A COMPARISON OF THE PATHOGENESIS
OF CHALLENGE AND SINGLE INFECTIONS
WITH FASCIOLA HEPATICA IN SHEEP

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A COMPARISON OF THE PATHOGENESIS OF CHALLENGE AND SINGLE INFECTIONS WITH *FASCIOLA hepatica* IN SHEEP.

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SUMMARY

An experiment was designed to investigate differences in the pathogenesis of fascioliasis in sheep receiving two infections, as compared to those receiving only one infection. The materials used, and the methods applied to monitor the clinical pathological changes during the course of the infections are detailed.

Changes in the haematological picture and serum biochemistry during the course of the experiment were consistent with those described by other workers as occurring during infection with *Fasciola hepatica*. In particular, elevated eosinophil counts were recorded, reaching peak values at 9 to 10 weeks post infection. Marked increases in serum enzyme levels were also observed. These reached a peak at either five or eight weeks post infection, depending on the group.

Gross pathological changes in the livers of the experimental animals are described in detail. There was marked individual variation in this parameter in that the livers of two of the sheep showed severe parenchymal changes while those of the other sheep showed only mild changes. This variation could not be attributed to the number of flukes recovered from each liver.

Differences in the numbers of flukes recovered and in the degree of development of those flukes, between the challenge and control groups were not seen.

No evidence for acquired resistance in sheep to *F. hepatica* infection was demonstrated, but there was
some reduction in the severity of the pathological changes during the early stages of challenge infection.
INTRODUCTION

_Fasciola hepatica_ (Linnaeus 1758) is a digenetic trematode most commonly found, in its adult form, in the lumen of the major bile ducts of the liver of its mammalian host. The species is very widely distributed, both geographically, and in the number of different definitive hosts it may parasitise. It has thus been found in rodents, pigs, herbivores, carnivores and primates over wide areas of the world (Dunn, 1969). However, it is most commonly a parasite of domestic cattle and sheep, and it is in these two hosts that the greatest economic losses resulting from the pathogenic effects of fascioliasis occur.

The life cycle of _F. hepatica_ is indirect and involves a period of development and multiplication of larval stages in an intermediate molluscan host. Over wide areas of the world, this host is commonly the amphibious snail _Lymnaea truncatula_ (Muller). The sequence of discoveries and events which led up to the elucidation of the life cycle in the intermediate host has been described by Taylor (1964). Development of _F. hepatica_ to its adult form also involves an extensive migration through the tissues of the final host. The controversy surrounding the actual route of this migration and the way in which this controversy was resolved has been described by Dawes & Hughes (1964), in a review article on the invasive stage of _F. hepatica_ in mammalian hosts.

Several authors (Ross, Dow and Todd, 1967, Urquhart 1956, Pantelouris 1965) have described the pathological
changes associated with the migration of the immature flukes through the liver parenchyma. Dawes and Hughes (1964) describe the reaction of the bile ducts to the presence of adult flukes in their lumen. Two clinical entities have been described which correspond to these two phases in the life cycle of the liver fluke in its final host. Thus the term Acute Fascioliasis refers to the clinical and pathological changes caused by the wanderings of the immature flukes in the liver parenchyma. Chronic Fascioliasis relates to the clinical pathological changes associated with the presence of adult flukes in the bile ducts.

Both forms of disease may result in death of the host if the infection is sufficiently severe, or in ill-health, depressed weight gain, or poor productivity, if the infection is less severe. These losses, can represent a serious loss of efficiency in animal production and much effort has been directed towards ways of minimising them.

Conventional control measures have been directed against the snail intermediate host or against the fluke, once it has become established in the final host. These methods, coupled with management procedures to prevent access of the final host to habitat occupied by the intermediate host, have afforded a reasonable degree of control in many instances. An alternative aspect of control would be the establishment of a population of final hosts which was resistant to the establishment or pathological effects of *F. hepatica*. 
Until recently there has been little evidence that the phenomenon of resistance to re-infection occurs in *F. hepatica* infections (Dawes and Hughes, 1964; Sinclair, 1967). Recent work involving laboratory animals (Goose and Macgregor, 1973; Hayes, Bailer and Mitrovic, 1973, 1972; Lang, 1968, 1967; Fortmeyer, 1973); cattle (Doyle, 1971, 1972, 1973; Ross, 1967 a & b) and sheep (Sinclair 1971 a, 1973; Ross 1967 b) has tended to show that protective immunity to re-infection may indeed occur. This opens up the possibility of using immunization techniques in the control of the disease.

Some of the recent work involves evidence for a protective response to infection with *F. hepatica* in sheep. It was therefore considered worthwhile to investigate the clinical, pathological and biochemical changes produced by an infection of *F. hepatica* in sheep which had had previous experience of the parasite, as compared with the infection in sheep which had not. It was hoped that further evidence, for or against, the existence of a protective immunity in sheep would be obtained.
1) Aspects of Pathogenesis

The literature on the pathogenesis of fascioliasis in sheep and other animals is extensive and has been reviewed by several recent authors (Sinclair 1967, Pullan 1968, Hammond 1971). This review will be confined to a discussion of the parameters used in this work to follow the course of the disease produced in sheep given single and challenge infections.

The primary factors which dictate the type and severity of the disease caused by *F. hepatica* are the size of the infecting dose of metacercariae, the infectivity of the metacercariae and consequently, the number of flukes becoming established in the bile ducts as adults. Thus Pullan, Sewell and Hammond, (1970) found that the administration of between 5,500 and 12,000 metacercariae to twelve sheep resulted in two distinct disease syndromes, which they termed Acute and Sub-Acute. These different syndromes were explicable in terms of the numbers of flukes recovered from the livers at post mortem examination. Conversely, Sewell, Hammond and Dinning (1968) produced chronic fascioliasis by the administration of either 2,400 or 6,000 metacercariae to sheep.

The percentage of metacercariae administered in experimental infections which become established in the livers of the recipient sheep is very variable, and depends on many factors, some of which have been detailed by Dawes and Hughes (1964). Examples of percentage
recoveries from experimental infections in sheep were cited by Pullan (1968) and varied from 37.4 percentage (Montgomerie, 1928) to less than 30 percentage (Sinclair, 1964, 1965).

The size of the initial infective dose is also important in determining the rate of development of the flukes which become established, since there is ample evidence (Ross, 1965; Taylor, 1964; Ross, Todd and Dow, 1966; Kendall, Herbert, Parfiit and Pierce, 1967; Roberts, 1968; Montgomerie, 1928; Sewell, 1962), that infection with large numbers of metacercariae can lead to overcrowding or competitive inhibition and consequent stunting of the flukes.

The technique of monitoring the levels of "liver-specific" enzymes in the serum or plasma of infected animals, as a means of assessing the degree of liver parenchymal damage caused by migrating liver flukes, has been widely used. The enzymes which have been most commonly studied have included glutamic oxaloacetic transaminase (S.G.O.T.) and glutamic dehydrogenase (G.D.) (Pullan et al 1970, Sewell, 1967b, Thorpe and Ford, 1969). In addition, Ornithine carbamyl transferase (O.C.T.) and Sorbitol dehydrogenase (S.D.H.) have recently been used to monitor Fasciola infections in goats (Treacher, Hughes and Harness, 1974). Ford (1967) has shown that S.D.H. activity is primarily situated in the liver parenchyma in sheep. The same worker, (Ford, 1965) has demonstrated a similar finding for O.C.T. in sheep. Keller (1973) examined several enzymes for their relative proportions in sheep tissues, and concluded that S.D.H. is a good indicator of acute liver cell lesions, having a high
liver specificity.

Haematological changes associated with fascioliasis have been studied by numerous workers. The most consistent findings have been an increase in total white blood cells, an absolute eosinophilia, and an anaemia of variable severity.

The eosinophilia has been described by Cameron (1951), Pantelouris (1965) and Ross et al (1966, 1967) and is considered to be a consistent finding during the migratory stage of the disease. Sinclair (1962) and Pullan (1968), found that the leucocytosis which was evident early in the infection, was primarily due to these raised levels of eosinophils.

Anaemia has been reported to be associated with both acute and chronic fascioliasis, but the origins of the anaemia in each case have been the subject of controversy. Taylor (1964) considered the anaemia of acute fascioliasis to be due to a toxin or to extensive destruction of liver tissue, while Symons and Boray (1967) and Ross et al (1967) stated that it is due to haemorrhage into the liver parenchyma or peritoneal cavity. Roberts (1968), although supporting this latter view, presents evidence for a dyshaemopoietic element in the aetiology, while Pullan et al (1970) thought it likely that an excessive breakdown of red cells, associated with severe liver disease was occurring. A similar divergence of opinion has occurred over the origins of anaemia in chronic fascioliasis. Cameron (1951), Taylor (1964) and Sinclair (1962, 1964, 1965) state that dyshaemopoiesis is the primary factor. However, more recent work and in particular, that using radioactive isotope tracer techniques for
labelling red cells, has provided evidence that loss of blood via the bile ducts, due to the feeding activities of the adult flukes, is mainly responsible (Holmes, Maclean and Mulligan 1971; Symons and Boray 1967; Sewell 1967; Sewell et al 1968; Dargie, Holmes, Maclean and Mulligan 1968).

Changes in absolute levels of serum proteins and in the relative proportions of the various protein fractions during fascioliasis have been described by many workers. Ross et al (1967) recorded a severe drop in serum albumin levels in the acute disease. Sinclair (1962) found that, although there was an absolute drop in albumin content, the total protein content of the serum was elevated at this stage of the disease, due to an increase in globulin, the gamma globulin fraction being particularly raised. Sinclair (1962) found the later stages of infection to be characterised by hypoalbuminaemia. Dawes and Hughes (1964) held the opinion that this could hardly be due to simple blood loss or haemodilution, and was likely to be due to impaired synthesis or abnormal loss as a result of exudation from damaged bile ducts. Dargie et al (1968) substantiated this last suggestion when they demonstrated a disproportionate loss of plasma protein, presumably via the bile ducts. However, Nansen (1971) found that, in cattle, despite massive hepatic fibrosis, there was a normal synthetic rate of albumin.
2) **Evidence for Immunity**

Several authors (Dawes and Hughes 1964; Taylor 1964; Sinclair 1967) have concluded that there was little evidence for the existence of an acquired immunity to *F. hepatica* infections. From field studies, Pontelouris (1965) came to the same conclusion, with regard to sheep.

However, it is well recognised that the different mammalian hosts of *F. hepatica* vary in their susceptibility to the adverse effects of this parasite and in their reaction to combat it. Ross (1968) examined this phenomenon, and classified the various hosts he studied into groups with low, medium and high resistance to infection. He attributed the differences in pathogenicity between these groups to differences in the fibrous structure of the liver of the individual species in each group and to variation in the fibroblastic response to infection. These differences may make comparisons of results between different species dubious. For example, Boray (1968) challenged sheep which had had a previous experimental *F. hepatica* infection removed by anthelmintics. He found that the challenged animals died, as did control animals, and that there was no reduction in the numbers of flukes becoming established. On the other hand, in a parallel experiment with cattle, fewer flukes developed on challenge and the pathological changes were less severe. He attributed this apparent "resistance" in cattle to fibrosis of the previously infected livers causing a mechanical barrier to the migration of the flukes.
Sinclair (1967) similarly described a "non-specific" resistance in cattle, due to the extensive bile duct calcification seen in that species.

The differences in host resistance to the parasite (or host specificity on the part of the parasite) described above, coupled with the need for a reliable method of diagnosing pre-patent infections, has stimulated an intense interest in the mechanisms of the immunology of \textit{F. hepatica} infections. This has resulted in the demonstration of a wide range of serological responses to infection. These immunological methods have been described by Sewell (1962), Pontelouris (1965) and Sinclair (1967). In a comparative study, using six of the methods available, Bénex, Guilhon and Barnabé, (1973) showed that antibody could be detected at two to three weeks post infection and rose in titre until six to eight weeks post infection, after which the rise levelled off and persisted, if the infection was untreated.

Gurdlach (1971) demonstrated a rise in titre after challenge. Sinclair (1967) held the view that these circulating antibodies had no protective properties. This was supported by Corba, Armour, Roberts and Urquhart, (1971) and Dawes and Hughes (1964) who failed to confer protection in rats and calves by the administration of serum from infected animals. Nevertheless, Dargie et al (1973) reported the passive transfer of protection to cattle, rats and sheep by the use of serum. Dawes and Hughes, (1964) thought that the likelihood of being able to immunize against \textit{F. hepatica}, with the present state
of knowledge, was low.

The involvement of cell mediated mechanisms of immunity in fascioliasis has been investigated by several workers. Lang, Larsh, Weatherly and Goulson, (1967) succeeded in passively conferring a degree of resistance to mice which had previously received peritoneal exudate cells from other mice infected with *F. hepatica*. These workers postulated that the mechanism involved in this resistance was a delayed hypersensitivity reaction. Corba, Armour, Roberts and Urquhart, (1971) also succeeded in conferring protection against a primary infection in isogenic recipient rats, by the transfer of lymphoid cells from previously infected rats. The same workers demonstrated a similar protection by the transfer of lymphoid cells between monozygous twin calves. Flagstad, Andersen and Nielsen, (1972), using calves with a congenitally deficient cellular immunity, demonstrated that these animals failed to show a normal specific response to infection with *F. hepatica*. Further evidence was provided by Dodd and O'Nuallain (1969) who demonstrated suppression of the normal cellular response to infection, and increased pathogenicity, in rabbits which had been given anti-rabbit lymphocytic sera prior to infection. On the other hand, Sinclair (1971), working with sheep, failed to demonstrate the provision of resistance by the transfer of lymph node and spleen homogenates from infected to uninfected sheep. Sinclair attributed a non-specific retardation of fluke
development, seen in the spleen homogenate-treated group to homograft reaction. In spite of this, other studies by the same author, involving splenectomised sheep (Sinclair 1970), and corticosteroid-treated lambs (Sinclair 1968, 1970), resulted in enhanced growth and increased pathogenicity of the infecting dose of *F. hepatica*, thus giving circumstantial evidence for the involvement of cell-mediated immunity in the response of sheep to this parasite. Dargie et al (1973) have reported the successful passive immunisation of sheep by the transfer of lymphoid cells.

Recently, more specific indicators of cell-mediated immune responses have been investigated. Genchi, Locatelli and Sartorelli, (1973) in cattle, and Aalund, Nielsen and Eriksen (1972) in goats and sheep have demonstrated strongly positive specific reactions to the Macrophage Migration Inhibition Test using cells, both from the peripheral bloodstream and from the local lymph nodes, of animals infected with *F. hepatica*. Similarly, Bolbol, Urquhart and Sewell (1974) have demonstrated specific *in vitro* transformation of peripheral lymphocytes of rabbits infected with *F. hepatica*. Both tests are considered to be evidence of antigen-specific lymphocyte activation, which is a requirement for cell-mediated immune responses (Soulsby 1972).

Platzer (1970) states in his review that attempts at artificial immunization using worm tissues, excretion products and X-irradiated metacercariae have been generally
unsuccessful. Kerr and Petkovitch (1935) demonstrated the development of a protective immunity in rabbits injected with saline extracts of dried worms. However other workers (Urquhart, Mulligan and Jennings 1954; Healey 1955; Dawes and Hughes 1964) could not confirm this finding in rabbits or sheep. The findings using X-irradiated cercariae are equally conflicting. Thorpe and Broome (1962) found that rats vaccinated with such cercariae developed lower burdens on challenge, compared with controls. In contrast, Hughes (1962) using rabbits and mice, and Dawes and Hughes (1964), using sheep, rabbits and mice, showed that X-irradiated cercariae elicited no protective response.

The results of studies using normal cercariae as an immunising agent are numerous and conflicting in some cases. Evidence for a protective mechanism to challenge infections has been demonstrated in the rabbit (Fortmeyer 1973; Ross 1967b), mouse (Lang 1967, 1968), rat (Hayes et al 1972, 1973; Goose and Macgregor 1973), cattle (Ross 1967a; Doyle 1971, 1972, 1973) and sheep (Ross 1967b; Sinclair 1971, 1973).

Although Ross (1966, 1967b), working with rabbits, cattle and sheep, found some evidence for an acquired resistance in these animals on challenge, there was extremely high individual variation within the groups and the level of significance was low. Kendall et al (1967) discussed a further problem in the experimental design of such challenge experiments. They considered that the apparent retardation of development, or reduction in
numbers becoming established, of flukes in animals given a challenge infection, could be attributed to a competitive inhibition of fluke establishment and development in a host which was already carrying a primary fluke burden. Several workers (Hayes et al 1972, 1973; Goose and Macgregor 1973; Doyle 1971) found that although there was a marked inhibition of the challenge population of flukes in their experimental animals, the existing primary population was apparently unaffected. Goose and Macgregor (1973), discussing this, suggested that it could be due either to the adult flukes in the bile ducts being inaccessible to the hosts' defence mechanisms, or to the existence of host mimicry by the parasite in this situation. However, work by Hughes and Harness (1973a, 1973b) in which they surgically transferred adult and immature flukes into recipient animals which had been previously immunised against the tissues of the donor animal, tends to discount the existence of host mimicry by F. hepatica.

The possibility that an existing burden can cause competitive inhibition of a challenge infection and the fact that the fibrosas produced by a primary infection can lead to non-specific inhibition of challenge infections (Sinclair 1967), created problems in the design of experiments set up to examine the existence of acquired immunity in fascioliasis. However, with the advent of an anthelmintic (Diamphenethide) known to have a high degree of efficiency against immature flukes (Kingsbury and Rowlands 1972), it became possible to terminate an experimental immunising infection at a stage
where liver fibrosis was minimal, but exposure of fluke antigen should have been sufficient to allow the production of an immune response, if one was present.

Sinclair (1971, 1973) used this technique of terminating a primary immunising infection by the use of drugs one, four, or nine weeks after infection. He found that in sheep, there was evidence for an acquired immunity which was manifested as a temporary retardation of fluke development, possibly associated with delayed entry into the bile ducts. The total numbers of flukes recovered from challenge and control groups was similar. Sinclair postulated that the mechanism involved in this resistance was a hypersensitivity reaction.
MATERIALS AND METHODS

1) Experimental animals

Six nine-month-old male Cheviot lambs with a mean body weight of 76 pounds were obtained from a farm where they had been born and reared under conditions designed to keep them free of helminth parasites (Animal Breeding Research Organisation, West Mains Road, Edinburgh). These animals were brought indoors and dosed, prior to the experiment, and on two subsequent occasions, with 100 mg Thiabendazole per Kilogram body weight. They were placed in pens with concrete floors and a litter of wood shavings which was replaced twice weekly so as to minimise re-infection with parasitic nematodes. A ten-month-old male Scottish Blackface lamb was obtained at a later date and, after an initial quarantine period, was included with and treated identically to the other experimental sheep. All the animals were castrated eight weeks before the start of the experiment. The sheep were fed on a daily ration of water and hay ad lib and received, in addition, 2 pounds per head per day of a cereal/protein/mineral mixture which was compounded on the university farm and an analysis of which is detailed over:-
**Quantities**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Item</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 cwts</td>
<td>Oats</td>
</tr>
<tr>
<td>1 cwt</td>
<td>Protein supplement (31% Protein)</td>
</tr>
<tr>
<td></td>
<td>6% Fibre</td>
</tr>
<tr>
<td></td>
<td>4.5% Oil</td>
</tr>
<tr>
<td>14 lbs</td>
<td>Mineral supplement</td>
</tr>
<tr>
<td></td>
<td>37% Magnesium</td>
</tr>
<tr>
<td></td>
<td>5.7% Calcium</td>
</tr>
<tr>
<td></td>
<td>3.5% Phosphorus</td>
</tr>
<tr>
<td></td>
<td>5.5% Na Cl</td>
</tr>
<tr>
<td></td>
<td>1% Iron</td>
</tr>
<tr>
<td></td>
<td>Trace Cu, Co, Mn, I, Zn, Vit D &amp; Vit A</td>
</tr>
</tbody>
</table>

**Analysis**

2) **Preparation of Infective Doses**

Metacercariae were obtained from the Helminthology Department, Centre for Tropical Veterinary Medicine, Roslin, Midlothian. They had been produced from laboratory cultures of *Lymnaea truncatula* which had been infected with miracidia from *F. hepatica* eggs obtained from the gall bladders of naturally infected sheep. The techniques of snail culture and metacercariae production have been described by Sewell (1962) and Pullan (1968). The metacercariae were stored at 10°C in water in the polythene bags in which they had been caused to encyst.

The infective doses of 1000 metacercariae were obtained as follows: The metacercariae were scraped off the polythene on which they were encysted into a petri dish, and the number of apparently viable (i.e. morphologically normal) cysts in each batch counted under a stereo microscope. These cysts were then pooled to give a total of 4,000 metacercariae suspended evenly in a known
volume of water in a measuring cylinder. This volume was split up into four equal volumes after thorough mixing. Several two ml, samples of the fluid in each of these four volumes were taken and the number of viable cysts again counted, to give an estimate of how many viable cysts were present in each volume. Each dose of approximately 1000 metacercariae was then trapped on filter paper in a Buchner funnel. The filter paper, bearing the cysts, was then folded loosely and administered to the sheep with a balling gun.

3) **Experimental Design**

a) Infection and treatment of sheep

The six sheep were divided randomly into three groups each containing two animals. The sheep were infected with metacercariae and treated with Diamphenethide according to the following plan:

<table>
<thead>
<tr>
<th>GROUP NAME</th>
<th>SHEEP NUMBER</th>
<th>PRIMARY INFECTION (CERCARIAE)</th>
<th>DIAMPHENETHIDE TREATMENT</th>
<th>CHALLENGE AND CONTROL MORTEM INFECTION (CERCARIAE)</th>
<th>POST MORTEM INFECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREATMENT</td>
<td>5</td>
<td>1000</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>CONTROL</td>
<td>19</td>
<td>1000</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>INFECTION</td>
<td>7</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>CONTROL</td>
<td>16</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>CHALLENGE</td>
<td>10</td>
<td>1000</td>
<td>+</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1000</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Animals number 19 and 16 died shortly after the first treatment with diethylcarbamazine. Animal number 19 was not replaced, but animal number 16 was replaced by sheep number SBF eight weeks post infection. Treatment with diethylcarbamazine five weeks post infection was at the dose rate of 250 milligrams per Kilogram body weight. This was reduced to 100 milligrams per Kilogram eight weeks post infection in case toxic effects of the drug had been responsible for the death of sheep number 16. 100 milligrams per Kilogram is the maximum dose rate recommended by the manufacturers.

b) Sampling

The sheep were weighed once a fortnight from the time they were first housed. Faecal samples were taken weekly and examined for the presence of helminth eggs or larvae. Blood samples were taken weekly, both into E.D.T.A. for haematological procedures, and into plain tubes for serum production. Evacuated glass tubes ("Vacutainer" Becton, Dickinson - France) and 21 gauge needles were used. All the above sampling procedures were carried out between 9.00 a.m. and 10.00 a.m.

A visual assessment of the animals' apparent general health, eating habits and environment was made at least twice weekly. This was coupled with a complete clinical examination of particular animals when necessary.
4) **Haematological Methods**

The following examinations were performed weekly on blood samples which had been thoroughly mixed using a mechanical mixer (Matburn LTD., 20-24 Emerald Street, London)

a) **Total White Blood Cell Counts:** These were performed by the method of Archer (1965), using a Neubauer counting chamber and one per cent Acetic acid as diluent. One side of the counting chamber was used for each estimation.

b) **Absolute Eosinophil Counts:** These were performed by a slight modification of the method described by Archer (1965). A Neubauer counting chamber was used and a one in 20 dilution of blood in one per cent aqueous Eosin as diluent. Both sides of the chamber were used for each estimation.

c) **Packed Cell Volumes:** These were determined, in duplicate, using a Hawksley micro-haematocrit centrifuge and reader (Hawksley & Sons, Lancing, Sussex). The samples were centrifuged at approximately 6,000 r.p.m. for 10 minutes on each occasion.

5) **Serum Biochemistry**

The following estimations were performed on serum which had been obtained weekly, by a slight modification of the method described by Hammond (1971). The serum was stored in plastic bijoux bottles, deep frozen at -20°C.
The estimations were thus performed on serum samples which had been stored in this way for periods ranging from 36 to eight weeks.

a) Total Serum Protein Concentrations: These were estimated by the Biuret method described by Henry, Sobel and Berkman, (1957), using an E.E.L. colourimeter (Evans Electroselenium LTD., Halstead, Essex) with a green filter (Reference number 0GRI) "Lab-Trol" (D.A.D.E., Division American Hospital Supply Corporation, Miami, Florida, U.S.A.) was used as a standard for comparison purposes.

b) Serum Immune Globulin Concentrations: These were estimated by the Zinc Sulphate turbidity test described by McEwan, Fisher, Selman and Penhale, (1970), using an E.E.L. Colourimeter with a red filter (Reference number ORI). "Versatol" (Warner - Chilcott Laboratories, Morris Plains, New Jersey, U.S.A.) was used as a standard for comparison purposes.

c) Serum Sorbitol Dehydrogenase (S.D.H.) Activities: These were estimated using the spectrophotometric technique described by Bergmeyer (1965). The changes in optical density of the final solution were measured using an SP 1800 Ultraviolet spectrophotometer with an AR25 automatic linear recorder (Pye Unicam LTD., Cambridge, England). The SDH activities of the serum samples were expressed in the units defined by Bergmeyer (1965).
d) **Ornithine Carbamyl Transferase (OCT) Activities:**

These were estimated by a modification of the colorimetric technique described by Carper and Roester (1968) using an E.E.L. portable colorimeter with a green filter (Reference number OGRI). The results were expressed as micro Moles Citrulline synthesised per ml. of serum per hour.

6) **Helminthological Techniques**

The following examinations were made on fresh faecal samples taken weekly. The examinations were performed on a "standard suspension" made by macerating and washing approximately three grams of a faeces sample through a coffee strainer into a container marked at 45 ml and making the suspension up to that mark with water.

a) **Nematode Eggs and Lungworm Larvae:** examinations for the presence of these were made on 10 ml. volumes of the standard suspension by a centrifugal flotation technique using saturated salt solution.

b) **Fasciola eggs:** the presence of these was determined by the centrifugal flotation "Sellotape" technique described by Sewell and Hammond (1972).

7) **Post Mortem Techniques and Measurement of Flukes**

These were carried out at the termination of the experiment or, in the case of the two animals which died
during the course of the experiment, as soon as practicable after death.

a) Post Mortem Techniques: The animals were killed by stunning with a captive bolt pistol and bleeding out. The common bile duct was ligated before removing the liver and intestines intact. The liver was dissected free of its attachments and laid to one side to be weighed and examined. The intestines were opened and their contents and mucosae examined for the presence of gastrointestinal nematodes or their larvae by other workers (Messrs. Gamble, McGrane, Roeder and Touray), using standard helminthological techniques. The condition of the carcase and the internal organs of each animal was examined and any pathological changes noted and described.

b) Extraction of Flukes: after weighing the livers individually and recording any pathological changes present, the gall bladder and major branches of the bile ducts were opened and any flukes present removed and washed in warm physiological saline. The flukes in the smaller bile ducts and parenchyma were removed by slicing the liver in sections approximately \( \frac{3}{4} \) inch thick and squeezing these sections between the fingers to expel the flukes. These sections were then soaked in warm saline overnight and any remaining flukes extracted by cutting up the liver tissue into sections no larger than \( \frac{3}{4} \) inch square and squeezing and sieving these sections in saline.
All the flukes obtained in this way were washed several times in physiological saline to remove excess blood. They were then left overnight in saline in a refrigerator at 4°C, to allow them to relax completely. The flukes were then stored at -20°C. until they were required.

(c) Measurements on flukes: (i) Total numbers - the total number of flukes obtained from each sheep was obtained by counting heads and shoulders only. The total numbers found in the bile ducts and in the parenchyma were noted separately for each sheep. (ii) Wet weight - the total wet weight of the flukes obtained from each sheep was measured after slight blotting on filter paper, using a Mettler P161 balance (Mettler, Greifensee – Zurich). (iii) Dimensions - the individual length and maximum breadth of each fluke was measured after placing the flukes on a flat glass surface, using a plastic ruler marked in millimetres. The product of the length and breadth of each fluke was calculated to give an estimate of the area of the rectangle into which each fluke would fit. (iv) Protein content - an extract was produced from either 100 or 50 flukes selected at random from those obtained from each sheep as follows: The flukes were placed in a McCartney bottle and four ml. of normal saline added. They were then macerated.
in this solution using a Silverson homogeniser (Silverson Machines LTD., Waterside, Chesham, Bucks.) for 10 minutes at mark 5. The resulting homogenate was then treated in an M.S.E. 100 watt ultrasonic disintegrator (Measuring and Scientific Equipment LTD., London) (Catalogue number 7100) for 10 minutes at 25 Kc/second, with an amplitude of 8 microns, peak to peak. The resultant suspension was centrifuged at 3,000 r.p.m. for 10 minutes, and the supernatant collected. All samples were treated identically. The protein content of the supernatant fluid was estimated using the two versions of the micro-method variation of the Biuret technique described by Goa (1953), for solutions with a low protein content. Version A involves treatment of the protein suspensions with trichloracetic acid. Version B does not. From this data, an estimate of the average amount of protein extracted from individual flukes from each sheep was calculated.
RESULTS

1) Clinical Condition and Growth Rate

a) Clinical Examination

Observation of the experimental sheep in the pre-infection period was carried out twice weekly. At about two weeks prior to infection, an abnormality in the sheep's respiratory rates and rhythms was noticed. The animals showed rapid, shallow respirations with marked involvement of the rib cage. This dyspnoea became even more marked on handling the animals and was accompanied by loud nasal sounds. The animals did not appear dull and were not off their food, although they showed a tendency to lie down for longer periods than was thought normal. Clinical examination revealed elevated body temperatures in the region of 106 - 107°F and auscultation over the area of the lungs revealed harsh bronchial sounds with occasional fluid rales. A diagnosis of broncho-pneumonia was made and all six animals were treated with Oxytetracycline at seven mg per Kilogram body weight, given by intra-muscular injection on three consecutive days. The clinical condition of all six animals improved after this treatment, but approximately ten days later, the condition of sheep number 15, which had been one of the most severely affected, deteriorated and the dyspnoea described above became evident again. A further three days antibiotic therapy resulted in another temporary improvement, but subsequently further relapses occurred. No further antibiotic therapy was given and the animal's respiratory condition improved over the course of the experiment. In fact, this animal showed the most rapid increase in live weight of all the animals during
the early part of the experiment, and its breathing had returned to normal mid way through the experiment.

Evidence of slight abdominal pain, manifested by some reluctance to move, was noticed in some of the animals between two and three weeks post infection. No difference in this respect was noticed between the challenge group and the infection control group. Several of the sheep adopted a "dog-sitting" position while resting. This was noted in the second half of the experiment, but could not be related to any specific group or phase of infection.

Two animals died within the experimental period. Sheep number 16 was found dead, with no previous signs of ill health, three days after the animals had been drenched with the first dose of diamphenethide. Unfortunately, it was not possible to perform a post mortem examination immediately, and the carcase had undergone such rapid post mortem change that no definite cause of death could be attributed.

Findings at Post Mortem of Sheep number 16

The carcase was in excellent condition and showed no evidence of dehydration. There was marked frothing from the mouth and nasal orifices. This froth, mixed with food debris, was present in the nasal cavity and extended down the length of the trachea and its branches, to the level of the bronchioles. Cardio-vascular changes included acute hyperaemia of the mucosa of the trachea along its whole length and extending into the major
bronchi. There was severe engorgement of both lungs with hyperaemia, discolouration and some infiltration, but no consolidation or collapse. Excess blood-stained fluid was present in the pericardial, thoracic and abdominal cavities and there was a fibrinous pleurisy present. There were marked sub-epicardial haemorrhages and severe endocardial hyperaemia and haemorrhage. The liver showed severe putrefactive changes but no flukes were seen. Both kidneys were liquifactive. The fore-stomachs and small and large intestines appeared normal, as did the brain. Apart from these findings, advanced putrefactive and post mortem changes were the most obvious features. Two possible causes of death were postulated - either an acute clostridial enterotoxaemia, or gangrenous pneumonia and septicaemia following aspiration of diamphenethide.

A few days after this animal died, it was noticed that sheep number 19 was not eating. Clinical examination revealed that the animal appeared depressed and was very reluctant to move, huddling into one particular corner. The eyes were bulging and apparently "unseeing" although the animal was not blind. Body temperature was 103.8°F. Respirations were normal in rate, but rather shallow with marked bronchial sounds. The heart sounds were faint. There was obvious abdominal pain on palpation and the abdomen was distended, apparently with fluid. The ruminal movements were weak. There was sub-cutaneous oedema of the rear legs, particularly on the anterior aspects of both meta-tarsals above the
fetlocks. At this time the presenting signs were considered to be evidence of acute liver damage and it was thought that the animal was showing some adverse reaction to the anthelmintic or to the dead flukes which had been killed while actively migrating through the liver. After a few days, the animal started scouring and passed quantities of altered blood in its faeces. At this time, the animal's body temperature was 102°F and the visible mucous membranes were pale. It was thought undesirable to instigate any treatment other than the injection of a multi-vitamin solution and the removal of the protein supplement from the animal's diet. The animal was thus moved to a pen on its own where it would not be bullied by the other sheep, and offered water and hay ad lib. The animal started to eat after this and appeared to improve slightly but was found dead one morning, ten days after it first became ill.

**Findings at Post Mortem of Sheep number 19**

There was a small area of blood-stained wool round the prepucial orifice. The prepuce, surrounding connective tissue and penis showed gross inflammation, gelatinous oedema and infiltration with fluid, which from its colour and smell, appeared to be urine. The area of the sigmoid flexure showed marked haemorrhage, bruising and necrosis. White irregular brittle calculi were found in the urethra at the sigmoid flexure and vermiciform appendage. On opening the abdomen, the bladder was not distended but showed marked haemorrhage of its
mucosal surface. There was oedema and gelatinous thickening of the bladder wall and surrounding tissues. There was no evidence of gross trauma to the bladder, but an organising blood clot on the ventral serosal surface which was adherent but not involved in an obvious tear in the bladder wall. There was a large volume of turbid yellow sanguineous flocculent fluid in the abdominal cavity and small blood clots on the omentum. The kidneys were grossly swollen and degenerate. The right kidney particularly showed cystic formation and dilatation of the renal pelvis. The ureters were not apparently dilated. The liver showed a degree of autolysis and was swollen with a pale yellow/orange colour. The bile ducts were slightly prominent on the visceral surface. Both surfaces showed hundreds of short, dark, narrow tracts under the capsule. The margins of the lobes particularly contained these tracts and had a blue/grey colour. The gall bladder was distended with thick dark bile. There was a small volume of blood-tinged fluid free in the thoracic cavity. Marked passive congestion of the lungs, heart and trachea was evident.

Helminthological examination: No adult flukes were found in the bile ducts. No immature flukes were found in the liver parenchyma and the bile in the gall bladder was negative for fluke eggs. The abomasum and small and large intestines were negative for gastro-intestinal nematodes. Microscopic examination of the fluid from the abdominal cavity revealed large numbers of hexagonal crystals and numerous large mobile bacilli. A diagnosis
of urolithiasis with urethral obstruction and possibly subsequent pin-point rupture of the bladder, or urethral rupture was made.

At the time of the death of sheep number 19, the faeces of the remaining sheep became soft, greyish and pale. This was attributed to a change in the protein supplement of the diet at that time. Removal of this supplement for a few days resulted in the faeces returning to normal within a week.

Twelve days after the first dose of diamphenethide it was noticed that in sheep number 7, there was a degree of wool separation and shedding at the skin level over the back. This slowly progressed, and during the course of the experiment, this animal gradually shed its complete fleece but had regrown a reasonably thick underfleece by the end of the experiment.

b) Growth Rate

The changes in mean body weight of the animals in each experimental group are shown in Figure 1. The animals in all three groups gained weight in a fairly uniform fashion. The average fortnightly increases in the mean live weights of animals in each group during the primary infection period and during the secondary infection period, are shown in table II.
Fig. 1 Variation in the group mean live weights during the course of the experiment.

\[\text{LIVE WEIGHT (lbs.)}\]

\[\text{WEIGHT POST INFECTION}\]

**I<sub>1</sub>** t.c.g. and c.g. infected with 1000 metacercariae

**I<sub>2</sub>** i.c.g. and c.g. infected with 1000 metacercariae

**D<sub>1</sub>** all animals dosed with diamphenethide (250 mg/Kg)

**D<sub>2</sub>** all animals dosed with diamphenethide (100 mg/Kg)

* t.c.g. represented by one animal from this point

▲ sheep number 16 replaced by sheep number SBF
Average fortnightly increases in body weight, in pounds, for each group ± one Standard Deviation.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>WEEKS</th>
<th>POST</th>
<th>INFECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-5 to +5</td>
<td>9 to 21</td>
<td></td>
</tr>
<tr>
<td>TREATMENT</td>
<td>2.7 ± 0.8</td>
<td>1.7 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INFECTION</td>
<td>4.3 ± 0.7</td>
<td>2.0 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHALLENGE</td>
<td>3.0 ± 1.8</td>
<td>1.6 ± 3.2</td>
<td></td>
</tr>
</tbody>
</table>

From these results it can be seen that the growth rate during the second part of the experiment appeared to be slightly less than that during the first part, but with considerably more individual variation.

2) **Haematology**

   a) **Total White Blood Cell Counts**

   The changes in mean total white blood cell counts of the animals in each group, are shown in Figure 2. There was considerable individual variation within the groups. The mean counts for each group also show variation from week to week. Following the administration of metacercariae to each group, a rise in mean total white cell numbers was recorded. This rise reached a peak from two to six weeks post infection depending on the group. After treatment with diaminphenethide, the mean white cell counts tended to drop back to their original levels. The challenge and Control infections tended to result in a biphasic increase in white cells. The rise seen soon after infection was followed by a second rise in the mean
Fig. 2 Variation in the group mean total white blood cell counts during the course of the experiment.

$I_1$ t.c.g. and c.g. infected with 1000 metacercariae
$I_2$ i.c.g. and c.g. infected with 1000 metacercariae
$D_1$ all animals dosed with diamphenethide (250 mg/Kg)
$D_2$ all animals dosed with diamphenethide (100 mg/Kg)
* t.c.g. represented by one animal from this point
▲ sheep number 16 replaced by sheep number SBF
white cell count at about eight to ten weeks after the administration of metacercariae.

b) Eosinophil Counts

The changes in mean eosinophil counts of the animals in each group are shown in Figure 3. Administration of metacercariae to each group resulted in a considerable increase in the mean eosinophil count. This rise was very rapidly reversed after treatment with diamphenethide. Challenge and control infections resulted in the rise in total eosinophils being maintained to some extent with a second rise in their numbers being evident nine to ten weeks after the administration of the metacercariae. This second rise was most pronounced with the Challenge group.

c) Packed Cell Volumes (P.C.V.)

The changes in mean P.C.V. values for the animals in each group are shown in Figure 4. No dramatic changes in this parameter were seen during the course of the experiment. Infection of any particular group with metacercariae was followed by a slight drop in the mean P.C.V. for that group. Differences in the levels between groups were not striking.

3) Serum Biochemistry

a) Sorbitol Dehydrogenase (SDH)

The changes in mean serum SDH activity of the animals in each group are shown in figure 5. Following infection with metacercariae, a very rapid and substantial
Fig. 3 Variation in the group mean absolute eosinophil counts during the course of the experiment.

1. t.c.g. and c.g. infected with 1000 metacercariae
2. i.c.g. and c.g. infected with 1000 metacercariae
D1 all animals dosed with diamphenethide (250 mg/Kg)
D2 all animals dosed with diamphenethide (100 mg/Kg)
* t.c.g. represented by one animal from this point
△ sheep number 16 replaced by sheep number SBF
Fig. 4 Variation in the group mean packed cell volumes during the course of the experiment.

- $I_1$: t.c.g. and c.g. infected with 1000 metacercariae
- $I_2$: i.c.g. and c.g. infected with 1000 metacercariae
- $D_1$: all animals dosed with diamphenethide (250 mg/Kg)
- $D_2$: all animals dosed with diamphenethide (100 mg/Kg)
- $\star$: t.c.g. represented by one animal from this point
- $\Delta$: sheep number 16 replaced by sheep number SBF
Fig. 5 Variation in the group mean serum sorbitol dehydrogenase activities during the course of the experiment.

1. t.c.g. and c.g. infected with 1000 metacercariae
2. i.c.g. and c.g. infected with 1000 metacercariae
D1: all animals dosed with diamphenethide (250 mg/Kg)
D2: all animals dosed with diamphenethide (100 mg/Kg)
* t.c.g. represented by one animal from this point
△ sheep number 16 replaced by sheep number SBF

--- infection control group (i.c.g.)
--- treatment control group (t.c.g.)
--- challenge group (c.g.)
rise in the mean level of activity of this enzyme was seen in the sera of the affected animals. Treatment with diamphenethide resulted in a very rapid return to pre-infection levels. Challenge and control infections resulted in a slight prolongation of the rise in SDH activity, followed by a rapid drop in the mean level of activity. An unexplained high level of activity in the serum of sheep number 5 (Treatment Control Group) was recorded at week 19.

b) Ornithine Carbamyl Transferase (O.C.T.)

O.C.T. estimations were performed on sera taken at fortnightly intervals. In general, the changes in mean O.C.T. activity in the sera for each group paralleled those of SDH. Statistical analysis revealed that there was a significant linear correlation between the values of SDH and O.C.T. for any particular serum sample ($p \leq 0.01$)

c) Total Protein Concentrations

The changes in mean serum total protein concentrations for the animals in each group are shown in Figure 6. No dramatic changes in the values for this parameter were seen during the course of the experiment. Following infection with metacercariae, a slight rise in mean total protein concentration was seen for each infected experimental group. Treatment with diamphenethide was followed by a period during which the mean total protein values showed increased variability. Infection with metacercariae at week 12 resulted in a similar small rise
Fig. 6 Variation in the group mean serum total protein and immune globulin concentrations during the course of the experiment.

- $L_1$: i.c.g. and c.g. infected with 1000 metacercariae
- $L_2$: i.c.g. and c.g. infected with 1000 metacercariae
- $D_1$: all animals dosed with diamphenethide (250 mg/Kg)
- $D_2$: all animals dosed with diamphenethide (100 mg/Kg)
- $\star$: t.c.g. represented by one animal from this point
- $\Delta$: sheep number 16 replaced by sheep number SBF
in total protein values. During this untreated infection, this rise was more prolonged than previously and was more obvious in the Infection Control group.

d) Serum Immune Globulin Concentrations

The changes in mean immune globulin concentrations for the animals in each experimental group, are also shown in figure 6. The Zinc Sulphate turbidity test used to determine these values was performed on serum samples taken at fortnightly intervals. Changes in mean immune globulin concentrations, as measured by this test, were very small during the course of the experiment. The initial infection with metacercariae at week 0 was followed by virtually no alteration in the mean values of this parameter. Infection at week 12 resulted in a small rise in mean immune globulin concentration which became evident at week 18 and was most obvious in the Infection Control group.

4) Helminthological Data

a) Salt Flotation

Examination of faecal samples taken from the sheep before the start of the experiment demonstrated low levels of strongyloid eggs. These were present at levels ranging from 0 to 145 per gram of faeces. After treatment with Thiabendazole, weekly examinations failed to demonstrate nematode eggs in significant numbers. Occasionally the eggs of Nematodirus spp. and Trichuris spp. were seen but never at levels of more than 60 per gram.
Because of the continued occasional presence of *Nematodirus* eggs, Thiabendazole was administered to the sheep on two occasions after the start of the first experiment. On each occasion, the eggs disappeared from the faeces but became detectable again in samples taken several weeks after the anthelmintic dose.

b) Zinc Sulphate Flotation

No fluke eggs were found in faeces from any of the sheep during the preinfection period and up until 20 weeks post infection. The numbers of *Fasciola* eggs found by this technique in the faeces from individual sheep taken during the period 19 to 23 weeks post infection is shown in Table III.

**TABLE III**

*Fasciola* eggs per gram of faeces, detected in the faeces of individual sheep during the weeks 19 - 23 post infection.

<table>
<thead>
<tr>
<th>SHEEP NUMBER</th>
<th>WEEKS 19</th>
<th>POST 20</th>
<th>POST 21</th>
<th>POST 22</th>
<th>POST 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>LARGE NUMBERS</td>
</tr>
<tr>
<td>SBF</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>LARGE NUMBERS</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>LARGE NUMBERS</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
5) **Pathological Findings**

   a) General Post mortem features

   All five animals at post mortem were in reasonably good bodily condition. Sheep number 5 was best in this respect, with large amounts of sub-cutaneous, omental and peri-renal fat. Sheep numbers 7 and SBF were possibly in slightly better bodily condition than numbers 10 and 15.

   Apart from the liver lesions, the only obvious pathological changes seen during the course of the post mortem examination were several small, consolidated areas of chronic pneumonia, in the lungs of three of the sheep. Thus, in sheep number SBF, the left cardiac lobe was affected. In sheep number 15, lesions were present in both diaphragmatic lobes, while in sheep number 7, both cardiac and diaphragmatic lobes were involved. The degree of involvement, in all cases, was not considered to be significant to the animals' health.

   Parasitological examination of the gut and its contents, belonging to each animal, demonstrated five specimens of *Trichuris ovis* in the caecum of sheep number 7, and one specimen of *Trichuris ovis* in the caecum of sheep number 15. In addition, three cysts, thought to be of *Cysticercus tenuicollis* were found adherent to the mesentery of sheep number SBF.

   All other internal organs were normal.
b) Liver Pathology

The total weight of each liver, including the gall bladder, is shown for each animal in table IV.

<table>
<thead>
<tr>
<th>SHEEP NUMBER</th>
<th>7</th>
<th>SBF</th>
<th>10</th>
<th>15</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEIGHT OF LIVER (g.)</td>
<td>950</td>
<td>820</td>
<td>850</td>
<td>1025</td>
<td>550</td>
</tr>
</tbody>
</table>

The gross pathological changes recorded for the liver of each animal, are described below.

Sheep Number 5

The liver was fairly small in size and a normal dark reddish-brown colour. Both diaphragmatic and visceral surfaces showed many small elongated areas of depression and contracture of the liver capsule. The ventral lobe was most severely affected and in addition, showed gross evidence of fibrosis, cellular infiltration and tract formation. The ventral border of this lobe was particularly involved. The cut surface appearance was fairly normal except for a few pale yellow areas, both on the surface of the liver and extending into its substance. The bile ducts were not very conspicuous and the gall bladder was normal in size and content. The hepatic lymph nodes were enlarged and hyperactive.

Sheep Number 7

The liver had a pale orange/yellow colouration with many roughly circular areas, four to five cms. in diameter which were markedly more yellow in colour and had the appearance of areas of cellular degeneration. The small
depressed areas of pitting described previously were present in large numbers and in addition, there was evidence of a massive invasion by liver flukes, particularly in the ventral lobe. This was seen as zones of sub-capsular haemorrhage and green areas which had the appearance of areas of cellular infiltration and necrosis which extended into the parenchyma. In addition there were areas of fibrosis and thickening of the liver capsule plus fibrin tags and organised adhesions on to surrounding structures. The major bile ducts were prominent on the visceral surface and the gall bladder was grossly enlarged and distended with dark bile. The hepatic lymph nodes were grossly enlarged.

Sheep Number SBF

The liver was dark brown in colour and the ventral lobe was particularly dark. There were a few areas of pale yellow discolouration on the diaphragmatic surface. An abscess approximately three cms. in diameter was present on the surface and extended into the substance of the ventral lobe. This abscess contained yellow caseous material. The ventral lobe contained a mass of fluke tracts and gross evidence of cellular infiltration. Contracture of the liver capsule was very obvious with thickening of the liver capsule and fibrin tags on its surface. The gall bladder was slightly enlarged, as were the hepatic lymph nodes. The bile ducts were slightly tortuous and prominent.
Sheep Number 10

The liver had a pale yellow/orange colour. Irregular pale areas having the appearance of cellular degeneration were numerous on both surfaces of the liver and extended into the substance. The ventral lobe particularly showed gross evidence of massive cellular infiltration and fibrosis. The surface showed numerous areas of thickening and contracture of the capsule. There were numerous small reddish and greenish areas of necrotic tissue obvious on the surface of the liver and extending into the substance. On cut section, the liver was very firm in consistency and showed massive gross evidence of cellular infiltration. In addition, there were numerous small areas of inspissated greenish necrotic material found in the parenchyma or blocking the lumen of some of the small bile ducts. Microscopic examination of this material could not demonstrate that these areas were tiny dead flukes. It was thought that they probably represented areas of tissue and cellular debris of both host and parasite origin which had become encapsulated. The gall bladder was normal in size and the local lymph nodes were grossly enlarged. The bile ducts were not conspicuous on the visceral surface.

Sheep Number 15

The liver was fairly dark and normal in colour but there were a few areas of pale discolouration on the diaphragmatic surface. Small areas of contracture of the liver capsule were seen, especially on the ventral lobe but these were fewer in number than in other animals.
The liver was swollen and the edges rounded. The ventral lobe was particularly enlarged. There was evidence of a few fluke tracts in the ventral lobe. The cut surface of the liver was fairly normal in appearance. The hepatic lymph nodes were swollen but the bile ducts were not very obvious. The gall bladder was slightly enlarged.

6) **Fluke Data**

   a) Total numbers recovered and wet weight

The total numbers of flukes obtained at post mortem from each animal, is shown in Table V. The wet weight of the flukes obtained from each animal is also shown, as is the percentage of the challenge infection of metacercariae administered to each sheep recovered as flukes.

**TABLE V**

<table>
<thead>
<tr>
<th>SHEEP NUMBER</th>
<th>NUMBER OF FLUKES IN BILE DUCTS</th>
<th>NUMBER OF FLUKES IN PARENCHYMA</th>
<th>WET WEIGHT (g)</th>
<th>PERCENTAGE OF METACERCARIAE RECOVERED AS FLUKES</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>189</td>
<td>0</td>
<td>7.7</td>
<td>18.9</td>
</tr>
<tr>
<td>SBF</td>
<td>222</td>
<td>0</td>
<td>18.6</td>
<td>22.2</td>
</tr>
<tr>
<td>10</td>
<td>84 NUMEROUS* DEAD</td>
<td>0</td>
<td>3.5</td>
<td>8.4</td>
</tr>
<tr>
<td>15</td>
<td>202</td>
<td>0</td>
<td>19.3</td>
<td>20.2</td>
</tr>
</tbody>
</table>

* The numerous dead flukes in the parenchyma refers to the
numerous areas of necrotic material referred to earlier in the description of the pathological features of the liver from sheep number 10.

b) Dimensions of flukes

The mean length and breadth of the flukes from each sheep is shown in Table VI, together with their appropriate standard deviation. The means and standard deviations of the products of these measurements for the flukes from each animal, is also shown.

**TABLE VI**

Dimensions of flukes from individual sheep ± one Standard Deviation.

<table>
<thead>
<tr>
<th>SHEEP NUMBER</th>
<th>MEAN FLUKE LENGTH (m.m.)</th>
<th>MEAN FLUKE BREADTH (m.m.)</th>
<th>MEAN OF LENGTH X BREADTH (m.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>13.8 ± 3.4</td>
<td>5.0 ± 1.3</td>
<td>72.6 ± 33</td>
</tr>
<tr>
<td>SBF</td>
<td>18.4 ± 3.4</td>
<td>7.8 ± 1.4</td>
<td>145.6 ± 44.5</td>
</tr>
<tr>
<td>10</td>
<td>11.7 ± 4.0</td>
<td>4.5 ± 1.4</td>
<td>57.4 ± 36.3</td>
</tr>
<tr>
<td>15</td>
<td>19.1 ± 3.5</td>
<td>7.6 ± 1.3</td>
<td>147.3 ± 43</td>
</tr>
</tbody>
</table>

c) Protein extraction

The mean values for the amount of protein extracted from each fluke for individual sheep are shown in Table VII.
TABLE VII
Average amount of protein extracted from individual flukes from each sheep.

<table>
<thead>
<tr>
<th>SHEEP NUMBER</th>
<th>NUMBER OF FLUKES USED</th>
<th>PROTEIN EXTRACTED PER FLUKE (mg)</th>
<th>A. with T.C.A. extraction*</th>
<th>B. without T.C.A. extraction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>100</td>
<td>0.36</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>SBF</td>
<td>100</td>
<td>1.35</td>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>0.96†</td>
<td>1.43†</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>1.66</td>
<td>1.83</td>
<td></td>
</tr>
</tbody>
</table>

*The method of extraction refers to the two variations of the Biuret technique described by Goa (1953) which were used to estimate the protein concentration in the supernatant fluid.

† The values obtained for the mean fluke protein extraction for sheep number 10 are considered to be elevated. This is because only 50 flukes were used in the extraction and it is considered that the protein extraction process was more efficient with this smaller number of flukes than in the case of the other animals where 100 flukes were used.
DISCUSSION

Due to financial, managemental and other limitations, the number of animals involved in the experiment had to be confined to an initial total of six. This necessitated experimental group sizes of two animals per group. This small group size and the high degree of variability inherent in biological, and in particular helminthological, systems set limits on the amount of information which could be obtained from the experiment. Thus, although dramatic differences between groups would be detected by this experimental system, the inability to apply statistical methods to the data from such a system, and the high within-group variation, meant that fine differences between groups would be masked. In fact, one of the most obvious features of the results was the high individual variation of the animals. Interpretation of the results must, therefore, take these inherent limitations into account. Nevertheless, the results demonstrated some interesting features of the pathogenesis of fascioliasis.

A consistent feature of the infections administered during the experimental period was the mildness of the clinical entity which ensued. Slight evidence of abdominal pain was the most apparent clinical manifestation. At no time was anaemia, jaundice or obvious ill thrift clinically apparent. An explanation of this situation involves several factors. a) The initial infecting dose of metacercariae was fairly low.
Taylor (1949) stated that 10,000 metacercariae were required to produce acute fascioliasis. Pullan et al (1970) used between 5,500 and 12,000 metacercariae to produce the clinical signs of the acute and sub-acute disease. b) The numbers of metacercariae which became established as adults was relatively low. Pullan et al (1970) found between 497 and 2,290 flukes present in the livers of their sheep which were showing clinical signs of sub-acute fascioliasis. c) The sheep were killed eleven weeks after the secondary infection given at week 12. This period is considered insufficient to allow the development of obvious clinical signs of chronic disease, given the number of adult fluke present. Sewell et al (1968) followed the development of the chronic disease for 30 weeks after infection with between 600 and 2,400 metacercariae. Dramatic changes in parameters were only recorded during the latter half of this period and the sheep were found to be harbouring between 44 and 367 flukes at post mortem examination.

The factors discussed above also help to explain the lack of obvious differences in the rate of live weight gain of the animals in the various experimental groups. However, there is some evidence that infection with metacercariae in all cases, resulted in a slight depression of the animals' subsequent rate of live weight gain. This occurred at an age when the rate of live weight gain for young animals should have been increasing (McDonald, Edwards and Greenhalgh, 1972).

The death of sheep number 16 was thought to be
explicable in terms of either an enterotoxaemia or aspiration pneumonia as described previously. Obstructive urolithiasis, the cause of death in sheep number 19, is often seen in castrated male sheep housed indoors and fed on heavy concentrate rations (Blood and Henderson, 1974). Water deprivation, which may also be a precipitating factor, was unlikely in this case although it is possible that this animal had been eating more of the concentrate ration than its neighbours, leading to an increased phosphorus intake, which can also be an associated factor in the aetiology of urolithiasis.

Diamphenethide has been shown to have an efficiency of between 99.9 per cent and 100 per cent against liver flukes aged one day to six weeks old when given to sheep at a dose rate of 100 mg/Kg (Annen, Boray and Eckert, 1973). This high degree of efficiency was borne out in this present experiment. No adult or immature flukes were found in the livers of the Treatment Control animals numbers 5 and 19. In the case of sheep number 19, this was true 16 days after the initial dose of diamphenethide had been given. Annen et al (1973) reported that dose levels of up to 400 mg/Kg were well tolerated in sheep. The only adverse effect which these workers recorded was wool shedding in one in ten of their animals. This feature has also been described by Kingsbury and Rowlands (1972) and is probably the explanation of the wool loss in sheep number 7. Diamphenethide does not appear to have the hepatotoxic
properties possessed by earlier drugs used against liver fluke. For example, there was no elevation of the serum SDH levels, after this drug had been given. On the contrary, after administration of the first dose of the drug, a dramatic drop in S.D.H. levels in the infected animals occurred and the pre-infection levels of this enzyme were maintained after the second dose of the drug.

The peaks in the total white blood cell count for each animal coincided approximately with the peaks in the eosinophil counts for that particular animal. This suggests that the leucocytosis which followed infection with *F. hepatica* was primarily a reflection of the rise in total circulating eosinophils at that time. This agrees with the observations of Sinclair (1962) and Pullan (1968). Following the challenge infection, the eosinophil response of animals in the Challenge group was different from that of animals in the Infection Control group. Eosinophil levels in the former group reached a low early peak after infection but then fell back to a relatively low level which was maintained until they rose dramatically at weeks 21 and 22. Levels in the Infection Control group rose slightly more rapidly to a higher but later initial peak. This raised level was maintained until weeks 21 and 22 when a further slight rise also occurred. These secondary rises in eosinophil levels coincided with the time when the infections were first becoming patent. They thus followed the period when the flukes were leaving the liver parenchyma and penetrating the bile ducts to lie in their lumens. Sinclair (1973) recorded an accelerated and more pronounced eosinophilia
in his challenged sheep compared with his control animals. However, in previous work the same author (Sinclair, 1971) recorded a reduced eosinophil response in his animals on challenge. It would seem in this present experiment, that in the Challenge group, the normal eosinophil response to the fluke infection was suppressed until the flukes gained access to tissue which had not been exposed to the previous infection. Soulsby (1972) states that the function of the eosinophil is not well understood, although the eosinophilia associated with parasitic infections, allergic diseases and following an anaphylactic reaction is well documented. Barbaro (1972) considers that eosinophils play a secondary role in the immediate-type hypersensitivity response and that their appearance is the consequence of an antigen-antibody reaction.

The lack of significant changes in the packed cell volume values of each group may be attributable to the low level of the infective dose administered and to the early termination of the infection as discussed previously. There is some evidence from the results that a more profound drop in P.C.V. values was occurring towards the end of the experiment. The mean values for both the Challenge group and the Infection Control group at weeks 22 and 23 were the lowest recorded for both those groups during the whole experiment. A more rapid drop in the mean P.C.V. was seen in both these groups from week 19 onwards. This corresponds approximately, with the period
when the flukes were entering the bile ducts. Sinclair (1962) and Holmes et al (1971) also recorded that the most obvious falls in P.C.V. commenced after entry of the flukes into the bile ducts. However, there appears to be no obvious difference between the severity of the anaemia produced in the Challenge and Infection Control groups.

A comparison of the changes in S.D.H. activity between the Challenge group and the Infection Control group shows features which are similar to the eosinophil changes discussed above. The Challenge group mean levels showed a reduced and delayed rise in comparison with the Infection Control group and the former had a sharp peak in activity at week 20. This occurred again at the time when the flukes were presumably entering the bile ducts.

Increased hepatic destruction has been recorded by Sinclair (1969) as occurring at the time of bile duct penetration by the flukes. This is presumably due to the large size of the flukes at that time or to increased vascular damage associated with trauma to the blood vessels which run alongside the bile ducts. The drop in S.D.H. and O.C.T. levels to near pre-infection levels at the end of the experiment agrees with the findings of Sewell (1966) who states that as soon as the flukes have entered the bile ducts, parenchymal repair is rapid and dysfunction detectable by liver function tests soon returns to normal. However, Treacher et al (1974) in an experiment in which they surgically placed immature flukes into the bile ducts, reported that liver damage and raised Glutamic dehydrogenase (G.D.), O.C.T. and
S.D.H. levels were produced without a liver migration phase. Sinclair (1973) recorded an earlier and more marked increase in G.D. activity in the serum of his challenged animals compared with his control animals. He attributed this finding and the enhanced eosinophilia mentioned earlier, to the existence of a hypersensitivity reaction to the challenge infection of metacercariae in an already sensitised animal. The findings in this present experiment do not support this hypothesis. Indeed they indicate that the degree of eosinophil response and liver damage in animals receiving a challenge infection was less than in animals receiving a primary infection.

High individual variation in the values for serum total protein make interpretation of these results difficult. The rise in total protein was most marked during weeks 16 to 22, and this rise was at least partly due to the rise in immune globulin levels recorded at this time. The rise in this latter parameter is more obvious in the Infection Control group than the Challenge group, but the overall changes were not dramatic. The increased gamma globulin seen in acute and chronic fascioliasis has been attributed by Nansen (1970) to an increased intra-hepatic synthesis of IgG which he suggested may be a function of liver damage and possibly be related to the production of antibody against the host's own tissues. The use of the zinc sulphate turbidity test did not allow an accurate estimate of the albumin concentration, by difference. Since changes in
globulin fractions other than gamma globulins have been reported (Furmaga and Gundlach, 1967) any changes in these would mask changes in albumin content if the latter was simply obtained by subtracting the immune globulin level from the total protein value. It is not, therefore, possible to state if a significant hypoalbuminaemia was produced.

Kendall and Parfitt (1962) stated that the minimum pre-patent period for F. hepatica in sheep was 55 days. The results in this experiment correspond with this figure since the first eggs were demonstrated in the faeces of sheep number 10 at 56 days post infection. _Fasciola_ eggs were not demonstrated in the faeces of sheep number 7 until 70 days post infection. However, Taylor (1964) states that because of the uneven expulsion of eggs from the gall bladder, the appearance of eggs in the faeces is very variable. It therefore seems unlikely that there is any evidence from this data of a retardation in the development of the flukes in the challenged animals.

The pathological changes described in the liver of each sheep show the highest individual variation of any parameter studied. Thus, one animal from each group infected at week 12 (sheep numbers SBF and 15) showed relatively few pathological changes associated with the presence of the flukes. Similarly one animal from each of these groups (sheep numbers 7 and 10) showed very marked changes. However, except in sheep number 10, these differences in liver pathology were not reflected
in differences in the numbers of flukes present in a particular liver. In sheep number 10, there appeared to be a massive cellular response to the invasion of the challenge dose of metacercariae. This had apparently resulted in inhibition and death of a large number of the immature flukes as they migrated through the parenchyma. The mechanism of this inhibition is of interest, because although there were extensive areas of green infiltration into the areas of fluke migration and inhibition, the changes in circulating eosinophils and S.D.H. levels in this animal were not greater than those in animals which showed a less severe hepatic cellular reaction. This would tend to suggest that the cellular response was not primarily eosinophilic, since Dow, Ross and Todd (1968) have shown that eosinophilia is mirrored by an increase in eosinophils locally in and near fluke tract lesions in the liver. The mechanism of immediate hypersensitivity and associated liver damage as a mechanism of resistance to challenge as postulated by Sinclair (1973) would not appear to be supported by the findings in this animal.

In contrast, the marked degree of gross liver damage seen in sheep number 7 was associated with very markedly raised levels of S.D.H. and O.C.T. activities and of circulating eosinophils. However, the degree of apparent cellular infiltration in this animal's liver was less than that of sheep number 10 and the pathological changes appeared to be more the result of the damage caused by fluke migration than a massive response to infection as
was the case in sheep number 10.

It can be postulated that, since eosinophilia is a response both to the presence of the parasite and to the tissue damage caused by the parasite, the reduced peripheral eosinophil response seen in sheep number 10 was a result of the early death of the flukes due to intense host reaction. This premature death meant that little tissue damage occurred and the host therefore showed an eosinophil response only to the presence of the parasite and not to liver cell damage. The green lesions seen in the liver of this animal thus represented a very localised intense eosinophilic response. In contrast, the liver of sheep number 7 was subjected to much greater tissue damage and this animal showed a response to both the presence of the parasite and to this tissue damage resulting in a marked peripheral eosinophilia. Because the flukes survived and were not inhibited, the areas of localised eosinophil response were less extensive.

The livers of sheep numbers SBF and 15 showed remarkably low grade pathological changes considering the number of flukes which they contained. The capacity of the liver to recover after fairly severe acute damage was illustrated by the almost normal appearance of the liver from sheep number 5.

A consistent finding in all of the livers examined was that the majority of fluke tracts and the areas of maximum cellular infiltration, fibrosis and local peritonitis were concentrated in the ventral lobe. This apparent preferential migration of the flukes through this
lobe has been described by Taylor (1964), and Ross et al (1966). It would seem possible that if a challenge infection of young flukes also migrated preferentially through this lobe, then the cellular infiltration and fibrous tissue reaction to a previous infection could physically hinder the development of the flukes in such a relatively confined area.

Apart from sheep number 10, which had a reduced percentage recovery of metacercariae, the percentage recovery of flukes was approximately equivalent to that recorded by Sinclair (1964, 1965) however, it was much less than that reported by Boray (1967) who had a 48.2 per cent recovery in 12 sheep given 1,000 metacercariae each. There is insufficient evidence to suggest that there was a difference in recovery rates between the Challenge group and the Infection Control group.

The fact that all the viable flukes recovered were found in the bile ducts and none in the liver parenchyma at 11 weeks post infection, would indicate a relatively early maturity and rapid rate of development of the flukes. Dawes and Hughes (1964) quote Schumacher (1938) as stating that a minimum period of eight weeks was required in sheep, before the flukes started to enter the bile ducts. It is possible that with a relatively low infective dose, there was no competitive inhibition between the flukes and they were able to develop and enter the bile ducts at an early date.

Evidence as to the degree of maturity and development
attained by the flukes in each sheep can be obtained from the data on fluke wet weights, dimensions and protein content. These data show great individual variation but there appears to be no consistent difference in the degree of fluke development between the two groups. The data on wet weights, dimensions and protein extraction tend to show that the flukes from sheep numbers 7 and 10 had not developed to the same extent as flukes from sheep numbers 15 and SBF. This may be associated with the increased local cellular response seen in sheep number 10 and to the marked hepatic damage seen in sheep number 7 which resulted in adverse local environmental conditions in each case.
CONCLUSIONS

The following conclusions may be drawn from the results of this work:

1) There was considerable individual variation in the values for the parameters studied.

2) The safety and efficacy of diamphenethide as an anthelmintic against immature liver flukes was upheld.

3) Serum levels of liver specific enzymes reflected the degree of liver damage caused by migrating liver flukes. Their usefulness as an aid to the diagnosis and prognosis of acute and sub-acute fascioliasis was re-emphasised.

4) Serum ornithine carbamyl transferase levels appeared to be as sensitive an indicator of liver damage as those of sorbitol dehydrogenase. The estimation of the former enzyme has the advantage that it can be performed without the use of ultra-violet spectrophotometric equipment.

5) No differences in the number of flukes recovered or in the degree of development of those flukes were demonstrated between the challenge and control groups.

6) Fluke development appeared to be affected by host response or gross hepatic damage in individual animals.

7) Reduced and delayed rises in the values of serum sorbitol dehydrogenase activity and in the numbers
of circulating eosinophils in the challenged animals during the first eight weeks of the challenge infection were recorded. These differences suggest a reduction in the severity of the pathological changes occurring during the early stages of the challenge infection as compared with the control infection.

8) No direct evidence for acquired resistance to reinfection with *F. hepatica* in sheep was seen.

9) Administration of 1,000 metacercariae to sheep resulted in a relatively mild disease with no obvious clinical signs during the early stages of the infection.

10) The necessarily small group sizes used during this work meant that other less conspicuous differences between the experimental groups may have been masked by the high individual variation.
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