mice with those found in the intestinal mucosa in cattle (see plates 19 to 24, pages 133 to 135), shows a striking similarity, amounting, in essentials, to identity. It also appears that, as in the natural infection of cattle, a marked organotropism for the intestinal mucosa and/

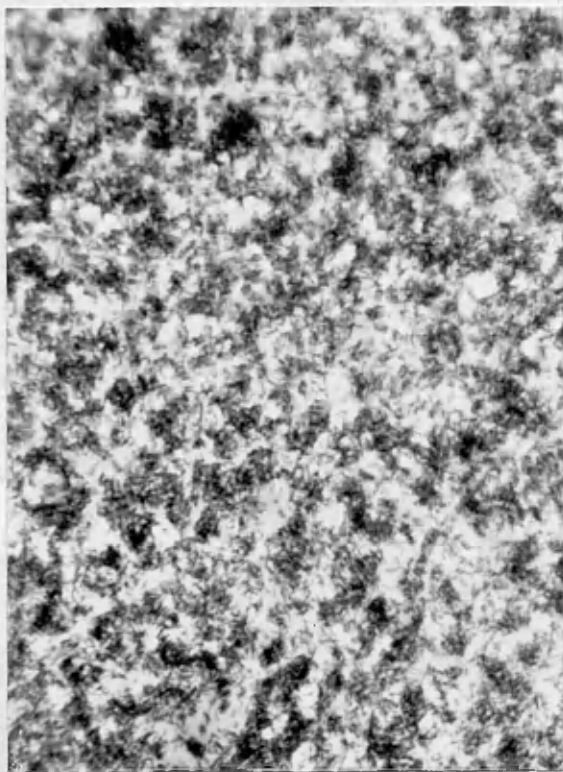


Plate 18. Smear of intestine of mouse which died of Johne's disease. No organisms other than acid-alcohol-fast bacilli are to be seen. Stained by Ziehl-Neelsen. Magnification x800.



Plate 19. Section of intestine of mouse which died of Johne's disease 14 months after the intraperitoneal inoculation of 1.96 mgm. of Myco. johnei. Note the gross dilatation of the villi which contain groups of large multinucleate macrophages. Stained by haematoxylin-eosin. Magnification x25.

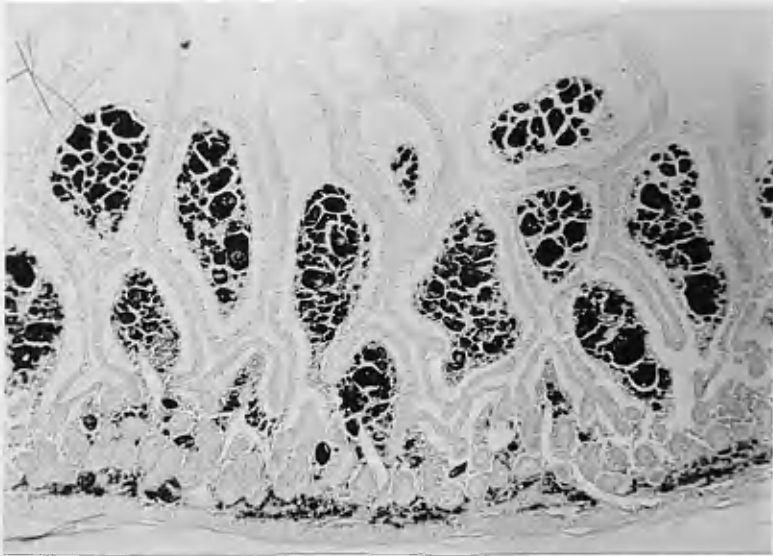


Plate 20. As Plate 19. Note masses of acid-alcohol-fast bacilli in the dilated villi. Stained by Ziehl-Neelsen. Magnification x25.



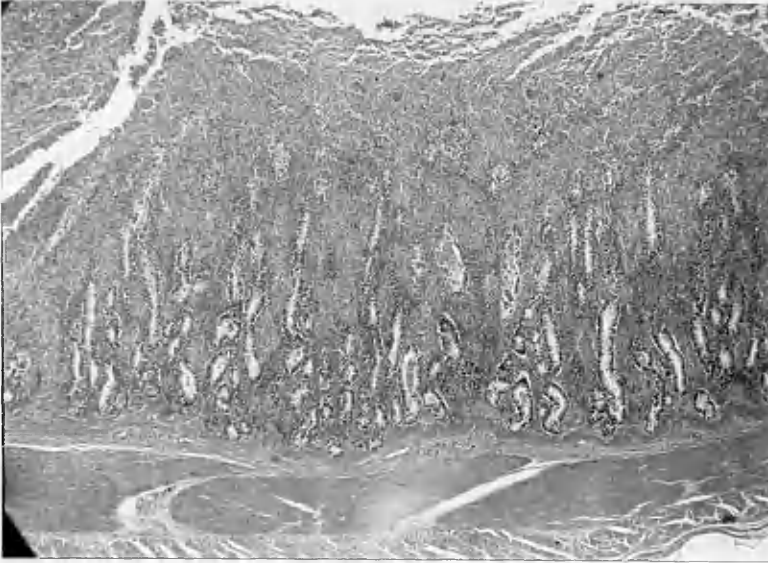


Plate 21. Johne's disease in cattle. Section of lightly infected bovine intestine showing giant cells in mucosa; there is some superficial ulceration. Stained by haematoxylin-eosin. Magnification x25.

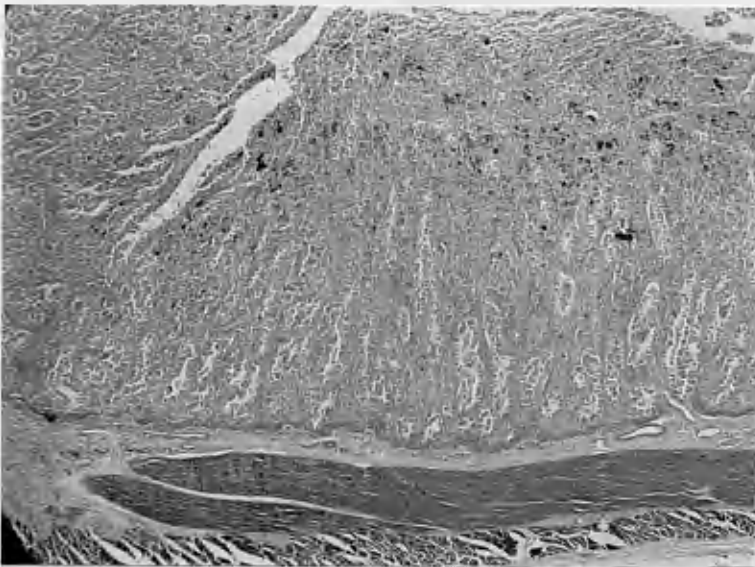


Plate 22. As Plate 21. Note acid-alcohol-fast bacilli, which appear as small dark groups at the free border of the mucosa. See also Plate 24. Stained by Ziehl-Neelsen. Magnification x25.

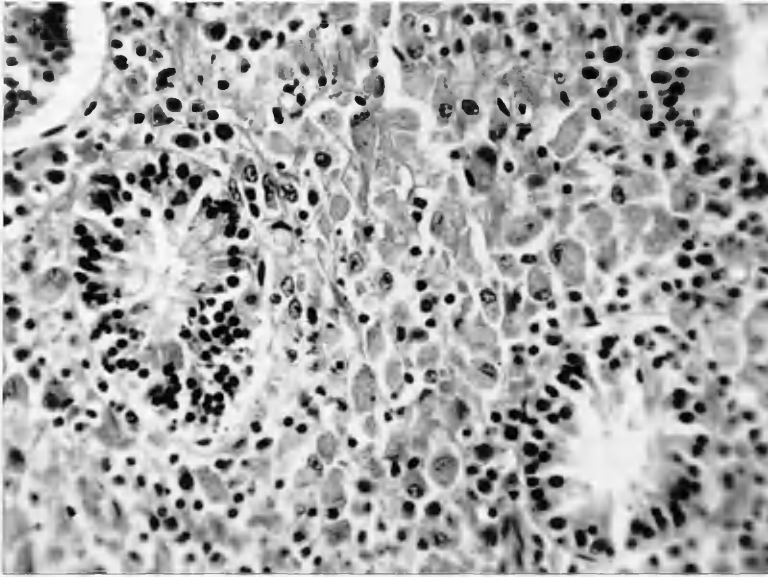


Plate 23. Johne's disease in cattle. Section of heavily infected bovine intestine showing mucous glands separated by large macrophages. Stained by haematoxylin-eosin. Magnification x600.

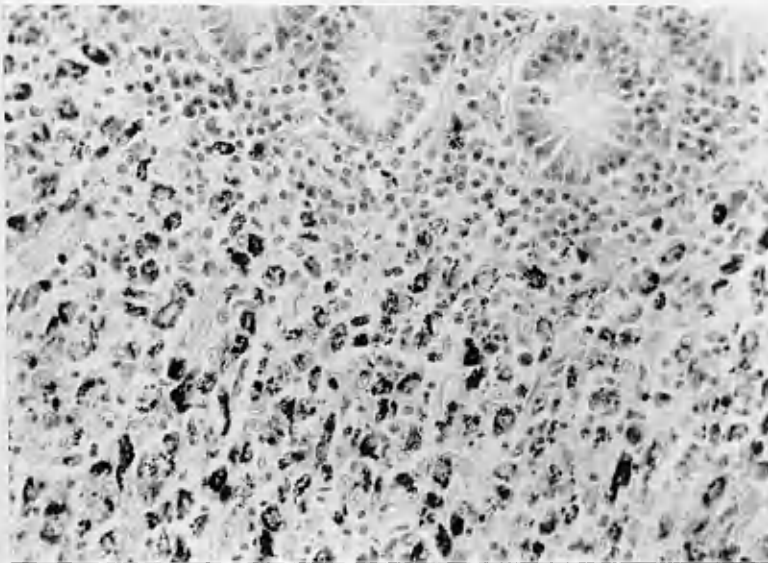


Plate 24. As Plate 23, showing acid-alcohol-fast bacilli within the macrophages. Stained by Ziehl-Neelsen. Magnification x400.

and mesenteric glands characterises Myco. johnei in the mouse. Lesions of the spleen and the liver, though possibly more common in the mouse than in cattle, are less extensive than those of the intestinal mucosa. There are no lesions in the kidneys or in the lungs of the mice, even after intravenous inoculation.

Cultures of Myco. johnei were grown during life from the faeces, and post mortem from the intestine, liver, and spleen, of the last 7 mice in this experiment; the organisms grew only in the presence of the Myco. phlei growth factor and were identified, therefore, as fully exacting strains of Myco. johnei. Thus, apart from proving that the organisms seen in smears from various sources were indeed Myco. johnei, the experiments showed that a single passage through the mouse had not affected their growth-factor requirement.

Considering the extent of the lesions, the age of the mice, and the absence of intercurrent infection, it seems reasonable to attribute the death of these 7 animals to Johne's disease, and to consider the macroscopic and microscopic lesions in these animals as representing the terminal stage of experimentally induced Johne's disease in mice. The extent of lesions leading to the death of an animal may, of course, depend on the individual, and it is possible/

possible that some of the earlier deaths in this group were also due to Johne's disease.

The results also show that the time required for the disease to develop in young mice is similar to that in adult mice, provided the latter live long enough. The age-immunity phenomenon, if it exists at all in the mouse, has not been demonstrated. Finally, and this is important for future experiments in view of the difficulty of obtaining adequate quantities of cultures, the experiment proves that infected bovine intestinal mucosa is pathogenic for the mouse: there is a hint - no more than this - that it is even more infective than cultures.

It has already been seen (see figure 1, page 124; and page 123) that the presence of acid-alcohol-fast bacilli in the faeces of experimental mice is an indication of the existence of histological lesions in the intestine. Moreover, it is possible to correlate the number of organisms in the faeces with the extent of the intestinal lesions. Thus, in the earliest stages of the experimental disease, the examination of faeces will reveal only isolated organisms. At this stage, lesions may easily be overlooked (Andersen, 1922); if, however, a thorough histological examination of the whole intestinal mucosa is made, one or more foci of infection may be found. On the other hand, the whole focus  
may/

may well be removed in preparing smears for bacteriological examination. Unless there is another focus nearby, the result of the bacteriological examination will be positive - clumps of organisms present - and that of the histological examination negative.

In later stages, when the numbers of bacilli have risen to about 50 per 50 microscopic fields, there is more likelihood of finding extensive histological lesions. However, it may still be necessary to examine a large number of sections on various levels of the intestine.

Finally, with a further rise in the number of organisms (200 or more organisms per 50 fields), it is practically certain that any longitudinal section of either the small or the large intestine will reveal large numbers of acid-alcohol-fast bacilli and extensive histological lesions in the mucosa.

Because of the similarity of the nature and location of the lesions produced by Myco. johnei in cattle and in mice, it was important to consider whether other features of Johne's disease in cattle could be observed in mice. In clinical features (diarrhoea, emaciation, polydypsia, and ravenous appetite) the species differ markedly. It was thought of interest to find out whether pregnancy has the same effect in/

in mice as it has in cattle, namely an exacerbation of the disease. The experiment was carried out as follows.

Three heavily infected female mice, 6-months-old litter mates, and 6 uninfected female mice of the same age were mated continuously for 9 months with 5 uninfected males. The same males were used for mating throughout the experiment, and were changed from box to box at intervals of 5 weeks to ensure impregnation of the females, which had not previously been mated.

Comparing the numbers of organisms in the faeces of the infected females with those in the faeces of simultaneously inoculated male litter mates, it appeared that pregnancy in no way intensified the disease. The striking result in this experiment was the effect of the disease on fertility (see table 6, page 140). During the 9 month period of observation litters of from 6 to 14 were born every 3 to 4 weeks to the non-infected females, whereas the litters of the infected females became smaller in number and were born less frequently until no more arrived, when it was assumed that the females were sterile. The mechanism of this effect has not been investigated.

Thus in cattle and in mice there seems to be no similarity in the relationship between the disease and pregnancy. The disease in mice was neither exacerbated nor brought/

TABLE 6

Influence of Johne's Disease on  
fertility of female mice.

		Infected			Uninfected
		1	2	3	Controls*
First Litter	Number in litter	6	7	4**	9
	Weeks after first mating	4	6	10	3
Second Litter	Number in litter	5	3	-	7
	Weeks since previous litter	13	15	-	4
Third Litter	Number in litter	-	-	-	9
	Weeks since previous litter	-	-	-	4

\* Average of 6 mice

\*\* These mice were eaten by the mother

- No litter born

brought to a fatal termination by pregnancies. Indeed, the converse seemed to be true - pregnancies were arrested by the course of the disease. This, incidentally, may also occur in cattle (Hagan & Zeissig, 1933b; Downham, 1951b;) - as an exception rather than the rule.

#### A FEEDING EXPERIMENT.

Since Johne's disease in cattle is probably contracted per os, I was surprised by my inability to produce the infection in mice by feeding Myco. johnei. The result was the more disappointing as Myco. johnei was introduced into the mice from birth, which is earlier than any other experimental procedure allows.

The food given to a pregnant female was soaked in an aqueous suspension of Myco. johnei, strain C, and the bovine intestinal mucosa from which strain C was isolated; some of the suspension was also added to the drinking water. The animal's nipples and abdomen were painted daily with the same material suspended in milk; this was continued throughout suckling and weaning. After weaning, the 5 young mice in the litter and the mother were fed on contaminated food and drinking water for a further 5 weeks. Throughout this period examination of the faeces of all the animals revealed acid-alcohol-fast bacilli; shortly after returning to/



to normal feeding the organisms disappeared from the faeces. When the animals were killed, 7½ months after the birth of the litter, none of them showed either intestinal lesions or organisms.

Since the strain of Myco. johnei used in this experiment was later shown to be pathogenic for mice, it is possible that the animals may have been killed during the incubation period. In view of what has been learned in the course of this work, animals in any later feeding experiments should be kept alive much longer.

#### THE MINIMAL INFECTIVE DOSE OF MYCO. JOHNEI

An attempt was next made to determine the minimal infective dose of Myco. johnei: this is difficult to assess because of the length of the incubation period. As will be seen later, the problem is further complicated by strains differing in their pathogenicity for the mouse, and because passage appears to enhance mouse-pathogenicity; both factors affect the minimal dose.

The information concerning the infective dose was derived from various experiments, not always designed with this end in view; some were carried out with cultures, some with bovine viscera, some with mouse viscera. Nevertheless, as a result of these experiments, a dose was found which, with

a/

a number of qualifications, can be considered the minimal infective dose.

The criteria by which the infective dose was assessed were the time between inoculation and the appearance of acid-alcohol-fast bacilli in the faeces, their subsequent increase in numbers, and the presence at post mortem of histological lesions of Johne's disease.

The most complete information was obtained from an experiment in which 36 mice were inoculated, some intraperitoneally, some intravenously, with strain M (see table 7, page 144). Fourteen months after inoculation the animals which received 0.5 mgm. are passing large numbers of acid-alcohol-fast bacilli in the faeces. Another 15 animals (not shown in table 7) received the same dose and were killed at regular intervals, the last one 8 months after inoculation; organisms were fairly numerous in the intestine, liver, and spleen, and there were single organisms in the faeces, but histological lesions of Johne's disease were not found in any of them. In contrast to this, 16 animals which received 1 mgm. were killed after 9 months, and 7 were found to have Johne's disease. Of the 13 receiving more than 1 mgm., 2 died shortly after inoculation; the remainder, with one exception, were killed, or died, 4 to 16 months after inoculation and 11 had Johne's disease. On the whole, it appears/

TABLE 7

Time required by various doses  
of Myco. johnei, strain M, to  
produce infection in mice

Number of mice inoculated	4	9	8	8	7
Dose in mgm.	2.64	1.96	1.0	1.0	0.5
Route of inoculation	I.P.	I.P.	I.V.	I.P.	I.P.
Time in months required to produce Johne's disease	7	8 to 12	9 (in 4 of 8 mice	9 (in 3 of 8 mice	14

appears that the time in which the mice developed the infection is in inverse proportion to the dose they received.

Only one dose, 1 mgm., of strain C was used (see table 8, page 146). The 5 animals in this group were killed after 9 months and 3 of them had Johne's disease.

With another strain, strain Fass, the effect of only 2 doses, 0.5 and 1 mgm., is shown (see table 8, page 146); in one of 5 mice, 1 mgm. produced Johne's disease in 9 months, the animals inoculated with 0.5 mgm. are alive 13 months after inoculation, and those inoculated intravenously are passing fair numbers of acid-alcohol-fast bacilli in the faeces.

Small inocula, 0.36 and 0.2 mgm. of a 4th strain, strain L, were used (see table 8, page 146). Twelve months after inoculation the mice which received the smaller dose are passing isolated organisms in the faeces; 3 of this group died of intercurrent infection 9 to 12 months after inoculation, and showed no lesions of Johne's disease post mortem; a few acid-alcohol-fast bacilli were seen in smears of the intestine, liver, and spleen. The mice which received the larger dose 10 months ago are still alive and passing isolated organisms in the faeces.

All mice which lived long enough after the inoculation of 0.5 mgm. of Myco. johnei developed Johne's disease; 1 mgm. produced/

TABLE 8

Numbers of acid-alcohol-fast bacilli (A.A.F.B.)  
in the faeces of mice after the inoculation of  
various strains of Myco. johnei suspended in  
various agents

Number of mice inoculated	6*	5**	2	9	4	2	2	10
Dose in mgm.	1.0	1.0	0.5	0.5	0.36	0.2	0.2	0.2
Strain of <u>Myco. johnei</u>	C	Fass	Fass	Fass	L	L	L	L
Suspending Agent	S	S	S	S	LP	GM	GM	GM
Route of inoculation	I.V.	I.P.	I.V.	I.P.	I.P.	I.V.	I.V.	I.V.
No. of A.A.F.B. in faeces per 50 oil immersion fields	***	***	10	10	10	10	10	10
Months after inoculation when A.A.F.B. appeared in faeces.	***	***	15	15	10	14	14	14

\* Killed after 9 months; Johne's disease in 3 of 6.

\*\* Killed after 9 months; Johne's disease in 1 of 5.

\*\*\* Not known.

S Saline

LP Liquid paraffin B.P.

GM Modified Glover's medium (see page 81)

produced the infection in a proportion of mice in all experiments after 9 months. In the experiments where an even larger dose was used, a larger proportion of the animals developed lesions, there was a shortening of the incubation period, and in some cases, death from Johne's disease. For practical purposes, it is essential that the disease should develop in mice before death from old-age becomes a factor of importance. This can be achieved by using either very young mice or an appropriately large inoculum, or both; the largest inoculum used in the present work was 2.64 mgm. It was found that doses of Myco. johnei greater than 1 mgm. could be tolerated only if given intraperitoneally.

Small inocula may well be capable of producing the disease; indeed, all mice inoculated with small doses of Myco. johnei pass acid-alcohol-fast bacilli persistently in the faeces, though in very small numbers, for as long as a year after inoculation. The significance of this is difficult to interpret. It is impossible to decide whether the inoculum was below the infective dose or whether it would have produced the disease after a long incubation period - one possibly exceeding the life of the mouse. It is also possible that small inocula are incapable of producing histological lesions. In either case, the animals could be looked upon as a type of carrier. It is known that Myco. johnei/

Myco. johnei can be recovered many months after inoculation from the tissues of laboratory animals which have not developed lesions of Johne's disease (Twort, 1914). Whether the bacilli simply persist in the tissues, or multiply slowly, is a matter for conjecture.

In the preceding experiments 1 mgm. of each of 3 strains of Myco. johnei produced Johne's disease in the mouse, but it should not be assumed from these results that all strains of Myco. johnei are of equal virulence. Indeed, the next group of experiments seems to show that strains of Myco. johnei differ in their intrinsic mouse pathogenicity.

#### THE PATHOGENICITY OF MYCO. JOHNEI FOR THE MOUSE

In these experiments (see table 9, page 149), 4 specimens of bovine intestinal mucosa were used; 2, from which strains F and R were subsequently isolated, contained numerous acid-alcohol-fast bacilli; the other 2, from which strains E and L were subsequently isolated, contained relatively few organisms.

The inocula were prepared by oxalating (see page 97), washing, and suspending 2 ml. of the resulting suspension of bovine intestinal mucosa in 10 ml. of saline. A total of 35 mice was inoculated; 6 mice, 7 days old, were inoculated with 0.2 ml. per inoculation of strain E mucosal suspension; 6 adult/

TABLE 9

Infectivity of 4 specimens  
of bovine intestinal mucosa

Mucosa	E	K	F	R
Acid-alcohol-fast bacilli in specimens of bovine intestinal mucosa	Scanty	Scanty	Numerous	Numerous
Number of mice inoculated	6	15	8	6
Acid-alcohol-fast bacilli appeared in faeces after	4 months	4 months	7 months	7 months
<u>Johne's disease post mortem</u>	+	L	L	L

All mice received 2 inoculations of 0.3 ml. of a 2 per cent saline suspension of oxalated, washed, bovine intestinal mucosa; the second inoculation was given 7 days after the first.

\* The corresponding strains of Myco. johnei have since been isolated from these specimens.

L Still alive and under observation.



6 adult mice received 0.3 ml. per injection of strain F mucosal suspension; 17 mice, 11 days old, received 0.3 ml. per injection of strain K mucosal suspension, and 6 adult mice received 0.3 ml. per injection of strain R mucosal suspension. All mice received 2 injections, the second 7 days after the first.

Four months after inoculation the mice inoculated with the lightly infected specimens E and K were passing acid-alcohol-fast bacilli in the faeces, and at 7 months all those inoculated with specimen E were histologically confirmed cases of Johne's disease. After 7 months the mice which received the more heavily infected specimens F and R are passing only small numbers of organisms in the faeces.

If the length of the incubation period in this experiment is taken as a measure of the virulence of a strain of Myco. johnei, it must be concluded that strains differ in their virulence for the mouse. This conclusion is strengthened because, of the 4 specimens of mucosa examined, the more virulent ones contained fewer organisms.

Not only were there differences in the intrinsic mouse pathogenicity of strains; it seems that the virulence of pathogenic strains can be enhanced by passage. Cultures were not used to demonstrate this effect because a sufficient bulk of/

of organisms was not available; suspensions of viscera from a heavily infected mouse were used instead.

Two litters (see table 10, page 152) each of 7 mice, litter A 7 days old and litter B 8 days old, were given 2 injections of 0.2 ml., the second 7 days after the first. The inoculum was a saline suspension of oxalated (see page 97) washed viscera of a mouse of a previous experiment, inoculated with strain M (mouse No.3 in tables 4 & 5, pages 122 & 127); there were large numbers of organisms in smears of the viscera of this mouse in which Johne's disease was confirmed histologically. Litter A received the saline suspension as such, and litter B a 1 in 4 saline dilution of it; the volume of the inoculum, 0.4 ml., represented approximately 0.04 gm. dry weight of material. Such a mass of material contained about 1 mgm. of organisms, which corresponds roughly to the dose used in the experiment described on pages 107 to 121.

Six months after inoculation large numbers of acid-alcohol-fast bacilli were present in the faeces of the mice which had received the undiluted suspension, and 2 to 3 months later 5 of them died of Johne's disease. Fourteen months after inoculation the mice which received the 1 in 4 saline dilution of the original suspension were passing only small numbers of organisms in the faeces (5 to 10 per 50 fields)/

TABLE 10

The effect of the size of the inoculum on  
the production of Johne's disease in mice

	Group A*	Group B**
1st inoculation at age of	7 days	8 days
2nd inoculation at age of	14 days	15 days
Mouse number	1 2 3 4 5 6 7	1 to 7
Number of acid- alcohol-fast bacilli in faeces during life	More than 1000 organisms per 50 oil immersion fields after 6 months	5 to 10 organisms per 50 oil immersion fields after 14 months
Time in months after inoculation when animal died of Johne's disease	8 8 11 15 9 9 8	Still alive after 14 months

\* Group A received a suspension of the viscera of a heavily infected mouse.

\*\* Group B received a 1 in 4 saline dilution of this suspension.

The inoculum, 0.2 ml., was given intraperitoneally.

fields): these mice are still alive and possibly still in the incubation period.

If, as it appears, the mouse pathogenicity has indeed been increased by passage, it would be logical to expect the minimal infective dose to have been reduced. This is not confirmed by the results obtained with litter B. Here, it is striking that approximately 0.25 mgm. of a strain of enhanced virulence failed to produce the infection in 14 months, whereas 0.5 mgm. of the parent strain produced the infection in the same time. It seems that even when the mouse pathogenicity has been increased by passage - and the incubation period thereby shortened - the infective dose cannot be reduced to half of its original value. It is possible, of course, that the infective dose has been reduced to some extent, but the reduction in this experiment is certainly not perceptible. The results with litter A, however, suggest that the enhancement of virulence may be reflected in a shortened incubation period.

The conclusion that passage enhances the virulence seems reasonable if one compares the results of earlier experiments (see pages 121 to 138), in which doses of 2 to 2.64 mgm. took on an average 7 to 10 months to produce the infection, with the results from litter A in the present experiment, in which approximately 1.0 mgm. of the same strain produced/

produced the infection in 6 months - and killed most of the inoculated mice in 9 months.

An attempt was made to enhance the mouse pathogenicity of small inocula of Myco. johnei, and thereby reduce the length of the incubation period by using as the suspending agents mucin and Liquoid-treated bovine serum (see page 98). It has already been shown that gastric mucin did not enhance the virulence of fairly large inocula of cultures of Myco. johnei (see tables 1 & 2, pages 108 & 109). In the present experiments, it was thought that either gastric mucin or Liquoid-treated serum might enhance the virulence of organisms contained in infected viscera, especially since the impression was formed earlier (see page 137) that such inocula might be more infective than cultures. The inoculum consisted of washed, oxalated (see page 97) viscera of mice which had died several months after inoculation with strain M: they were probably in the incubation period and their viscera contained only small numbers of Myco. johnei; as expected, they had no histological lesions.

One group of mice received a saline suspension of viscera, a second a suspension in Liquoid-treated bovine serum, and a third a suspension in a final concentration of 1.75 per cent gastric mucin. Of the 32 mice in these experiments/

experiments, 9, representing all 3 groups, have so far died of intercurrent infection. Two died 2 months after inoculation and the other 7, 5 to 12 months after inoculation; all were passing small numbers of acid-alcohol-fast bacilli in the faeces, and at post-mortem there were small numbers of organisms in the intestine, liver, and spleen; the results of histological examination were negative. The remaining 21 mice are still alive 10 to 12 months after inoculation, and are passing small numbers of organisms (5 to 10 per 50 fields). It was concluded that the suspending agents tested in this experiment had not obviously increased the mouse pathogenicity, at least not of such small inocula.

Apart from typical strains of Myco. johnei, (i.e. strains requiring the growth factor), experiments involving 55 mice were carried out with the 2 non-exacting strains, M.143 and Teps (see table 11, page 156). Strain Teps grows without the growth factor and is used for the production of johnin in the Royal Veterinary College, Streatley, Berkshire. Strain M.143, according to Allen (1953), is also non-exacting, but we have so far been unable to grow it in the absence of the growth factor.

Two mice were inoculated intraperitoneally with strain M.143; one died shortly after inoculation and the other, of Johne's/  
/

TABLE 11

Details and results of the inoculation  
of mice with 2 non-exacting\* strains  
of Myco. johnei

Number of mice inoculated	2**	32	1	9	10	1
Route of inoculation	I.V.	I.V.	I.P.	I.P.	I.P.	I.V.
Dose in mgm.	not known	0.17	0.17	1.0	2.0	0.5
Volume of inoculum in ml.	0.3	0.3	0.3	0.2	0.2	0.2
Suspending Agent	Saline	Saline	Saline	Liq.Par. B.P.	Saline	Saline
Strain	ML43	Teps	Teps	Teps	Teps	Teps
Months after inoculation when A.A.F.B. appeared in faeces	9	nil at 11	nil at 10	nil at 10	nil at 5	nil at 5
Johne's disease post mortem	+	L	L	L	L	L

\* These strains do not require the growth factor.

\*\* One died from intercurrent infection shortly after inoculation.

L Alive and under observation.

Johne's disease, 11 months after inoculation. The dose these mice received is unknown as the inoculum contained a proportion of egg-yolk medium.

Fifty three mice received up to 2.0 mgm. dry weight of strain Teps intraperitoneally or intravenously. Eleven months after inoculation there are no acid-alcohol-fast bacilli in the faeces of any of the animals which received 0.17 to 1.0 mgm.; those which received 2.0 mgm. have been under observation for 5 months and are not passing organisms in the faeces. Some have died and at post mortem organisms were not seen in smears of any of the organs.

This confirms the findings of Twort (1914), who infected mice, rabbits, and rats, with a non-exacting strain of Myco. johnei. It also indicates that strain Teps, in addition to being non-exacting, is probably of greatly attenuated virulence, if not avirulent.

#### CONTROL EXPERIMENTS WITH MYCOBACTERIA OTHER THAN MYCO. JOHNEI

It was important to ascertain whether mycobacteria other than Myco. johnei could produce in the mouse a picture resembling macroscopically and microscopically, that of Johne's disease. In the experiments to this end 4 strains of mycobacteria were used: (i) culture N.C.T.C. 6926, described as a strain of "Myco. johnei" capable of growing on/



on meat-extract agar in 48 hours at 37°C: obviously, in spite of its label, this is not to be accepted as a strain of Myco. johnei; (ii) an unidentified, orange-pigmented mycobacterium isolated by A.J. O'Hea in this Department from the pleural cavity of a patient in the Western Infirmary: this strain grows very slowly on Lowenstein-Jensen medium at 37°C, not at all on meat-extract agar, and it is not pathogenic for the guinea-pig; (iii) Myco. phlei N.C.T.C. 525; (iv) an unnamed mycobacterium maintained in this Department, which grows on meat-extract agar in 48 hours at 37°C.

(i) "Myco. johnei" N.C.T.C. 6926. Ninety mice were inoculated intraperitoneally or intravenously with this organism, the inoculum ranging from 0.001 to 4.0 mgm. dry weight of organisms. Death followed inoculation after from 4 days to 8 months, according to the dose, and was due to multiple pyogenic lesions in the kidneys.

Less than 0.006 mgm. intravenously was without effect; 0.006 to 0.03 mgm. caused death in 4 to 6 weeks; at post mortem there were white abscesses, about 1 mm. in diameter, in the kidneys. The inoculation of from 0.03 to 1.0 mgm. led to death in 4 to 7 days; the only observable lesions were abscesses in the lungs.

Intraperitoneal inoculation of less than 0.1 mgm. was without/

without effect. Inoculation of from 0.1 to 5.0 mgm. was followed by death in from a week to 8 months. Less than 1.0 mgm. gave rise to small, gritty nodules in the peritoneum and abscesses in the kidneys. More than 1.0 mgm. resulted in adhesions, generally involving the small intestine, kidneys, spleen, and the site of inoculation; the larger the inoculum, the more extensive were the adhesions which often contained green abscesses. Some animals died as the result of the abscesses rupturing the abdominal wall. Strain N.C.T.C 6926, was the only organism isolated from the lesions.

Histologically, the kidney abscesses were characterised by a large central mass of organisms - sometimes poorly acid-alcohol-fast in sections - surrounded by inflammatory cells, mainly polymorphonuclear leucocytes, while lymphocytes predominated in older lesions; there was extensive destruction of the adjacent renal parenchyma. There were no intestinal lesions.

(ii) Orange-Pigmented Mycobacterium from Pleura. Two mice received a large unmeasured inoculum of this mycobacterium, one intraperitoneally and the other intravenously. The mouse inoculated intravenously is still alive 12 months after inoculation; there are no acid-alcohol-fast bacilli in the faeces. The one inoculated intraperitoneally remained in good condition until it died suddenly 4 weeks after inoculation.

At/

At post-mortem the liver and lungs appeared normal, the spleen and one kidney were enlarged, and the other kidney was small, pale, and granular in appearance. There were numerous small, white, hard, gritty nodules in the mesentery and pleural cavity. Smears of these nodules and of the kidneys, liver, lungs, and spleen, revealed acid-alcohol-fast bacilli. There were no organisms in the faeces or intestine.

Histologically there was a local tissue reaction in the spleen, and in the pleural and peritoneal nodules; macrophages with intracellular organisms were observed. There were no lesions in the intestine.

(iii) Myco. phlei N.C.T.C. 525. Forty-eight mice were inoculated intraperitoneally or intravenously with doses of this mycobacterium ranging from 0.1 to 2.0 mgm. dry weight. The intravenous inoculation of 1.0 mgm. killed mice in 24 hours; the cause of death was not investigated; 0.5 mgm. was well tolerated and the animals were killed after 2 weeks. At post mortem unclumped acid-alcohol-fast bacilli were seen in the kidneys, liver, and spleen.

An attempt was made to determine the fate of Myco. phlei inoculated intravenously. A number of mice received 0.1 mgm. and were killed at weekly intervals for 4 weeks, then at monthly intervals for 8 months. Until the fourth or fifth month after inoculation the organisms could be detected in smears/

smears of various organs, and unclumped acid-alcohol-fast bacilli were seen now and again in the faeces. Between the fourth and fifth month after inoculation the organisms began to disappear from the organs and, coinciding with this, very large clumps of organisms appeared in the faeces and persisted there for about 2 weeks. Thereafter, organisms could be found neither in smears of tissue nor of intestinal mucosa.

A number of experiments was also carried out with heat-killed Myco. phlei, 0.1 mgm. of which was inoculated intravenously. The same striking, delayed appearance of the organisms in the faeces was observed.

It would seem that the appearance of Myco. phlei in the faeces reflects a mechanism of excretion, and does not represent an active process of localisation and multiplication in the intestine.

The intraperitoneal inoculation of 1.0 to 2.0 mgm. of live organisms was followed by the appearance of organisms in the kidneys, liver, and spleen; the mice were first examined 2 to 3 weeks after inoculation. In animals killed 6 months after inoculation organisms were not seen in these sites. The faeces of these animals were not examined, and it is impossible to say whether the same mechanism of excretion operated as in animals inoculated intravenously.

Neither/

Neither in animals inoculated intravenously - even when excreting large clumps of organisms in the faeces - nor in animals inoculated intraperitoneally, did histological examination reveal intestinal lesions. There was no tissue reaction in any other organs.

(iv) Unnamed Mycobacterium. A group of 11 mice was inoculated with a small dose, 0.1 mgm., of this organism intravenously and with large doses, 1.0 to 4.0 mgm., intraperitoneally. The animals were killed 5 months after inoculation. No organisms were seen in smears of the organs and no lesions were detected in the intestine, kidneys, liver, lungs, and spleen.

The experiments with 4 strains of mycobacteria other than Myco. johnei may be summed up thus: none of the organisms gave rise to an entity resembling Johne's disease in mice; one strain was highly pathogenic and caused multiple pyogenic lesions; another strain was not tolerated intravenously in large doses, and smaller doses led to the transient appearance of the organisms in the faeces; even in this case, however, lesions were not found in the intestinal tract. Thus it appears that of the mycobacteria investigated, only Myco. johnei can produce Johne's disease in mice.

## DISCUSSION

Experimental Johne's disease in mice lacks some of the impressive clinical features of the natural disease, and it has not been possible to produce the infection per os. However, experimental infection not uncommonly differs from the natural infection - for example, diphtheria and tuberculosis in man and in the guinea-pig. The clinical picture of naturally acquired Johne's disease is seldom seen in the experimental infection of the natural host (Wilson Taylor, 1949). On the other hand, there is a basic similarity between Johne's disease in cattle and in mice in respect of the localisation and nature of the lesions, and of the course of the infection. In my opinion the similarities between the natural and the experimental infection, particularly the similarity in lesions, are more suggestive than the differences - mainly clinical in nature - and the results of the work described in this thesis support the view that Johne's disease has been reproduced in mice.

Since, on the whole, Johne's disease is considered not to be transmissible to animals other than the natural hosts, it is of interest to compare, in some detail, the experimental findings with those in the natural infection.

It/

It is generally believed that cattle contract the infection per os. Experimentally all 3 routes, oral, intraperitoneal, and intravenous, have been used successfully to produce the infection in cattle. In contrast to this, in mice, the infection has been produced so far only by the intraperitoneal and intravenous routes. The daily oral administration both of cultures of Myco. johnei and of infected bovine intestinal mucosa, over a period of 8 weeks, failed to produce Johne's disease as judged by the absence of intestinal lesions in the mice killed  $7\frac{1}{2}$  months after the start of the experiment; in the light of later results, this may have been too soon after the start of the experiment.

The experiment was thought to have failed, however, not only because of the absence of lesions, but because there were no acid-alcohol-fast bacilli in any of the organs. As will be seen later, Myco. johnei is always found in the viscera of mice after the intraperitoneal and intravenous inoculation of even small inocula which produce no lesions. In mice fed Myco. johnei, acid-alcohol-fast bacilli were present in the faeces only so long as the inoculum was fed. It is possible, in view of this finding, that Myco. johnei neither penetrated the mucosa nor multiplied; bacilli which lodged in the mucosa were probably destroyed or eliminated by the host. Since Twort (1914) managed to infect only one of/

of 12 mice by the oral route, it seems that, on the whole, the intestinal mucosa of the mouse is more susceptible to infection with Myco. johnei from the bloodstream than from the lumen of the intestine. This, of course, is in marked contrast to the susceptibility of bovine intestinal mucosa in experimental infection.

Another difference between Johne's disease in cattle and in mice is the absence in mice of the clinical features, polydipsia and ravenous appetite, diarrhoea, and emaciation, which mark the advanced stages of the disease of cattle. Diarrhoea, on the whole, appears to be a rarity in mice, and I have seen it only once; this was in 4 young stock mice and it lasted 2 days. In mice with Johne's disease, diarrhoea was observed only just before death.

Emaciation of the type found in cattle was not seen, but a hollowing of the perineum, described by Lominet (1936) as characteristic of Johne's disease in cattle, was observed consistently in infected mice: the animals, too, had little or no fat, one of the less common signs in cattle (Bang, 1906), and in that sense, compared with normal mice of the same age, they could be considered emaciated. On the other hand, the weight at death of even heavily infected mice was 30 to 40 gm., while the weight of uninfected controls was 40 to 50 gm. The almost normal weight of the infected mice might be accounted for/



for by the fact that the small intestine was seen post-mortem to be grossly distended and full of fluid. This might also account for the deceptive external appearance of good condition presented by these mice. The atrophy of lumbar muscles, so characteristic of the late stages of the disease in cattle, appears to be absent in the mouse.

The age-immunity phenomenon, a feature of Johne's disease in cattle, was not demonstrated in mice. A further point of difference may be of degree rather than of kind; in infected cattle, calving may be followed by an exacerbation and the death of the animal in 3 or 4 months; surviving animals are occasionally sterile. Hagan & Zeissig (1933a) believe that, while the improved nutritional condition in pregnancy delays the advance of the disease, its sudden progress in the puerperal stage is favoured by the extra demand upon the mother.

In my experiment with mice pregnancy and parturition did not affect the course of the disease, but the disease as it progressed appeared to affect sterility. The number in litters decreased, the litters became less frequent, and breeding ceased prematurely. Thus, in both species, sterility may be an outcome of Johne's disease.

If Hagan and Zeissig are correct in their view that pregnancies delay the course of the disease, it is possible that/

that in mice the short time between pregnancies during the incubation period, combined with their liberal food allowance, may be a delaying factor to the progress of the infection. There is not, as yet, enough evidence either to prove or to disprove this suggestion.

As already mentioned, there are a number of similarities between the natural and experimental infection: two observable during life are the very long incubation period and the presence in faeces of acid-alcohol-fast bacilli.

The incubation period in cattle may be assumed to end with the onset of diarrhoea, which is also when acid-alcohol-fast bacilli are generally first sought in the faeces. There is no diarrhoea in mice to indicate the end of the incubation period, but if the sudden increase in the numbers of acid-alcohol-fast bacilli in the faeces is taken as marking its end, then in mice too there is an incubation period remarkable, as in cattle, in its duration. In cattle, with a life-span of 15 to 20 years, it is 18 months to 2 years; in mice, which live 2 to 3 years, it is 4 to 12 months. The incubation period seems to be independent of the life-span of the species, and in rabbits, which normally live 2 to 4 years, Andersen (1922) found lesions in the intestine 12 to 18 months after inoculation, although the length of the incubation period is not known with certainty.

Acid-/

Acid-alcohol-fast bacilli are found in the faeces both of infected cattle and mice. In cattle, a clinical diagnosis of Johne's disease is confirmed by the presence in faeces of densely packed clumps, or nests, of acid-alcohol-fast bacilli. Only in recent years (Doyle & Spears, 1951; Smythe, 1951; Soltys, 1951;) has the presence of organisms in other than the characteristic arrangement come to be regarded as possibly indicative of Johne's disease. In the course of the present work I have diagnosed Johne's disease in cattle on the basis of single acid-alcohol-fast bacilli in the faeces, and these results were confirmed, both by complement-fixation tests carried out by Mr. N.H. Hole, at Weybridge (McIntyre, 1953, personal communication), and by post-mortem examination. Since Wilson Taylor (1951b) has isolated Myco. johnei from bovine faeces many months before it could be seen in films, it seems probable that Myco. johnei first occurs as single acid-alcohol-fast bacilli which increase in number and eventually appear in the characteristic clumps.

This is what appears to happen in the faeces of mice, which were probably more methodically examined in the present work than, as a rule, are bovine faeces. In mice, Myco. johnei was seen first as single bacilli and, as the disease advanced, numbers increased and clumping followed. The organisms/

organisms in such advanced cases in mice were generally longer than those seen in bovine faeces, but regardless of size, they were Myco. johnei: they were isolated and identified as such by their requirement for the Myco. phlei growth factor, in which respect mouse passage did not seem to have affected them. In advanced stages of the infection in mice, there are even more organisms in the faeces than I have seen in any specimens from cattle. This is understandable because the invasion of the intestine in cattle is rarely as extensive as that observed in mice.

The fundamental point of similarity is the localisation and nature of the lesions both in cattle and mice; it should be borne in mind that even after intravenous inoculation the lesions in mice are found chiefly in the intestinal mucosa and associated lymph glands, with involvement of the liver and spleen. The initial lesions in cattle are found in the vicinity of the ileo-caecal valve; at later stages of the infection lesions have occasionally been reported extending to the rectum, and even to the duodenum (Houthuis, 1932). In mice the initial lesion is not necessarily situated around the ileo-caecal valve; in advanced cases, the stomach and the whole of the intestine are affected. The intestine is enlarged, the wall thickened, and the whole appearance similar to that in cattle. Andersen (1922) remarked that the intestinal/

intestinal infection in the rabbit was a replica of that seen in cattle; this comment applies equally well to the intestinal infection in mice. The marked organotropism of Myco. johnei for the intestinal mucosa is a striking characteristic both of the natural and the experimental infection.

In cattle and in mice the lesions consist of macrophage infiltration of the deep mucosal and submucosal layers, the macrophages being densely packed with innumerable acid-alcohol-fast bacilli. A number of polynucleate macrophages of the giant-cell type are also present; there is no caseation and there are very few polymorphs. The epithelium of the intestine shows no obvious lesion of continuity.

Another similarity lies in the death rate. Among cattle the great majority of animals which develop clinical symptoms die; in mice it is difficult to determine the death rate because the incubation period is, relatively, much longer, and old age becomes a complicating factor. But for that, the death rate would probably approach 100 per cent, as indeed it was in one experiment.

It is true, of course, that some mice inoculated with Myco. johnei failed to develop Johne's disease. In those which received the minimal infective dose or more, the number of/  
of/

of organisms in the faeces increased considerably from about the sixth month and intestinal lesions developed. Those which received smaller inocula continued to pass small numbers of organisms until they died, usually 12 to 16 months after inoculation, and post mortem there were fair numbers of bacilli (5 to 10 per microscopic field) in the intestinal mucosa, mesenteric lymph glands, liver, and spleen, and smaller numbers in the kidneys and lungs. There were no lesions of Johne's disease in any of these mice, but it seemed that some of them were in the early stages of infection and, were it not for the short life-span of the mouse, would probably have developed Johne's disease.

The presence of Myco. johnei in the intestinal mucosa and mesenteric lymph glands, without lesions of Johne's disease, is not without its parallel in cattle. Wilson Taylor (1949, 1952a) isolated Myco. johnei from the ileo-caecal lymph nodes of 101, or 15 per cent, of 665 cattle, being "a random sample of graded animals slaughtered for human consumption". He considered this finding indicated that Myco. johnei might be "only potentially pathogenic" for cattle, although more infective than generally believed. Wilson Taylor's finding, moreover, indicated that many animals might harbour Myco. johnei for a long time as a commensal without showing symptoms of the disease: only under/

under certain conditions the organism might become pathogenic. Similarly, mice harbouring Myco. johnei may be considered sub-clinical cases or carriers. Such mice may never become clinical cases. Pregnancy, which, with its demand on the reserves of the mother, directly or indirectly potentiates Johne's disease in cattle, did not seem to affect the course of the disease in mice in the experiment reported here. This work did not reveal any other conditions capable of precipitating the development of the infection in mice.

In spite of the many similarities between the infection in mice and in cattle, it might still be argued that the entity experimentally produced in mice is not Johne's disease.

It could be said, in view of the size of the inoculum required to produce the infection (approximately 0.5 mgm.), that the mycobacteria have not multiplied but are simply being excreted into the intestinal mucosa, possibly through the liver, and ingested on the way by macrophages; and furthermore, that the histological changes in the intestine, consisting mainly of macrophage infiltration, by itself not characteristic of any particular infection, are merely the result of the presence of large numbers of acid-alcohol-fast bacilli which may, or may not, be Myco. johnei. However, there/

there are counter-arguments which render this point of view untenable and I have little doubt that an infection, accompanied by multiplication of the causative agent, had indeed taken place.

Firstly, in the intestinal mucosa of a heavily infected mouse there is an enormous number of organisms. Supposing these to be the inoculum, it is inconceivable that such large numbers could escape detection even if distributed throughout the tissues; yet in mice examined shortly after inoculation only small numbers of organisms were found in any of the tissues. It seems improbable that these small numbers, concentrated in the abdominal viscera, would account for the numbers found in the intestinal mucosa, the liver, and the spleen. Moreover, there is the difficulty of explaining how the elimination of Myco. johnei into the intestinal tract is delayed for several months and then proceeds at an ever increasing rate; at the same time there is an increase in the numbers of the organisms not only in the intestinal mucosa, the liver, and the spleen, but also in the kidneys and the lungs.

In the second place, it is certain that Myco. johnei multiplies in the mouse. Accepting Johne's bacillus as roughly cylindrical, 1.0  $\mu$ . long and 0.5  $\mu$ . in diameter, and assuming its specific gravity to be 1.045 - the average of the values given for the tubercle bacillus by Dilg and Nebel (quoted/



(quoted by Calmette, 1922) - its weight can be calculated as approximately  $2.10^{-10}$  mgm., i.e. there are roughly  $5.10^9$  organisms per mgm. Thus, the largest inoculum used, 2.64 mgm., contained no more than  $13.10^9$  organisms. This number refers to true dry weight of organisms, whereas the inoculum used in this work was simply dried in the incubator for 48 hours and was thus bound to contain fewer organisms.

The number of organisms passed by mice in the faeces - disregarding those passed during the incubation period and attempting to count them only in the more advanced stages of the disease - can be very roughly calculated\* as  $4.5 \times 10^9$  single/

\* In the more advanced stages of the infection there are approximately 1000 single organisms per 50 oil-immersion fields, i.e. there are 20 single organisms per field. One oil-immersion field covers about 0.00025 sq. cm. With the method of making smears of faeces, one pellet of faeces can be spread over roughly 56 sq.cm., i.e. over 225,000 oil-immersion fields.

Therefore, one pellet contains approximately  $20 \times 225,000 = 4.5 \times 10^6$  organisms.

Since mice were observed to pass, on an average, 6 pellets of faeces per hour, i.e. 1008 per week, it was estimated that heavily infected mice pass approximately  $4.5 \times 10^6 \times 1008 = 4.5 \times 10^9$  organisms per week.

single organisms per week; some mice were passing organisms at this rate for as long as 15 weeks. This calculation does not include the numerous clumps accompanying the single organisms. There is roughly one clump of organisms to 10 single organisms, and although it is practically impossible to count the organisms in a clump, the number generally exceeds 20. If the clumps are included in the calculation, the total number of organisms excreted per week becomes  $13.5 \times 10^9$ . Thus, as opposed to  $13.10^9$  organisms in the inoculum, a mouse may excrete in the faeces in 15 weeks roughly  $20.10^{10}$  organisms. This leaves uncounted the truly immense number of organisms in the mucosa and submucosa of the whole intestine, in the liver, and in the spleen. Smears made from these sites and stained by the Ziehl-Neelsen method appear red to the eye, and microscopically, look like badly prepared smears from a culture.

More convincing proof of multiplication is provided by the passage experiments. A liver lobe, from a mouse which died 7 months after inoculation, was homogenised in saline and injected into 7 other mice. Within 5 months these mice were passing large numbers of acid-alcohol-fast bacilli in the faeces, and 9 months after inoculation 6 died of Johne's disease. Smears of the viscera of these mice were indistinguishable, qua number of organisms, from the smears of the viscera of the original mouse. If all of the 2 mgm. of/  
of/

of Myco. johnei which this mouse received were contained in the liver lobe used as the inoculum for the other 7 mice, the maximum each mouse could have received was approximately 0.3 mgm. But it is known that 0.5 mgm. of this strain of Myco. johnei does not produce Johne's disease in the mouse in under 14 months, and that passage of the strain, though shortening the incubation period, does not reduce perceptibly the minimal infective dose. It is obvious, therefore, that apart altogether from any increase in the virulence of the strain, Myco. johnei must have undergone multiplication. It is difficult, incidentally, to conceive of an increase of the virulence of an organism without multiplication.

In mice which received less than the minimal infective dose, and which continued to pass acid-alcohol-fast bacilli in small numbers, Myco. johnei probably underwent some multiplication too, although it cannot be so convincingly demonstrated as in the mice which received larger doses and developed the infection. Thus, in the organs of a group of mice - each of which received 0.5 mgm. Myco. johnei, strain M, and some of which were killed one at a time after intervals of one day, one week, 2 weeks, 3 weeks, 4 weeks, and thereafter every fourth week for 8 months - there were more organisms to be seen after 8 months than after 24 hours. There was almost certainly multiplication, but not sufficient to,

to cause lesions within the period of observation. The 7 surviving mice developed lesions 14 months after inoculation!

It is remarkable that after even the smallest inocula - 0.2 mgm. of culture, and probably less of infected mouse viscera - Myco. johnei seems seldom to disappear from the tissues of a mouse; isolated acid-alcohol-fast bacilli can generally be found. If Myco. johnei were devoid of pathogenicity for the mouse, it would certainly be reasonable to expect the host to destroy and eliminate such small numbers within 16 months which is, after all, more than half the life of the mouse.

Lastly, the nature of the intestinal lesions and identity of the organisms therein must also be considered. However non-specific, the histological changes in cattle and in mice are practically identical, and in both species large numbers of Myco. johnei are associated with them. It would seem improper to term the entity in cattle Johne's disease and to seek another explanation for the same picture in mice, unless the acid-alcohol-fast bacilli seen in the faeces and the organs of mice could be other than Myco. johnei, for example, saprophytic mycobacteria ingested with the food or derived from the bedding. Even then it would have to be shown that such mycobacteria can reproduce in mice the same picture as Myco. johnei.

Proof/

Proof that the vast numbers of acid-alcohol-fast bacilli in the faeces and viscera of infected mice are Myco. johnei lies in the fact that, as already mentioned, Myco. johnei has been isolated both from the faeces during life and from the viscera post mortem; in all cases, a confluent growth was obtained. No mycobacterium other than Myco. johnei has ever been isolated from any of the infected mice. As to the possibility that the acid-alcohol-fast bacilli in the faeces could be other than Myco. johnei, I have never seen acid-alcohol-fast bacilli in the faeces of uninfected, control mice.

In the faeces of mice inoculated intraperitoneally or intravenously with mycobacteria other than Myco. johnei, small numbers of isolated organisms were present in the faeces and then only for a few months after inoculation. With Myco. phlei, very large clumps of organisms appeared in the faeces 4 to 5 weeks after inoculation, persisted for about 2 weeks, and disappeared; even massive doses of Myco. phlei fed continuously for 6 months, gave rise during feeding to only small numbers of unclumped organisms in the faeces. On the other hand, feeding smaller doses of Myco. johnei, although not leading to a progressive infection, resulted in the appearance in the faeces of fair numbers of acid-alcohol-fast bacilli throughout the period of feeding. There/

There seems to be no doubt that the short-lived presence of large numbers of acid-alcohol-fast bacilli in the faeces, following the inoculation of Myco. phlei, is a different phenomenon from the persistence and multiplication of Myco. johnei. With Myco. phlei, it can be assumed that the organisms are eliminated; with Myco. johnei one has to accept a multiplication in situ.

So far as tissues are concerned, non-pathogenic mycobacteria when inoculated into mice disappeared rapidly from the organs, unlike Myco. johnei. Pathogenic mycobacteria, however, multiplied and caused lesions in the kidneys and lungs, organs where Myco. johnei has not so far been observed to produce lesions. Furthermore, these lesions were entirely different histologically from those produced by Myco. johnei. None of the 4 mycobacteria investigated produced a picture resembling that produced by Myco. johnei.

The transmissibility of Johne's disease to small laboratory animals is probably not accepted by authors of standard text-books because reported failures so greatly outnumber successes. My concern here is not with the non-acceptance of successful experiments but with the reasons for so many failures to produce Johne's disease in laboratory animals. In view of the ease and constancy with which/

which my results were obtained, I think that some explanation for these failures is called for. Here it may be noted that neither Francis himself (1953), nor Glover (1953), was able to reproduce Francis's earlier (1943) results.

There are probably many reasons for such a failure, and the most important may well be the lack of a criterion, short of post-mortem findings, by which the success of inoculations can be gauged. Reference to the publications of Andersen (1914,1922), Twort & Ingram (1913a), Twort (1913, 1914), Boquet (1925), and others, and to my own results, makes it clear that in the experimental infection not only is the clinical picture of the natural infection absent, but other untoward symptoms may not be observed. Thus, it is very difficult to know, without killing them, which animals have become infected, yet without this knowledge results can only be assessed post mortem. It seems surprising, despite the known long course run by the infection in cattle, that so many workers should expect a rapid infection in laboratory animals. Wilson Taylor (1951a) killed guinea-pigs and rabbits 7 weeks after inoculation: the results, as expected, were negative. It seems probable that most workers killed their animals without knowing whether or not they were infected, and almost certainly killed them during the incubation period.

The/

The examination of faeces for acid-alcohol-fast bacilli, introduced in the course of this work, enables the progress of the disease to be followed, and in its quantitative application indicates when an animal may be killed with a reasonable expectation of positive histological findings. Indeed, all animals with fairly large numbers of organisms in the faeces (more than 100 per 50 fields) had lesions in the intestinal mucosa; it has also been my experience that once an animal becomes infected there is no regression of the lesions.

Without the regular examination of faeces for acid-alcohol-fast bacilli, positive histological findings could have been obtained only if the animals had been kept alive for what may appear to be, compared with other experimental infections, an unusually long time - a year or more. It is noteworthy that, in the past, the few successes reported were achieved when the period of observation was exceptionally long. The surest way to obtain results seems to be to leave infected animals to die, either of Johne's disease or of old-age.

Some experimental infections may have failed because the animals received too small an inoculum; unfortunately, few workers indicate the dose. From my experiments, however, the size of the inoculum emerges as an all important factor.

All/



All mice inoculated with 0.5 mgm. or more of Myco. johnei developed Johne's disease; 0.5 mgm. produced the infection in 15 months, 1 mgm. in 9 months, and 2 to 2.64 mgm. in 7 months or more. Since the length of the incubation period appears to be inversely related to the dose, and the life of the mouse is short, an incubation period of not more than 9 months is desirable; for practical purposes, therefore, 1 mgm. is suggested as a minimal infective dose. The dose, of course, may vary with the strain of organism, the strain of mice, and as the result of passage. It should, however, be remembered that, although one mouse passage enhanced the virulence of a strain, the enhanced virulence was not reflected in a perceptibly reduced infective dose but rather in a shortened incubation period. Even so, a large number of mouse passages might well reduce the dose.

The inoculum of 1.0 mgm., recommended as a working infective dose as opposed to the minimal infective dose of 0.5 mgm., is rather large. However, the use of large inocula in experimental infections is fairly common and in experimental Johne's disease of cattle large doses either of cultures or of infected bovine intestinal mucosa are invariably used, the smallest known figure being 100 mgm. moist weight of culture, recently used by Wilson Taylor (1953). This amount was chosen arbitrarily and is probably greater than/

than the minimal infective dose. In the natural infection of cattle the infective dose is not known but it is fair to assume that it is small, certainly less than 100 mgm.; in the experimental infection too it might be expected to be small since Myco. johnei is probably very highly adapted to cattle. Whether the unusually low infective dose for voles of 0.0001 mgm. reported by Levi (1950) is also the result of adaptation is a matter for speculation - as long ago as 1913 Twort thought that wild rabbits and mice may "take the disease naturally" and act as reservoirs of Myco. johnei.

It is conceivable that some failures may have been due to the use of avirulent strains. That strains of Myco. johnei differ in virulence for the mouse is shown by my experiments. The incubation period in mice, following the inoculation of cultures and using the presence of large numbers of organisms in the faeces as the indicator of infection, was 8 months with one strain and 12 months with another; it was later reduced from 8 months to 6 months by mouse passage. In another experiment, mice were inoculated with suspensions of bovine intestinal mucosa; 4 specimens were used, 2 containing considerably larger numbers of acid-alcohol-fast bacilli than the others. The mice inoculated with the specimens containing fewer organisms were passing large numbers of Myco. johnei in the faeces within 4 months, whereas the others had not done so in 8 months. These results can be most/

most easily explained in terms of degrees of virulence. If so small a number of strains differ so considerably in virulence, it is possible that with larger numbers the differences would be even more marked - the pathogenicity of Myco. johnei for the mouse may, indeed, range from very high to nil. This may account for the results obtained by Boquet (1925), who inoculated mice intraperitoneally with as much as 5 to 30 mgm. of Myco. johnei, and achieved only hypertrophy of the abdominal and tracheo bronchial glands without intestinal localisation: like so many other workers, however, Boquet did not indicate how long he kept his animals before killing them, and again, the period of observation may have been too short.

The characters by which Myco. johnei is defined - requirement for growth factor, slow rate of growth, and ability to produce Johne's disease in cattle - do not exclude the possibility that the entity comprises, if not more than one species, at least several varieties. Non-exacting strains represent a variety of Myco. johnei not covered by the definition, and my results with 2 such strains deserve comment. Strain Teps, used for johnin production, in my hands grows in a chemically defined medium without growth factor: it appears to be avirulent, as much as 2 mgm. dry weight of organisms disappearing fairly rapidly from the tissues./

tissues. On the other hand, strain M143, according to Allen non-exacting, is pathogenic for the mouse. Twort (1914) also produced Johne's disease in mice with a non-exacting strain of Myco. johnei, so that it would appear that the mouse pathogenicity of Myco. johnei is not related to its growth-factor requirement. If this is true, strain Teps is probably more accurately described as an attenuated, non-exacting strain of Myco. johnei.

Lastly, breeds of mice probably differ in their susceptibility to infection with Myco. johnei. It is known not only that different strains of mice vary in susceptibility to infection, but that even the most highly inbred strain is not necessarily homozygous in respect of susceptibility either to infection or to toxins (Wilson & Miles, 1946). The strain of W-Swiss mice, which proved so valuable in the present work, is no exception. In litter mates of this strain, Lominski (1953) showed that the lethal dose of diphtheria toxin ranges from 20 to 200 guinea-pig M.L.Ds. As far as susceptibility to infection with Myco. johnei is concerned, the length of the incubation period differed from litter to litter, and even within the same litter.

Few workers specify the breed of mice used, or if they do so, do not mention the particular strain used. It is/

is possible, although experimental proof is lacking, that some of the failures in the past were due to the use of insusceptible breeds of mice. This point could be settled either by inoculating various breeds with virulent strains of Myco. johnei, or, where known virulent strains are not available, by inoculating various breeds, including a known susceptible one, with cultures or suspensions of infected bovine intestinal mucosa. The advantage of the latter would be to allow strains of Myco. johnei to be simultaneously isolated and tested for virulence.

It is possible, of course, that in many cases a combination of some or all of the above factors - size of inoculum, virulence of strain, length of incubation period, susceptibility of mice - was responsible for failure, especially as they are all to a large degree interdependent. For instance, a short period of observation, as is so often the case, following the inoculation of a strain of low virulence into a resistant breed of mice is almost bound to lead to negative findings. The case of Francis in particular, who was unable to repeat his own results in mice, might well be accounted for in this way.

In conclusion, it seems clear that Johne's disease has been reproduced in mice in the present work since the experimental/

experimental infection resembles the natural infection in localisation, in histological reaction, and in its prolonged course and since the essential postulates of Koch have been fulfilled. To some extent it also seems possible to account for the failure of so many workers in the past.

In the present work, the W-Swiss mouse has proved to be the ideal experimental animal; apart from its susceptibility to infection with Myco. johnei, it is easily bred, easily accommodated, easily handled, and is resistant to intercurrent infection. It is also possible that with the information gained on the importance both of the size of the inoculum and of the length of the incubation period, Johne's disease may be produced in other breeds of mice and in other laboratory animals.

SUMMARY

The methods for the isolation of Myco. johnei from intestinal mucosa and media for its cultivation are discussed; 30 bovine and 2 pigmented ovine strains of Myco. johnei have been isolated. A new method for the isolation of the organism from faeces has been devised.

A vitamin-K compound was found to stimulate the growth of a strain of Myco. johnei not requiring the growth factor supplied by Myco. phlei. This compound did not support the growth of 3 typical strains of Myco. johnei requiring the growth factor. Other aspects of the metabolism of Myco. johnei were also investigated.

The main object of this study was to reproduce Johne's disease in mice. For this purpose, 6 strains of Myco. johnei were inoculated as cultures: of these, 4 were virulent, one was avirulent, and one was tested only with what may have been sub-minimal inocula. Five strains were inoculated as suspensions of infected viscera: of these, 3 were virulent, and the virulence of the other 2 is as yet unknown. The infection was produced by the intraperitoneal and intravenous, but not by the oral route.

Johne's disease in mice is characterised by a very long/

long incubation period - 8 to 16 months, depending on the size of the inoculum - at the end of which Myco. johnei appears in the faeces and rapidly increases in numbers as the infection progresses. The disease is almost asymptomatic, yet in some experiments the death-rate was very high. Although it was possible to produce the infection in approximately 14 months with 0.5 mgm. dry weight of organisms, the use of larger doses shortened the incubation period. The shortest incubation period observed was 6 months.

Post-mortem examination of infected mice reveals thickening of the intestine and enlargement of the mesenteric lymph glands; microscopically, innumerable organisms are seen in smears of the viscera. The lesions, infiltrated by macrophages packed with acid-alcohol-fast bacilli, and sometimes by giant cells, were found in the intestinal mucosa and submucosa, and in the mesenteric lymph glands, liver, and spleen; the intestinal lesions are a replica of those seen in cattle, although generally more extensive.

Myco. johnei was re-isolated from the faeces and organs of experimentally infected mice, and the infection was reproduced by inoculation of the viscera of such animals.

Of a total of 205 mice inoculated with cultures, 51 received sub-minimal inocula, 53 received the avirulent strain/



strain, and 55 were killed for histological examination within 4 weeks of inoculation. Of the remaining 46, 27 were killed after 9 months; of these 27, 11 had Myco. johnei in the faeces and 5 of the 11 examined histologically had lesions. Another 12 of the 46 had Myco. johnei in the viscera 12 to 16 months after inoculation and 11 of them, examined histologically, had lesions. The remaining 7 of the 46, still alive, have large numbers of Myco. johnei in the faeces.

Of 54 mice inoculated with bovine intestinal mucosa, 7 died shortly after inoculation, 25 are in the incubation period, and 22 developed the infection. Of these 22, 12 are still alive and 10 are dead, of which 7, examined histologically, had lesions.

Of 74 inoculated with mouse viscera, 12 died shortly after inoculation, 25 still alive have large numbers of Myco. johnei in the faeces, 18 are in the incubation period, 4, inoculated 11 months ago, probably received a sub-minimal dose, and 15 developed the infection. Of the 15, 7 examined histologically had lesions.

Reasons are suggested for the failure of other workers to transmit Johne's disease to laboratory animals. The size of the inoculum, the length of the observation period, and differences in virulence of strains of Myco. johnei and in the susceptibility of animals, appear to be the main factors.

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## ACKNOWLEDGMENTS

My thanks are due firstly to the Medical Research Council for the studentship which enabled me to carry out the work herein described; I must also thank the Trustees of the Rankin Research Fund of the University for provision towards the expenses of the research.

It is quite inadequate to say simply, "Thank you", to Doctor Iwo Lominski, yet to say more would be to say not enough. At all times, as my friend and supervisor, he has been helpful, critical, and above all, patient. To him, in the hope that he realises all its implications, I say, "Thank you".

For extending to me the hospitality of the Bacteriology Department of the University and Western Infirmary, and for continued interest and encouragement in my research my thanks are due to Professor C.H. Browning and to Professor J.W. Howie.

The histology of the mice and much of the photography is the work of Doctor G.B.S. Roberts, to whom I am greatly indebted; I should also like to thank Mr. Kerr for his share of the photography.

For assistance in the preparation of media I have to thank the Technical Staff of the Bacteriology Department, and/

and to both the Medical and Technical Staffs I should like to extend my thanks for making my stay in the Department so pleasant.

Outwith the Bacteriology Department I have to thank Professor W.L. Weipers of the Veterinary Hospital for offering me facilities to observe many natural cases of Johne's disease. I should also like to thank Mr. W. Jarrett of the Veterinary Hospital for teaching me, in the course of many post-mortem examinations, the pathology of Johne's disease in cattle and in sheep.

To Professor R.E. Glover, Liverpool, Doctor J.T. Stamp, Edinburgh, Doctor A. Wilson Taylor, Edinburgh, Mr. H.R. Allen, Streatley, Mr. T.M. Doyle, Weybridge, Mr. W.G. Johnston, Auchincruive, and Mr. I.M. McIntyre, Veterinary Hospital, I am greatly indebted for specimens of infected bovine intestine and faeces, and for cultures of Myco. johnei, without which, of course, this work would not have been possible.