SOME ASPECTS OF IMMUNITY TO THE LIVER FLUKE FASCIOLA HEPATICA IN SHEEP AND CATTLE

A thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine of the University of Glasgow

by

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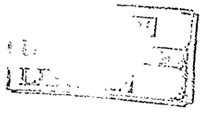

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GENERAL SUMMARY

The studies in this thesis were designed to investigate the comparative immunological responses of sheep and cattle to primary and secondary F. hepatica infection in order to more accurately define the reasons for the observed differences in susceptibility to the parasite in the two ruminant species. The effect of previous exposure to nematode and cestode infection in the course of F. hepatica infection in sheep was also studied.

In Chapter I an extensive literature survey covering relevant aspects of the host-parasite relationship was carried out, and in Chapter II the materials and methods used in the thesis were described.

In Chapter III the course of primary and secondary experimental F. hepatica infection in sheep was monitored and compared with that in experimental calves. This was done by performing various haematological, biochemical and parasitological procedures, including estimation of the liver enzymes G.L.D.H. and G.G.T. and detection of circulating antibody using D.I.D. and I.H.T. In this connection the microplate Enzyme-linked-immunosorbent assay (micro-E.l.i.s.a.) was investigated for early diagnosis of F. hepatica infection in ruminants. Problems associated with this technique were discussed in some detail. In addition the protective effect of serum from ruminants infected with the parasite was investigated in passive transfer studies in the laboratory model of fascioliasis.

Following primary infection sheep rapidly became anaemic and remained susceptible to reinfection although serum from infected animals afforded a degree of protection when passively transferred to rats. In contrast, no anaemia was detected in experimental calves. The liver enzymes G.L.D.H. and G.G.T. were found to be reliable indicators of liver cell necrosis and bile duct damage, respectively, following primary infection in the two ruminant species, but proved of limited value on reinfection. D.I.D. and I.H.T. were

found to be reliable diagnostic aids for detection of serum antibody, the former as a qualitative test and the latter as an early, sensitive quantitative indicator. E.l.i.s.a. was found to be both unreliable and idiosyncratic in this system.

Chapter IV described the response of peripheral lymphocytes of sheep and cattle to stimulation with various mitogens as well as F. hepatica somatic lipid-free antigen (L.F.E.) was monitored during experimental primary and secondary F. hepatica infection, using the Lymphocyte Transformation Test (L.T.T.). The patterns of responses obtained from infected and reinfected animals were compared with those of control animals. Differences observed in such patterns were noted and discussed in the context not only of the modifying effect of infection and reinfection on such responses, but also in terms of inherent differences in pattern of response between the two ruminant species.

An immunosuppressive effect of F. hepatica infection was observed for the first time in experimental sheep, characterised by a reduction in the transforming ability of peripheral lymphocytes in response, not only to non-specific mitogenic, but also to specific antigenic (L.F.E.) stimulation. Such an effect was apparent not only in impaired responses of once-infected animals compared with control animals, but also when reinfected animals were compared with once-infected animals, thus suggesting that reinfection had reinforced the immunosuppressive effects. No such effects were demonstrable during experimental infection and reinfection of cattle in which mitogenic and antigenic responses of infected and control animals was of a similar order. Possible reasons for the observed differences were discussed.

The effect of prior nematode and cestode infection of sheep on the course of experimental F. hepatica was studied in Chapter V. The cross immunising properties of these parasites per se and in addition the modification

of any protective effect conferred by the immunomodulating compound L tetramisole (Levamisole) were investigated. In addition the effect of passive transfer of serum from sheep infected with the cestode T. hydatigena to rats simultaneously infected with F. hepatica was investigated and the relative protection afforded by such serum compared with that obtained from fluke infected sheep and cattle was determined.

It was recorded that no protection was conferred on sheep against F. hepatica infection by virtue of prior infection with any helminth species per se, including C. tenuicollis and in addition that serum from sheep infected with the larval cestode C. tenuicollis afforded no protection against the fluke when passively transferred to rats. However, a degree of immunity to F. hepatica was recorded, for only the second time, in sheep primed with helminth infections prior to treatment with levamisole. This is the first demonstration of a beneficial effect conferred by levamisole by virtue of its immunomodulatory properties, in ruminants infected with any helminth species. Resistance was evidenced by a 47% reduction in flukes removed from sheep treated in this way. There appeared to be a slight reduction in S.D.H. values of partially resistant sheep, when compared with control animals.

Possible reasons for the stimulation of resistance were discussed with a view to incorporating levamisole in a prophylactic regime.

In the final chapter of this thesis (Chapter VI) the various differences observed in the course of F. hepatica infection in the two ruminant species were described in some detail. The immunological shortcomings of sheep during F. hepatica infection were discussed with a view to harnessing the therapeutic properties of such compounds as levamisole and "transfer factor" in a prophylactic regime incorporating the most recently developed specific immunogens in protecting not only sheep, but also cattle against fascioliasis.

LIFE CYCLE OF FASCIOLA HEPATICA

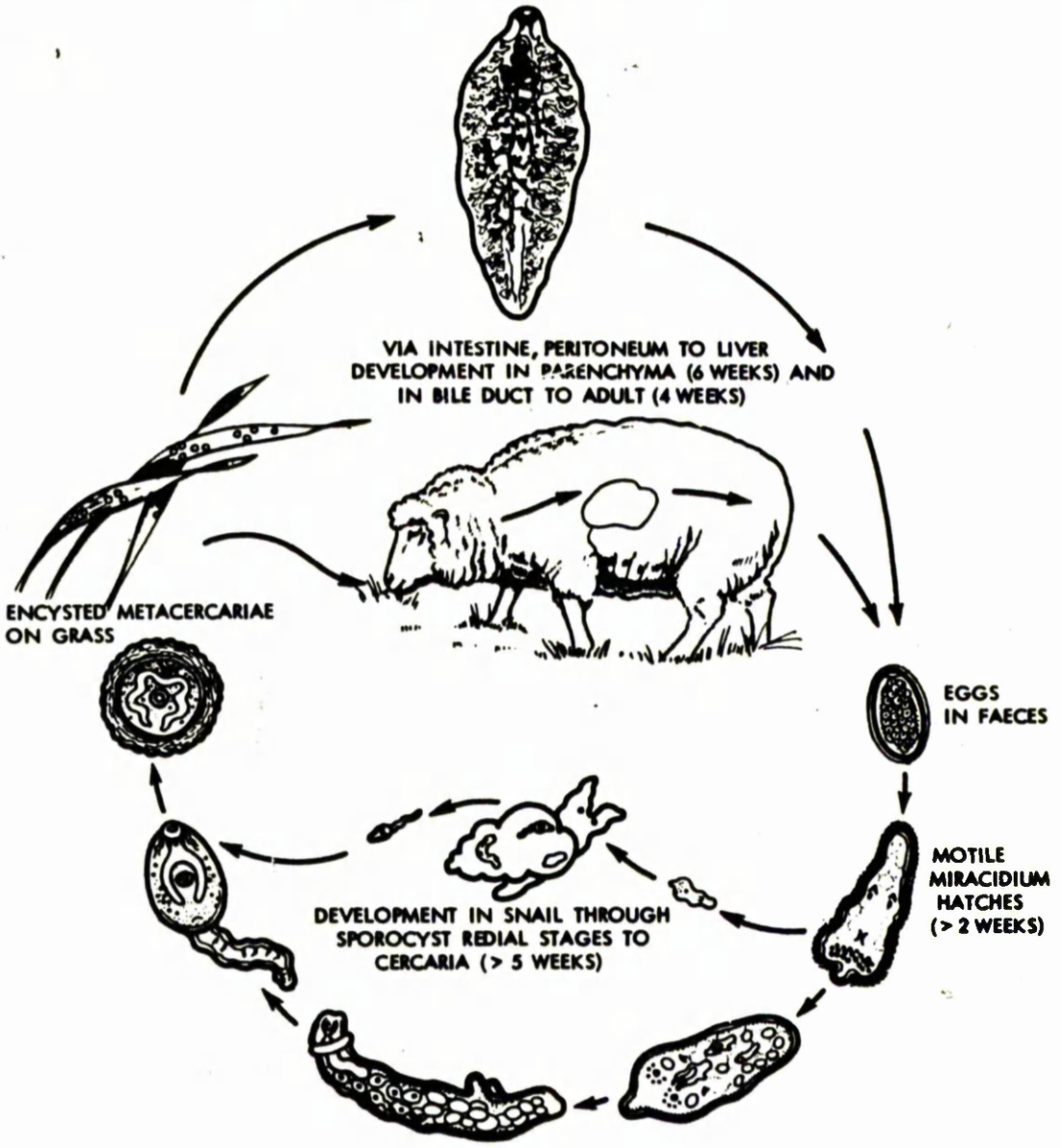


Plate 1

Historical

Disease caused by the trematode Fasciola hepatica has been recognised since very early times, indeed the eggs of F. hepatica have been demonstrated in archaeological excavations from the early Viking period (750-800 A.D.) in Denmark (Nansen and Jorgensen, 1977), and fluke eggs along with shells of Galba (Lymnaea) truncatula were found in material excavated from a "terp" (refuge mound) in North-West Germany believed to be 1800-2000 years old (Over and Jansen, 1962). Reference to disease caused by the parasite may date back as far as the 10th century, in the Gwentian Code of Wales comprising laws enacted by Howel the Good (Anon, c.1000). Another early reference, (Anon, c.1200) asserted: "teythy davat blys (sic) ac oen ay goruod hyd kalanmey rac erauat enykafo teyrguala or tavaul neuuyt, "which may be construed as "the attributes (or essential characteristics) of a sheep are milk, and a lamb, and to be guaranteed until the calends of May against the liver-rot until it has had three sufficiencies of the new herbage." Jean de Brie, one of the best breeders of cattle and sheep in France, in a treatise ordered by Charles V in 1379 declared that the fluke was not the principal cause of liver-rot, but that worms engendered from decay due to the corruption of the liver by certain plants, completed its destruction.

The first recognisable account of the liver fluke, and its connection with so-called "liver-rot", appeared in "A newe Tracte of Treatise moost profytable for all Husbandemen" by Sir Anthony Fitzherbert in 1573, quoted by Smith (1919), who pointed out that "flooded land be bad for sheep", and "white snails be ill for sheep in pasture." Some time later in 1575 Cornelius Gemma, a prominent physician and astronomer in the Netherlands, described a dreadful epidemic of "duva" or liver fluke sickness, which he

felt represented a manifestation of divine justice, in the following terms: "Anno 1562.....lues quoque infanda percoris in Hollandia, natis vermibus passim circa hepatis regionem", which translates as "in the year 1562 there occurred an unspeakable pestilence of flukes in Holland, arising everywhere through worms in the region of the liver." Similar outbreaks of "sheep-rot" were documented in Germany in 1663 and the Netherlands in 1674, which were also explained in terms of sheep feeding on particular plants. In Britain, Frank Nicholls, a London physician and England's leading anatomist presented a report to the Royal Society in 1755 on "Worms in Animal Bodies". His remarks included reference to "flooks" which, in his opinion, caused "a species of dropsy" in bullocks and sheep by building "a wall of stone for their defence". Up to this time this condition had been variously thought of as due to "particles of soil (gruft) adhering to grass after flooding; luxuriant growth of plants in moist weather; grazing on butterwort, white-rot and sundew; poor diet and drinking too much water" (1792, quoted by Simonds, 1880) or a poisonous effluvia emitted from marshy soil (Harrison, 1804). The "flooks" which some authors had linked with the "liver-rot" were given the definitive Latin name "Fasciola hepatica" by Linnaeus, 1758, who considered them to be accidentally swallowed leeches whose proper habitat was under stones in fresh water.

The Revelation of the Life-cycle of Fasciola hepatica

This remarkable piece of scientific investigation is contained in the excellent review articles of Taylor (1937) and Reinhard (1957), quoted by Pantelouris (1965), who pointed out that although the existence of the adult fluke had been known for many years it was not until 1752 that the first record was made of any other stage in the life cycle having been observed, when the Dutch mircroscopist Swammerdam, while dissecting snails came upon moving objects,

which he called worms and which from his drawings were recognisable as cercariae. These were also described by Muller (1773), who coined the name cercariae, and Mehlis (1881). Around the same time Professor Ludwig Bojanus (1818) noticed that pond snails harboured "royal-yellow worms", now called rediae.

Meanwhile foundation stones of a different type were being laid, and towards the end of the 18th century it became recognised in certain quarters that some parasites divided their life-cycle between 2 or more hosts, and Johannes Steenstrup, a young Norwegian-Danish scholar (he was 28 years old at the time), developed the idea of an alternation of generations in trematodes, among other animal species, which he defined as follows: "alternation of generations is the remarkable phenomenon of an animal producing an offspring which at no time resembles its parent, but which, on the other hand, itself brings forth a progeny which returns to its form and nature to the parent animal so that the maternal animal does not meet with its own resemblance in its own brood, but in its descendants of the second, third or fourth degree of generation". The first suggestion of a definite connection between a specific fluke and a specific cercariae appeared, however, to have been made some ten years after Steenstrup's publication by Professor Rudolf Leuckart (1852) in Munich, who professed that a certain fluke found in the intestine of predacious fish was specifically indentical to an encapsulated cercariae found in the gills of other small fish, on which the final host fed. When La Valette (1855) demonstrated that certain encysted cercariae from water snails developed into adult flukes in birds, and Wagener (1857) observed the penetration of the miracidium, (first described by Mehlis (1831), into the snail, and its subsequent development into the rediae, the last links in the chain had been completed, and it only remained for the chain to be unravelled.

Plate 2. Sir Algernon Phillips Withiel Thomas (1875-1937).

Photograph taken on his 80th birthday.

The impetus for the final elucidation of the life cycle of F. hepatica (illustrated in Fig. 1) was provided by a severe epidemic of fascioliasis in the British Isles, during the winter of 1879 - 80, in which 3 million sheep were lost. As a result of this the Royal Agricultural Society of England decided to offer a grant for the investigation of the natural history of the parasite that caused the disease. The grant went to a 23 year old graduate of Balliol College, Oxford, named Algernon Phillip Withiel Thomas (see Plate 1), who had just been appointed demonstrator in biology at the University Museum. At the same time the great Leipzig Zoologist, Professor Rudolf Leuckart, was also undertaking experiments to describe the developmental history of the liver fluke. The work of both men culminated in the publication of two series of papers (Leuckart, 1881, 1882 a) & b); and Thomas, 1881, 1882 a) & b), 1883 a) & b)), in which the snail Lymnaea truncatula was identified as the intermediate host, and the successive larval stages were bred and described. In the opinion of Taylor (1937), "the raw recruit", in the shape of A.P. Thomas, should receive the greater part of the credit for this discovery, since Leuckart, "the great grey general", had confused the identity of the intermediate host with Lymnaea peregra, in which an abortive development takes place.

The work of Thomas and Leuckart was supplemented by Lutz (1892, 1893), 1893), who demonstrated the infectivity of cercariae and Sinitsin (1914), who established the route followed by metacercariae from gut to liver in the definite host.

The life history of the liver-fluke Fasciola hepatica (Plate 2) was thus elucidated more than 150 years after the first observation of the cercariae.

Disease due to Fasciola hepatica and its economic significance

Present day descriptions of fascioliasis in domestic species (see

literature review) indicated that fascioliasis is manifest as a clinical entity in two quite distinctive conditions. Firstly, acute fascioliasis which is characterised by sudden death, caused by an acute haemorrhagic anaemia, with at post mortem, the presence of large numbers of immature flukes in the liver parenchyma and secondly, at the other end of the clinical spectrum, chronic fascioliasis which is characterised by a progressive loss of condition and development of anaemia, resulting in failure to thrive, and debilitation. In this case the fluke population is present principally as adult flukes in the bile ducts. These two clinical conditions represent the two extremes of fascioliasis and other, intermediate sub-acute disease states may present, as well as sub-clinical debilitatory conditions of particular importance in adult milking cattle, due to infection with smaller numbers of flukes.

The importance of all the manifestations of fluke infection in terms of the annual cost to the agricultural industry in Great Britain may be judged from Tables 1, 2 and 3, based on 1974 figures released by I.C.I. (in Fluke Disease, ICI 1974).

Type of stock	Infection rate (%)
Cows	40
Heifers and steers	17
Adult sheep	13
Lambs	5

Table 1: Average incidence of F. hepatica in Great Britain

Type of stock	Percentage liver condemnation	Loss of revenue
Cattle	29	£2 million
Sheep	8	£15,000

Table 2: Average losses due to liver condemnation

Type of stock	Nature of loss	Loss of revenue
Dairy cattle	Lower milk yields, lower milk quality	£4 - 6 million £2 - 4 million
Beef cattle	Higher feed costs, lower carcass values.	£5 - 7.5 million
Sheep	Poor lamb growth and longer finishing periods	£0.75 - 1 million

Table 3: Average losses from low-level fluke infection

As well as the losses outlined above other losses due to mortality in sheep and poor wool yields and fleece quality boosted the total loss in revenue caused by the liver fluke (1974) to a figure in excess of £50 million. It must also be stressed that fascioliasis is of world-wide importance and as such constitutes a major economic problem.

It is of no little interest to discover that in most parts of the world, including Great Britain, only the chronic condition is seen commonly in cattle, although acute fascioliasis has been described in calves in Ireland by Ross (1966a) and an outbreak of sub-acute fascioliasis in cattle was reported by Eckert et al. (1977) in Switzerland; in contrast acute, acute/sub-acute and sub-acute/chronic outbreaks of fascioliasis have been documented in sheep (Ross et al., 1967a);

Reid et al., 1970). In those studies the different clinical entities corresponded to the numbers of flukes, the stage of development and also the time of year.

From this evidence it would appear that cattle exhibit a decreased susceptibility to, or tolerance of Fasciolia hepatica infection, and experimental studies by Ross (1966b); Boray (1967a) and Doyle (1971) have clearly shown that cattle can successfully resist secondary infection with F. hepatica, as demonstrated by a lowering of the number of flukes recovered, compared with control animals given only a primary infection. However, recent studies by Van Tiggele and Over (1975) do not wholly support this. It has also been clearly established that sheep remain susceptible to infection with F. hepatica, and would appear incapable of acquiring resistance as measured by numbers of flukes recovered. This has emerged in many studies including those by Boray (1967b); Sinclair (1971a) and Rushton (1977) and only the very recent work by Campbell et al. (1977), which will be discussed later would tend to dispute this basic premise.

The reasons for this difference in susceptibility to F. hepatica infection between sheep and cattle have not as yet been fully determined despite many and varied studies over a period of several years. One of the most basic questions to be answered is the relative importance of immunological and non-specific factors in the phenomenon of resistance. Dargie et al. (1974) presented evidence to show that although physical barriers such as hepatic fibrosis could not be discounted in host resistance to fascioliasis immune mechanisms were unquestionably involved; these mechanisms were shown to involve both humoral and cell mediated immunity, and the degree of protection achieved was not related to the extent of hepatic fibrosis.

Despite this apparent immunity to fascioliasis in cattle, attempts to immunise sheep and cattle against fascioliasis have proved disappointing although recent work involving the use of antigens elaborated during periods of in vitro cultivation of the fluke (Lang, 1976; Lang and Hall, 1977) encourage some hope for the future. Recently, however, Campbell et al. (1977) reported that sheep became immune to reinfection with metacercariae of F. hepatica following infection with the larval cestode Cysticercus tenuicollis. This apparent cross-immunisation begged the question of immunisation against fascioliasis using generically unrelated parasitic extracts.

Objectives of this thesis

In this thesis the literature pertaining to relevant aspects of Fasciola hepatica infection was reviewed, with particular reference to the disease in ruminants. In addition an attempt was made to elucidate the reason(s), immunological or otherwise for the observed difference in susceptibility between sheep and cattle to infection with the liver fluke Fasciola hepatica. This was done by monitoring primary and secondary infections with F. hepatica in the two species. As well as parasitological, biochemical, and haematological studies, a number of detailed immunological procedures were carried out to detect humoral and cell mediated immune responses in the infected animals. In this connection the serological studies carried out in sheep and cattle stimulated an ancillary investigation to find the serological test most suitable as an early diagnostic aid in detection of fluke infection, and, for this reason the Enzyme-linked immunosorbent assay (E.l.i.s.a.) first described by Engvall and Perimann (1971) was investigated and its various merits described.

Finally, preliminary studies designed to determine the possible cross-immunising properties, of other helminths, against F. hepatica infection in sheep, and the effect of the immunomodulatory compound L. tetramisole (levamisole) on these properties, were undertaken.

CHAPTER 1

LITERATURE REVIEW : FASCIOLIA HEPATICA INFECTION, WITH PARTICULAR REFERENCE TO SHEEP AND CATTLE

INTRODUCTION

The presentation of this review is divided into sections on different aspects of the host-parasite relationship. Any literature not reviewed in depth in this chapter, but of specific importance to the subject matter of another chapter, will be found in more detail in the introduction of the relevant chapter. The results of contemporaneous studies, i.e. those published in the past 3 years, are included in the relevant discussion sections.

SECTION 1

CLINICAL AND POST MORTEM STUDIES

Sheep

Clinical diseases due to F. hepatica infection in sheep was first classified as acute and chronic by Ollerenshaw (1958). However, the first detailed description of these diseases was provided by Ross, et al., (1967 a)) who documented the clinical and post mortem findings under conditions of natural and experimental infection with F. hepatica. These authors classified the disease into 3 forms, namely, acute (Type I and II), subacute, and chronic. In the acute Type 1 condition death occurred without clinical signs being present 7-8 weeks after experimental infection with 5,000 metacercariae, at which time 2,000-2,500 flukes were recovered from infected livers. In field cases between 1,000 and 2,500 flukes were found. In this type of fascioliasis 60% of the flukes were found migrating in the liver parenchyma and 40% in the bile ducts.

Post mortem findings were typically those of an acute haemorrhagic anaemia. The abdomen was filled with a large quantity of blood stained fluid. The liver was enlarged, congested and subcapsular haemorrhages were frequent. Histologically, it was found that very few areas of the liver

parenchyma escaped damage from migrating parasites. Characteristic lesions included large areas of confluent fluke tracts consisting of a central area of haemorrhage surrounded by a border of eosinophils and lymphocytes together with damaged hepatocytes.

The acute type II form of the disease also presented as sudden death and essentially similar pathological changes were found at post mortem. However, many more distinct fluke tracts were evident and congestion of the liver was less severe. Typically 700-1,000 flukes were present, a larger percentage as mature flukes in the bile ducts than in the type 1 disease.

In sub-acute fascioliasis as described by Ross, et al. (1967 a)) loss of condition, pallor, evidence of abdominal pain, severe anaemia and hypoalbuminaemia was found for a period of 1 - 2 weeks prior to death which occurred 12 - 20 weeks after infection. This condition was consistent with a fluke burden of between 800 and 1,500, of which 30-60% were present as adults in the bile ducts.

The latter picture is consistent with that reported by Reid et al. (1970), in a study of naturally acquired fascioliasis in sheep. This work described the sequential development of sub-acute fascioliasis in a flock grazing known infected pasture, and the clinical signs noticed were similar to those described by Ross et al. (1967 a)). In the naturally acquired infection fluke burdens varied from 110 - 1,628 and the percentage of immature flukes was 90% at the beginning of the outbreak in October, and gradually decreased through the winter to March when 87% of the flukes were present as adults in the bile duct.

Sub-acute fascioliasis as described by Ross et al. (1967 a)) presented the following picture at post-mortem: large numbers of flukes were present

in the bile ducts. Ascites was not a feature of this condition although the liver was congested and numerous fluke tracts were distinguishable as raised red or yellow areas in the liver parenchyma.

The final clinical condition described in this work by Ross et al. (1967 a)) was chronic fascioliasis. Clinical features included firstly unthriftiness resulting in debility, loss of weight and finally emaciation. Death may occur from 20 weeks post infection and as in the sub-acute disease moderate to large numbers of fluke eggs may be present in the faeces. At post mortem numerous fluke tracts were evident along with varying amounts of fibrosis and thickening of the biliary mucosa.

Cattle

As discussed previously fascioliasis in cattle, unlike the situation in sheep, normally only occurs as a chronic condition. However isolated incidents have been reported where outbreaks of acute (Ross, 1966 a)), and subacute (Ross, 1966 a); Eckert et al., 1977), facioliasis have occurred in cattle. The acute disease presented the following clinical and post mortem features (Ross, 1966 a)); calves aged 5 - 8 months at grass between September and November, on farms with a history of heavy fluke infection, lost condition rapidly, became dyspnoeic, showed evidence of abdominal pain, and developed a dry cough. Calves died shortly after symptoms developed. Fluke eggs were absent from the faeces, but eosinophilia and hypoalbuminaemia were present. Anaemia was not a feature of this condition, although surviving calves developed a severe haemorrhagic anaemia.

At post-mortem examination the liver was enlarged and severely engorged and typically 1,000 or more immature flukes were present in the liver parenchyma.

Sub-acute fascioliasis (Ross, 1966 a); Eckert, 1977) displayed the following clinical and post mortem features: yearling animals were affected, usually between November and March. Animals lost condition, developed pallor, but retained a normal appetite until extremely weak, when the worst affected animals became recumbent. 15-30% mortality occurred and in surviving animals a severe haemorrhagic anaemia, hypoalbuminaemia, a variable eosinophilia and high faecal egg counts were noted. At post mortem an enlarged haemorrhagic liver was evident, along with a fibrinous peritonitis, with typically between 500 - 1,000 flukes, the majority of which were adults in the bile ducts.

This general picture is consistent with the findings of Eckert et al., (1977) in an outbreak of subacute disease in Switzerland, and Reid et al., (1972) who reported the occurrence of subacute fascioliasis in experimentally infected grazing calves.

Chronic fascioliasis, the predominant form of fascioliasis found in cattle was described in detail by Ross (1966 a)). This condition unlike the acute and subacute conditions surfaced clinically as a herd problem, morbidity frequently being 100%. Where acute or subacute incidents had occurred, chronic fascioliasis developed in the remainder of the herd 1 - 6 months later. The condition was most severe in animals 9 months to 2 years of age, and the author also considered stress factors such as pregnancy important in determining the severity of the condition. Clinically the disease occurred between January and July, and was characterised by progressive loss of condition, hypoalbuminaemia, eosinophilia and severe haemorrhagic anaemia. The liver may be palpably enlarged and fluke eggs were demonstrable in the faeces. At post mortem examination the livers of affected animals were enlarged and cirrhotic, with severe fibrosis in the

bile ducts in which calcification was an invariable feature. Variable degrees of emaciation and oedema were present in the carcass and peritonitis occurred in animals with heavy infections.

This clinical and post-mortem picture was confirmed in a number of experimental and field studies, notably by Sewell (1965); Dow et al. (1967) and Rahko (1969).

Finally, of economic importance in cattle is the occurrence of sub-clinical fascioliasis in milking cattle. This subject has been treated by several authors including Ross (1970); Liddel (1972); Black and Froyd (1972); Armour (1975). In this condition low or moderate levels of infection result in debilitation which may or may not be recognised by the farmer (Ross, 1970). According to Armour (1975), sub-clinical disease is characterised by lowered productivity reflected by inadequate food conversion rates, poor carcass conformation and reduced milk yields, particularly solids not fat, without major haematological changes. Hence this situation in particular, in the adult animal, presents the problem of diagnosis, as will be discussed later.

SECTION II

HAEMATOLOGICAL STUDIES

Another major feature of Fasciola infection in ruminants is the anaemia initially normocytic, normochromic, which occurs in all of the types of fascioliasis described above. Generally, haematological changes in Fasciola infections in ruminants include a peripheral eosinophilia, lowered packed cell volume, lowered haemoglobin, lowered number of red cells and an increase in total number of white cells. The cause of the anaemia in Fasciola infection has been the subject of fierce controversy over a long number of years. Various theories have been put forward over the years by various authors viz: some authors considered that the anaemia could be explained by the haematophagic activities of the adult fluke resulting in blood loss (Jennings et al., 1956; Todd and Ross, 1966; Symons and Boray, 1967); others suggested that dyshaematopoiesis due to Fe or Vit B12 deficiency may be an important factor (Sinclair, 1964); while other suggestions included the release of toxic substances by the flukes, which may either depress haemopoiesis or cause increased haemolysis of erythrocytes (Sinclair, 1967). More recently studies with ^{51}Cr labelled red cells (Holmes et al., 1967; Sewell et al., 1968; Holmes et al., 1968; Dargie and Mulligan, 1970; Holmes and Dargie, 1975) have indicated that anaemia develops initially because of haemorrhage within the parenchyma of the liver, and latterly because of excessive losses of red cells into the digestive tract. This may be due to the blood sucking activities of the fluke from vessels in the wall of the bile duct, as first suggested by Urquhart (Ph.D. thesis, 1955) and Jennings et al. (1956), and confirmed by the recent work of Holmes and Dargie (1975).

SECTION III

BIOCHEMICAL STUDIES

Plasma Protein Studies

Changes in plasma protein content in animals of different species, infected with F. hepatica have been well documented over a lengthy period of years.

Considering first ovine fascioliasis, a number of authors have mentioned the association of F. hepatica infection with alterations in plasma protein levels. These include Balian (1940); Jennings et al. (1956); Noguchi et al. (1950); Ibrovic and Gall Palla (1959); Nikolic et al. (1962); Sinclair (1962); Furmaga and Gundlach (1967); Jennings et al. (1968); Holmes et al. (1968); Nansen and Neilsen (1968); Holmes (1969); Dargie (1975 a) & b)); Nansen et al. (1975); Sinclair (1975).

Early work by Jennings et al. (1956) indicated that from 5 weeks after experimental F. hepatica infection there developed a hyperproteinaemia due mainly to an increase in γ globulin, and further that from the 10th week post infection there was a progressive fall in total protein, albumin and globulin in the plasma due to loss of protein into the gastrointestinal tract. These results were consistent with the findings of Sinclair (1962) who suggested that loss of albumin may be partly due to loss in inflammatory exudates in the damaged liver. However, studies involving radioactive labelling of protein by Holmes et al. (1968) indicated that hypercatabolism of albumin in fluke infected sheep was due to leakage of the protein into the gut via the bile caused by the feeding activities of the flukes. Dargie et al. (1968) working in the rabbit demonstrated that amounts of plasma considerably in excess of those expected from losses of whole blood, pass into the gut of infected animals. This loss was explained by Murray (1969), in terms of

leakage through the junctional complexes of the biliary mucosa, which became permeable to macromolecules in the hyperplastic, parasitised mucosa.

The nature of the hypergammaglobulinaemia in F. hepatica infected sheep (as well as cattle) was the subject of a study by Nansen et al. (1975). These workers drew attention to the fact that in infected sheep IgG_2 and IgA levels remained unaltered while IgG_1 increased significantly in the late pre-patent period and remained elevated during the chronic period of infection. The authors thought it worthy of mention that this immunoglobulin was associated with the exocrine (mucous membrane) immune system.

As far as cattle are concerned similar plasma protein changes have been described by several authors, including Ross et al. (1966); Reid et al., (1972); Flagstad and Eriksen (1974); Nansen et al. (1975).

Pioneer work in this field by Ross et al. (1966) described a hypoalbuminaemia from the 5th week after experimental infection with F. hepatica accompanied by variable elevations in all the globulin fractions, particularly in $\alpha + \gamma$ globulins from the 3rd week after infection. These findings were substantiated by the work of Reid et al. (1972). The mechanisms resulting in hypergammaglobulinaemia were investigated by Flagstad and Eriksen (1974), who observed that the livers of calves infected experimentally with F. hepatica, unlike that of the normal animal, participated in immunoglobulin synthesis, as demonstrated by the presence of immunoglobulin producing cells in the liver parenchyma. In cattle given single infections only, the cells in question were mainly of the IgA type, unlike livers with repeated infections which presented a picture of IgG producing cells as the predominant type. Examination of the portal lymph nodes revealed abundant IgG_1 producing cells in both cattle with single and repeated infections. The authors believed the lymph node reaction to be largely

responsible for the rise in plasma globulin concentration.

Liver Function Studies

Among the earlier ancillary aids to liver function employed in experimental F. hepatica infection, the following tests are worthy of note:- Bromosulphalein excretion test (B.E.T.) (Urquhart, 1954) and the Iodine Test (I.T.) (Lloyd, 1957; Pyper, 1965; Ross et al. 1966);

Bromosulphalein excretion test B.E.T. was used by Urquhart (1954) to confirm that biliary obstruction was not a feature of chronic fascioliasis in the rabbit. The author recorded that values for B.E.T. in fluke infected rabbits were similar to those of normal rabbits and were in no way comparable to those found in rabbits with biliary obstruction.

Iodine test. A modification of the I.T. described by Lloyd (1957) and Pyper (1965) was employed by Ross et al. (1966) as an indicator of liver function in calves experimentally infected with F. hepatica. The author reported a steady rise in readings commencing 10 weeks after infection in heavily infected calves to a peak 16 weeks post infection, thereafter readings decreased steadily and became negative 23 weeks after infection. It was the opinion of the author that because the mechanism of the I.T. was not fully understood, in the absence of similar alterations in liver enzyme levels, interpretations of I.T. must be made cautiously.

Liver Enzyme Studies

Of the biochemical changes occurring in ruminants infected with F. hepatica the release of liver enzymes into the blood in response to liver injury has provided not only the basis for early diagnosis of fascioliasis by enzyme assay, but also valuable information regarding the activities of the flukes at various times after infection. The following enzymes have been used to indicate liver damage due to F. hepatica infection:

aspartate aminotransferase (Asp.T.), formerly serum glutamic oxaloacetic transaminase (S.G.O.T.)

alanine aminotransferase (Al.T.), formerly serum glutamic pyruvictransaminase (S.G.P.T.)

alkaline phosphatase (A.L.P.)

gamma glutamyl transpeptidase (G.G.T.)

glutamate dyhydrogenase (G.L.D.H.)

lactate dehydrogenase (L.D.H.)

leucine aminopeptidase (L.A.P.)

sorbitol dehydrogenase (S.D.H.)

pseudo-cholinesterase (Ch.E.)

ornithine carbamoyl transferase (O.C.T.)

Of these the most commonly used currently in diagnosis of fascioliasis are G.G.T., G.L.D.H. and S.D.H.

High levels of S.G.O.T. and S.G.P.T. occur when hepatocellular damage is present, A.L.P. has a poor reputation as a test of liver function, although high levels may indicate liver damage (Paton, 1969). Raised enzyme levels alone however do not constitute a specific response to fluke infection, and it has been shown (Boyd, 1962 and Ford, 1965 & 1967) that carbon tetrachloride-induced liver necrosis in sheep produced significant increases in three of the enzymes mentioned above, namely, G.L.D.H., O.C.T. and S.D.H. Simesen et al. (1973) comparing the relative values of G.G.T. and S.G.O.T. in bovine fascioliasis, concluded that G.G.T. is superior in this respect.

Detailed studies by Anderson et al. (1977) in response of calves to infection with fascioliasis suggested that G.L.D.H. and G.G.T. serum activity are the most sensitive indicators. The authors reported a maximum of mean activity of infected calves for G.L.D.H. and G.G.T. of 18 and 13 respectively times greater than uninfected animals. This compared with

five and six fold increases in the case of S.D.H. and O.C.T. respectively, which responses were in turn much greater than those of AspT and L.D.H. Results from the same study indicated that G.G.T. and O.C.T. levels began to rise later than the other enzyme levels. This finding is consistent with the theory that the G.G.T. response is due to damage to bile duct cells as suggested by Anderson et al. (1977) or biliary stasis as suggested by Ford (1974).

Of the other enzymes used in the past in diagnosis of fascioliasis S.G.O.T. levels in experimental infections in calves were studied by Ross et al. (1966) who found slight increases 5 weeks post infection in all infected animals, but found that activity of this enzyme remained within the normal range during infection which ranged from 5 - 25 weeks. However Doyle (1971) following experimental infections of calves obtained an increase in S.G.O.T. 2 weeks post infection, and a significantly elevated level throughout 16 weeks of infection. Reid (1968) examined levels of S.G.O.T., A.L.P. and S.G.P.T. following experimental infections in sheep, and concluded that only S.G.O.T. and A.L.P. were of value in diagnosis, and although S.G.P.T. levels were raised no particular pattern was evident.

Jaime et al (1970/72) reported that higher activities of S.G.O.T. were observed in fluke-infected cattle than normal while S.G.P.T. appeared to have no value in diagnosis of bovine fascioliasis.

More recently studies by El Harith (1977) on experimental fascioliasis of sheep recorded that S.G.P.T. activity started to rise in the second week post infection and thereafter fluctuated in irregular fashion, and similarly that S.G.O.T. levels increased at 2 weeks post infection, as did S.D.H. levels while G.G.T. levels began to rise at 8 weeks post infection.

In summary it has been collectively demonstrated that S.D.H. and

G.L.D.H. appear to be the most sensitive and earliest indicators of hepatocellular damage due to fluke migration in the liver parenchyma, although doubts have been expressed regarding the suitability of G.L.D.H. for this type of assay because of its instability (Anderson et al., 1977). G.G.T. appears to be an excellent guide to bile duct changes caused by the flukes.

SECTION IV

IMMUNOLOGICAL STUDIES

Serological Studies

The detection of circulating antibody in the serum of ruminants infected with F. hepatica has been extensively documented using a wide range of serological techniques. These include, in alphabetical order:-

Complement Fixation (C.F.)

Counter-immuno-electrophoresis (C.I.E.)

Defined antigen substrate spheres system (D.A.S.S.)

Double immunodiffusion (D.I.D.)

Enzyme-linked immunosorbent assay (E.l.i.s.a.)

Flocculation test (F.T.)

Fluorescent antibody test (F.A.T.)

Intradermal skin test (I.S.T.)

Latex-agglutination (L.A.)

Passive-haemagglutination (P.H.)

Complement Fixation. C.F. tests have been widely applied to sera of fluke infected animals. Studies using this test include those by Frick (1970), Yakhontov (1970), Renz (1973), Kapp-Burzynska et al. (1974), Lalic et al. (1976). This latter study included a comparison of C.F., D.I.D., and I.S.T. in which it was found in a trial of 226 serum samples from cattle infected with F. hepatica that only 133 were positive by C.F. In addition C.F. gave false positive results in approximately 10% of samples tested. The authors therefore concluded that complement fixation was of limited value in diagnosing F. hepatica infection in cattle. This is in agreement with many other authors, therefore this test has not been widely used in diagnosis of fascioliasis.

Counter-immuno-electrophoresis. C.I.E. has been used by several workers, including Nansen (1970) and Benex et al. (1973). The latter, in studies on experimental infections with F. hepatica in sheep, showed that infection was detectable at 3 weeks with this test. Van Tiggele and Over (1976) also used C.I.E. to monitor antibody levels in fascioliasis in both field infections and experimentally infected sheep. They found C.I.E. to be more sensitive than D.I.D., but suggested that quantitative determinations by serum dilution in C.I.E. was too time consuming and recommended the use of P.H. where quantitation of antibody was desired.

Double immunodiffusion. D.I.D. along with other forms of agar gel diffusion has been widely used for the diagnosis of fascioliasis. Studies using these techniques include those by:- Urquhart et al. (1954); Cuperlovic and Lalic (1970); Kapp-Burzynska et al. (1971, 1973); Horchner (1973); Doyle (1973b); Hillyer (1976), Van Tiggele and Over (1976). Kapp-Burzynska et al. (1971) reported that D.I.D. gave 54% positive results in naturally infected cattle, while Doyle (1973b) found that precipitating antibodies were first detected at 2 - 4 weeks post infection in experimentally infected calves and found that 100% of samples analysed were positive. Horchner (1973) suggested that agar gel procedures gave better results in sheep than in cattle. Generally D.I.D. was reported to be reliable, in that few false positive reactions occurred, but on the other hand sensitivity of the test was found to be low.

Enzyme-linked immunosorbent assay. E.l.i.s.a. first described by Engvall and Perlmann (1971) has now been applied to a wide range of helminth and protozoan diseases (Voller et al., 1976). Recently E.l.i.s.a. was used in the diagnosis of bovine fascioliasis by Grelck and Horchner (1977).

This study employed frozen sections of F. hepatica as antigen and compared the relative merits of E.l.i.s.a. and F.A.T. in diagnosising

fascioliasis in naturally infected cattle. It was reported that F.A.T. was more sensitive than E.l.i.s.a., but the authors suggested that because of the rapidity of the E.l.i.s.a. technique and its requirement for only very small volumes of serum, it was well suited for routine qualitative testing of bovine serum for fascioliasis.

Fluorescent Antibody Test. F.A.T. has been frequently used for serological diagnosis of fascioliasis. Studies using F.A.T. include those by:- Movsesijan and Borojevic (1970); Movsesijan (1971); Benex et al. (1973); Borojevic et al. (1973); Flagstad and Eriksen (1974); Person (1974); Horchner et al. (1976); Hanna and Jura (1977). Movsesijan and Borojevic (1970) demonstrated that F.A.T. detected antibodies to F. hepatica 2 weeks after experimental infection. This was in agreement with Benex et al. (1973) who obtained a rise in antibody titre at a similar time after experimental infection of lambs with F. hepatica. In a study of naturally infected cattle Horchner et al. (1976) showed that 87% of infected animals were detected by F.A.T. with only 4% false positives.

Intradermal Skin Testing. I.S.T. has received a considerable amount of interest as a diagnostic aid in bovine fascioliasis. Doyle (1973c) carried out I.S.T. in experimentally infected calves using a lipid-free antigen and found that I.S.T. was positive as early as 3 weeks post infection; the size of the weal elicited increased with the duration of infection, but could not be correlated with either the magnitude of the acquired fluke burden or to reinfection. Quiroz et al. (1973) in a study of 155 cattle naturally infected with F. hepatica reported that the skin test was positive in 96.7% of all cattle positive by faecal examination. Horchner (1973) suggested that I.S.T. was one of the serological methods practical for large scale field investigations

of fascioliasis. However, he pointed out that antigen was not yet available in sufficient quantities for such a project. Falagiani et al. (1973) reported that a crude extract of F. hepatica proved more sensitive in I.S.T. than other somatic and metabolic antigens. Blancou et al. (1976), using a highly purified extract of F. hepatica as antigen compared I.S.T. with C.F. and D.I.D. in bovines naturally infected with F. hepatica and found that I.S.T. was the most sensitive technique, detecting 60% of animals found to be infected at meat inspection, but the test also gave positive reactions in a number of uninfected animals.

Latex Agglutination Test. L.A. was considered by Horchner (1973) to be the simplest and cheapest method for diagnosis of fascioliasis, but its sensitivity required further study. Horchner et al., (1976) later reported that when L.A. was applied to the serum of 504 animals of which 32 were known to be infected, only 19 of the latter gave a positive result, and there were more than 20% false positive reactions. Benex et al. (1973) found that L.A. detected antibodies in the serum of experimentally infected sheep around 2 weeks after infection, whereas C.I.E. and D.I.D. did not detect antibodies until 3 weeks post infection. Gladysz-Pawlak (1977) reported positive reactions in 81.7% of cattle naturally infected with fascioliasis. This compared with 50 - 60% positive reactions using other diagnostic tests.

Passive Haemagglutination. P. H. has been extensively used in detecting fluke infections in both sheep and cattle. Ross et al. (1966) reported that positive titres in P.H. occurred 4 weeks after experimental infection of cattle with F. hepatica. Poluektova (1975) found that 14 - 21 days after experimental infection of sheep with F. hepatica P.H. demonstrated circulating antibodies. This is in agreement with the findings of Benex et al. (1973) who also were able to detect serum antibodies by P.H. 2 weeks after experimental infection of sheep with F. hepatica. Satisfactory results

were also reported by Van Tiggele and Over (1976) in one of the few serological studies which attempted to relate serological findings to the field situation, as well as recording antibody titres in experimentally infected sheep and cattle. In this latter situation, in both species P.H. titres were first detected at 2 - 3 weeks post infection. P.H. was also found useful in diagnosing field infections in sheep at various levels of infection (Van Tiggele and Over, 1976) although some weak false positive reactions were reported.

In summary it emerged from these various studies that D.I.D. , F.A.T. and P.H. were reliable aids to the diagnosis of fascioliasis in sheep and cattle and that the E.l.i.s.a. technique merited further study, particularly in ovine fascioliasis where the test has yet to be assessed. Therefore, in the present experiments P.H. and E.l.i.s.a. were used to quantitate circulating antibody levels, while D.I.D. was used as a qualitative test.

Serum Transfer and F. hepatica Infection.

Although there have been a large number of investigations into the humoral response to F. hepatica infection, there have been relatively few reports concerning any protective effects conferred by the antibody produced.

In this connection it is pertinent to examine the work of Dargie et al. (1973); Armour and Dargie (1974) and Hayes et al. (1974 a) & c)). The former authors were able to transfer immunity to F. hepatica in rats using serum from different domestic species as well as donor rats. Thus serum from fluke infected cattle, sheep and rats conferred a significant degree of protection against challenge with F. hepatica, when injected intraperitoneally into the rat. However, it was pointed out that this protective effect was a function of the volume of serum injected and the time of injection. It was also observed that protection increased with the volume

of serum which was administred intraperitoneally simultaneously with, and two days after fluke challenge.

These findings were supported by Hayes et al. (1974 a) & c)), who were also able to protect rats from F. hepatica infection by intraperitoneal injection of serum from infected donor rats. These latter authors confirmed that serum transfer was only protective if carried out at the same time as challenge infection. It was also noted that the transferred serum had an "all or nothing" effect, in that flukes surviving the action of transferred serum were in no way inhibited in their development. While asserting that this evidence of passive immunity must be in part due to serum antibody, the authors took the view that other factors e.g.,"transfer factor": the leucocyte dialysate described by Liburd et al. (1972), Maddison et al. (1976) which will be discussed later, may also be implicated in this protective effect.

It must also be mentioned at this juncture that other authors, notably Wikerhauser (1961) in studies involving transferring immune bovine serum to guinea pigs, and Hughes (1962) using immune rabbit serum transferred to mice, reported failure of passive transfer to influence, in any way, F. hepatica infection.

The functional effects of immune serum on F. hepatica featured largely in a number of interesting studies, including those by Lang (1974, 1976) and Howell et al. (1977).

The effects of immune and normal sera on the viability of young flukes transferred to normal recipient mice were examined by Lang (1974). It was recorded that incubation of young F. hepatica up to 24 days old for 4 hours in immune mouse sera, followed by intraperitoneal transfer to normal mice, resulted in a significant reduction in fluke burden compared to treatment with

heat activated immune sera and normal serum.

The same author (Lang, 1976) also investigated the effects of mouse antisera against products from the incubation of juvenile F. hepatica, and was able to demonstrate that such antisera debilitated migrating flukes in recipient mice, thus reducing host mortality and fluke recovery.

A combination of some of the in vivo and in vitro techniques mentioned above was described by Howell et al. (1977). This illuminating piece of work reported that significant protection against infection with F. hepatica was conferred on naive rats by the passive transfer of serum from infected rats. This immune serum did not have a pronounced effect on the mortality of metacercariae in vitro, yet its presence was associated with the formation of a precipitate, containing rat antibody, on the surface of metacercariae maintained in culture medium. Metacercariae maintained in culture medium containing immune serum failed to develop. The authors were quick to add the proviso that there also appeared to be impairment of development in metacercariae maintained in the presence of normal serum and stressed that as yet no relationship had been established in vivo between precipitin formation and impaired viability of metacercariae.

In the present studies serum from F. hepatica infected cattle and sheep was employed in passive transfer studies in the rat, in an attempt to correlate the relationship between antibody production and protective immunity.

Cell-mediated Immunological Studies

The relationship between F. hepatica infection and cell mediated immunity has been the subject of a wide range of investigation both in vivo and in vitro.

Experiments in vitro stemmed from the early work of Lang et al. (1967)

in the mouse. These authors demonstrated that the transfer of peritoneal exudate cells from isologous donors not only caused a more rapid response to infection in terms of loss of weight and white blood cell levels than controls, but also resulted in a significant reduction in numbers of flukes recovered after challenge infection. On the basis of the timing and degree of lymphocytic infiltrations in, and the histopathology of livers of mice immunised in this way it was suggested that a delayed hypersensitivity response played a prominent role in the immunity stimulated. The opinion was expressed that such a response preceded a non-specific allergic inflammation (with polymorphonuclear leucocytes as the dominant cell) and accompanying striking biochemical changes in the liver environment which compelled the flukes to migrate to the common bile duct. This theory was put forward to explain earlier migration of flukes in mice immunised with peritoneal exudute cells.

Shortly after this study, work by Dodd and O'Nuallain (1969) highlighted the role of cell mediated immunity in F. hepatica infection. These experiments were designed to investigate the effect of inhibition of the normal cellular response on the reaction caused by flukes migrating through the livers of experimentally infected rabbits. Anti-rabbit lymphocyte serum, prepared either in the sheep or horse was used to inhibit the cellular response. The results of these experiments demonstrated that all rabbits which had received anti-lymphocytic sera either of ovine or equine origin died from acute hepatitis following challenge with 200 F. hepatica metacercariae. The cellular response in the area of migrating flukes in the livers of such rabbits was minimal compared with control rabbits none of which died during the 28 days following challenge. Liver histopathology in rabbits which received anti-lymphocytic serum indicated that only a minimal attempt was made either to contain the flukes or repair the damage they had

caused compared with control rabbits which remained healthy and mounted on intense cellular reaction against migrating flukes. The authors concluded that the normal cellular response is essential in limiting the damage caused by F. hepatica as it migrates through the liver parenchyma

These findings were supported by those of Flagstad et al. (1972) who monitored the course of experimental F. hepatica infection in Black Pied Danish cattle of Friesian descent which had a spontaneous defect in cell-mediated immunity. This study recorded the apparent inability of calves with a functional and morphological thymus defect to mount a specific response to F. hepatica infection as evidenced by absence of peripheral eosinophilia, lack of skin test reaction to fluke antigen after infection, and diminished cellular reaction to migrating F. hepatica in the liver. This work emphasised the importance of the cell-mediated immune reaction in the response of the normal animal to F. hepatica infection.

Direct evidence that immunity to F.hepatica could in fact be transferred using cells of the lymphoid series was first provided by Corba et al., (1971). These experiments indicated that lymphoid cells from rats experimentally infected with F. hepatica when transferred to isologous recipients could confer a high degree of protection (60-100%) against a challenge infection. For resistance to be conferred a minimum period of 8 weeks infection of donors was required before transfer of cells, presumably to allow for recruitment of sufficient numbers of suitably primed cells. A similar result (80% protection) was obtained when lymphoid cells from a calf experimentally infected with F. hepatica were transferred to its monozygous twin.

Contemporaneous studies by Sinclair (1971b) described attempts to transfer resistance to F. hepatica in sheep with lymph node and spleen

homogenates. These experiments provided no evidence of resistance in terms of establishment or effects of F. hepatica infection in sheep given homogenates of lymphoid cells. Retarded development of flukes was recorded in sheep given homogenates of lymphoid cells from infected sheep, but also in animals given placebo cells from non-infected animals. The author considered that a non-specific homograft type reaction was involved which necessarily invalidated any immunological conclusions drawn from this work.

However further studies by Armour and Dargie (1973) confirmed successful transfer of immunity to F. hepatica by lymphoid cells. The results of these experiments also indicated that the degree of the antigenic stimulus imparted to recipient animals was important, as the adoptive transfer of lymphoid cells from donors with high fluke burdens (7 - 8 flukes) conferred significantly greater protection than cells from rats harbouring only a few flukes. The authors discussed the possible role of the transferred lymphocytes and suggested that lymphocytes sensitised against parasitic antigens (and perhaps autoantigens formed in the damaged liver) initiated a cell-mediated type of immunity by congregating at sites of antigen deposition, thus triggering an inflammatory response capable of destroying the parasite and damaging hepatocytes. This theory broadly agreed with that of Lang et al. (1967) who were of the opinion that acquired immunity in murine fascioliasis was expressed as a delayed type hypersensitivity reaction. These experiments (Armour and Dargie, 1973) also demonstrated that serum from infected donor rats was protective when transferred to recipient rats, whose livers, unlike control rats were completely normal. It was therefore suggested that both humoral and cell mediated mechanisms were involved in acquired immunity to F. hepatica, the latter possibly acting as a second line of defence.

As well as the in vivo studies described above a number of in vitro studies have been carried out by various authors into the role of cell mediated immunity in F. hepatica infection in various species.

Aalund and Nansen (1972) and Aalund et al. (1972) reported a significant inhibition of migration of peripheral leucocytes from liver fluke infected rabbits when tested with both whole fluke extract and an egg antigen of F. hepatica. A period of approximately 4 weeks stimulation of migration occurred before the phase of migration inhibition which occurred between 42 and 105 days after F. hepatica infection.

An interesting series of studies was undertaken by Genchi et al. (1973), Genchi and Sartorelli (1974) in which peripheral leucocytes from F. hepatica infected cattle were investigated in vitro. This series of studies examined the relative amounts of histamine released by, and migration inhibition of leucocytes from non-infected and fluke infected cattle. The results of the first study (Genchi et al., 1973) showed that significantly more histamine was released by leucocytes from cattle infected with F. hepatica, after incubation with F. hepatica antigen, than by cells from uninfected cattle. In addition the migration inhibition test (M.I.T.) was positive in 71 out of 80 cattle infected with F. hepatica, and negative in 40 uninfected cattle. A later study (Genchi and Sartorelli, 1974) indicated that histamine release from leucocytes of non-infected cattle previously sensitised with fluke antigen was increased by exposure of such cells to plasma from fluke-infected animals.

The authors interpreted these results in the following way:-

a) that a serological factor possibly of a reaginic nature may be responsible for the release of histamine from sensitised leucocytes and that this may be important in an immediate type hypersensitivity reaction in

F. hepatica infection. This finding is in agreement with the work of Jarrett (1972), who demonstrated that F. hepatica infection in the rat resulted in a dramatic potentiation of the reagin response to ovalbumin in sensitised rats.

b) that the positivity of the M.I.F. implicated cellular factors responsible for a delayed type hypersensitive response.

On these grounds the authors concluded that the immune response to F. hepatica in cattle had both humoral and cellular components and should be considered "intermediate" in type.

Further evidence of the role of cell mediated immunity in F. hepatica infection was furnished by Bolbol et al. (1974), and Bolbol and Sewell (1975) in studies on antigen induced in vitro transformation of peripheral lymphocytes of rabbits infected with F. hepatica. It was reported that lymphocytes of rabbits infected with F. hepatica transformed in response to an antigen present in a supernatant extract from homogenised, packed adult flukes, as measured by uptake of tritiated thymidine (H3) by the cells. The antigen stimulated the cells to incorporate at least 6 times as much tritiated thymidine as other cells cultured in the absence of antigen.

Similar work was carried out by Eriksen (1975) using the goat as an experimental host for F. hepatica infection. She investigated the response of peripheral blood leucocytes from goats infected experimentally with F. hepatica, to antigen prepared with whole fluke extract in the hope of demonstrating migration inhibition. However, no significant difference was recorded in the migration index between infected goats and controls. Although the goat is regarded as a very susceptible host for F. hepatica in the opinion of the author (Eriksen, 1975) it was unsuitable for immunological studies possibly because of its very poor resistance and also because the blood of the normal goat contains a high percentage of lymphocytes, undesirable in leucocyte migration studies.

F. hepatica Infection and Immunosuppression

The work of Corba and Spaldonova (1974, 1975) and Sinclair (1968, 1974) investigated the relationship between immunosuppression and F. hepatica infection in rats and sheep respectively. Both studies were concerned with the action of various pharmacologically active compounds, most of which are immunosuppressive, on experimental infections with F. hepatica.

The former studies (Corba and Spaldonova, 1974, 1975) investigated the immunosuppressive effects of chlorambucil, cyclosphamide, azathioprine, amethopterine and a cortisone derivative of betamethasone on the development of experimental F. hepatica infection in the rat. Earlier migration of flukes to the common bile duct and earlier onset of egg production, took place in rats treated with these drugs, compared to control rats. Pathological changes in the liver were less marked in treated rats, but there was an increased pathogenicity of metacercariae resulting in a higher mortality rate within treated groups. The administration of immunosuppressants neither influenced the total number of flukes developing, nor the appearance of eosinphilia in the peripheral blood of treated rats. These findings were in broad agreement with those of Sinclair (1968, 1974) who recorded the effects of corticosteroid and the amine antagonist promezathine hydrochloride on the response of sheep to experimental F. hepatica infection. In accord with the work of Corba and Spaldonova (1974, 1975) in the rat, administration of corticosteroid to sheep resulted in accelerated growth of the flukes. However, in sheep, unlike the rat, the drug also caused a virtual abolition of the characteristic peripheral eosinophilia and hypergammoglobulinaemia, (Sinclair, 1968), but enhancement of the pathogenic effects of the fluke as evidenced by severe traumatic hepatitis, occurred to the same extent as in the rat.

The results of treatment with promezathine hydrochloride (Sinclair, 1974) were strikingly different. This compound had an overall attenuating effect on the pathogenicity of the liver flukes. This was apparent in the delayed onset of liveweight loss and less severe liver damage in treated sheep, in terms of consistently lower levels of plasma glutamate dehydrogenase (GLDH). Promezathine treatment like corticosteroid therapy inhibited the initial development of peripheral eosinophilia. The author explained his findings in terms of suppression by the drug of the inflammatory response to migrating parasites. He further speculated that the lack of peripheral eosinophilia may be due to blockage of release of amines which may mediate the hypersensitivity response in which these cells have a part, and also that release of such pharmacologically active amines may play a prominent role in the natural resistance of sheep to F. hepatica infection.

Evidence for the primary involvement of the host immune system in immunosuppression was furnished by two interesting studies, referred to elsewhere in this text, in cattle and in the rabbit respectively, into the course of F. hepatica infections in animals with a natural or artificially impaired immune system.

The former work described the course of experimental F. hepatica infection in Black Pied Danish Cattle which had a deficient cellular immune system due to a genetic defect. Such cattle were unable to mount a specific cellular response to experimental F. hepatica infection and failed to show either peripheral eosinophilia or intradermal reaction to fluke antigen. At post mortem the cellular reaction to migrating flukes in the liver was greatly reduced in terms of numbers of lymphocytes, neutrophils and eosinophils present. In all affected calves there was an accelerated growth of liver

flukes and patency occurred earlier than in other animals although there was no increase in the absolute number of flukes recovered.

This clinical and pathological picture was strikingly similar to that of rabbits injected with anti-lymphocytic sera before and during experimental infection with F. hepatica (Dodd and O'Nuallain, 1969). All rabbits treated in this way had died within 27 days of infection, whereas all control rabbits, similarly challenged with 200 F. hepatica metacercariae survived the length of the experiment. At post mortem rabbits given anti-lymphocytic serum were found to have acute hepatitis with subcapsular haemorrhages. Microscopically cellular infiltration into migrating fluke tracts in the liver was minimal compared with the intense cellular reaction in the livers of control rabbits.

The most direct evidence of immunosuppression in F. hepatica infection was provided by Goose (1976) who suggested that immunosuppression may affect the longevity of F. hepatica in some species. These experiments were carried out in the rat, and it was recorded that rats infected with F. hepatica, as well as rats injected intraperitoneally with excretory/secretory (E.S.) antigens of F. hepatica produced a significantly lower haemagglutinin response to a T dependent antigen, namely, sheep red blood cells (SRBC). It was further reported that the numbers of spleen plaque forming cells to SRBC was also significantly lower in F. hepatica infected rats. In the author's opinion these findings may in part explain the persistence of F. hepatica in rats resistant to challenge infection (Goose and McGregor, 1973), and possibly increased susceptibility of fluke infected cattle to invasion by Salmonella dublin (Aitken et al., 1976).

In summary it was felt that the role of immunosuppression in

F. hepatica infections merited some attention and consequent provision was made in the present studies.

SECTION V

COMPARATIVE HOST RESISTANCE TO F. HEPATICA INFECTION

It has been well established by various authors: Ross (1966 b), 1967 a), b) and c); Boray (1967 a), 1969); Doyle (1971, 1972, 1973); Nansen (1975); Van Tiggele and Over (1975) that cattle, unlike sheep are able to develop some resistance to F. hepatica infection. Possible reasons for this difference were investigated in comparative studies of experimental fascioliasis in cattle, sheep and pigs described by Ross (1967 a)) who concluded that compared to sheep, cattle showed increased fibrosis, minimal or absence of liver cell regeneration in response to primary infections, resulting in restriction and inhibition of parasites migrating in the liver parenchyma. Excessive fibrosis and calcification of the bile ducts were presented as additional factors which reduced the possibility of clinical disease occurring in cattle. These conclusions were based on a series of studies by the author into the response of cattle to experimental infection with F. hepatica. The first of those early studies investigated the course of single primary experimental infections in calves (Ross et al., 1966), the life span of F. hepatica following single infections (Ross, 1968) reinfections (Ross, 1966b), and acquired immunity in cattle (Ross, 1967 a) b) & c). The initial study (Ross et al., 1966) of experimental infections in calves of 200, 300 and 1300 metacercariae demonstrated that a percentage of each infection was retarded in the liver parenchyma as long as 23 weeks after infection. In the same year Ross (1966b)described an investigation into reinfection in which calves, previously infected with 200 metacercariae were challenged either 3 or 18 weeks later with 300 metacercariae. A resistance to challenge was evidenced by a reduction in the mean total

percentage of flukes established to 17% in both reinfected groups compared with 31% in groups given a primary infection. The author reported that in the group challenged 18 weeks after initial infection the second infection resulted in a clearance of the initial patent infection from the bile ducts, and further suggested that this was an example of the phenomenon of "self-cure" previously reported in Haemonchus contortus infections in sheep (Stewart, 1950). The term "acquired self-cure" was suggested as more appropriate in this case, as the self-cure was induced, as opposed to occurring naturally. This study also drew attention to the fact that following secondary challenge infections, migrating parasites in the liver parenchyma were more prone to inhibition of development than in comparable single infections. Further evidence for the theory of "acquired self-cure" in bovine fascioliasis was provided by the same author (Ross, 1968) in a study of the life span of F. hepatica infections in cattle. It was recorded that with low level single infections of around 200 metacercariae 75% of those would be lost between the 5th and 21st month after infection but in larger experimental infections between 2,500 and 15,000 metacercariae the survival time was reduced to around 6 - 7 months. Where challenge infections were involved the life span of infection could be as short as 4 - 5 months, and in this situation the "acquired self-cure" phenomenon with a turnover of the adult fluke population was though to be involved.

The views of Ross (1966b) on "acquired self-cure" were contested by Boray (1969) who suggested that such an expression should only be used for a clearly immunological phenomenon and this may not be justified in the case of bovine fascioliasis. The same author also expressed the view that resistance to fascioliasis in cattle was due to an immediate tissue reaction, which along with a chronic reaction including severe fibrosis and bile duct calcification resulted in the formation of mechanical barriers to infection.

The author could find no convincing evidence of an immune reaction which would influence challenge infections in cattle. This is in direct contradiction to the views of Dargie et al. (1973, 1974) who concluded that immune reactions which may include humoral, cellular and hypersensitive components were operative in the acquisition of resistance to F. hepatica in cattle.

Qualified support for the views of Ross (1966 b), 1967) was expressed by Doyle (1971, 1972, 1973a) who demonstrated that calves developed immunity both to single experimental infection (Doyle, 1972) and reinfection (Doyle, 1971). The first study in this series (Doyle, 1971) provided significant evidence that cattle previously exposed to adult stages of F. hepatica, by virtue of 16 week old infections, developed immunity to reinfection in agreement with the findings of Ross (1966 b), 1967) and Boray (1967 a), 1969). Doyle (1971) also concluded in further agreement with Ross (1968) that cattle do not maintain a population of flukes derived from an initial infection, but that there is a significant loss of these flukes, accompanied by a fall in faecal egg output. Ross (1968) had described such a fall in faecal egg count of infected calves 8 months after infection with F. hepatica associated with a 75% reduction in the fluke population, between the 5th and 21st month of infection. However, Doyle (1971) described the loss of 83% of the fluke population, derived from a single infection, between 16 and 30 weeks after infection, associated with a decrease in faecal egg output over this period. This discrepancy was explained by Doyle (1971) in terms of different levels of infection (Doyle (1971) used 750 metaceracriae and Ross (1968) 200 metacecariae), different times of infection and possibly the fact that the experiments of Ross (1968) involved very small groups of calves.

Doyle (1971) demonstrated that only 16% of the fluke burden recovered from calves given a single infection with 750 metacercariae, was recovered

from calves reinfected with 1,650 metacercariae which had received a similar initial infection. This is in close agreement with the findings of Ross (1966 b)), although the author (Doyle, 1971), suggested that in Ross's study (1966b)) the fact that control calves for the initial infection were killed considerably before the reinfected calves, may account for the apparent reduction in the initial fluke population recovered from the reinfected calves, which formed the basis of the "acquired self-cure" theory.

Doyle's second study (1972) demonstrated a significant reduction in the fluke burden of calves killed 24 weeks after infection with a single experimental dose of metacercariae, compared with that of a similarly infected group killed at 4 weeks after infection. The author also demonstrated a reduced biological activity of the flukes, as indicated by a fall in faecal egg counts, accompanied by a fall in S.G.O.T. levels and a rise in mean serum albumin levels, prior to their expulsion.

Further studies by Doyle (1973a) were directed towards investigating the relationship between duration of a primary infection with F. hepatica and subsequent development of acquired resistance to infection, in calves. He demonstrated a significant resistance, as evidenced by a 75% reduction in fluke burden, compared with once infected animals, to reinfection with 1,300 metacecariae in calves infected 12 weeks previously with 750 metacercariae. However, he was unable to obtain resistance in calves similarly reinfected 7 weeks after initial infection. It was concluded that the degree of resistance conferred to reinfection was related to the duration of the initial infection. These findings were consistent with the earlier work of Boray (1967) and Doyle (1971). Doyle (1973) also noted that in this study an "acquired self-cure" as described by Ross (1966b) did not occur, as a considerable number of adult flukes from the initial infection were still present in reinfected animals. The author went on to

discuss possible mechanisms of acquired resistance to reinfection. These included the mechanical barrier theory proposed by Boray (1967a), 1969) due to the degree of fibrosis and cholangitis present, as described earlier in this section, and also the theory of acquired immunity per se. In this connection the author cited work by Corba et al. (1971) in transferring immunity to fascioliasis with lymphoid cell suspensions using identical twin calves.

This study noted that a minimum of 8 weeks exposure to infection was required for successful transfer of immunity to be induced, in the absence of adult parasites. Doyle (1973a) suggested that this time factor may be necessary to allow the host to mount a competent immune response from the limited lymphoid stimulus as young fluke pass quickly through the gut wall.

Further information on the acquired immunity to bovine fascioliasis was provided in studies in the Netherlands by Van Tiggele and Over (1975) on the interaction between primary infections and reinfections. This work investigated these interactions using three different infection schedules as follows: long interval reinfections of 6 or 12 months; short interval reinfections of 3, 6 or 9 weeks and finally single infections at different levels.

It emerged from the initial study using long periods (6 or 12 months) between infections, firstly, that different rates of fluke expulsion occurred in different age groups of animals, older animals appearing to expel flukes more quickly. However, the authors pointed out that this evidence was not conclusive because of high variance between small groups of animals. This study also concluded that immunity to reinfection at 12 months was demonstrable, but not at 6 months after primary infection, at which time only a slower development of flukes could be shown. An incidental finding of interest in this study (Van Tiggele and Over, 1975) was a sharp increase in faecal egg count

1-2 months after reinfection of calves at 12 months after a primary infection. The author suggested that this was due to retarded flukes from the first infection recommencing development. This, of course, directly contradicts Ross (1966b) whose postulated "acquired self-cure" depended on reinfecting flukes causing loss of the initial infection. This study also demonstrated a degree of resistance to triple infection with 6 monthly intervals.

The second study in this series employed shorter intervals of 3, 6 and 9 weeks between infections. An interesting result of this regimen of infection was that calves reinfected at 3 weeks and 6 weeks with 1,000 metacercariae after primary infections of 1,000 metacercariae showed enhanced egg output compared with calves singly infected with 1,000 metacecariae. In both cases detection of eggs in faeces occurred earlier than following a primary infection. The authors correlated these findings with serological studies employing complement fixation and passive haemagglutination. Antibody titres after reinfection were found to be generally higher than after primary infection and it was suggested that this may be associated with the enhanced development of existing infection mentioned above.

The final study in this series utilised 3 different primary infections viz:- 1,000 normal metacercariae, 10,000 "normal" metacercariae and 1,000 metacercariae encysted in 2% glucose solution. It was assumed that the metacercariae encysted in 2% glucose were "high-energised", although this was not backed up by biochemical analysis and likely to result in good synchronisation of infection. This was supported by the fact that the "glucose" metacercariae gave sharp, early peaks in S.D.H. and G.G.T. levels. On the evidence that normal metacercariae gave several peaks of liver enzyme levels and other parameters, it was suggested that this was due to desynchronisation of infection. It was further postulated that if a "barrier" is elicited by first

invading flukes against a further infection, this "barrier" would be encountered by slower invading flukes, which could either migrate round this "barrier" thus extending it, or wait for the "barrier" to be lifted.

In summary it was concluded that immunological interactions occurred in bovine fascioliasis not only between primary and secondary infections, but also between "slow" and "fast" flukes derived from one single infection.

SECTION VI

THE ROLE OF FIBROSIS IN IMMUNITY TO F. HEPATICA

In view of the speculation regarding the role of fibrosis as a mechanical barrier to F. hepatica infection and reinfection it is pertinent, at this stage, to review the relevant subject matter to date.

Among the multitude of opinions expressed on this subject are those of the following authors: Urquhart (1956); Ross et al. (1967 b); Sinclair (1967); Dow et al. (1967, 1968); Kendall et al. (1967); Boray (1967a) Kendall and Sinclair (1971); Doyle (1972); Eriksen and Flagstad (1973); Rushton et al. (1974); Dargie et al. (1974, 1975); Murray and Rushton (1975); Murray and Armour (unpublished data); Wensvoort (1975); Isseroff et al. (1977); Rushton and Murray (1977).

The fibrosis accompanying chronic fluke infection was first described in detail by Urquhart (1957) who described a five-fold process which included healing of fluke tracts, chronic cholangitis and other proliferative changes on the periphery of healed fluke tracts, resulting in cirrhosis in the livers of experimentally infected rabbits. Similar descriptions of fibrosis in chronic F. hepatica infection in sheep, cattle and pigs were provided by Ross (1967 a) & b), 1968). In the opinion of the latter author the essential basis for differences in immune status to F. hepatica infection of cattle, sheep and pigs lay in the fibrous nature of the various livers. It was postulated that because the liver of cattle is more fibrous than in the sheep and also because there is an apparently greater fibroblastic response in cattle than in sheep, cattle may have more innate host resistance than sheep to F. hepatica infection. This view was supported by Boray (1967a), in studies on experimental fascioliasis in cattle, who considered that the severe fibrotic reaction in the liver of

infected cattle presented a physical barrier to establishment of a further population of flukes, and that this was the basis of resistance to F. hepatica expressed in cattle. Further support for this view was expressed by Kendall and Sinclair (1971), working in the rabbit. The opinion was expressed that previous infection with F. hepatica was partly responsible for an effect on the liver environment, detrimental to invading flukes, i.e. that a physical barrier to reinfection had been set-up.

This theory was contested by a number of authors, notably Eriksen and Flagstad (1974 a) & b); Dargie et al. (1974 a) & b); Murray and Rushton (1975). The first named authors (Eriksen and Flagstad, 1974 a) & b) working in the rat demonstrated 50% protection to challenge with F. hepatica in rats implanted subcutaneously with adult flukes. These authors pointed out that the liver of protected animals showed no pathological change, which precluded the involvement of any physical barrier to infection such as fibrosis, and suggested the possible involvement of immunological mechanisms. The findings of this study were confirmed and extended by Dargie et al. (1974 a) & b), who recorded that active immunisation procedures achieved considerable levels of protection to challenge with F. hepatica, in rats, sheep and cattle, completely independent of hepatic fibrosis. This work described a number of different types of fibrosis occurring in experimental studies of chronic ovine fascioliasis, and reported that fibrosis may occur at some distance from fluke tracts and may also be more marked in cases of reinfection, which is in agreement with the findings of Rushton (1977) who found delayed fluke migration to the bile ducts with consequently greater fibrotic change in the livers of reinfected sheep. Dargie et al. (1974 a) & b) suggested that certain forms of fibrosis may be due to an immune reaction either of a humoral or cellular nature possibly directed against the liver. This was supported by the work of Rushton (1976) who

demonstrated auto-antibodies in sheep experimentally infected with F. hepatica. These antibodies were shown to react against sheep liver and other tissues, although they were neither organ nor species specific. The same author (Rushton, 1977) subsequently observed, in studies involving reinfection with F. hepatica in experimentally infected sheep, that a factor, possibly of immunological origin, influenced the progress of flukes through the liver parenchyma. Further weight to the argument for immunological involvement in resistance to F. hepatica infection was added by Murray and Armour (unpublished data presented at Lelystad, 1975). These workers speculated that an immunological hypersensitivity mechanism may be operative in the aetiology of fibrosis in experimental bovine fascioliasis. It was further suggested that the eosinophilic phlebitis found in infected bovine livers may be of immunological origin, and may lead to diffuse fibrosis in reinfection.

A further theory regarding the possible mechanisms of fibrotic change in chronic F. hepatica infection was provided by Wensvoort (1975). In a study of chronic F. hepatica infection in cattle the author described various forms of fibrosis occurring in this form of the disease. He suggested that fibrosis may result from functional changes in the liver physiology accompanying infection. One of the functional effects described was cholestasis due to obstructive processes or tissue debris. It was suggested that temporary periods of cholestasis may provide a locus for areas of concentric fibrosis, and if intermittent cholestasis occurred in various lobes of the liver this would contribute to a diffuse fibrotic change. Recent support for the theory that liver changes may be induced indirectly by F. hepatica infection in contrast to the findings of Eriksen and Flagstad (1973) was provided by Isseroff et al. (1977) in experimental studies in rats. This work showed that biliary changes could be induced by adult

F. hepatica implanted intraperitoneally. The author suggested that such changes may be induced chemically.

SECTION VII

ARTIFICIAL IMMUNISATION IN F. HEPATICA INFECTION

In view of the immunity developed by some animal species to F. hepatica infection it seems logical that it may be possible to induce immunity to F. hepatica by artificial means, and indeed many different vaccination regimes have been devised to this end. These will now be discussed under the three broad headings outlined below.

Vaccination with Live Parasites in Abnormal Sites

Studies using this immunisation regime include those by Lang and Dronen (1972); Fortmeyer (1973, 1974); Goose and McGregor (1973); Hughes et al. (1975, 1976 a) & b), 1977 a); Eriksen and Flagstad (1974 a) & b); Anderson et al. (1975).

The first of these studies by Lang and Dronen (1972), examined the resistance conferred by flukes of different ages when transferred from infected mice to normal recipient mice. The authors were able to report that immunisation with 8 and 16 day old flukes produced a significant reduction in fluke burdens in mice given a challenge infection of 2 metacercariae, compared with control mice given a similar challenge infection. From the results of this work it appeared that young flukes were capable of inducing acquired immunity during the entire liver migration period.

Similar studies in the rabbit by Fortmeyer (1973, 1974) documented the effects of different routes of infection with metacercariae, namely oral and intraperitoneal, on the subsequent development of a challenge infection with F. hepatica.

The first of these studies (Fortmeyer, 1973) indicated that rabbits receiving an oral primary infection followed by an oral challenge

infection had considerable reductions in fluke burdens compared to control rabbits. This was not the case in orally infected rabbits challenged intraperitoneally which showed no such reduction. The author interpreted this as evidence to support the theory that two kinds of immune mechanisms were involved in F. hepatica infections, one occurring in the intestinal wall, and the other during the migratory phase.

Hughes et al. (1975, 1976 a) & b), 1977a) also examined immunity induced by parasites introduced by abnormal routes, but used adult flukes as opposed to metacercariae. In the first of a number of similar experiments, adult living F. hepatica were implanted into the subcutaneous tissues of rats (Hughes et al., 1975). When implanted rats were challenged orally with 20 metacercariae a significant reduction in fluke burden was demonstrable compared with control rats. The next study in this series compared the relative immunogenic merits of subcutaneously implanted flukes with oral administration of metacercariae (Hughes et al., 1976 a) & b). In this study challenge with adult F. hepatica was carried out either subcutaneously or intraperitoneally. The authors reported oral sensitisation with normal metacercariae to be the more effective method of immunisation, but added that the full expression of immunity, as regards killing of adult flukes, was only seen in rats challenged intraperitoneally.

A quantitative assessment of the relative value of the two vaccination regimes described above, in terms of percentage mortalities of adult flukes of the challenge infection was presented by Hughes et al.(1976b). It appeared that rats immunised by prior exposure to an oral infection with metacercariae killed 71% of an intraperitoneal challenge with adult F. hepatica, compared with 23% in rats sensitised by implantation of adult flukes. The salient features of the ability of immunised rats to kill F. hepatica were described

by Hughes et al. (1977a). This ability was expressed regardless of the source of challenge flukes, was independent of the number of flukes present in the bile ducts of immunised rats, and developed within 2 weeks of an oral F. hepatica infection, i.e. while the sensitising infection was still migrating through the liver parenchyma. These authors, in agreement with Lang and Dronen (1972), concluded that antigens associated with immature stages may be more important than those of adult F. hepatica in immunisation.

Confirmation of the protective influence of subcutaneously implanted adult F. hepatica on subsequent challenge infection was provided by Eriksen and Flagstad (1974 a) & b)) and Anderson et al. (1975). The former studies involved surgical implantation of adult F. hepatica obtained from sheep, goats and cattle into the subcutis of rats. Immunity was demonstrable in immunised rats in terms of a reduction in the number of F. hepatica established in implanted rats compared with controls.

Vaccination with Somatic or Metabolic Extracts of F. hepatica

A number of workers have employed various different parasitic extracts in an attempt to produce immunity against F. hepatica in different animal species, including Kerr and Petkovich (1935); Urquhart et al. (1954); Shibanai et al. (1956); Babadzhanov and Tukhmanynts (1950); Ershov (1959); Hughes (1962, 1963); Geyer (1967); Ross (1967 c), Dragneva (1972); Kozar (1974); Sinclair and Joyner (1974); Lalic et al. (1976); Lang (1976); Lang and Hall (1977). Notable among the early studies in this field is the work of Urquhart et al. (1954) on immunisation of rabbits against F. hepatica using an alum-precipitated protein extract of the parasite, injected intramuscularly. This vaccination procedure did not reduce fluke recovery compared with control animals, but did produce inhibition of development of the flukes. In the opinion of the authors a hypersensitive response may have been triggered by the immunisation

procedure, as reflected in the exaggerated cellular reaction to the parasites in the livers of vaccinated rabbits. Other authors (Kerr and Petkovic, 1955; Shibanai et al., 1956; Ross, 1967 c); Sinclair and Joyner (1974) also reported a measurable acquired immunity to F. hepatica in rabbits using extracts of adult fluke, while others were unable to confirm this finding (Healy, 1955; Hughes, 1962, 1963; Thorpe and Broome, 1962; Lalic, 1976). Dragneva (1972) was also unable to obtain a significant degree of immunity in rabbits immunised with lipid-free extracts or lyophilised homogenates of F. hepatica, but did record immunity in rabbits hyperimmunised with metabolic antigens of the fluke.

In sheep Ershov (1959) claimed to have produced immunity using a polysaccharide albumen complex antigen, but Hughes (1962), was unable to substantiate this finding using a similar technique. Ross (1967 c) was able to demonstrate a slight degree of resistance to challenge in sheep vaccinated subcutaneously and intramuscularly with extracts of immature flukes, in terms of retardation of fluke development and decreased fluke recovery, although it was pointed out that the reduction in fluke recovery in the vaccinated sheep following challenge was less than that in calves following experimental challenge infection.

The same author (Ross, 1967c) used a similar technique in an attempt to vaccinate calves against F. hepatica infection, and was able to report a reduction in fluke recovery and retardation in fluke development following vaccination procedures.

A number of vaccination techniques employing parasitic extracts have also been carried out in the laboratory model of F. hepatica in the rat and mouse. An interesting study by Kozar (1974), examined the effect of immunising rats with antigens obtained from the intermediate host. The

authors injected rats with whole-body antigens of Galba (Lymnaea) truncatula or Lymnaea tomentosa and subsequently challenged with F. hepatica metacercariae obtained from one or other snail species. Reduction in intensity of infection was observed as well as reduction in pathological changes in the liver and inhibition of fluke development in vaccinated rats, compared with controls especially when both antigen and metacercariae were of G.(L.)truncatula origin. It was concluded that snail host antigen occurred in metacercariae and that F. hepatica was phylogenetically better adapted to G.(L.)truncatula than to L. tomentosa. Also in the laboratory model of fascioliasis Lang (1976) and Lang and Hull (1977) successfully vaccinated mice against F. hepatica infection using various parasitic products. Lang (1976) injected mice intraperitoneally with an antigen elaborated from the in vitro maintenance of juvenile flukes for a period of 24 hours. Mice injected several times with this material and challenged 30 days later with 2 metacercariae, were protected in terms of reduction in mortality and fluke recovery compared with control mice. The same author (Lang and Hall, 1977) reported a study of the immunising effect of culture antigens (Lang, 1976) and a crude sonicated extract of juvenile flukes. Two injections with the culture incubated antigen afforded the best protection (up to 87.5%) reduction in fluke recovery, compared with controls, although significant reductions in recovery were also obtained in mice given a single injection. A single injection of the sonicated fluke extract resulted in an 86% reduction in fluke burden compared with controls. Double injections with this material reduced the fluke burden further, but increased the mortality rate. It was suggested that this may be due to a hypersensitivity reaction in view of the severe pathological changes in the livers of mice treated in this way.

Vaccination Procedures involving Irradiated Metacercariae

Irradiation has featured largely in a large number of studies by various authors over several years, including Hughes (1962, 1963); Thorpe and Broome (1962); Lagrange (1963); Dawes (1964); Sokolic (1968); Tewari (1970); Corba et al. (1971); Armour et al. (1974); Kozar (1974); Nansen (1974, 1975); Harness et al. (1975, 1976 a) & b), 1977); Chiriboga et al. (1976).

One of the earliest of these studies (Thorpe and Broome, 1962) indicated that rats vaccinated orally with irradiated metacercariae developed a detectable degree of resistance to challenge with normal F. hepatica metacercariae. It was noted that a period of 11 weeks was required between vaccination and challenge, for any immunity to develop.

Similar work by Hughes (1963) and Dawes (1964), however, in the mouse failed to demonstrate any immunity to challenge in terms of either delayed maturation or reduced fluke burdens compared with controls, in mice vaccinated orally with irradiated metacercariae. These authors challenged only 22 days after vaccination, which may partly explain this discrepancy.

Further work in the laboratory model of F. hepatica infection in the mouse was carried out by Harness et al. (1975, 1976 a) & b), 1977 a) & b). The first in this series of studies (Harness et al. 1975, 1976 a), described immunisation of mice with 1 or 2 doses of 20 irradiated F. hepatica metacercariae, followed by oral challenge with 100 normal metacercariae 3 weeks later. It emerged that the numbers of immature flukes recovered 2 days later from the peritoneal cavity of immunised mice was significantly lower than those recovered from control mice. These results were interpreted by the authors as evidence for a pre-hepatic immune response operating either in the intestinal mucus or the gut wall. On further examination of this phenomenon

(Harness et al., 1976 b), 1977 a) this theory was discarded in favour of an explanation along the following lines: that the differences in numbers of flukes recovered from vacinnated mice compared to controls 2 days post-challenge was in fact due to more rapid migration of the flukes from the peritoneal cavity in the former. This theory was based on the observation that the number of flukes recovered from the livers of vaccinated and control mice 12 and 14 days after challenge was similar, indicating that no protective immune mechanism was operative in the intestine or peritoneal cavity. The effect of withholding the challenge infection in vaccinated mice, until maturity of the sensitising infection was the subject of subsequent studies by the same authors (Harness et.al., 1977b). It was clear that this ruse resulted in abolition of the observed increase in migration rate to the liver (Harness et al., 1976b), but at the same time caused a reduction in the numbers of flukes recovered from the livers of vaccinated mice 14 days after challenge. An ancillary finding in this study was that due to increasing mortality there was no advantage to be gained from dosing with more than 2 metacercariae, if mice with adult flukes in their bile ducts were required. These workers also warned of the danger of assessing immunity in mice given this number of metacercariae, bearing in mind that one third of the mice will fail to become infected.

An interesting series of studies by Armour et al. (1974) attempted to vaccinate rats and calves using irradiated metacercariae administered orally. These workers recorded that rats immunised at weekly intervals with 2 or 3 oral doses of 20 Kirradiated metacercariae became significantly resistant to subsequent challenge with 20 normal metacercariae, compared to control rats, in terms of a reduction in fluke recovery. Following these preliminary results the same authors described a similar vaccination regime in cattle, and were able to demonstrate a 30% reduction in fluke recovery in

a group of 7 cattle challenged 4 weeks post-vaccination, and a 70% reduction in a similar number of cattle challenged 8 weeks post-vaccination, compared with control cattle. The authors were quick to point out however that despite these marked reductions in fluke burdens in the latter group of cattle, the liver lesions present in these animals would certainly have resulted in liver condemnation at meat inspection.

Contemporaneous studies in cattle by Nansen (1974, 1975) confirmed the findings of Armour et al. (1974). The former author obtained a 71% reduction in fluke numbers recovered from calves vaccinated orally with irradiated metacercariae, compared with control animals. Unlike the previous study, however, calves were not challenged orally with normal metacercariae, but were turned out on to fluke-infected pasture, following vaccination. In addition to reduced fluke burdens resistance in vaccinated calves was also reflected in the stability of GGT levels compared with control animals.

In summary, from the various immunisation regimes tried to date, with the possible exception of the use of antigenic material from in vitro culture, which held out some hope for the future, the prospects for an effective fluke vaccine were rather bleak. One of the major problems is that for any vaccine material to be successful, its efficiency would necessarily have to be 100%, for if even a few flukes reached the liver, their migratory activities would neutralise any beneficial effect of the vaccine, as this would probably result in liver condemnation at the abattoir, in addition to impaired productivity due to liver damage.

CHAPTER II

MATERIALS AND METHODS

SECTION I

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Sheep

Sheep used in these experiments were of the Finn-Dorset breed unless stated otherwise, purchased from a local farm and reared indoors until 10 - 12 weeks of age. After purchase they were housed in concrete pens cleaned out weekly and sheep were bedded daily on oat straw. All sheep were fed hay and water ad libitum, and in addition concentrates were offered at the rate of $\frac{1}{2}$ - $\frac{3}{4}$lbs per sheep, twice daily. Regular examination of faecal samples proved consistently negative for parasitic eggs or larvae.

Calves

Calves used in these experiments were castrated males of the Friesian breed reared indoors until 12 - 14 weeks of age, when they were obtained from a local farm. After purchase they were housed in concrete pens cleaned out weekly and calves were bedded daily on oat straw. All calves were fed hay and water ad libitum, and in addition concentrates were offered at the rate of 2 - $2\frac{1}{2}$lbs per calf, twice daily. Regular examinations of faecal samples, prior to the commencement of any experiments, proved consistently negative for parasitic eggs or larvae.

Rats

Rats used in these experiments were males of the Hooded Lister strain purchased from Animal Suppliers, Welwyn, Herts, England, when they were between 150/200 grams in weight, unless otherwise stated.

Weighing

All animals were weighed using an Avery scale correct to 1 kg before commencement of any experiments.

SECTION II

HAEMATOLOGICAL ANALYSIS

Blood samples were collected weekly directly from the jugular vein, into both heparinised and unheparinised evacuated glass containers (Vacutainer No. 3204, Becton, Dickinson & Co., Rutherford, NJ, U.S.A.). Samples collected in unheparinised tubes were allowed to stand overnight on the laboratory bench. Serum from these clotted samples was centrifuged at room temperature, at 3,000 r.p.m. for 20 minutes in a Mistral 44 centrifuge (Measuring Scientific Equipment, London, England) and the supernatant pipetted into capped polypropylene tubes which were immediately stored at -20°C. Samples collected in heparinised tubes were used immediately in haematological determinations.

Packed Cell Volume (P.C.V.)

The packed cell volume percentage was determined by the microhaematocrit method, using a microhaematocrit centrifuge and reader (Hawksley & Son Ltd., London, England).

Haemoglobin

The haemoglobin concentration was determined using the cyanohaemoglobin technique as described by Hallmann (1966).

Cell counting

Erythrocytes and leucocyte counts were carried out using a Coulter counter (Model D) (Coulter Electronics Ltd., Dunstable, England).

Eosinophils

Eosinophil counts were performed on Giemsa stained blood smears. 100 cells were differentiated in each count.

Haemoglobin typing

Before the commencement of cross-immunity experiments the haemoglobin type of experimental sheep was carried out by a modification of the method of Smithies (1955), as follows:

Separation of haemoglobins by multi-microband electrophoresis

Solutions used

1. Stain 0.2% Ponceau -S in 3% Trichloro-acetic acid
2. Buffer A (pre-buffer) pH 9.0 containing:

   16.1 gms. "Tris" (Hydroxymethyl-amino-methane)

   1.56 gms. Disodium ethylene-diamino-tetra-acetic acid (E.D.T.A.)

   0.93 gms. Boric acid

   1,000 mls. Distilled water
3. Buffer B (pH 8.5) containing:

   1.8 gms. Barbitone

   10.3 gms. Sodium barbitone

   1,000 mls. Distilled water
4. Wash 5% Acetic acid

Method

Preparation of tank

200 mls. of buffer B was poured into an electrophoresis tank (Shandon Southern Instruments, Frimly Road, Camberley, Surrey), on a level laboratory bench, ensuring that the liquid level was the same in each compartment.

Preparation of electrophoresis strips

4 pieces of filter paper (Whatman No. 1) were cut 8 cm. x 5 cm. for use as wicks. Strips were then floated (Shandon Celagram Electrophoresis strips, 78 x 150 mm) in the tank and allowed to soak for 5 minutes before blotting dry between clean tissues. Strips were then marked for identification, on the bottom right-hand side. Using a 1 ml. syringe 2 drops of distilled water were then placed on each location of the application plate.

Application of the test sample

A small drop of the heparinised blood samples under test was then added to the distilled water, again using a 1 ml. syringe. The syringe was washed several times with distilled water, after each application. The teeth of the applicators were then dipped into the samples, ensuring that the whole area of the groove in each tooth was in contact with the sample.

Electrophoretic separation

The applicator was then applied vertically to the strip about 2.5 cm. from the cathode end, and the strip placed in the tank so that the ends overlapped the wicks. The rods were then placed over the edges of the strips, and moved in a direction away from the centre. The lids were then replaced and the voltage set at 150v. after checking the polarity of the tank.

Washing and staining of strips

After 30 - 40 minutes the strips were removed and treated as follows:-

1. 5 minutes in 5% T.C.A.
2. 5 minutes in Ponceau -S in 3% T.C.A.

The strips were then washed with 5% acetic acid until the background appeared white.

Determination of haemoglobin type

Haemoglobin type was then determined by comparing test strips with those of sheep of known haemoglobin type.

SECTION III

BIOCHEMICAL ANALYSIS

Liver Enzymes - Glutamate dehydrogenase (GLDH) and Sorbitol dehydrogenase (SDH) Estimation

GLDH and SDH serum activity was estimated according to kinetic methods using Boehringer reagents (Boehringer & Co., Bell Lane, Sussex,

England). Spectrophotometric measurements were made at 340 mm on a Pye Unicam SPG500 spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, England). Non specific creep reactions were measured for each serum sample analysed and the resultant value altered accordingly. Before each batch of sera were analysed the accuracy of the test method was checked using a Seronorm reference serum (BDH Chemicals Ltd., Poole, England). Only when the reference serum value was within acceptable limits was determination of unknown samples undertaken.

Gammaglutamyl transpeptidase (G.G.T.) Estimation

Gammaglutamyl transpeptidase serum activities were kindly carried out by the University of Glasgow Veterinary School, Biochemistry Department, using an LKB reaction rate analyser at 37°C (LKB Instruments Ltd., Addington Road, South Croydon, Surrey, England).

SECTION IV

SEROLOGICAL ANALYSIS

Preparation of F. hepatica lipid free extract (L.F.E.)

Mixed mature and immature flukes collected from sheep livers were chopped finely with scissors. After blotting dry this material was weighed into Universal bottles in 2 gm amounts and freeze-dried overnight in a freeze drier (Speedivac, Edwards Vacuum Components, Sussex, England). The following day approximately 5 mls of cooled diethyl ether was added to each bottle and the mixture homogenised in a Silverson homogeniser (Silverson Machines Ltd., London, England) at -15°C (using dry ice) for 3 periods of approximately 20 seconds. The ether was decanted after centrifugation for 10 minutes at 2,500 r.p.m. and -20°C, and the residue dried by suction from a vacuum pump. 5 mls of 0.15M PBS pH 7.2 was then added to each bottle

and the contents homogenised, on ice, in a Silverson homogeniser for 3 periods of 20 seconds, and the homogenate obtained extracted overnight in 0.15M PBS pH 7.2 at 4°C on a magnetic stirrer. The extract was then centrifuged at 2,500 r.p.m. and 4°C for 30 minutes, the supernatant decanted and stored at -20°C. The sediment was resuspended in a further 5 mls of 0.15M PBS (pH 7.2) and the processes of homogenisation, extraction, and centrifugation repeated as described above. The second supernatant obtained was decanted, pooled with the first, centrifuged at 2,500 r.p.m. and 4°C then ultracentrifuged at 20,000 r.p.m. in 4°C and sterilised by passage through 0.45 and 0.22 u Millipore filter (Millipore UK Ltd., Abbey Road, London) when a sterile preparation was required. The protein content of the extract was estimated by the method of Lowry et al. (1951) before being finally dispensed in appropirate aliquots, and stored at -20°C until required.

Enzyme linked immunosorbent assay (E.l.i.s.a.) for analysis of ovine serum

This test was used in a microtitre system as described by Ruitenberg et al. (1975). It was chosen initially because of its sensitivity, cheapness and relative simplicity. The antigen used was a lipid-free extract (L.F.E.) of F. hepatica prepared as previously described. For use in the test L.F.E. was diluted to a suitable concentration in sodium carbonate buffer (0.1 M) pH 9.6.

Antigen coating

The assay was performed as follows:- disposable, flat bottomed microtitre plates (Dynatech Labs., Sussex, England. Batch No. M.129A) were coated with antigen by adding 150 µl of the dilution of antigen required to each of the 96 wells and incubating at 37°C for 1 hour. Plates were then covered and placed overnight in a refrigerator at 4°C. The following day the plates were emptied and washed 3 times with a solution of 0.85% saline containing 0.05% Tween 20.

Addition of antiserum and conjugate

Washing was carried out using a 50 ml syringe, and between each wash plates were allowed to stand on the bench for 2 - 3 minutes. 150 µl of an antiserum appropriately diluted in 0.15M PBS pH 7.2 containing 0.05% Tween 20 was then added to each well and the plates again incubated at 37°C for 1 hour. At the end of this period plates were again washed as described above and 150 µl of an appropriate dilution of rabbit-anti-sheep IgG conjugated to horseradish peroxidase (H.R.P.O.) (Nordic Immunochemicals, Maidenhead, Berkshire, England.) in 0.15 M PBS pH 7.2 containing 0.05% Tween 20 was added to each well.

Substrate Preparation and Use

After a further period of one hour incubation at 37°C, washing was again carried out as described above, and 150 µl of an appropriate substrate, in this case ortho-phenylene-diamine (O.P.D.) was added to each well. This substrate was preferred to the more commonly described 5 Amino salicylic acid (5AS), because results with the latter were inconsistent. O.P.D. was prepared as follows:-

35 mgs O.P.D. was dissolved in approximately 95 mls of phosphate/citrate buffer (P.C.B. pH 6.0). The solution was then filtered, using Whatman's No. 1 filter paper, 0.167 ml of Hydrogen peroxide (H_2O_2) was added and the solution made up to 100 mls with P.C.B. pH 6.0. During preparation the substrate was protected from the light as much as possible.

Reading of the Test

30 minutes after addition of the substrate to the plates, the reaction was stopped by adding 0.025 ml of 1M Aristar sulphuric acid (H_2SO_4) to each well.

The test was read using 10 mm microcuvettes in a spectrophotometer (Pye Unicam SP 600, Pye Unicam Ltd., Pork Street, Cambridge, England) at 460 mm.

Concentration of Reagents

For analysis of ovine serum antigen was used at a concentration of 5 µg protein N/ml, peroxidase conjugate was diluted 1:1000 and sera were diluted 1:50.

Controls for E.l.i.s.a.

For each test carried out controls were set up according to the following schedule:-

	F. hepatica L.F.E.	F. hepatica infected ovine serum	Normal ovine serum	Conjugate	Substrate
Positive-serum control	+	+	-	+	+
Negative-serum control	+	-	+	+	+
Conjugate control	+	-	-	+	+
Substrate control	+	-	-	-	+

Standardisation of Results

It was found that, in this E.l.i.s.a. system, there was a variation in absorption values for the same sera, not only between different batches of microtrite plates, but also between individual microtite plates. This was presumably due to different antigen-binding capacities of the plates. To

circumvent this problem the E.l.i.s.a. results were expressed relative to a standard score of 1.0 absorbance for a known positive serum. In each case the value for the conjugate control was subtracted from the value for each test serum, before its final value was calculated. In order to confirm that consistent values for test sera were obtained the value of a positive reference serum was measured on each plate.

Indirect haemagglutination test (I.H.T.)

Fixation of Red Cells

Ovine erythrocytes were fixed by a modification of the technique of Herbert (1967), using glutaraldehyde. Freshly collected ovine red blood cells were washed 3 times in 0.15 M PBS pH 7.2. 25% glutaraldehyde neutralised with 10% sodium carbonate, diluted 3: 2: 10 by volume in PBS pH 8.0 and 0.85% (N) saline was added to a 50% suspension of erythrocytes, slowly with constant stirring over several hours, using a peristaltic pump, in an ice-bath. The mixture was stirred overnight at 4^{o}C on a magnetic stirrer, and the following day the cells were filtered through an opened gauze swab to remove gross debris. The fixed cells were then washed 4 times in 0.15 M PBS pH 7.2, being thoroughly resuspended between each wash, and made up to a 1% suspension. 10 ml. aliquots of cells were then transferred to Universal bottles.

Tanning and Coating of Red Cells

10 mls. of tannic acid solution (5 mg/50 ml. 0.15 M PBS pH 7.2) was added to each Universal and the mixture incubated for 15 minutes at 37^{o}C. After washing in 0.15 M PBS pH 7.2 the cells were precoated by mixing equal volumes of a 2% cell suspension with 0.5% bovine serum albumin (B.S.A.) in 0.15 M PBS pH 7.2 B.S.A. was removed by washing in 0.15 M PBS pH 7.2, and antigens were attached by adding 0.2 ml of fluke L.F.E. containing

6.5 mg protein N/ml (prepared as previously described) to a 1% solution of cells in PBS pH 6.4 before incubation at 37°C for 30 minutes. Antigen coated cells were then washed 3 times in 0.15 M PBS pH 7.2 containing 1% normal rabbit serum (N.R.S.).

Inactivation and Absorption of Rabbit Serum

N.R.S. was previously inactivated at 56°C for 30 minutes and absorbed at room temperature for 30 minutes with one quarter of its volume of a 2% suspension of cell from the same batch as those being coated.

Equilibration of Red Cells

After washing cells were incubated overnight with stirring at 37°C to facilitate settling of the cells, as described by Van Tiggele and Over (1976), and stored at 4°C in 0.15 M PBS pH 7.2 containing 1% inactivated and absorbed (I & A) N.R.S., until required.

Preparation of Serum for Test

Before use in the test all sera were inactivated at 56°C for 30 minutes and absorbed on the bench for 30 minutes in a 1: 10 solution of tanned uncoated cells from the same batch.

Microtitre Test System

Serial dilutions of sera in 0.15% PBS pH 7.2 with 1% N.R.S. beginning at 1: 20 were made using microtitre equipment (Dynatech Labs., Sussex, England) V bottomed microtitre plates were employed and 0.025 ml drops of cells and serum dilutions were titrated together.

Reading of the Test

End points were read after incubation on the bench for 16 hours.

Controls for I.H.T.

Known positive and negative serum controls were titrated for each batch of test sera.

Double immunodiffusion (D.I.D.)

Immunodiffusion in an agar-gel medium was carried out as follows:-

Preparation of agar - 500 mls of 2% Agar was prepared by adding 10 gms Oxoid No. 2 agar to 250 mls barbitone acetate buffer pH. 8.6 (B.A.B.) diluted in an equal volume of distilled water. 100 mgs merthiolate was added to the mixture which was heated to approximately 80^{o}C and dispensed in 50 ml flat medical bottles.

Preparation of Barbitone-acetate Buffer (pH 8.6) (B.A.B.) 1 litre of B.A.B. was prepared by dissolving 10. 31 gms sodium barbitone together with 4.1 gms sodium acetate, 1.342 gms diethylbarbituric acid and 100 mgs merthiolate. Before use the buffer was filtered using Whatman No.1 filter paper.

Preparation of Gel Layer

A 2% solution of agar in B.A.B. as described above was heated to melting point in a steam bath. This solution was then allowed to cool to approximately blood heat before being poured on to glass slides 7.5 x 2.5 cm, in a plastic slide holder (Gelman Hawksley, Northampton, England) to give a uniform layer of approximately 2 mm in depth. The slides used had been pretreated by dipping in an adhesive agar solution (0.1% Oxoid No.2 agar in distilled water containing 0.05 glycerol and 0.02% azide) to facilitate application of the gel layer. The agar was allowed to cool for several hours at room temperature before a series of seven wells were punched in the agar using a gel punch (No.71632 Gelman Hawksley, Northampton, England). One central well was punched surrounded by a circular arrangement of six further wells.

Each well measured 3.0 mm in diameter and peripheral wells were separated by a distance of 5 mm from central wells. The punched areas of agar were then removed using suction from a vacuum pump.

Application of Antigen and Test Samples

Approximately 40 µls of L.F.E. (6.5 mgs protein N/ml) was then added to each of the central wells using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). In the same manner 40 µl samples of antisera under test were added to the peripheral wells. Diffusion was then allowed to occur under moist conditions, at room temperature for a period of 48 hours.

Washing and Staining of Gel Layer

The gel layer was washed for a minimum period of 48 hours in several changes of 0.85% saline with sodium azide preservative, before being rinsed overnight in distilled water. The agar was then dried at approximately 70^{o}C under filter paper, before staining in 0.5% Coumassie brilliant blue in a solvent solution of methanol/water/acetic acid in the proportions 9: 9: 2 by volume, for 15 minutes. Destaining was then carried out in the same solvent solution described above, until a clear background was obtained.

Reading of the test

A positive result in the test was indicated by the presence of one or more precipitin lines, and a qualitative assessment was made of the intensity of the line(s) present, for each antiserum tested.

Passive transfer studies

Passive transfer technique. Rats were allocated to groups randomly before commencement of any experiments. Serum for use in these studies was obtained from appropriate animals as previously described. Relevant batches

of serum were pooled and stored at -20°C until required. Experimental rats were lightly anaesthetised using a glass jar containing cotton wool soaked in trichloroethylene, before an appropriate volume of serum (see specific experimental design for details), was injected intraperitoneally.

SECTION V

CELL-MEDIATED IMMUNOLOGICAL ANALYSIS

Lymphocyte transformation test (L.T.T.)

The following procedure was carried out in sheep experimentally infected with F. hepatica as well as non-infected control animals, by a technique similar to that described by Burrells and Wells (1977).

Separation, Counting and Viability of Lymphocytes

10 mls of blood was collected from each animal under test, directly from the jugular vein into sterile evacuated tubes containing 0.1 ml of sterile preservative free heparin (Evans Medical Co. Ltd., Liverpool, England).

The blood was carefully layered onto an equal volume of a Triosil (Triosil 440 Nyegaard & Co., Oslo, Norway), Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), distilled water mixture (specific gravity 1.075) and centrifuged at 400g for 45 minutes at room temperature, by which time a distinct band of lymphocytes had formed at the plasma/triosil/Ficol/distilled water interface. The lymphocytes were collected and washed three times by centrifuging at 400g for 10 minutes at room temperature. Between each wash the cells were resuspended in Hank's balanced salt solution containing 1% heparin to prevent clumping, and were finally resuspended in 1 ml RPMI

1640 medium (Gibco Biocult, Paisley, Scotland) supplemented with 20% foetal calf serum F.C.S. (Flow Labs, Irvine, Scotland), penicillin (100 iu per ml) streptomycin (100 mg per ml) mycostatin and 20 mM hepes buffer (Gibco Biocult, Paisley, Scotland).

An aliquot of the washed cells was diluted 1:10 with 0.1% Trypan Blue and the cells counted in an Improved Neubauer Counting Chamber. Cell viability was assessed on the basis of Trypan Blue dye exclusion.

All suspensions contained not less than 95% lymphocytes with 95% viability. The initial lymphocyte suspension was then adjusted to give a final concentration of 1×10^6 cells per ml by adding culture medium RPMI 1640 plus supplements.

Assessment of lymphocyte responsiveness

Lymphocyte responses were determined in medium containing F.C.S. alone or F.C.S. and 2 mercaptoethanol (M.E.) 5×10^{-5} M. Phytohaemagglutinin (P.H.A.) 2.5 µg/ml, Pokeweed mitogen (P.W.M.) 10 µl/ml, (Gibco Biocult, Paisley, Scotland), Concanavalin A (Con.A) 6.25 µg/ml (Difco Labs., Detriot, U.S.A.) and lipopolysaccharide of Eschericia coli (L.P.S.) were used to measure the proliferation of the lymphocyte population. All were supplied in the lyophilised stated and following reconstitution were dispensed in 0.2 ml aliquots and stored at -20°C, until use.

Preparation of F. hepatica - lipid-free antigen

F. hepatica lipid free extract (L.F.E.) for use in L.T.T. was prepared as described earlier. For use in L.T.T., L.F.E. was diluted in culture medium RPMI supplemented as outlined above. Optimal dilutions of L.F.E. suitable for stimulation of ovine lymphocytes were determined prior

to the commencement of any experiments. For ovine lymphocytes L.F.E. was used at dilutions of 19, 6, 3, 2, 1.25 and 1 µgm protein N/ml.

Preparation of microtitre plates

Mitogens and antigens diluted in RPMI 1640 medium containing 20% F.C.S., antibiotic, mycostatin, 20 mM hepes buffer and 1% heparin in 20 µl amounts were placed in the wells of sterile flat-bottomed microtitre plates (Gibco-Biocult, Paisley, Scotland). 200 µl of the lymphocyte suspension were then added to give 2×10^5 cells per well. All cultures were set up in triplicate with and without the addition of mercaptoethanol to the culture medium. The plates were covered with loose fitting lids and incubated in an air-tight moist container at 37°C for a period of 5 days.

Pulsing of microtitre plates

1 Ci Methyl (H_3) thymidine (specific activity 5mCi/m mol; The Radiochemical Centre, Amersham, England) in 20 µl of supplemented RPMI 1640 was added to each well 18 hours before terminating the cultures by freezing at -20°C. The cultures were stored in this temperature until harvesting.

Harvesting and counting of lymphocytes

After thawing, the lymphocytes were harvested onto glass-fibre filter paper (Skeltron, Norway), using a semi-automatic cell harvester (Titertek, Flow Laboratories, Irvine, Scotland). The papers were dried at 37°C and the filter paper discs transferred to scintillation vial inserts containing 1 ml of scintillation fluid (Nuclear Enterprises 243, Edinburgh, Scotland), for counting in an automatic liquid scintillation counter (Packard, England). Vials were counted for a period of 1 minute on three separate channels.

Lymphocyte transformation test (L.T.T.) using bovine cells

Essentially the same technique was used as described for ovine

lymphocytes. However, the dilutions of L.F.E. employed were slightly different viz 38, 19 and 2 µg protein N were the dilutions most often employed.

SECTION VI

PARASITOLOGICAL TECHNIQUES

Modified MacMaster Faecal Egg Count Method

Before commencement of experiments regular faecal samples were obtained for detection of eggs or larvae of helminth species. Gordon & Whitlock's (1939) modification of the MacMaster faecal egg counting technique was employed for this purpose as follows:-

3 grams of faeces were mixed with 42 mls of tap water and homogenised using an M.S.E. homogeniser (Measuring Scientific Equipment Ltd., London, England. The resultant mixture was passed through a sieve, (Endecotts Test Sieves Ltd., London, England) of aperture 250 µ and the liquid transferred to a 15 ml flat-bottomed test tube. After centrifugation at 2000 r.p.m. for 3 minutes the supernatant was decanted and the sediment shaken using a whirlimixer (Fison's Scientific Apparatus Ltd., Loughborough, Leicestershire). A saturated solution of sodium chloride was then added to the sediment and the tube inverted five times to ensure complete mixing. Using a pasteur pipette both chambers of a MacMaster counting slide (Hawksley & Sons, London, England) were filled and examined under the microscope. The dilution of faeces made was such that the total number of eggs or larvae found in both chambers of the counting slide multiplied by 60 gave the total number of eggs or larvae per gram of faeces. Alternatively, if small number of nematode eggs or larvae were likely to be present the following method was used.

Flotation Technique

The sample was treated as described previously and saturated salt

solution was added to the faecal suspension in a 15 ml flat-bottomed test tube as above. The tube was then placed in a bench centrifuge and saturated salt solution was again added until the tube was full and showed a positive meniscus. A thick 19 x 19 mm square cover slip was then placed on the tube which was centrifuged at 1,000 r.p.m. for 2 minutes. The cover slip was removed and placed on a microscope slide. The slide was then systematically searched using the 17 mm objective of the microscope and all eggs and/or larvae counted. Each egg or larva counted represented 1 per gram of faeces.

Boray Sedimentation Technique for detection of F. hepatica ova.

The following modification of the technique of Happich and Boray (1969) was employed for the detection of F. hepatica ova in the faeces of infected animals.

Preparation of samples

3 grams of faeces were homogenised in 50 mls tap water in an M.S.E. homogeniser (Measuring Scientific Equipment Ltd., London, England) and the resultant homogenate passed through a sieve (Endecott's Test Sieves Ltd., London, England) of aperture 100 μ. The retained debris was thoroughly washed with a fine water jet and the filtrate allowed to stand on the laboratory bench in a conical urine glass for a period of 2 minutes. The supernatant was then drawn off by suction from a vacuum pump and the sediment transferred to a 15 ml flat-bottomed test tube, which was again allowed to stand on the bench for 2 minutes.

Identification and Counting of F. hepatica ova

The supernatant was drawn off as before and 3 drops of 10% methylene blue solution added to the sediment which was transferred to a lined plate for identification and counting of F. hepatica eggs. The oval, golden brown eggs were easily visible against the blue stained background, when examined

under x12 magnification on a steroscopic microscope.

This method was used for detection of small numbers of eggs, where larger numbers were present a modification of the MacMaster flotation technique (Gordon & Whitlock, 1939) using zinc sulphate instead of saturated salt solution, was employed.

Lymnaea truncatula Culture

L. truncatula adults were maintained in petri dishes containing algae of Oscillatoria species, at a temperature of 20°C. Before transfer to fresh plates the snails were washed in a fine jet of distilled water to remove soil debris and other adherent material.

Egg masses from stock L. truncatula were kept in distilled water at room temperature until hatching when the small snails were transferred to petri dishes lightly seeded with algae.

Infection of L. truncatula with miracidia of F. hepatica

Adult L. truncatula, approximately 3 - 5 mm in length were individually infected by exposure to 4 F. hepatica miracidia for 2 hours, in distilled water, in the wells of microtitre plates (Dynatech Laboratories, Sussex, England). 5 weeks post-infection the snails were thoroughly washed in a fine jet of distilled water and placed on crushed ice in a petri dish lined by Visking dialysis tubing (Scientific Instrument Centre, London, England). During this period the infective cercarial stages of F. hepatica left the snails and encysted on the Visking tubing.

Faecal culture of Ostertagia circumcincta

Freshly collected faeces of sheep infected with O. circumcinta were bulked and placed in a number of honey jars. These were then left at room temperature for a period of 14 days, by which time all larvae should have reached the infective stage. During the culture period the jars were regularly

checked for fungal growth, and if any had taken place the faecal pellets were sprayed with a fine mist of tap water, whilst being thoroughly mixed to break up the fungal elements.

Recovery of O. circumcincta 3rd stage larvae

After 14 days incubation water was added to the jars containing faecal pellets. The contents were then filtered using a Buchner apparatus with vacuum pump attached. A Baermann apparatus was then set up, consisting of a large funnel filled with warm water, fitted with a short piece of rubber tubing closed with a spring clip. A wire mesh of aperture 0.15 mm previously wetted with tap water was placed in the funnel and water added until the level of the screen was reached. The material retained in the filter of the Buchner apparatus was then transferred to the screen of the Baermann apparatus. This apparatus was allowed to stand overnight, and the following day approximately 10 ml of fluid containing 3rd stage larvae was drawn off into a Universal container. Larvae obtained in this way were then examined under a stereoscopic microscope and viability on the basis of mortality was assessed. The larvae were then counted and appropriate doses made up in approximately 10 mls of distilled water.

Preparation of infective doses of F. hepatica metacercariae

Metacercariae were generally present on Visking tubing if obtained from laboratory snails or on cellophane if obtained from other laboratories. The same batch of metacercariae was used in individual experiments. The cellophane or Visking tubing was cut into thin strips of convenient size for counting using a scalpel. Such strips were transferred to petri dishes and flooded with distilled water. Metacercariae were assessed

individually for viability under x50 magnification on a stereoscopic microscope. The criteria used for viability was embryonation and only embryonated metacercariae were counted. When counted, metacercariae on appropriate sizes and numbers of cellophane strips were transferred to Universal bottles containing distilled water. Strips were dried by suction from a vacuum pump, blotted using Whatman No. 1 filter paper and finally wrapped in Whatman No. 1 filter paper, folded into an appropriate shape for oral dosing.

Preparation of infective doses of Taenia hydatigena ova

The following procedure was employed in the preparation of suitable numbers of T. hydatigena eggs for oral infection of sheep.

T. hydatigena eggs, suspended in 0.85% saline containing penicillin, streptomycin and mycostatin were thoroughly mixed using two 100 ml glass beakers. 20 samples, each of 0.025 were then removed from the suspension and spread along the surface of glass slides in a linear fashion using a micro pipette. Viable embryonated eggs were then counted under x25 magnification using a Wild dissecting microscope (M.S. Wild, Iteerbrug, Switzerland). The number of eggs per mls of suspension was found by calculating the mean number of eggs present in one 0.025 mls drop and multiplying this figure by 40. Doses containing the required numbers of T. hydatigena eggs for oral dosing were then prepared in Universal bottles containing approximately 10 mls of normal saline.

SECTION VII

POST MORTEM TECHNIQUES

Necropsy Procedure

Experimental animals were humanely killed with a captive bolt pistol and immediately bled out. The abdomen and chest were opened and the peritoneal

surface examined for the presence of flukes before the liver was removed. The liver was weighed and photographed and an arbitary classification on the basis of haemorrhage, degree of fibrosis and calcification was made, as illustrated later.

Recovery of F. hepatica

Any flukes adhering to the liver were removed, the gall bladder and main bile ducts opened and any flukes found removed. The liver was then sliced into thin (4 mm) slices. The sliced liver tissue was allowed to stand overnight in warm saline and again repeatedly squeezed to remove trapped flukes. This supernatant was removed and the tissue debris thoroughly searched in numerous aliquots in petri dishes for the presence of small flukes.

Measurement of F. hepatica

All flukes found were measured and allotted to one of three groups, viz:- 0-6 mm, 6-12 mm, greater than 12 mm. Not all flukes were intact, but where possible fragments of flukes were matched and assigned to the appropriate group. All measurements were made on flukes relaxed overnight in distilled water then fixed in 40% formaldelhyde.

In addition to procedures previously described for recovery of liver flukes, the following special procedures were carried out on animals infected either with eggs of T. hydatigena, or nematode larvae.

Examination for cysticerci of Cysticercus tenuicollis

At slaughter the abdominal cavity, mesentery and thoracic viscera were thoroughly examined for living or degenerate cysticerci of C. tenuicollis. The liver was removed along with any attached cysticerci. These adhering to the liver capsule were removed and counted individually. Degenerate cysticerci found during processing of liver tissue were noted, along with cysticerci adherent to abdominal or other viscera and to the body wall.

Examination of abomasum and small intestine contents for nematodes

Preparation of abomasum and small intestine

The abomasum and small intestine of nematode infected sheep were removed immediately after slaughter. The abomasum was opened along its greater curvature and the contents emptied into a plastic bucket. The mucosa was then thoroughly rinsed under a gentle stream of tap water and the bucket contents made up to 2 litres (4 litres for bovine material). The small intestine was treated in a similar fashion; being opened along its entire length using dissecting scissors and the mucosa passed between thumb and forefinger under a gentle stream of tap water to remove all contents, which were again made up to 2 litres with tap water. Duplicate 200 ml samples were then removed from each bucket, after thorough mixing, into honey jars, a small amount of 40% formalin added to each, and the jars stored at room temperature on the laboratory bench until required.

Identification and counting of nematode species

Approximately 2 - 3 mls of iodine/potassium iodide solution was added to the contents of each jar and 10 x 4 mls samples were withdrawn from each into lined Petri dishes for counting. Sodium thiosulphate was then added to each dish until a clear background was obtained against which the stained parasites would be clearly visible. The contents of each Petri dish in turn were then examined under the x12 objective of a stereoscopic microscope. Any nematode species found were identified by morphological characteristics and counted. The number of nematodes found in 10 x 4 ml samples was multiplied by 50 times to give a total count.

SECTION VIII

STATISTICAL METHODS

The following statistical analyses were performed on experimental data in these studies:-

1) Where observations were thought to come from a normal population Student's "t" test, as described by Fisher (1934), was used to determined the level of significance of a given result.

2) Where observations were thought not to come from a normal population or where sample variances were not of the same order in groups being compared, the non-parametric Mann-Whitney U test was used (Mann and Whitney, 1947).

CHAPTER III

COMPARATIVE STUDIES OF FASCIOLA HEPATICA INFECTION IN SHEEP AND CATTLE

INTRODUCTION

In view of the differences in response to F. hepatica infection in sheep and cattle discussed previously, it was decided that the logical approach in investigating such differences would be to monitor the course of primary and secondary fluke infection in the more susceptible ruminant species i.e. sheep and compare this response with that of a few cattle included in the experiment. To this end a wide range of biological parameters were recorded during infection of both species employing various haematological, biochemical, serological and parasitological techniques. These included estimation of serum G.L.D.H. and G.G.T. activity to indicate liver cell damage and bile duct changes respectively. Both quantitative and qualitative tests for detection of serum antibody were employed namely E.l.i.s.a. and I.H.T. as early indicators of the presence of serum antibody, and D.I.D. was employed purely as a qualitative test (see relevant review section p. 30) in order to assess the relative values of these tests as diagnostic aids. For reasons which will be discussed later E.l.i.s.a. was only performed on ovine serum. In addition studies involving passive transfer of serum from both ruminant species to rats in order to assess the comparative protective properties, if any, of such serum, against infection with the parasite were undertaken.

EXPERIMENTAL DESIGN

8 sheep and 5 calves were purchased from a local farm at birth and reared under parasite free conditions until 10 - 12 weeks of age. Unfortunately 2 of the calves died during the rearing period which necessitated a rearrangement of the original plans. Following rearing the experimental animals were randomised on a weight basis and infected as outlined in Table III.1. Negative control serum was obtained weekly from sheep Nos. S2, S3, S4 and S8 until

week 16 when these animals were given a primary infection, and calf No. C2 served as a negative control throughout the experiment.

Sheep No.	Weight (kgs)	(wk. 0) Day 0	(wk. 15) Day 102	(wk. 16) Day 111	(wk. 18) Day 195
S1	16	350 metacerc.	Anthelmintic treatment*	250 metacerc.	Kill
S5	22	350 metacerc.	Anthelmintic treatment*	250 metacerc.	Kill
S6	14	350 metacerc.	Anthelmintic treatment*	250 metacerc.	Kill
S7	19	350 metacerc.	Anthelmintic treatment*	250 metacerc.	Kill
S2	17	-	-	350 metacerc.	Kill
S3	14	-	-	350 metacerc.	Kill
S4	13	-	-	350 metacerc.	Kill
S8	18	-	-	350 metacerc.	Kill
Calf No.	Weight (kgs)	(wk. 0) Day 0	(wk. 13) Day 91	(wk. 14) Day 98	(wk. 26) Day 184
C2	146	1,000 metacerc.	Anthelmintic treatment*	1,000 metacerc.	Kill
C4	125	1,000 metacerc.	Anthelmintic treatment*	1,000 metacerc.	Kill
C3	152	-	-	-	Kill

*Rafoxanide 7.5 mg/kg (Flukanide, Merck, Sharpe & Dohme, Hoddesdon, England

Table III.1

Due to the problems mentioned previously it was unfortunately only possible to use 3 calves in the experiment and for reasons of economy it was not possible to include anthelmintic control sheep or cattle. The efficacy of the anthelmintic used against F. hepatica was assumed from the extensive studies carried out by Gibson (1975) and also from the recent review of Armour (1979), and assessed by faecal egg counts.

The respective doses of metacercariae given to experimental ruminants were chosen, when availability allowed, to permit justifiable comparison with recent authors, e.g. Doyle (1973a); Van Tiggele (1978). Heparinised blood along with serum samples were collected weekly during infection for haematological (P.C.V., haemoglobin, white and red cell counts and eosinophil counts), biochemical (estimation of the serum levels of the liver enzymes G.L.D.H. and G.G.T.) and serological (estimation of serum antibody levels by I.H.T., E.l.i.s.a. and D.I.D.) analysis. In addition faecal samples were examined weekly for F. hepatica eggs commencing 10 weeks after primary and secondary infections, and also weekly following anthelmintic treatment until all samples proved negative.

Passive Transfer Studies

A summary of the treatments of rats used in these experiments is shown in Table III.2.

Group No.	No. of rats	Type of serum	Challenge
1	8	*6 week	+
2	8	10 week	+
3	8	14 week	+
4	8	Normal	+
5	8	-	+

Table III.2: Passive Transfer Experiment I

*denotes week of infection of donor animals

Serum for these studies was obtained from sheep Nos. S1, S5, S6 and S7 at the appropriate times after primary infection (Table III.1). Serum from these animals was pooled to obtain sufficient volumes for transfer.

RESULTS

The mean values of all the various parameters monitored throughout infection and reinfection are illustrated with standard errors in the text of this chapter. Where it was thought that confusion due to error bars may have affected the worth of the illustrations, duplications omitting standard errors are also provided. Variation of the parameters in individual animals are illustrated in the appendix of this thesis.

Haematological Changes

The results of the haematological analysis of the changes accompanying experimental primary and secondary F. hepatica infections in ruminants are illustrated in Figs. 3.1 - 3.10 and Figs. A.1 - A.9 of appendix. Considering firstly alterations in packed cell volumes and haemoglobin it is apparent from Figs. 3.1 - 3.2 that a parallel fall in both parameters occurred following primary F. hepatica infection of experimental sheep. This change commenced 8 - 9 weeks after infection and both P.C.V. and haemoglobin reached minimum values 12 - 13 weeks after infection, i.e. around patency. Following treatment both parameters returned to levels comparable with control animals, but after reinfection a fall in both P.C.V. and haemoglobin was again observed.

This picture contrasted sharply with that found following experimental infection of calves (Figs. 3.3 & 3.4) where both P.C.V. and haemoglobin values closely paralleled those of non-infected control animals, and the only deviations from normal which were detected were slight falls in haemoglobin values around patency, i.e. 12 - 13 weeks following primary infection and

again 11 - 12 weeks after reinfection. It is noteworthy that the changes which occurred in experimental calves were much less marked than those in experimental sheep.

The results of monitoring numbers of white and red blood cells during primary and secondary F. hepatica infection in sheep and cattle are illustrated in Figs. 3.5 - 3.8. It emerged that a fall in the total number of red blood cells commenced 8 - 9 weeks after primary infection of experimental sheep and the level gradually fell to a minimum value 13 - 14 weeks after infection (Figs. 3.5 & 3.6). Following treatment the number of red blood cells was restored to within the normal range, but a slight fall was again observed 4 weeks after reinfection. A rise in the numbers of white blood cells was noted 6 - 7 weeks after primary infection. During reinfection this phenomenon was not repeated, indeed there was a slight reduction in numbers during this time.

Changes in blood cells following experimental F. hepatica infection in calves (Figs. 3.7 & 3.8), included a slight fall in numbers of red blood cells 7 - 8 weeks after primary infection. Much more dramatic however was a significant rise in the number of white blood cells also commencing 7 - 8 weeks after primary infection which reached a peak value around patency, i.e. 12 - 13 weeks after infection. Following reinfection the total number of white blood cells remained elevated compared with control values, but gradually returned to normal 9 - 10 weeks after reinfection. The primary rise in white cells was greater and more prolonged than that described following primary F. hepatica infection in experimental sheep.

A peripheral eosinophilia was recorded following fluke infection in both ruminant species (Figs. 3.9 & 3.10). In both cases a rise occurred

2 weeks after experimental infection. Peak values were recorded 4 - 5 weeks after primary infection in sheep and 12 - 13 weeks following bovine infection. Following treatment there was a rapid fall in eosinophilia in sheep and after reinfection a sharp increase again took place. In the bovine situation a peak value for eosinophils again occurred 2 - 3 weeks after reinfection. Thereafter there was a gradual decrease in numbers of eosinophils, although the level remained elevated after reinfection. In both species the changes in eosinophils paralleled those in total white cells, particularly in the bovine where a strikingly similar patter of elevation was evident 12 - 13 weeks after primary infection.

Biochemical Changes

Serum G.G.T. and G.L.D.H. activities are illustrated in Figs. 3.11 - 3.14 and Figs. A.10 - A.17 of Appendix.

The former illustrations (Figs. 3.11 & 3.12) which show changes in the above liver enzymes following F. hepatica infection in sheep show that serum G.L.D.H. began to rise 2 weeks after infection and rose steadily to reach a peak value around 10 weeks after infection. Following treatment G.L.D.H. levels fell sharply, but rose again 2 weeks after reinfection, and thereafter fluctuated at an elevated level. Peak values of G.L.D.H. during reinfection were found to be much lower than those reached during primary infection. In contrast serum G.G.T. activity did not rise until 6 - 7 weeks of infection, but thereafter rose steadily to reach a maximum value 9 - 10 weeks after infection. Following treatment the level of this enzyme also dropped sharply and following reinfection was slightly elevated before a rise occurred around 9 weeks after reinfection.

Figs. 3.13 & 3.14 record the changes in serum G.G.T. and G.L.D.H. following experimental infection of calves with F. hepatica. From Fig. 3.14 it can be seen that G.L.D.H. activity increased sharply 2 weeks after primary infection and continued to increase steadily prior to treatment, reaching 3 distinct peak values, the highest of which occurred around 10 weeks after infection. G.L.D.H. activity fell sharply following treatment and levels of the enzyme fluctuated at a slightly elevated level during reinfection. Serum G.G.T. activity increased around 9 - 10 weeks of infection then rose steadily to peak 5 - 6 weeks later. Again levels of this enzyme fell sharply on treatment and continued to decrease during reinfection, reaching normal values 6 weeks after reinfection. Thereafter G.G.T. activity remained within normal limits. As in the experimental ovine infection levels of both enzymes during reinfection were much lower than those reached during primary infection.

Serological changes

Double immunodiffusion

The results from primary and secondary infection of sheep and cattle with F. hepatica as outlined in Table III.1, are shown in Tables III.3 - III.6.

Table III.3 : Double immunodiffusion results

Ovine primary infection

Sheep No.	Weeks of infection												
	1	2	3	4	5	6	7	8	9	10	11	12	13
S1	-	-	-	-	+	+	+	+	+	+	+	+	-
S5	-	-	-	-	+	+	+	+	+	+	+	+	-
S6	-	-	-	-	+	+	+	+	+	+	+	+	-
S7	-	-	-	+	+	+	+	+	+	+	+	+	-

Table III.4 : Double immunodiffusion results

Ovine secondary infection

Sheep No.	Weeks after reinfection											
	1	2	3	4	5	6	7	8	9	10	11	12
S1	-	+	+	+	+	+	+	-	+	+	+	+
S5	+	+	+	+	+	+	+	-	+	+	+	+
S6	+	+	+	+	+	+	+	+	+	+	+	-
S7	+	+	+	-	+	+	+	+	+	+	+	+

Serum samples from non-infected control sheep proved consistently negative in this test.

Calf No.	Weeks of infection											
	1	2	3	4	5	6	7	8	9	10	11	12
C2	-	+	+	+	+	+	+	-	+	+	+	+
C3	+	-	-	-	-	-	-	-	-	-	-	-
C4	-	-	+	+	+	+	+	+	+	-	+	-

Table III.5 : Double immunodiffusion

Bovine primary infection

Calf No.	Weeks of infection											
	1	2	3	4	5	6	7	8	9	10	11	12
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	-	-	-	-	-	-	-	-	-	-	-	-
C4	+	+	+	+	+	+	+	+	+	+	+	+

Table III.6 : Double immunodiffusion

Bovine secondary infection

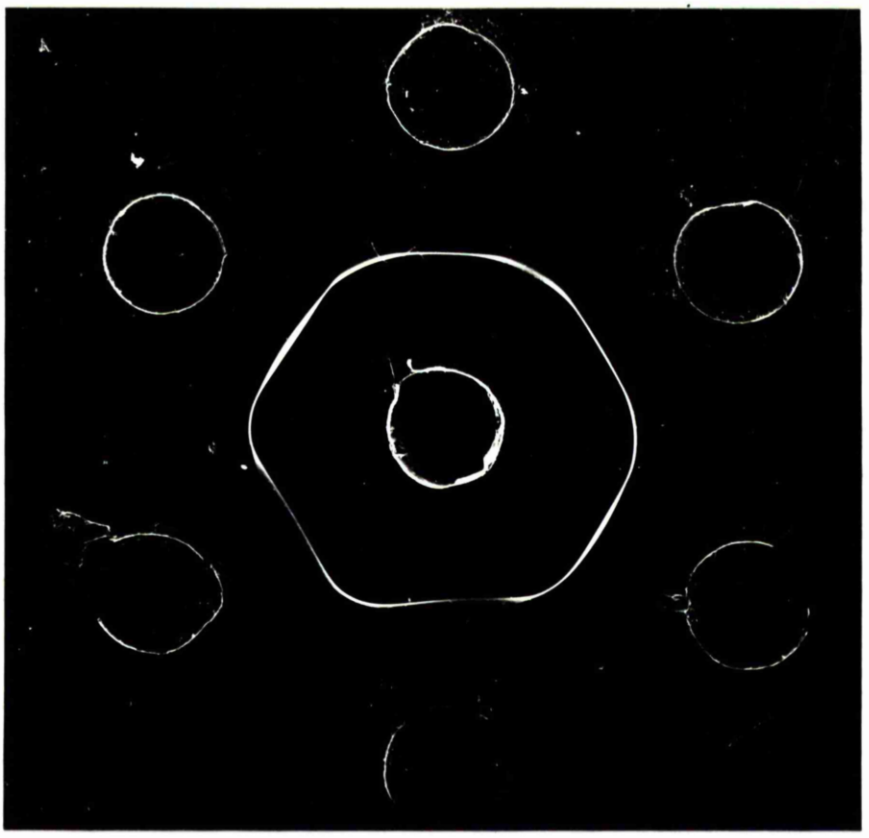


Plate 3. Double Immunodiffusion (D.I.D.): Precipitin lines obtained from lipid-free extract (L.F.E.) of F. hepatica and positive ovine serum.

Due to the speculation regarding the suitability of D.I.D. for use in the bovine (see Chapter I), C3, the non-infected control animal was monitored throughout primary and secondary infection. It was noted that on only one occasion (see Table III.5) was a false positive reaction obtained.

As can be seen from Table III.3 precipitins were first detected in the serum of infected sheep 4 - 5 weeks after primary infection, and were in evidence from this time until week 13 when all sera proved negative. On reinfection antibodies were detected, with one exception as early as 1 week after reinfection. Sera proved consistently positive throughout reinfection with a few exceptions (e.g. S1 and S5, 8 weeks after reinfection).

An essentially similar result was obtained from analysis of serum from infected and reinfected cattle (Tables III.5 & III.6). Following primary infection antibodies were detected at 2 - 3 weeks, and serum proved consistently positive throughout 11 weeks of infection. On reinfection after only 1 week a positive result was obtained, and all sera analysed were positive up to 12 weeks after reinfection.

It was noted that the lines obtained from D.I.D. performed on sheep sera were much sharper, before and after staining, than those obtained using bovine serum (Plate 3).

I.H.T. (Plate 4)

The results of I.H.T. performed on sera from experimentally infected sheep and cattle are shown in Figs. 3.15 & 3.16 respectively, and individual responses appear in Figs. A.18 - A.21 of Appendix. In order to increase the accuracy of the quantitative estimation of serum antibody content all sera were titrated against two separate batches of antigen-coated cells, one prepared by the author on the laboratory bench, and the other kindly supplied by Drs. H.J. Over and L.J. Van Tiggele, Lelystad, The Netherlands.

It is evident from Figure 3.15 that I.H.T. titres started to rise around 2 weeks after experimental infection of sheep and reached peak values at 4 - 5 weeks. After this time there was an appreciable fall in antibody levels which increased again to reach twin peaks at 7 - 8 weeks and 11 - 12 weeks of infection. After reinfection there was an immediate massive increase in I.H.T. titres which fluctuated at abnormal levels while gradually declining during the 12 weeks of reinfection. It was noted that I.H.T. titres were higher in all cases using cells prepared in the laboratory compared with the cells supplied in the Netherlands. The pattern of antibody production during infection and reinfection was found to be the same, however, regardless of the source of the antigen-coated cells.

Following primary infection of experimental calves with F. hepatica (Fig. 3.16) a similar pattern of antibody production was demonstrable. In this case antibody levels again rose 2 - 3 weeks following infection gradually increasing to reach twin peak values 9 - 10 and 12 - 13 weeks after infection. It was noticeable that no such fall in antibody titre occurred in cattle as was observed 5 - 6 weeks following experimental infection of sheep. Following reinfection of experimental calves there was a much more gradual increase in antibody level than that noted in sheep. It was again noted that cells produced in the laboratory gave higher I.H.T. titres than cells supplied by Dr. H. J. Over, although the overall picture of antibody production was similar. By comparison of Figs. 3.15 & 3.16 it is evident that more false positive reactions were obtained during analysis of ovine compared with bovine serum. This was felt to be due to sophistication in the laboratory technique employed since the analysis of bovine serum was carried out subsequent to the analysis of the ovine serum.

Enzyme-linked immunosorbent assay (E.l.i.s.a.) (Plate 5)

The results of E.l.i.s.a. following primary infection of experimental sheep are documented in Figs. 3.17 & 3.18. Antibody levels began to rise 2 - 3 weeks post infection, until 5 - 6 weeks when a fall in titre occurred. Thereafter antibody titres rose steadily to peak at 7 and 9 weeks before gradually decreasing for the remainder of the infection period. A limited number of results obtained on reinfection of sheep suggested that a dramatic rise in antibody titre occurred within 1 week of reinfection of experimental sheep, since absorbance values within the range 0.8 - 1.0 were obtained in this period. The difficulties associated with the above technique precluded the possibility of further quantitative values of antibody production being obtained, i.e. during the greater part of the reinfection period of experimental sheep, and for the duration of experimental infection in cattle. It is felt by the author however that discussion of the various difficulties encountered with this technique may prove of more practical value to other authors than mere presentation of results. For this reason what results as are meaningful in the opinion of the author are presented and the shortcomings of the technique are described in some detail in a special discussion section.

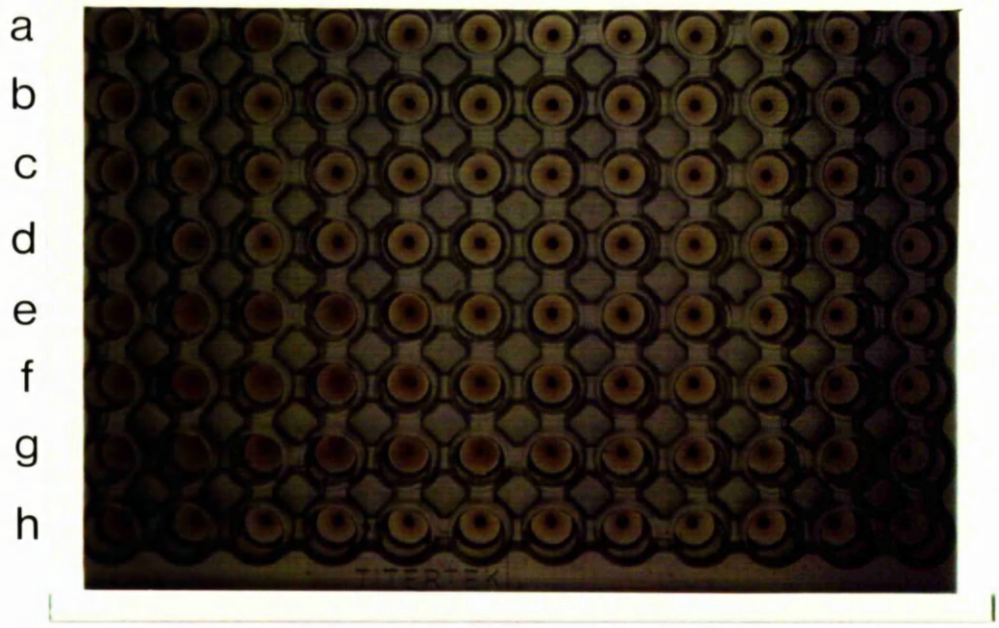


Plate 4. Indirect haemagglutination (I.H.T.): Titration of serum antibody in sheep undergoing primary F. hepatica infection (S1, S5, S6, S7 : Rows A, E, F. G) compared with non-infected controls (S2, S3, S4, S8 : Rows B, C, D, H).

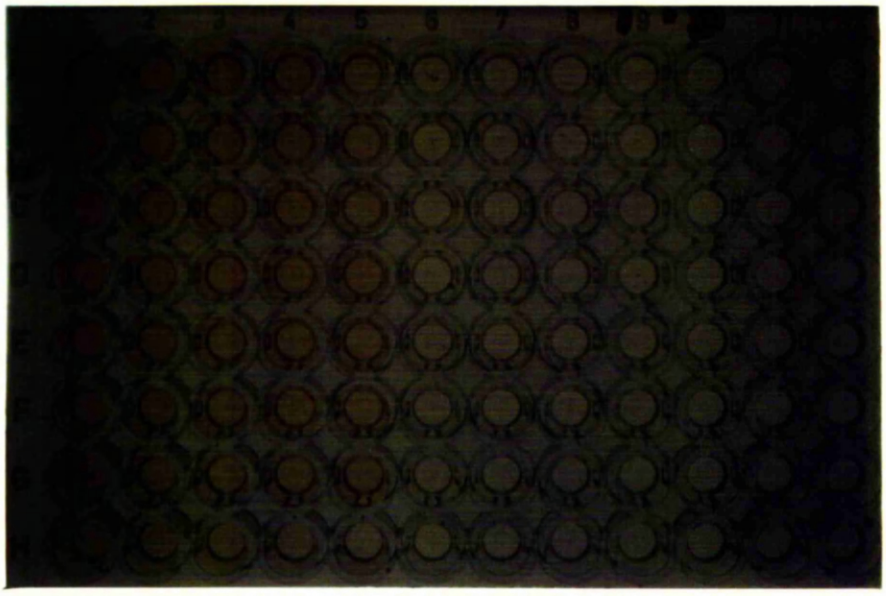


Plate 5. Enzyme-linked immunosorbent assay (E.l.i.s.a.): Chequerboard titration differentiating positive (Rows 1 - 5) and negative (Rows 6 - 10) sera.

Passive Transfer Studies

The number of rats examined for presence of flukes are shown in Table III.7. During the experiment 3 rats died, from which no flukes were recovered. One rat was lost from each of groups 1, 3 and 5. Fluke recoveries from the various groups are summarised in Table III.8.

Group No.	No. of rats	Type of serum
1	7	6 week
2	8	10 week
3	7	14 week
4	8	Normal
5	7	-

Table III.7 : Passive Transfer Experiment 1

As previously described the serum used was obtained from sheep at different times after F. hepatica infection. The numbers of flukes recovered from each group are shown in Table III.8.

Group 1	Group 2	Group 3	Group 4	Group 5
2	1	0	0	4
2	1	5	2	4
5	1	3	2	4
1	2	3	4	6
0	1	0	0	1
4	3	3	4	3
	1	2	2	3
	0		1	

Table III.8 : Passive Transfer Experiment 1

Individual fluke burdens

A statistical breakdown of this data appears in Table III.9

Group No.	Mean fluke burden ± S.D.	% Reduction vs control	Probability 'p'
1	2.3 ± 1.7	36%	*N.S.
2	1.3 ± 0.9	64%	p .01
3	2.3 ± 1.8	36%	N.S.
4	1.9 ± 1.6	47%	N.S.
5	3.6 ± 1.5		

Table III.9: Passive Transfer Experiment 1

Statistical breakdown of fluke recoveries

* N.S. = not significant

Percentage reductions in fluke recoveries are expressed relative to control group 5. Student's 't' test was used to compare individual treatment groups with the control group.

Parasitological Results

Faecal examination. Sheep. Following primary infection of sheep with F. hepatica metacercariae (Table III.1) ova were first detected in the faeces of all animals (S1, S5, S6, S7) at 12 weeks. Following treatment and reinfection, however, F. hepatica ova did not reappear until 11 weeks. It was noted that at this time no F. hepatica ova had been detected in the faeces of sheep given a primary infection, at the same time as reinfection was carried out i.e. secondary control animals (S2, S3, S4, S8 in Table III.1).

Cattle. F. hepatica ova were first detected in the faeces of experimentally infected calves, C2 and C4, (see Table III.1), 12 weeks after primary infection. After treatment and reinfection this period was again found to be 12 weeks.

Post-mortem findings

Sheep. An arbitrary classification on the basis of gross pathology of the livers of experimentally infected sheep is illustrated in Table III.10.

Lamb No.	Designation	Fibrosis	Haemorrhage	Calcification
S1	1^{o} & 2^{o}	+	-	-
S2	1^{o}	+	+	+
S3	1^{o}	+	-	-
S4	1^{o}	+	-	-
S5	1^{o} & 2^{o}	++	-	-
S6	1^{o} & 2^{o}	+++	-	-
S7	1^{o} & 2^{o}	+++	-	-
S8	1^{o}	+	-	-

Table III.10; Classification of liver lesions in fluke-infected sheep

1^{o} = Primary intection only of 350 metacercariae
1^{o} & 2^{o} = Primary and secondary infections.
Secondary infection = 250 metacercariae.

The numbers and relative sizes of the flukes recovered from experimental sheep are shown in Table III.11.

Lamb No.	Designation	No. of flukes >12 mm	6-12 mm	<6 mm	Total
S1	1^{o} & 2^{o}	32	8	2	42
S2	1^{o}	18	3	1	22
S3	1^{o}	10	14	0	24
S4	1^{o}	21	20	1	42
S5	1^{o} & 2^{o}	7	3	0	10
S6	1^{o} & 2^{o}	18	14	0	32
S7	1^{o} & 2^{o}	15	9	1	25
S8	1^{o}	8	3	1	12

Table III.11: Fluke recoveries

Experimental sheep

A statistical analysis of the fluke recoveries from these animals appears in Table III.12.

Group Designation	Lamb No.	Total No. Flukes	Mean ± S.D.	% Reduction vs. control	*Probability 'p'
Reinfected	S1	42	27 ± 13 S.E.7	+8.0	N.S.
	S5	10			
	S6	32			
	S7	25			
Primary infection	S2	22	25 ± 12 S.E.6		
	S3	24			
	S4	42			
	S8	12			
Reinfected	C2	151	109 ± 59 S.E.42		
	C4	67			
Non-infected control	C3	0			

Table III. 12: Statistical breakdown

Experimental sheep and cattle

* 'p' value using Student's 't' test
N.S. = Not significant

From Table III.12 it is clear that there was no reduction in numbers of flukes recovered from reinfected sheep, when compared with animals receiving a primary infection. Further, the data in Table III. 11, provided no evidence to suggest that the fluke populations of the two groups differed in respect of size distribution.

Cattle

Table III. 13 describes the gross pathological features of the livers of experimentally infected calves C2, C3 and C4 (see Table III. 2).

Calf No.	Liver wt (kgs)	Fibrosis	Calcification	Haemorrhage
C2	5.0	++	++	-
C3	3.7	-	-	-
C4	4.5	++	+	-

Table III. 13: Classification of liver lesions in fluke-infected cattle

It is noteworthy from this table that calcification was a feature of the bile ducts of infected calves.

A breakdown of the numbers and sizes of flukes recovered from experimentally infected calves appears in Table III. 14.

Calf No.	Designation	Nos. of flukes >12 mm	6-12 mm	<6 mm	Total
C2	Reinfected	119	30	2	151
C4	Reinfected	24	35	8	67
C3	Non-infected control	-	-	-	-

Table III.14: Fluke recoveries

Experimental calves

Clearly, from the limited number of animals available, and the need for a non-infected control animal, no conclusions can be drawn from the above tables regarding the resistant status of experimental calves, in terms of reduction in fluke recoveries. Similarly no conclusions can be drawn from the size distribution of flukes recovered from experimental calves.

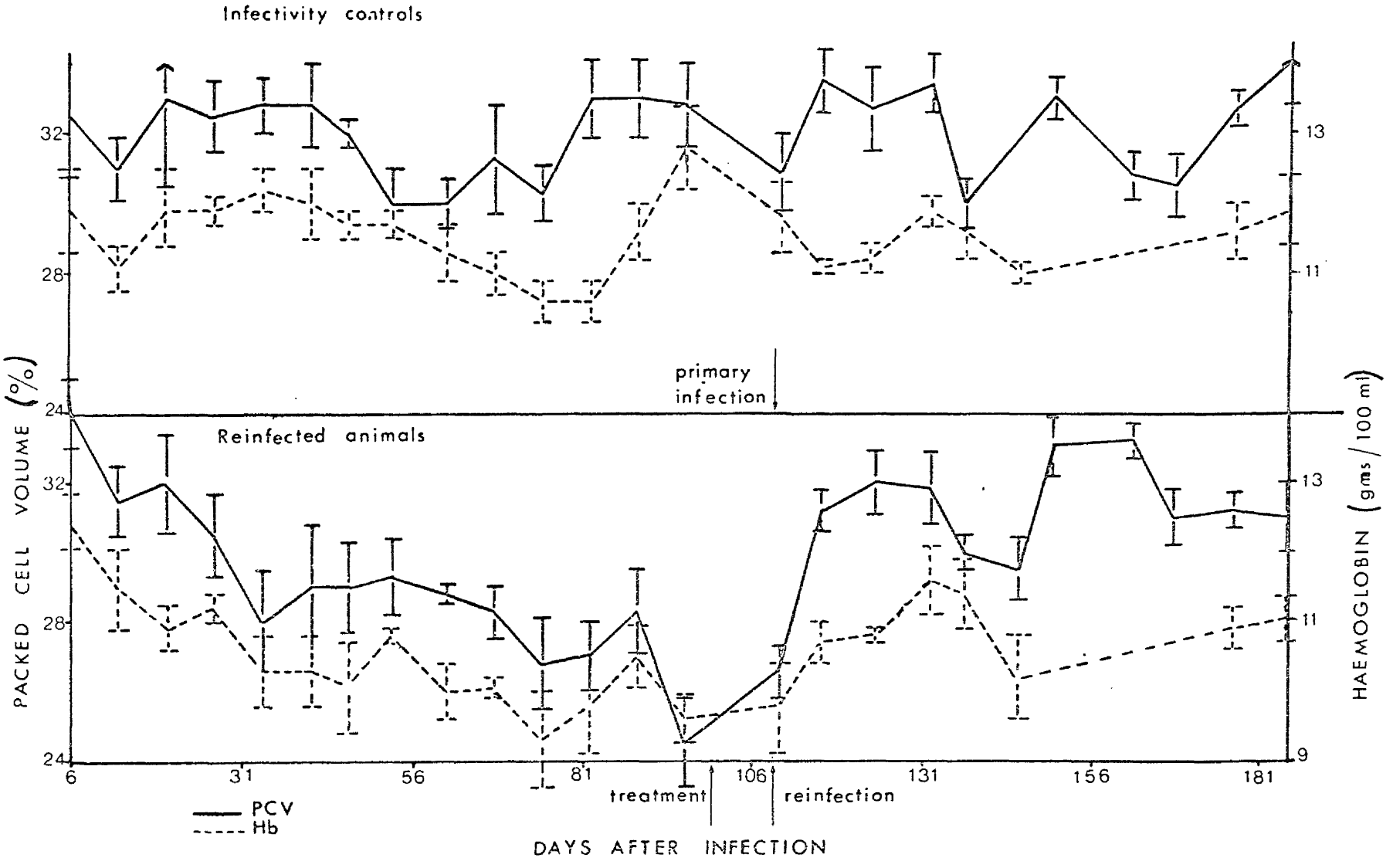


Fig. 3.1 Comparison of P.C.V. and haemoglobin in sheep given primary and secondary F. hepatica infections with a control group given a primary infection simultaneously with reinfection of the former, showing standard errors.

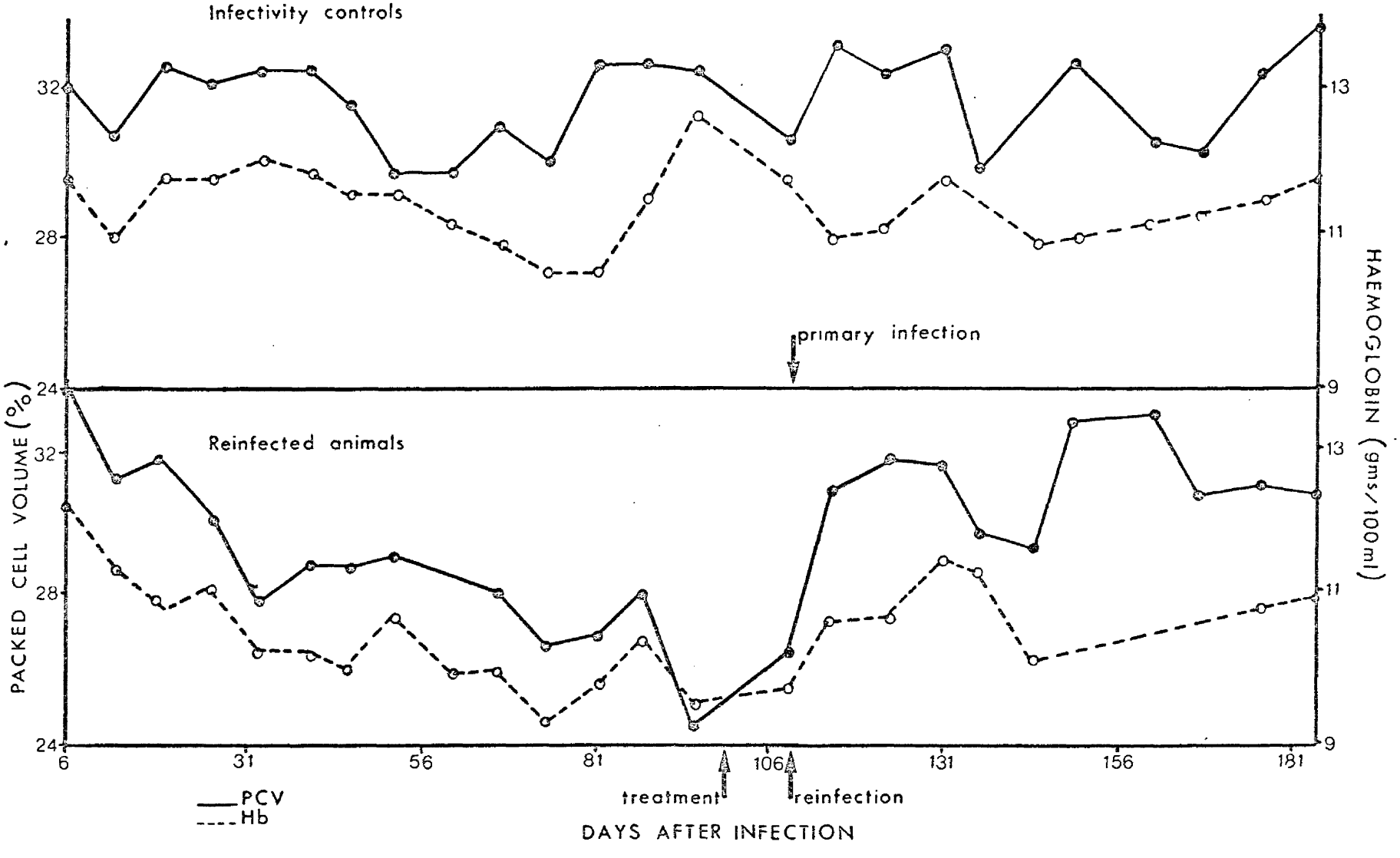


Fig. 3.2 Comparison of P.C.V. and haemoglobin in sheep given primary and secondary F. hepatica infections with a control group given a primary infection simultaneous with reinfection of the former.

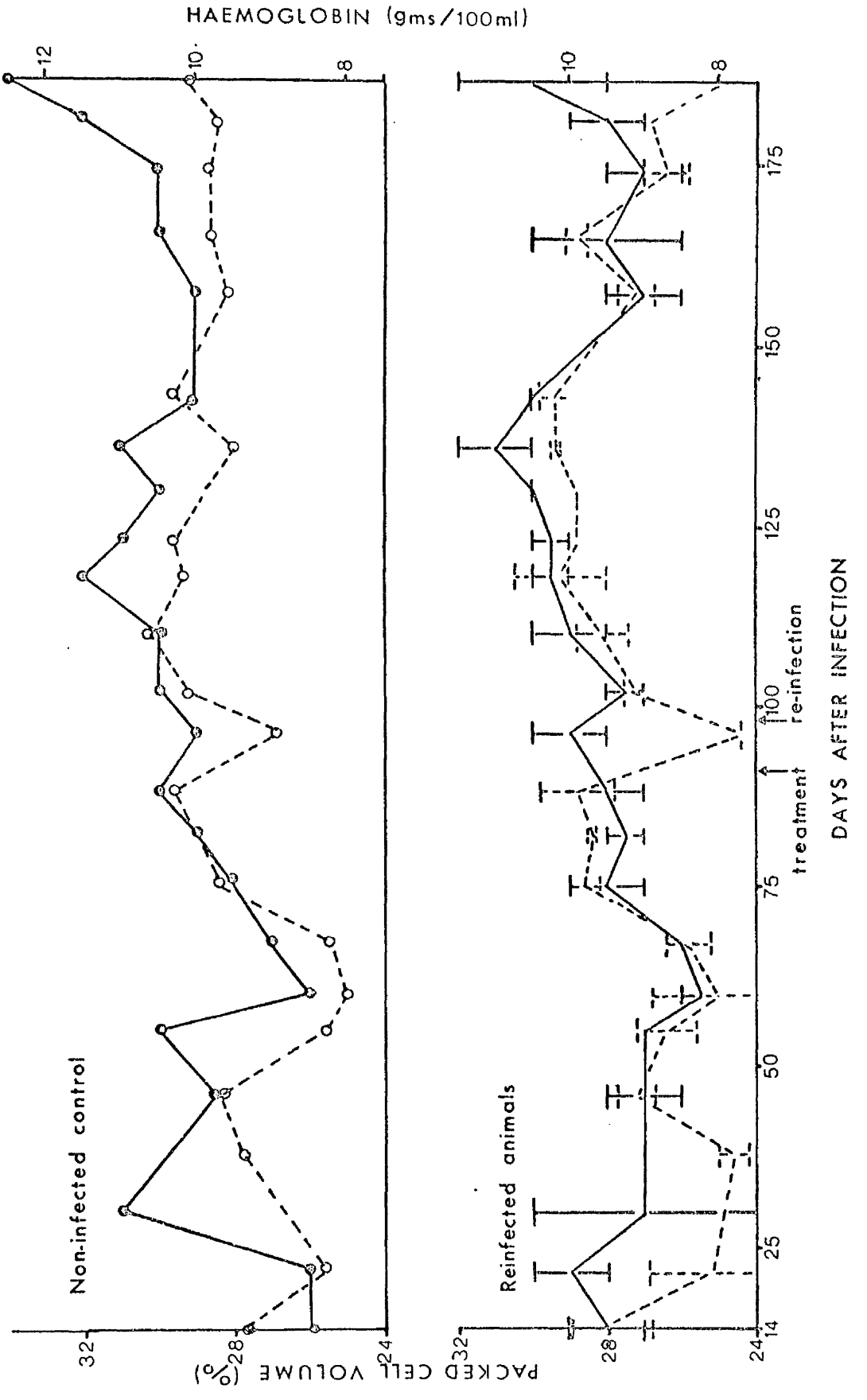


Fig. 3.3 Comparison of P.C.V. and haemoglobin in cattle given primary and secondary F. hepatica infections with non-infected control, showing standard errors.

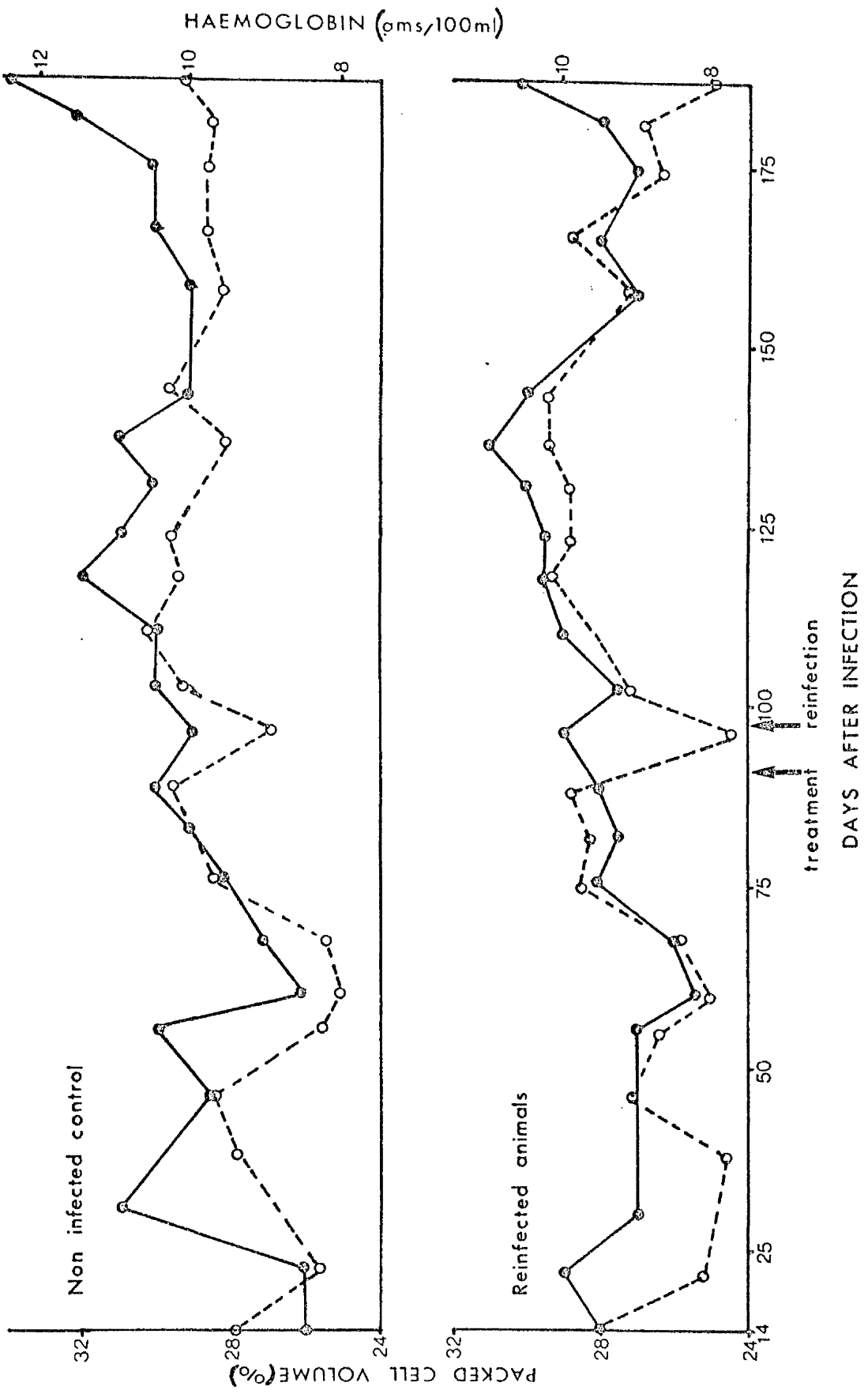


Fig. 3.4 Comparison of P.C.V. and haemoglobin in cattle given primary and secondary F. hepatica infections with non-infected control.

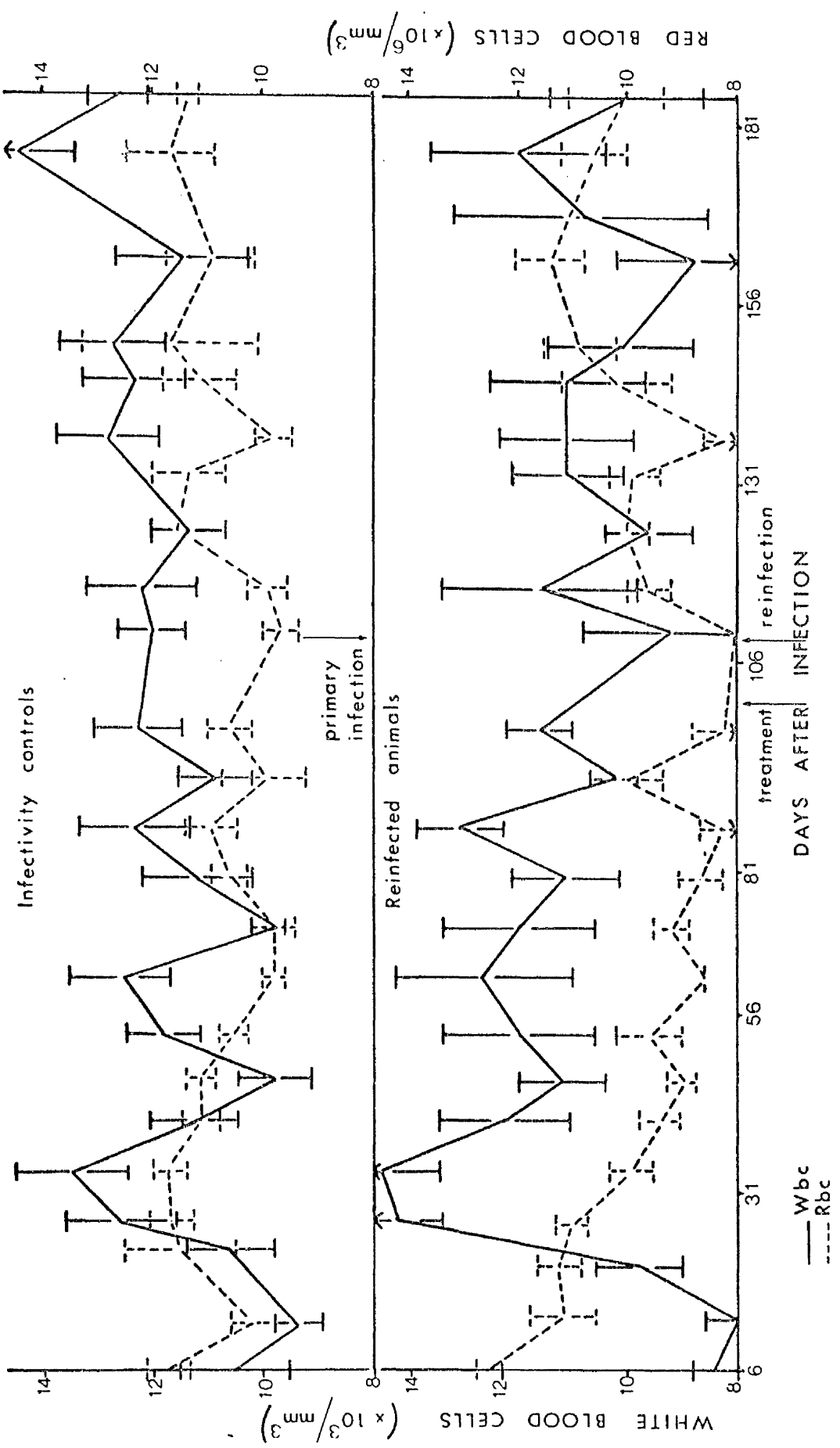


Fig. 3.5 Comparison of wbc and rbc counts in sheep given primary and secondary F. hepatica infections with a control group given a primary infection simultaneous with reinfection of the former, showing standard errors.

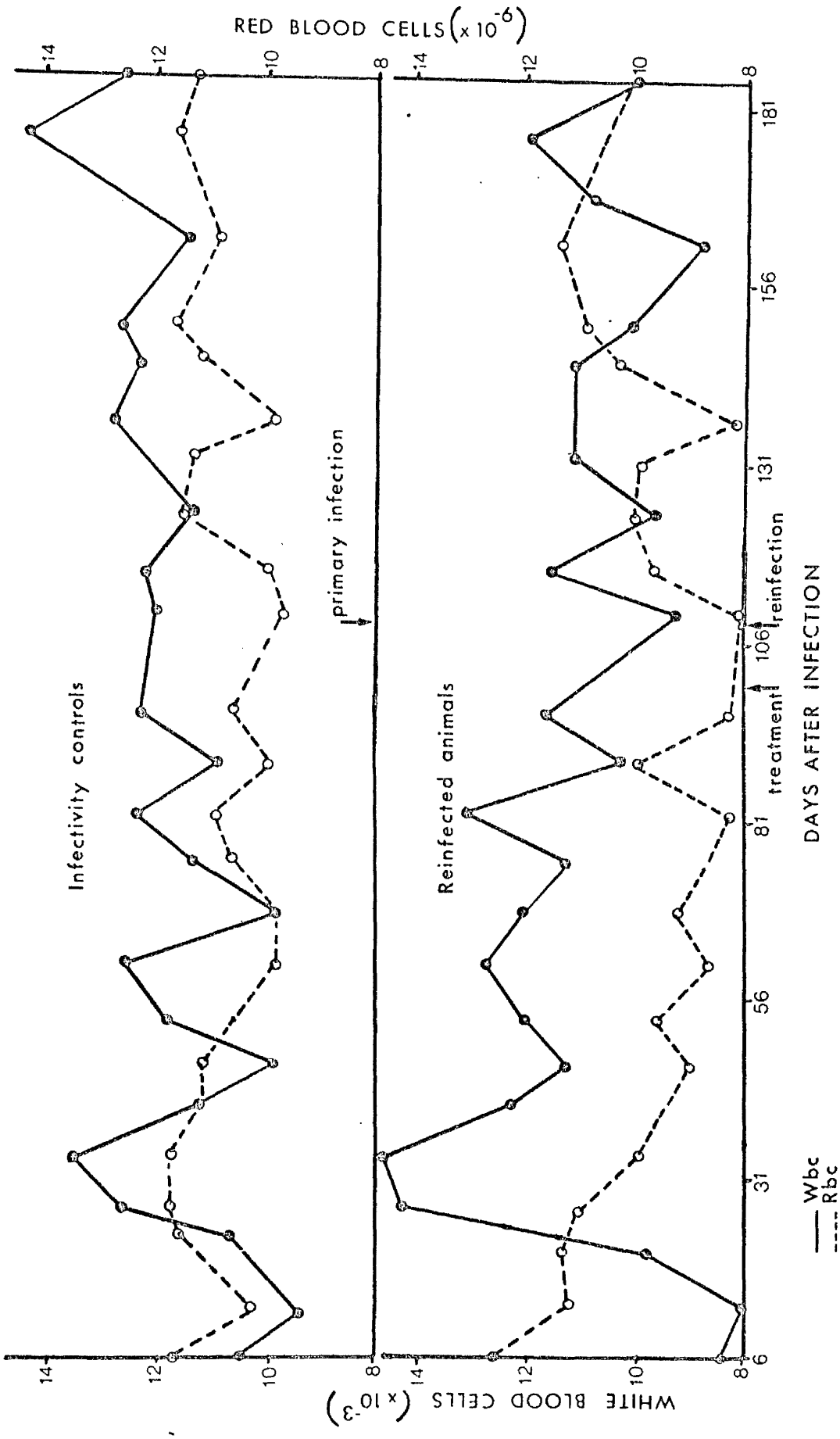


Fig. 3.6 Comparison of wbc and rbc counts in sheep given primary and secondary infections with a control group given a primary infection simultaneous with reinfection of the former.

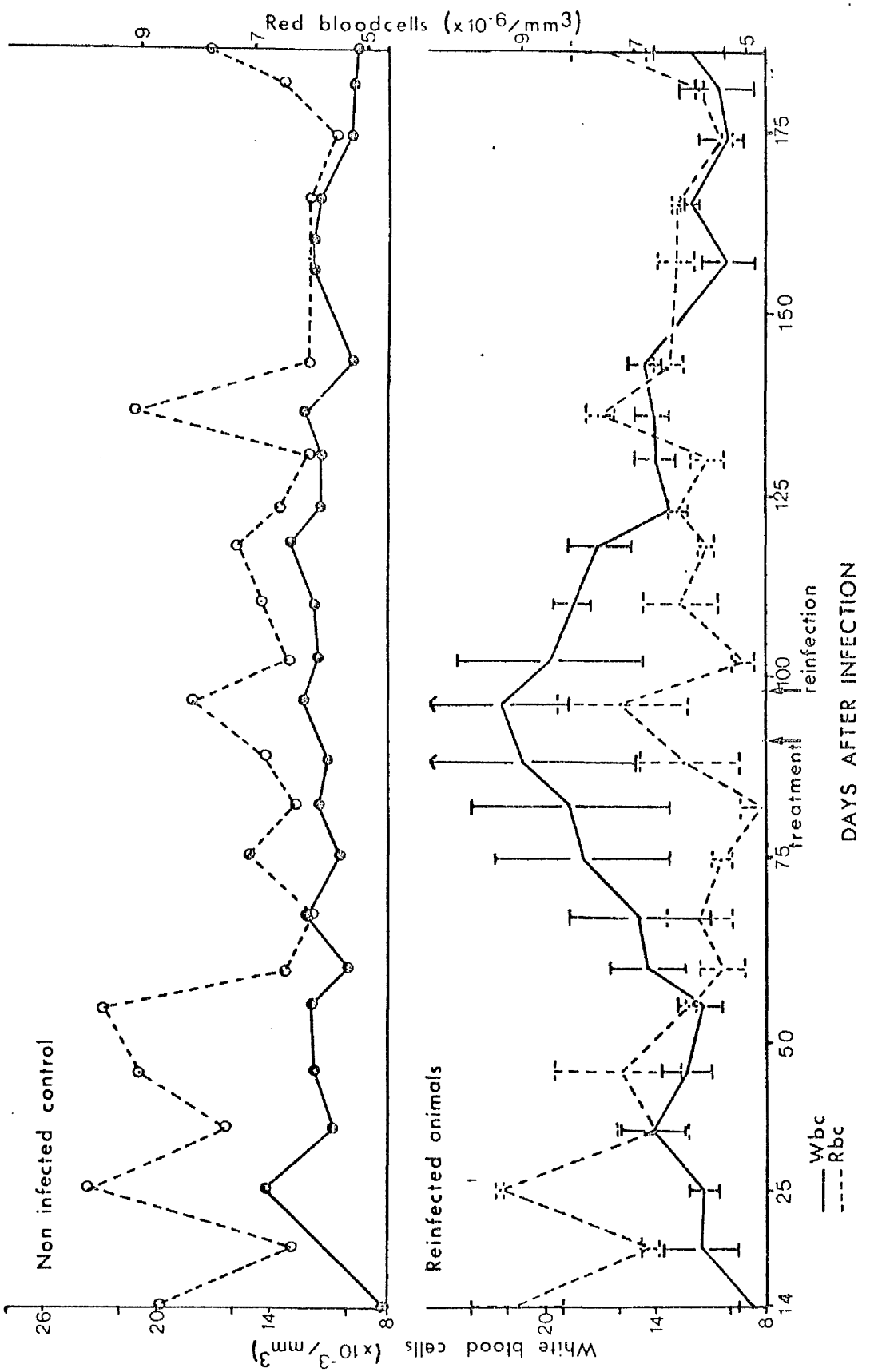


Fig. 3.7 Comparison of wbc and rbc counts in cattle given primary and secondary F. hepatica infections with non-infected control, showing standard errors.

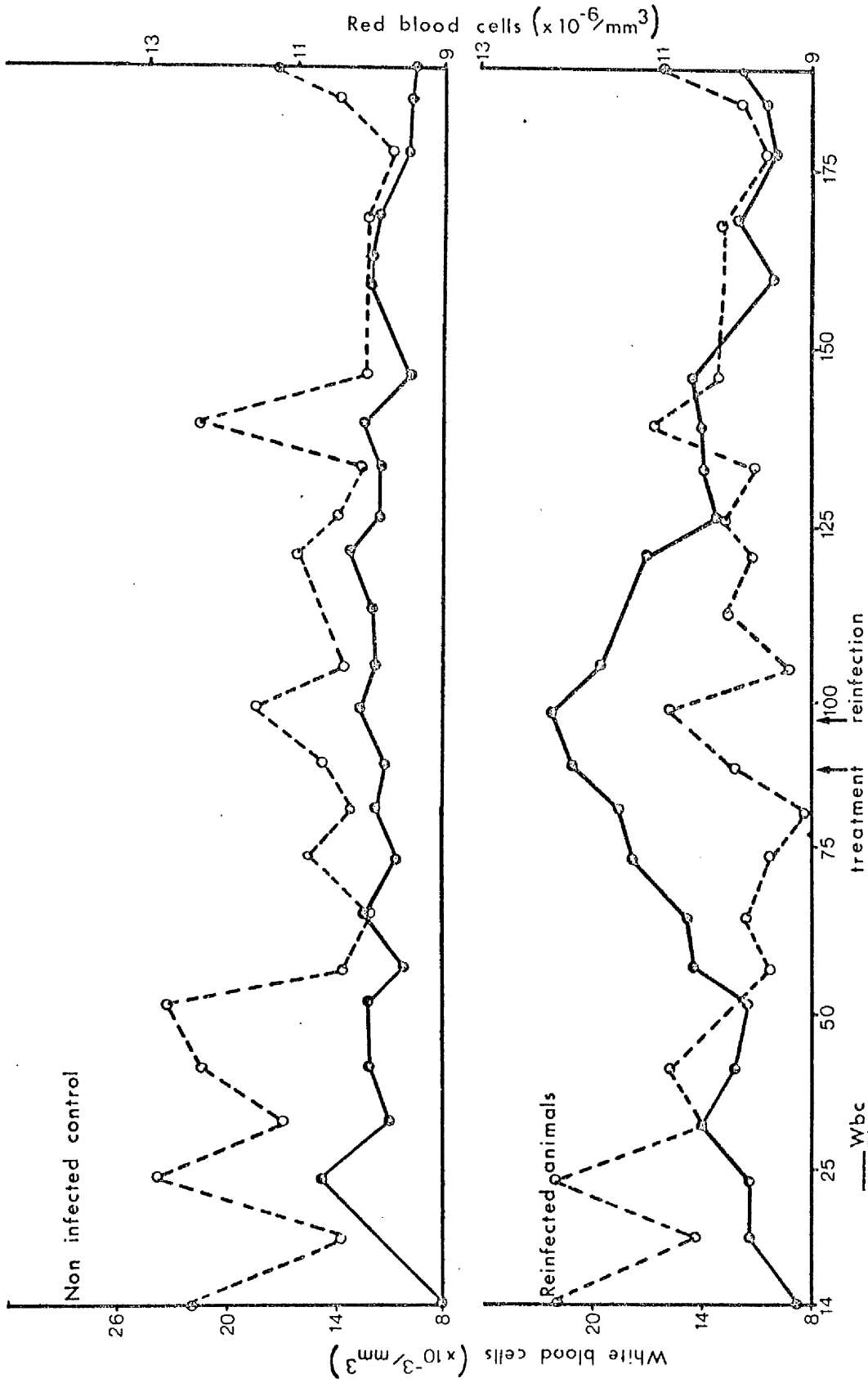


Fig. 3.8 Comparison of wbc and rbc counts in cattle given primary and secondary F. hepatica infections with non-infected control.

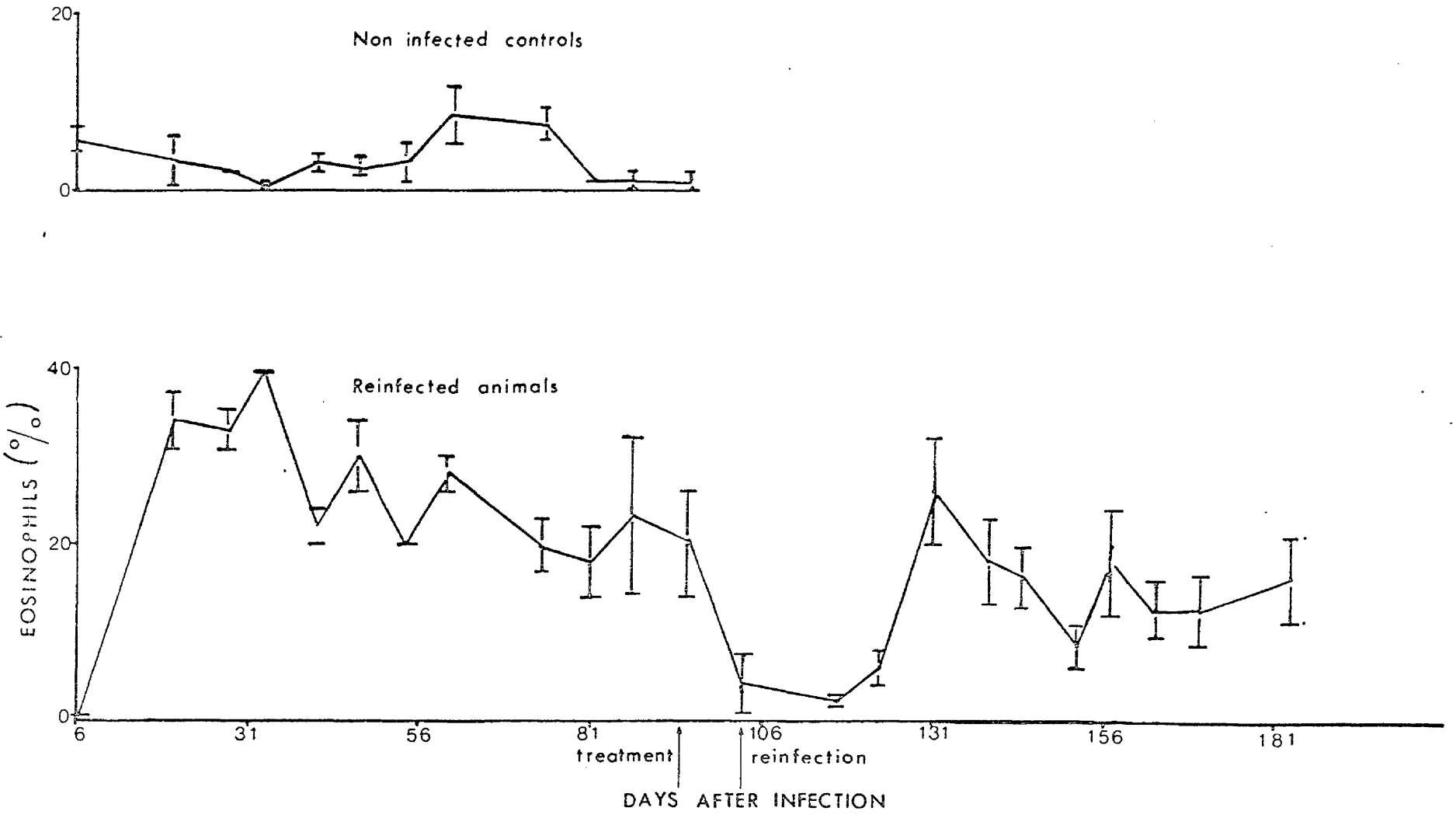


Fig. 3.9 Comparison of eosinophil counts in sheep given primary and secondary F. hepatica infections with non-infected controls, showing standard errors.

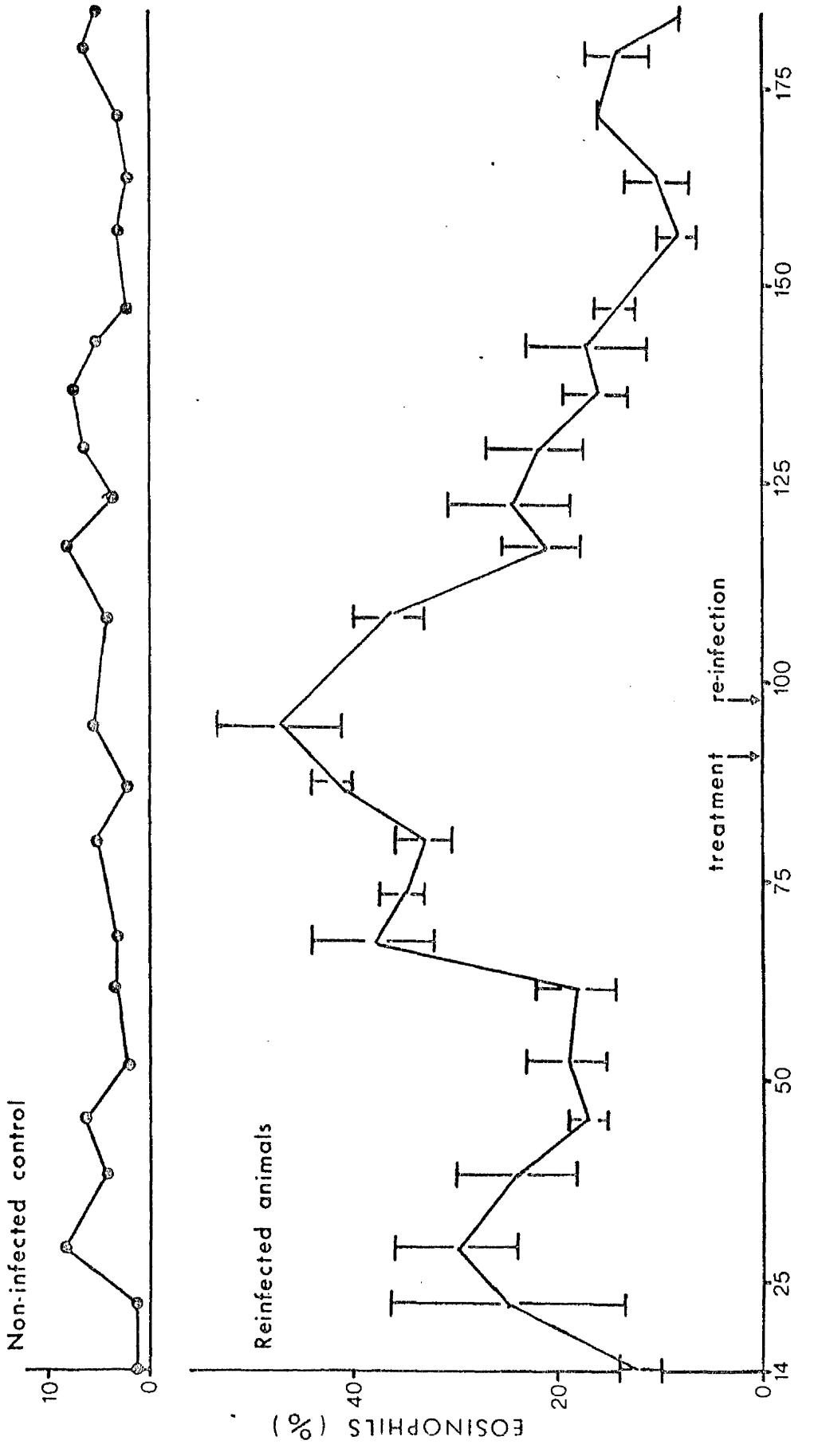


Fig. 3.10 Comparison of eosinophil counts in cattle given primary and secondary F. hepatica infections with non-infected control, showing standard errors.

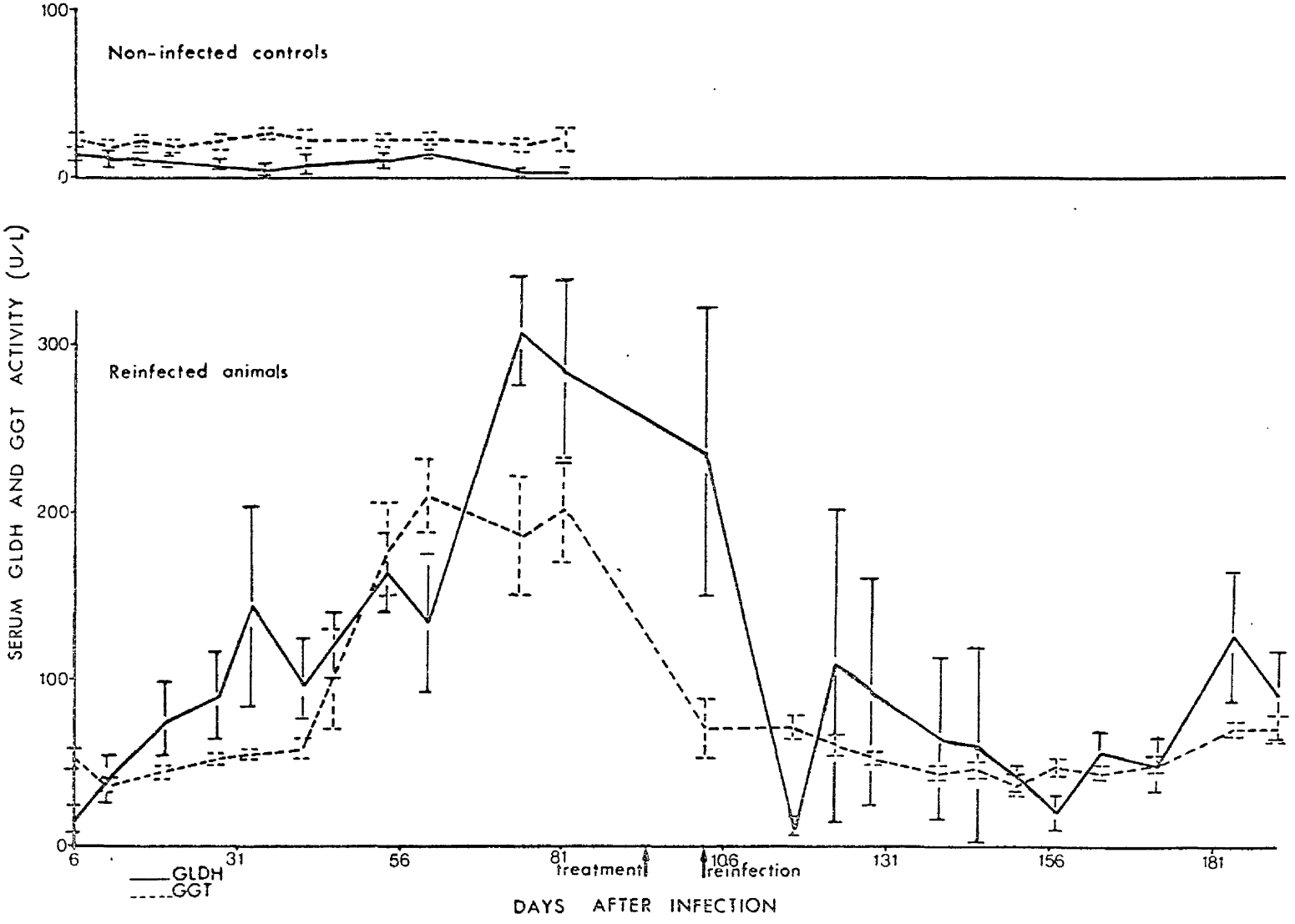
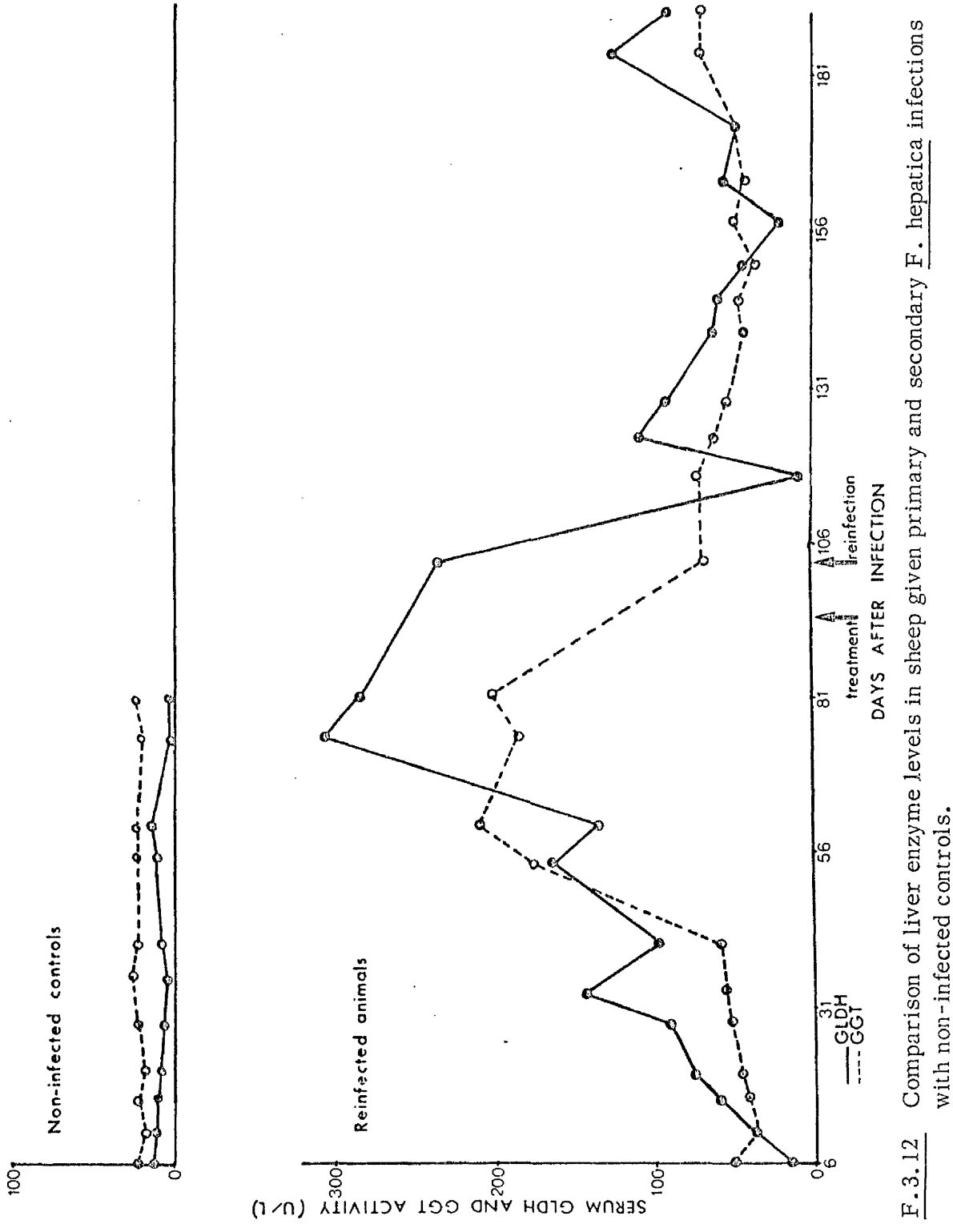


Fig. 3.11 Comparison of liver enzyme levels in sheep given primary and secondary F. hepatica infections with non-infected controls, showing standard errors.



F.3.12 Comparison of liver enzyme levels in sheep given primary and secondary F. hepatica infections with non-infected controls.

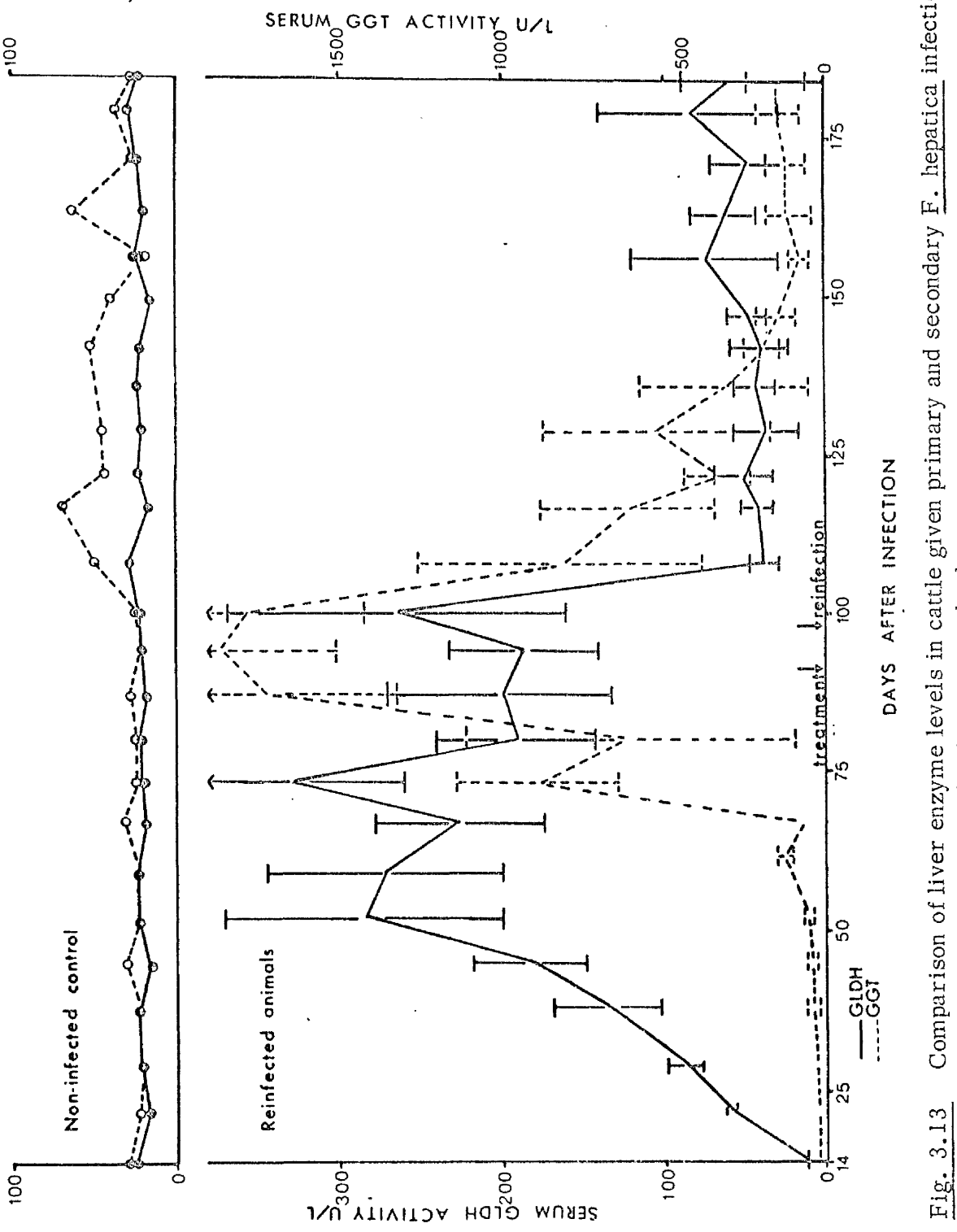


Fig. 3.13 Comparison of liver enzyme levels in cattle given primary and secondary F. hepatica infections with non-infected control, showing standard errors.

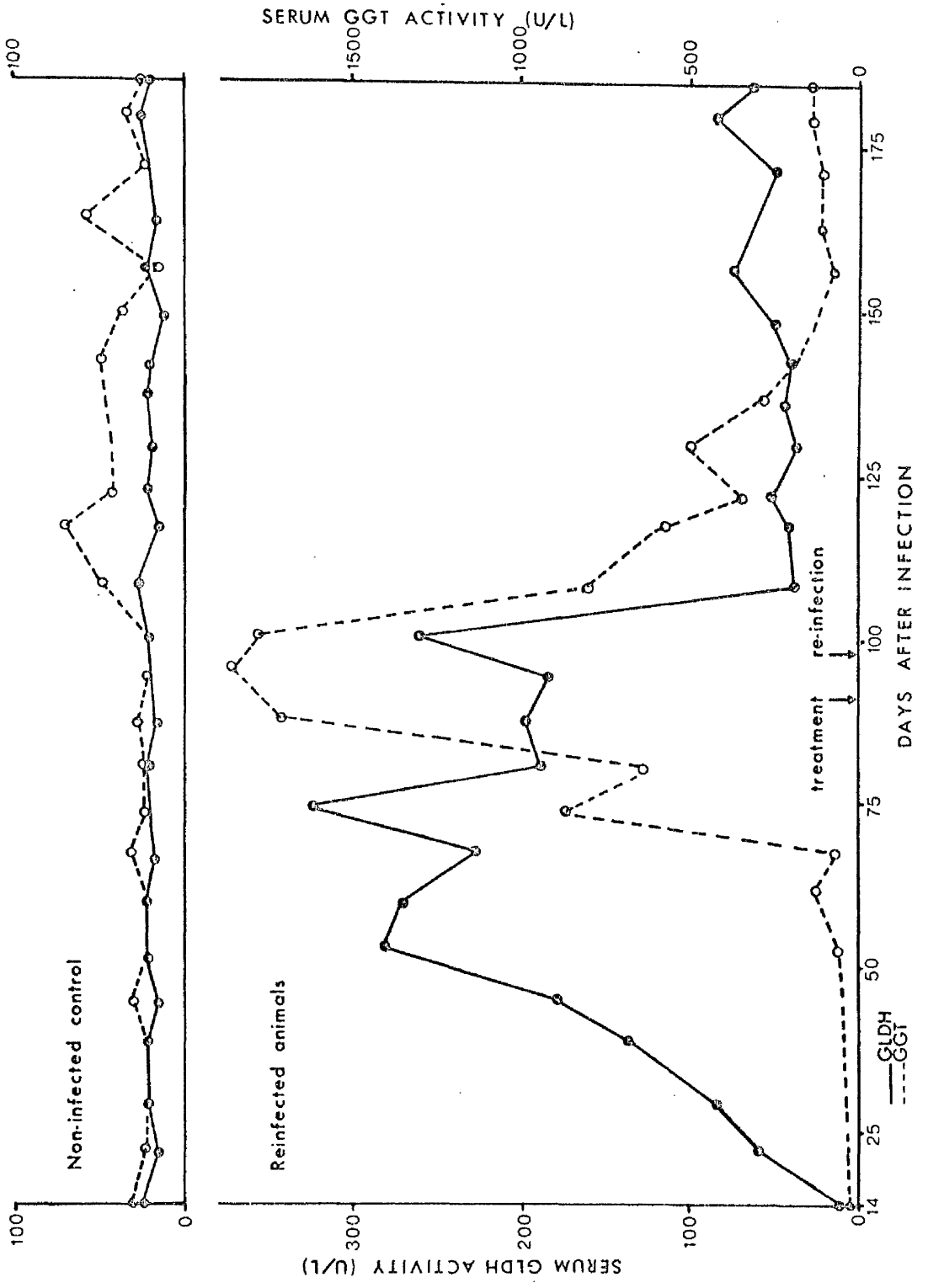


Fig. 3.14 Comparison of liver enzyme levels in cattle given primary and secondary F. hepatica infections with non-infected control.

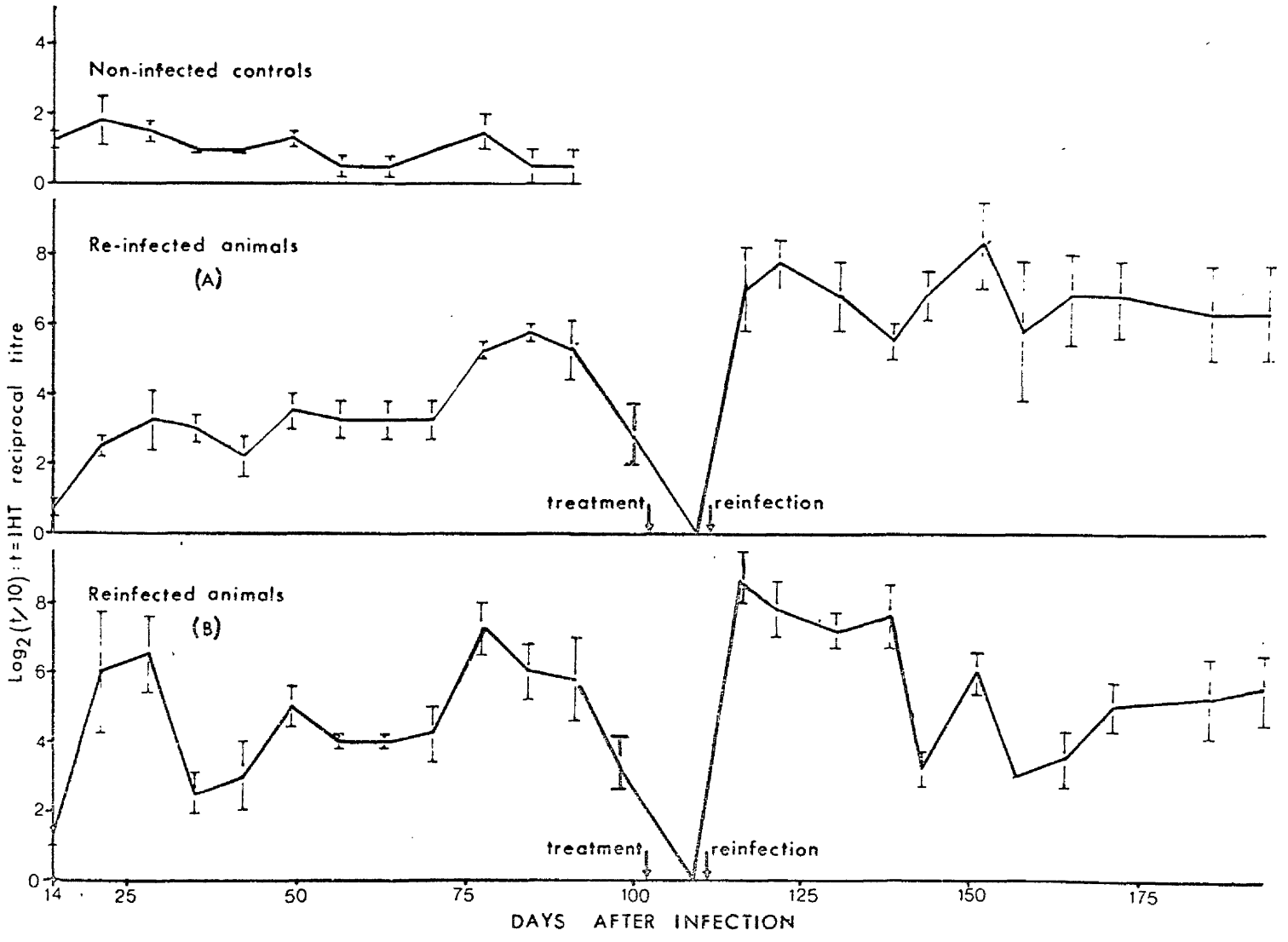


Fig. 3.15 Comparison of I.H.T. titres in sheep given primary and secondary F. hepatica infections with non-infected controls, showing standard errors.

(A) = cells prepared in Glasgow (B) = cells prepared in Rotterdam

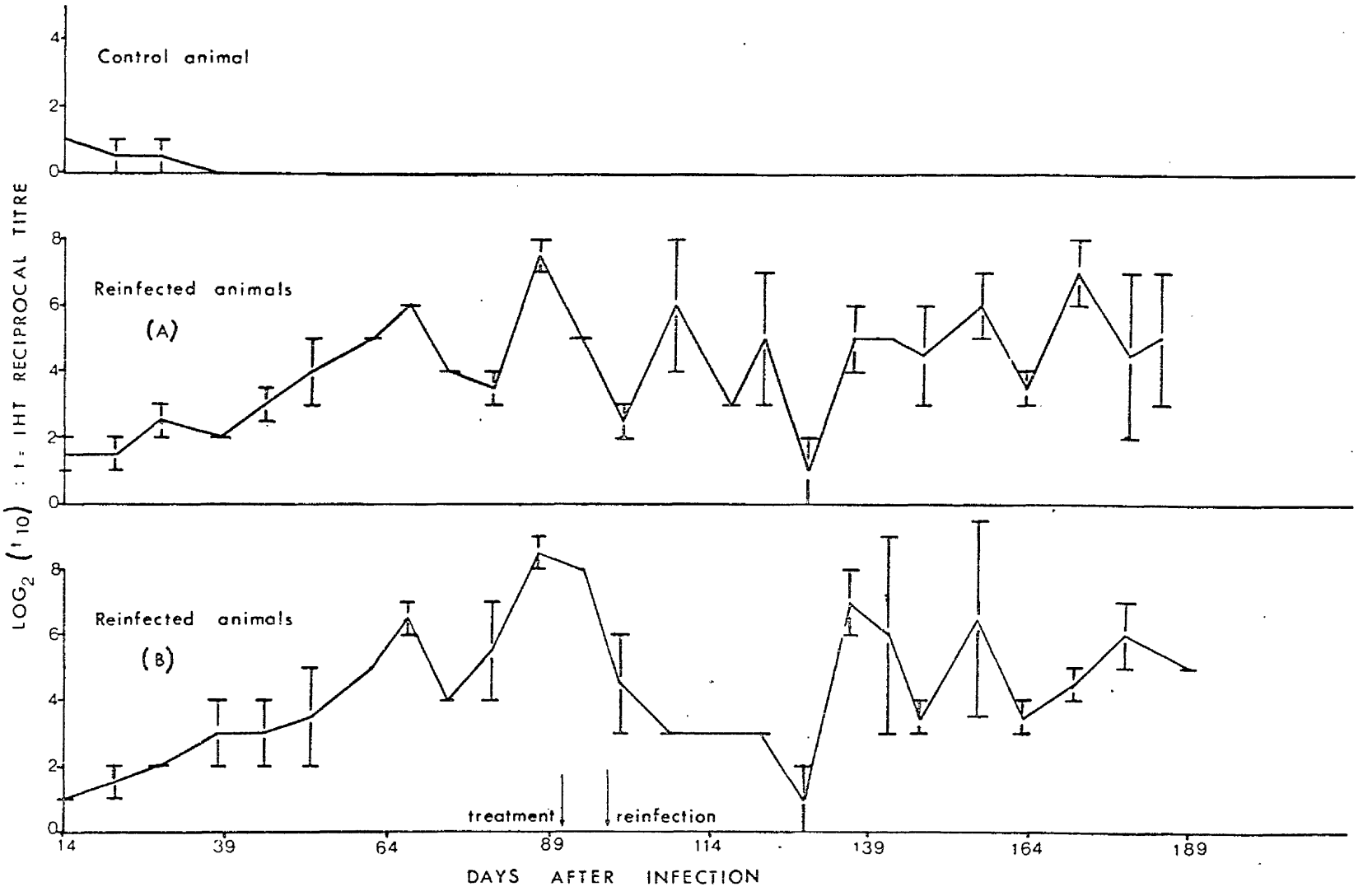


Fig. 3.16 Comparison of I.H.T. titres in cattle given primary and secondary F. hepatica infections with non-infected control, showing standard errors.

(A) = cells prepared in Glasgow (B) = cells prepared in Rotterdam

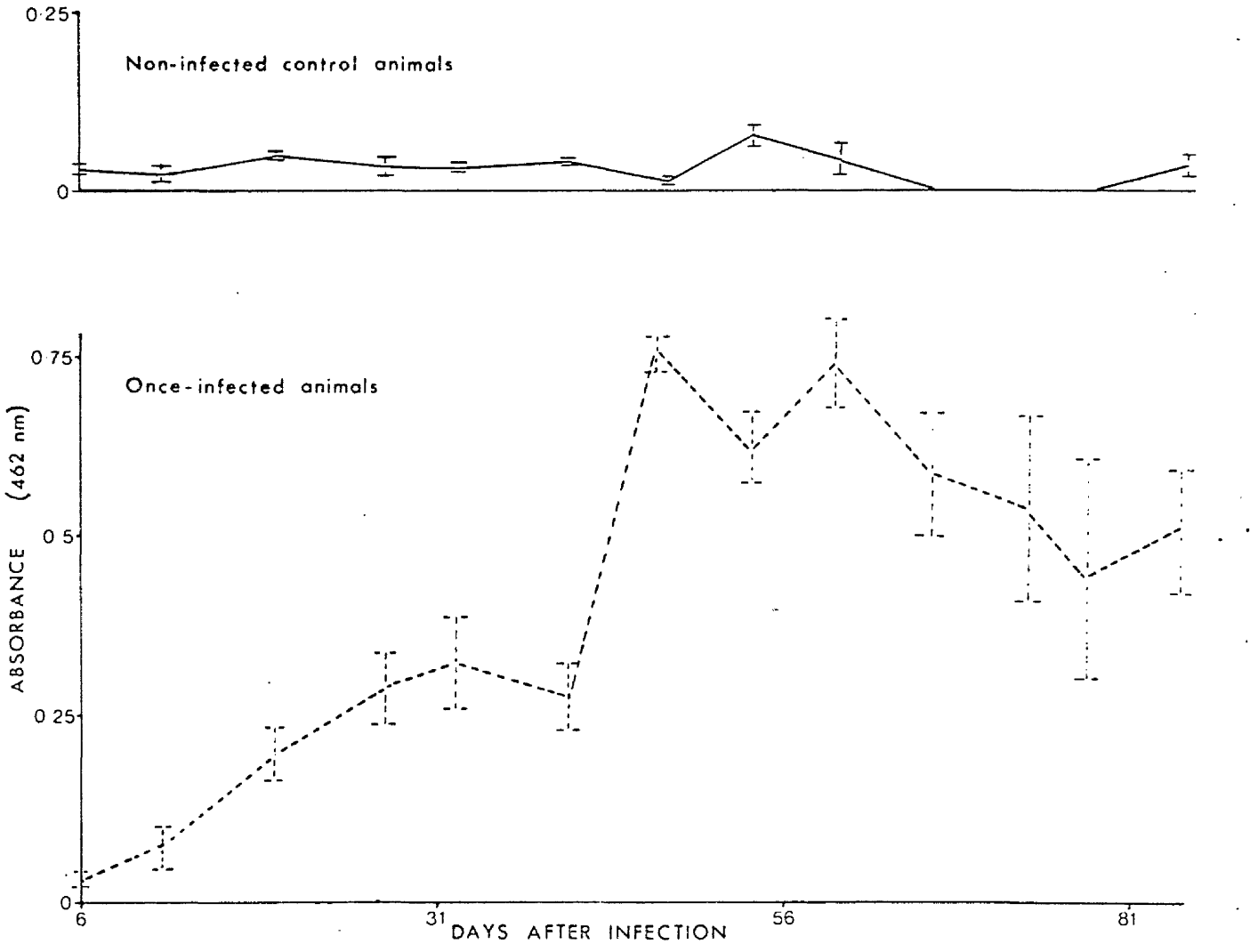


Fig. 3.17 Comparison of E.l.i.s.a. values in sheep given a primary F. hepatica infection with non-infected controls, showing standard errors.

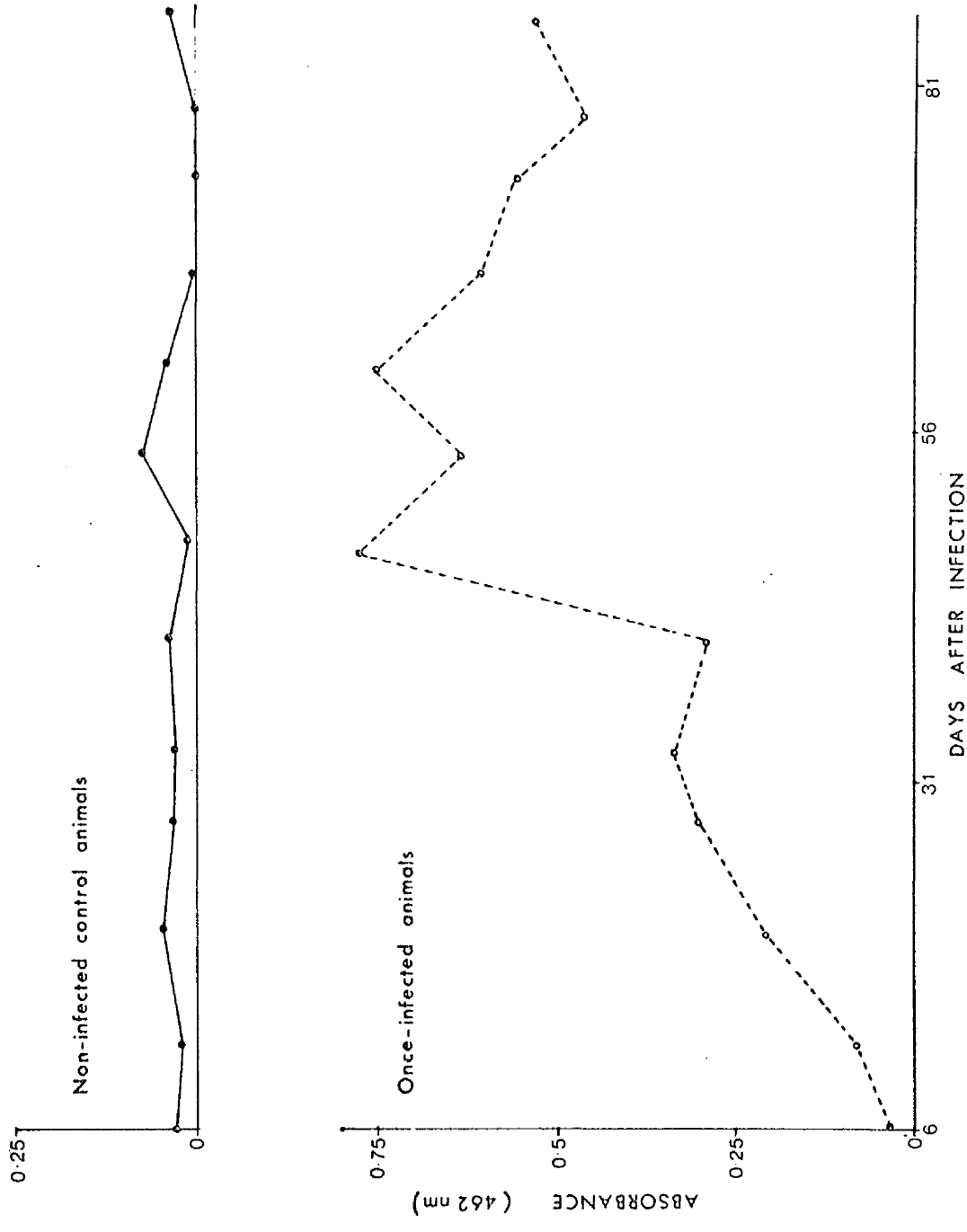


Fig. 3.18 Comparison of E.l.i.s.a. values in sheep given a primary F. hepatica infection with non-infected controls.

DISCUSSION

Anaemia

Sheep

In the present studies (Figs. 3.1, 3.2, 3.5 & 3.6) anaemia as reflected by lowered packed cell volume, haemoglobin values and erythrocyte counts was evident from 7 - 8 weeks after primary infection. This is in agreement with the classical descriptions of anaemia by Ross et al. (1967 a) and Reid et al. (1970) but is slightly earlier than noted by El Harith (1977). However, it has been established (Sinclair, 1964; Boray, 1967b) that the onset of anaemia is highly dose dependent, and the former author (El Harith, 1977) infected his animals with only 225 metacercariae, with an average of 34 flukes becoming established, compared with the dose of 350 metacercariae and an average of 27 flukes established, used in the present studies. Further evidence for the variability in time of onset of anaemia in experimentally infected sheep was recently provided by Van Tiggele, 1978. This author reported that reduction in the number of erythrocytes was most marked between 7 and 11 weeks after infection and was followed by a period of recovery or stabilisation thereafter. This picture is consistent with the findings of the present study.

The results of the eosinophil counts following primary infection of sheep were consistent with those of previous workers in that an initial rise occurred 2 weeks post-infection and after reaching peak values at 6 - 6 weeks gradually declined. These findings are in agreement with the recent studies of Van Tiggele (1978) who noted that numbers of eosinophils rose 2 weeks post infection, peaked around 6 - 7 weeks and gradually decreased together with the total number of white cells.

Cattle

Cattle under experimental conditions in the present study behaved significantly differently from sheep with respect to development of anaemia (Figs. 3.3, 3.4, 3.7 & 3.8). It was apparent that changes in the parameters reflecting anaemia i.e. P.C.V., haemoglobin and erythrocyte levels did not occur to an extent comparable with that in sheep. However, a slight fall was noted in haemoglobin and erythrocyte values around patency i.e. 10 - 12 weeks after primary infection. These results are consistent with the findings of El Harith (1977) and Van Tiggele (1978). Indeed, the latter author was unable to detect anaemia in a calf experimentally infected with 10,000 metacercariae which harboured more than 1,000 flukes, nor in calves infected initially with 1,000 metacercariae and reinfected at 16 weeks with 2,000 metacercariae which had a mean fluke burden of almost 200 in a regime comparable with that of the present studies (Table III.1). However, Reid et al. (1972) reported that cattle infected with 1,000 metacercariae developed anaemia from 12 weeks of infection. This apparent contradiction may well be explained by the fact that the animals in the latter study (Reid et al., 1972) grazed during the late autumn without supplement and consequently had a lower level of nutrition than calves in the present study, which were kept indoors, and fed ad libitum.

More difficult to explain however are the findings of recent studies by Furmaga and Gundlach (1978) which recorded that anaemia developed 10 - 12 weeks after infection of calves with 600 F. hepatica metacercariae, in terms of a drop in P.C.V., haemoglobin, erythrocyte and iron levels. It was further recorded that the anaemia appeared to depend on the number of flukes harboured by individual animals. The reasons for this difference are not apparent, although the plane of nutrition of the experimental animals would

be of considerable interest.

The numbers of eosinophils recorded increased 2 weeks following primary infection of cattle and elevated values, consistently higher than those recorded following ovine infection were recorded throughout primary and secondary infection. These findings are in agreement with that of Van Tiggele (1978) who noted that a gradual decrease in eosinophil levels occurred only in once-infected and not in reinfected animals. The reason for the greater eosinophil response in cattle compared with sheep can only be speculated upon since a protective role for the eosinophil has yet to be established in F. hepatica infection (Doy et al., 1978b).

Liver Enzymes

Sheep

The results of serum enzyme assays (Figs. 3.11 & 3.12) demonstrated that following primary infection of sheep with F. hepatica G.L.D.H. levels rose 2 - 3 weeks after infection and reached a peak value around 11 weeks, whereas G.G.T. levels did not rise until 6 - 7 weeks after infection, reaching peak values 10 - 11 weeks after infection. Those findings are in general agreement with the work of El Harith (1977) and Van Tiggele (1978), although both the latter workers reported a rise in G.G.T. slightly later than in the present studies, i.e. 8 - 9 weeks after primary infection. From Fig. 3.12 it is evident that levels of G.L.D.H. and G.G.T. following reinfection were much lower than those reached during primary infection. The reason(s) for this phenomenon are open to speculation, although a similar occurrence was reported in the recent thesis by Van Tiggele (1978), in a number of sheep 15 weeks after primary F. hepatica infection. Such chronically infected animals had low G.L.D.H. values and were found to have more extensive

damage to the ventral lobe of the liver than other animals. It is possible therefore that the release of G.L.D.H. into the blood stream may be self-limiting in an absolute sense, in that, a point is reached beyond which further liver damage will not be reflected as a rise in G.L.D.H. values. In the present studies, such a point may have been approached on reinfection of sheep resulting in only a moderate rise in G.L.D.H. levels. More difficult to explain, however, was the failure of reinfection to cause an increase in G.G.T. levels accompanying invasion by the flukes of the bile duct mucosa. One possible explanation of this is that animals were killed before G.G.T. levels could rise, at 12 weeks after reinfection. This view is supported by the slight delay in maturation of the infecting fluke population, as evidenced by the apparent increase in prepatent period of this infection, compared with the primary infection. Delayed migration on reinfection of sheep with F. hepatica has been previously reported by Rushton (1977), who found that reinfecting flukes developed more slowly and were therefore more destructive than flukes from a primary infection. The latter author considered this to be due to an adverse host response, possible of an immunological nature.

Cattle

A similar picture (Figs. 3.13 & 3.14) of serum enzyme activity was obtained on infection and reinfection of experimental calves. G.L.D.H. values again rose 2 - 3 weeks post-infection and peaked at 10 - 12 weeks, with G.G.T. levels following suit at 9 weeks and reaching a maximum at 12 - 13 weeks. Following reinfection enzyme levels were only slightly raised compared with control values. These findings are in general agreement with a number of recent studies including those of Anderson et al. (1978), and Van Tiggele (1978). The former study (Anderson et al. 1978) indicated that G.L.D.H. and G.G.T.

levels rose 2 - 3 weeks and 6 - 7 weeks respectively after primary infection with 1,000 F. hepatica metacercariae, and that no such effect was produced on reinfection 35 or 54 weeks later. The authors considered this to be due to the operation of a resistance mechanism which persisted for at least 54 weeks after primary infection. The nature of such a mechanism was thought to involve rejection of the invading fluke population at the level of the liver capsule, preventing or retarding the penetration and subsequent migration of the flukes through the liver substance. While it is tempting to employ a similar argument to that outlined above to explain the lower serum enzyme values obtained on reinfection in the present studies it is felt that such conclusions would not be valid in view of the difference in time interval between infections in the two studies viz 14 weeks compared with a minimum of 35 weeks.

In this connection the findings in the thesis by Van Tiggele (1978) in the only comparable study are of considerable interest. This author investigated the variation in a number of biological parameters including serum enzyme activities in groups of 4 calves given primary and secondary infections of F. hepatica 16 weeks apart and not treated with anthelmintic. On reinfection it was found that serum enzyme values generally were of a much lower order than following primary infection and that G.G.T. levels were not significantly elevated. The author interpreted this latter finding as meaning that no flukes of the second infection had passed the parenchymal stage, and that in his experiment reinfected animals had more adult flukes from the primary infection than animals receiving a primary infection only. Thus the secondary infection had in some ways enhanced the primary infection.

Whether any such conclusions are justified in the present studies, in

the opinion of the author, cannot be determined on the basis of serum enzyme activities alone and must be judged from consideration of all the information available from all the parameters measured during experimental infection of calves with F. hepatica (see Chapter VI) particularly serial parasitological results.

Serology

Double immunodiffision

The results of the present study (Tables III.3 - III.6) confirmed the findings of previous workers, notably Doyle (1973b); Van Tiggele and Over (1976); Kendall et al. (1978); Van Tiggele (1978). The latter author demonstrated that D.I.D. gave a positive reaction 2 - 3 weeks after experimental infection of sheep and cattle with F. hepatica. In the present studies a similar pattern of precipitin production was observed although in sheep the first detection of antibody was slightly later, i.e. 4 - 5 weeks post infection. Interestingly enough Van Tiggele (1978) reported the sudden disappearance of antibody titres at 10, 13 and 15 weeks after primary infection of cattle. This phenomenon was observed in the present studies at 8, 10 and 12 weeks after experimental bovine infection. In the opinion of the former author (Van Tiggele, 1978) this may be due to consumption of antibody in immune complex formation at those times.

Indirect haemagglutination (I.H.T.)

The results of I.H.T. following primary and secondary infection of ruminants with F. hepatica (Figs. 3.15 & 3.16) were in close agreement with a number of recent studies, notably by Poluektova (1975); Van Tiggele and Over (1976) and Van Tiggele (1978). The above-named authors recorded first detection of serum antibody using this test 2 - 3 weeks after experimental

infection of sheep and cattle with F. hepatica and a similar pattern of antibody production throughout infection with the parasite.

Several interesting points emerged from the present studies which have previously been noted by other authors during contemporaneous experiments at other laboratories. Firstly the dramatic fall in antibody titre detected 5 - 6 weeks following experimental infection of sheep. This fall was also evident from the micro-E.l.i.s.a. results, which of course measure solely IgG (Figs. 3.17 & 3.18) and coincided exactly with that reported by Van Tiggele (1978). It must be therefore concluded that such a drop was largely due to a fall in circulating IgG levels since the drop in titre recorded by E.l.i.s.a. was identical to that detected in I.H.T., thus precluding the involvement of the IgM component of the I.H.T. response. Such a phenomenon did not occur following primary infection of cattle, although a decrease in I.H.T. titres was recorded around 10 weeks of infection, i.e. coinciding with the disappearance of precipitins previously mentioned. The latter author (Van Tiggele, 1978) suggested that this remarkable suppression of antibody titre may be due to the rapid consumption of antibodies by antigens derived from the fast-growing flukes which cannot be compensated by an increase in the rate of antibody production, or to an expression of immunological tolerance or suppression (see also Chapter IV & VI, p. 181 & 233). Interestingly enough Over et al. (in press) reported an increased susceptibility to reinfection in these weeks (6 weeks post infection) compared to 3 and 9 weeks.

In the present studies it was also interesting to observe that antibody titres in experimentally infected sheep were consistently higher than those in comparable bovine infections. This phenomenon has been well documented (Horchner, 1973; Van Tiggele and Over, 1976) and was particularly apparent

in the present studies on reinfection of sheep when an immediate dramatic increase in antibody recorded by I.H.T. and micro-E.l.i.s.a. occurred. This was not apparent on reinfection of calves, where a much more gradual rise in antibody titre was recorded. The possible significance of this finding are discussed in more detail in the light of other experimental findings in Chapter VI.

Problems associated with Enzyme-linked immunosorbent assay microtechnique (micro-E.l.i.s.a.) for diagnosis of F. hepatica infection

Notwithstanding the considerable literature on the use of E.l.i.s.a. for diagnosis of a wide range of parasitic and other diseases, it was felt that a number of problems encountered by the author in the application of the technique to F. hepatica diagnosis were worthy of note, for the enlightenment of subsequent authors.

1. Microtitre plates. It was found that the results obtained from micro-E.l.i.s.a. varied not only with the type and batch of microtitre plates used, but there was also a degree of variation between individual plates. In the view of the present author and a number of colleagues (Smith, personal communication; Van Tiggele, 1978) this was due to differences in the antigen-binding capacities of various plates, possibly due to the fact that the bond between the antigenic material and the plate was not chemical, being purely electrostatic. This fact was amply illustrated by the work of Denmark and Chessum (1978) who investigated standardisation of E.l.i.s.a. for detection of Toxoplasma antibody. These authors recorded that when uniform dilutions of antigen, antiserum and conjugate were used in all 96 wells of a microtitre plate higher E.l.i.s.a. readings were recorded on the edges of the plate. It was reported that incubation of plates at room temperature did not overcome

this problem and that several batches of microtitre plates displayed similar properties. The authors concluded that this was due to the nature of the plastic of the plates and concluded that a standard plate was necessary for the micro-E.l.i.s.a. to be of diagnostic value. To circumvent this problem, as mentioned previously in the present studies, 2 positive control sera were examined on each plate, the first to standardise the values of test sera, and the second to check the antigen-binding capacity of the plate. This approach was workable, but Smith (personal communication) has suggested a more practical solution to the problem using paper discs instead of plastic as a vehicle for attachment of antigen, on the grounds that a much more stable bond was formed between the antigenic material and the paper, i.e. a similar system to Radio-immuno-assay using an enzyme instead of a radioactive label.

2. Optimal concentration of reagents. The requirement for accurate dilution of antigen, antiserum and conjugate for use in the test, though admittedly a prerequisite of most serological reactions, was found to be critical for a workable system. This necessitated 3 dimensional titration of the reagents, before using the system to estimate antibody content of test sera. The importance of accurate dilution of reagents was illustrated by the occurrence of "prozone" information during titration of antigen against antibody. This was evident as a reduction of micro-E.l.i.s.a. readings under conditions of considerable antigen excess, due presumably to consumption of antibody in antigen complexes which rendered the antibodies unavailable for detection in the test. Optimal dilution of the reagents was designed to give an absorbance value of 1.0 for positive control serum, and a minimal value (<0.1) for a negative control serum. It was found, however, that such optimal concentrations were only specific within very narrow limits, and any slight alteration in any of

the critical concentrations could result in values for test sera outside the desirable range. In addition, not surprisingly, a given test system was only applicable to serum from one particular ruminant species, i.e. sheep, and for analysis of bovine serum a new, unrelated set of values was required.

3. Choice of substrate. Considerable difficulty was also encountered regarding the choice of an appropriate substrate for the enzyme system. The substrate used widely with horseradish peroxidase, namely 5 Amino salicylic acid (5 A.S.) was found to be totally unsuitable due to its instability to light. This meant that values for test sera could not be obtained since the substrate had darkened to such an extent during reading of the test that the absorbance was too high to be measured. For this reason ortho-phenylene-diamine (O.P.D.) was preferred in the present studies, which gave more consistent results. Various authors have reported a degree of non-specific background colour using various substrates, particularly 5 A.S., and various methods have been used to reduce this undesirable effect. Notably among these is the use of Bovine serum albumin (B.S.A.) in the buffer used to dilute both antiserum and conjugate (Ruitenberg et al., 1975). This compound was purported to occupy any sites of attachment on the microtitre plates not occupied by specific antigen-antibody complexes, thus precluding the attachment of non-specific serum factors, resulting in a reduction in background reading. This ploy was attempted in the present studies and was found to be without value in the F. hepatica system.

4. Reading of the test. Major difficulties were encountered regarding the reading of the test, not only due to the choice of substrate as described previously, but also from a purely mechanical point of view. In the present context E.l.i.s.a. was necessarily being used to quantitate antibody production,

and therefore visual reading of the test was obviously not possible. Therefore microcuvettes were used into which the contents of individual wells were pipetted. This undertaking proved tedious and time-consuming in the extreme and was completely impratical for the number of serum samples which required to be analysed. Apparently the mechanisation of the reading of micro-E.l.i.s.a. presents a number of serious technical difficulties, and at the time of writing a reliable automatic reader is not commercially available.

Passive transfer

The following conclusions can be drawn from the results of Passive Transfer Experiment I, by reference to Tables III.7 - 9. Firstly, the only antiserum which afforded significant protection ($p < .05$) to F. hepatica infection in rats was that obtained from sheep infected 10 weeks previously with F. hepatica, although there were substantial reductions in fluke recoveries in other groups. This protection was manifest as a reduction of 64% in fluke numbers recovered from rats given this particular serum compared with control rats. These findings are consistent with the work of other authors, notably Hayes et al. (1974 a) & c)); Armour and Dargie (1974). The latter workers obtained a reduction of 66% in total flukes recovered from rats similarly injected with antiserum obtained from sheep infected 12 - 14 weeks previously with F. hepatica. From the results of these authors it was apparent that time and dose of antiserum determined the level of protection transferred and for this reason, in the present work, a similar regime of passive transfer was employed, i.e. 2 injections of 10 mls each of antiserum were injected intraperitoneally. In the opinion of this author the relatively large volume of serum used may have resulted in a non-specific deleterious

action of the serum on metacercariae of F. hepatica which may explain the reduction in fluke numbers obtained from rats given normal serum (Table III. 9) although no such finding was reported by Armour and Dargie (1974).

Thus, the present study, together with a number of other studies, the most recent of which was carried out by Rajasekariah and Howell (in press), would implicate humoral immunity in a protective role in F. hepatica infection. The last named authors showed conclusively that hyperimmune serum obtained from rats infected repeatedly with F. hepatica, consistently produced a significant degree of protection when administred immediately following challenge. It would appear therefore that protection between homologous and heterologous donor species can be achieved, although the situation in ovine fascioliasis would appear to be anomalous in some respects, in that sheep which were themselves susceptible to challenge infection (Table III. 12) were able to protect rats from infection, presumably due to a serum factor. As can be seen from Figs. 3.15-3.18 at 10 weeks of infection sheep were producing considerable amounts of antibody which was clearly non-functional, as far as protecting the animal from reinfection is concerned. Possible reasons for this situation are discussed in Chapter VI.

Pathological findings

Sheep

From Table III. 10 the gross pathological changes noted on infection and reinfection of experimental animals were no different from those classically described by previous authors. On the basis of the extent of fibrotic change in the livers of reinfected sheep there would appear to be some evidence of a delayed migration of reinfecting flukes, as purported by Rushton (1977) although as discussed previously this was not borne out by the results of liver enzyme estimation. Of incidental interest was the finding of a slight degree of

calcification in the liver of one sheep which received only a primary infection. The reasons for this curious observation are not apparent.

Cattle

As is evident from Table III.13 the liver lesions in reinfected cattle were consistent with those recorded by previous workers, notably Doyle (1971) with fibrosis and calcification being the main features of the pathological changes noted.

Parasitological findings

Sheep

Clearly, from an examination of the parasitological data on experimental sheep (Tables III.10 - 12), in agreement with the findings of many previous workers, there was no evidence to suggest that sheep develop any immunity to reinfection with F. hepatica. From Table III.10, on the basis of gross pathological changes it was evident that reinfected animals (S1, S5, S6, S7) suffered more severe liver damage in terms of fibrotic replacement of liver parenchyma than once-infected animals, but as can be seen from Tables III.11 and III.12 there was no difference either in total numbers of flukes recovered, or size distribution of flukes recovered from the 2 groups. Indeed, the mean number of flukes recovered from the 2 groups was remarkably similar (Table III.12).

As mentioned previously, there was some evidence, on the basis of faecal egg counts and serum enzyme activity to suggest that there was a delayed migration of reinfecting flukes compared with the primary infection. From this data alone it is impossible to comment on any resistance mechanism involved in such a phenomenon, as proposed by Rushton (1977), without considering a number of immunological parameters. This aspect will be

discussed at fuller length in Chapter IV.

The parasitological findings in experimental ovine fascioliasis of the present study are in close agreement with concurrent work by Meek and Morris (1979), in Australia. The latter authors examined the development of F. hepatica, in previously non-infected sheep compared with animals exposed to various regimes of infection. It must be pointed out that in the Australian study sheep were grazed on fluke-free pastures during the experiment, but in all other respects this study was analogous to the present work. It was reported that no appreciable differences were obtained in mean numbers, length, prepatent period or fecundity of flukes in reinfected sheep compared with once-infected animals. The authors therefore concluded that from their findings there was no evidence to suggest that sheep develop acquired immunity to F. hepatica.

Cattle

From Tables III.12 - 14, the following points are worthy of note. Firstly, calcification as classically described in bovine fascioliasis was found to be a feature in the livers of experimentally infected calves, in contrast to experimental ovine fascioliasis. Secondly, there appeared to be no increase in the prepatent period on reinfection, compared with primary infection. Thus there was no evidence of a delayed migration of reinfecting flukes as postulated in experimental infection of sheep.

Finally, there was a large variation between the numbers of flukes recovered from both infected animals. This is not surprising in view of the small group of animals involved, and, for this reason, no conclusions can be drawn regarding the development of acquired immunity in experimental calves, on the basis of fluke recoveries or size distribution. However, it is instructive

to consider the findings of recent studies by Kendall et al. (1978) and Van Tiggele (1978) for contemporary points of view on this matter. The former authors, Kendall et al. (1978), demonstrated firstly that a process of rejection in terms of a reduction in numbers and fecundity, as measured by total number and egg output, of flukes recovered, occurred with time, in animals given single infections. This phenomenon had been previously reported by a number of other workers including Kendall et al. (1967) and Doyle (1972). In addition it was reported that repeated infection of cattle produced a degree of resistance as evidenced by a reduction in fluke burden (up to 82%), fluke size and a fall in numbers of fluke eggs detected in the faeces. The nature of this resistance was not discussed in detail by these workers although an immunological mechanism was inferred.

These findings were partly substantiated by the work of Van Tiggele (1978) who obtained a reduction of 41% in total flukes recovered from reinfected animals compared with once-infected animals. This author also reported a reduction in numbers of flukes of intermediate size, in reinfected animals, which he explained as being due to inhibited development of the secondary infection. From the results of the latter study, it was proposed that resistance or acquired immunity in terms of reduction of numbers and fecundity of flukes may be due to a combination of purely immunological, purely mechanical and immunologically mediated mechanical factors.

Although as mentioned previously a comparison of the resistant status of once infected and reinfected cattle was outwith the scope of the present studies, the total numbers of flukes recovered (Table III.12) from calves infected, treated and reinfected as outlined in Table III.1 is worthy of note. The mean number of flukes recovered from these animals was 109

which was several times greater than the mean value reported in comparable studies by Doyle (1973a). The latter author infected his animals with 750 F. hepatica metacercariae and reinfected with 1,300 while 1,000 metacercariae were used on both occasions in the present studies. While it is recognised by this author that it is rather difficult to draw valid conclusions when experimental groups are rather small as in the present studies, it is felt that such a difference is worthy of comment and therefore possible explanations for this anomaly will be discussed at greater length in Chapter VI.

It has emerged from the above studies, in agreement with previous work in this field that sheep do not develop an acquired immunity to reinfection with F. hepatica in terms of decreased numbers or impaired viability of reinfecting flukes. The susceptibility of sheep to the parasite was also reflected in the development of anaemia which did not occur in cattle undergoing a similar regime of infection.

From the various parameters of infection investigated it was apparent that the liver enzymes G.L.D.H. and G.G.T. were of value as indicators of liver cell necrosis and bile duct damage following primary liver fluke infection, but proved to be of limited worth on reinfection. The early eosinophilia detectable in both ruminant species was also a useful ancillary aid to liver-fluke diagnosis, although the non-specificity of this reaction would necessitate confirmation by liver enzyme or serological analysis.

Of the serological tests used I.H.T. was considered to be a reliable sensititive method of early diagnosis of F. hepatica which could be used for quantitative study of the antibody response. D.I.D. was also found to be reliable, but of lower sensitivity and suitable only for qualitative procedures. E.l.i.s.a. was found to be the least reliable of the methods used.

CHAPTER IV

CELL MEDIATED IMMUNITY TO FASCIOLA HEPATICA INFECTION

INTRODUCTION

The importance of cell-mediated immunity in the host response to helminths has been recognised for some considerable time (reviewed by Larsh and Weatherly, 1975). However, the relevance of various laboratory techniques for estimation of the cell-mediated immune status of parasitised animals has been the subject of a great deal of controversy. Two of those which have received considerable attention are the leucocyte Migration Inhibition Test (M.I.T.) and the Lymphocyte Transformation Test (L.T.T.).

The former test was employed by Aalund et al. (1972); Aalund and Nansen (1972); Genchi et al. (1973) and Genchi and Sartorelli (1974); in F. hepatica infection in ruminants. The results of these studies have been considered in the relevant review section of this thesis (p. 39-40), and it is felt that further comment is not necessary at this stage.

The latter test (L.T.T.) has only been recently adapted for parasitological investigation in order to examine the response of lymphocytes from parasitised animals when exposed to mitogenic or antigenic preparations. The immunological significance of the proliferation of lymphocytes from man and other animals when cultured with specific antigenic and non-specific mitogenic material has been the subject of considerable discussion. It has been argued to reflect specific cell-mediated immune responsiveness because it accompanied delayed-type hypersensitivity reactions in vitro and could be observed in the absence of detectable serum antibody in cell donors (Mills, 1966; Oppenheim, 1968). However, Benezra et al. (1969) in studies involving immunisation of rabbits showed that lymphocyte transformation was not associated with either the antibody response or delayed-type hypersensitivity, but depended upon the intensity of either response. Adams and Cripps (1977) put forward the view

that antigen-induced proliferation of cultured lymphocytes may indicate the activity of cells committed to react to a particular antigen without necessarily being indicative of the immune response in vivo and may in fact only provide an estimate of the antigen reactive lymphocytes within cell populations.

However one wishes to interpret this transformation response L.T.T. has proved a useful tool in the investigation of disease caused by a number of different helminth species, including the cestode Echinococcus granulosus (Yusuf et al., 1975), the trematode Schistosoma mansoni (Colley, 1975; Colley et al., 1977 a) and b); 1978) and the nematodes Trichostrongylus colubriformis (Adams and Cripps, 1977) and Haemonchus contortus (Adams, 1978). The relevant findings of these recent studies will be discussed later in this chapter in the light of the findings of the present study.

In the present studies in vitro stimulation of both ovine and bovine peripheral lymphocytes by various mitogens was carried out using a modification of the technique described by Burrells and Wells (1977). In addition L.T.T. was employed to monitor the response of ruminants to somatic fluke antigen during active infection with F. hepatica. L.T.T. was preferred to other C.M.I. indicators in the present work as it was considered that more valuable information could be gained using this test regarding the precise action of the cell-mediated response in F. hepatica infection than, for example using M.I.T. It was hoped that monitoring both primary and secondary F. hepatica infections in the two ruminant species with L.T.T. would indicate not only the relative responses of lymphocytes from infected and non-infected animals to antigenic and mitogenic material, but also the role, if any, of immunosuppression or immunodepression in fascioliasis in sheep and cattle. It was considered that the results of such a study may well contribute to the overall understanding of

the relative importance of physical and immunological factors, which has been the subject of so much debate in the past, in the observed susceptibilities of sheep and cattle to fascioliasis.

EXPERIMENTAL DESIGN

The schedules for infections of sheep and cattle used in the experiment are shown in Table III.I. Peripheral lymphocytes were obtained for the L.T.T. from these animals at 2 - 3 weekly intervals during infection, and also prior to infection in order to assess base levels for mitogenic and antigenic stimulation. A number of different mitogens with various lymphocytic affinities were used throughout the experiment, namely, phytohaemagglutin (P.H.A.), pokeweed mitogen (P.W.M.), Concanavalin A (Con A) and lipopolysaccharide of E.coli (L.P.S.), in order to maximise the information obtainable regarding the various subpopulations of lymphocytes involved in the cellular response. In all cases lymphocyte cultures were set up with and without the addition of 2 mercaptoethanol (M.E.). The use of this compound as a macrophage substitute in lymphocyte culture is widely known (Leuke and Hans Georg-Opitz, 1976), and in the present studies, as in those of the former authors, 2 M.E. was incorporated to enhance or reinforce, the response of lymphocytes in culture to antigenic material.

RESULTS

For the purposes of the present study the results of L.T.T., as illustrated in the chapter, are presented as comparisons between different treatment groups within the two ruminant species. This method of display was adopted due to the wide variation in responses to mitogenic and antigenic stimulation encountered throughout the duration of the experiment. In the opinion of the author this variability inherent in the technique precluded the

meaningful presentation of results as a factor of time after infection. For this reason no allusions to particular times except whether before or after infection/ reinfection are made during this chapter and differences noted between various treatment groups are considered in terms of general trends. For ease of illustration ovine mitogen responses below 20,000 dpm, bovine mitogen responses below 10,000 dpm and antigen responses below 1,000 dpm are given in numerical form rather than as individual values.

Sheep

A diagrammatic representation of a comparison between sheep receiving primary and secondary F. hepatica infections and control animals on the basis of response to mitogenic and antigenic stimulation appears in Figures 4.1- 4.8.

Mitogen responses

Figures 4.1 illustrates the response of ovine lymphocytes from sheep given a primary infection of F. hepatica to 4 different mitogens, when cultured in medium containing foetal calf serum (F.C.S.) without the addition of M.E., compared to non-infected control animals. From this diagram it is clear that sheep infected with F. hepatica gave consistently lower responses than control animals to stimulation with 3 out of the 4 mitogens used, i.e. P.H.A., P.W.M. and Con A. As the responses to L.P.S. were of a much lower order than those of the other mitogens and were in fact comparable to the results of antigenic stimulation, L.P.S. responses are illustrated and described with antigen responses. When M.E. was added to the culture medium (Fig. 4.2) a similar result was obtained. In this case the trend was the same as in Fig. 4.1, i.e. infected sheep gave lower responses to all mitogens, although it must be pointed out that differences between the two groups were less obvious

than in Fig. 4.1. Under both sets of conditions (Figs. 4.1 and 4.2) the most marked difference between the 2 groups was in response to the mitogen Concanavalin A (Con A).

The effect of reinfection of sheep on mitogen responses is shown in Figs. 4.3 and 4.4. The responses of reinfected animals are compared, in this case, with animals which received a primary infection simultaneously with the administration of the reinfecting dose to the first group of animals. Again responses were measured in the presence and absence of M.E. From Fig. 4.3 it is clear that the responses of reinfected animals to mitogenic stimulation were lower than animals given a primary infection only. An identical pattern of responses is seen in Fig. 4.4 where M.E. was added to the culture medium. On the whole, differences between reinfected and once-infected animals were of similar magnitude or slightly more marked than those observed between once-infected and non-infected animals. In the former situation (Figs. 4.3 and 4.4) the most marked differences were found following stimulation by P.W.M. and Con A.

Antigen responses

Figs. 4.5 - 4.8 illustrate the results of stimulation of ovine peripheral lymphocytes from once-infected, reinfected and non-infected animals, by the mitogen L.P.S. and a lipid-free extract of F. hepatica antigen (L.F.E.), prepared as described in Chapter II. Various concentrations of antigen were employed since it was found that some stimulation was obtained over a range of different concentrations (6 µg - 1 µg/ml). In all cases antigen concentrations were based on the amount of protein N present estimated, as previously described, by the technique of Lowry et al. (1951).

From Fig. 4.5 it is evident that the responses of sheep given a

primary F. hepatica infection to L.P.S. stimulation did not differ from those of non-infected control animals, when lymphocytes from the 2 groups of animals were cultured in the absence of M.E. When M.E. was added to lymphocyte cultures (Fig. 4.6) L.P.S. responses were again of a very similar order. On reinfection (Fig. 4.7 and 4.8) L.P.S. responses from reinfected sheep were comparable to those of once-infected animals. Unfortunately, the number of L.P.S. responses measured was limited due to considerable difficulties in obtaining this mitogen during this period.

It is also evident from Fig. 4.5 which shows antigen responses in the absence of M.E. that there was a tendency for non-infected sheep to respond to a greater extent to L.F.E. than animals given a primary infection, particularly at antigen concentrations of 6 and 3 μg/ml. At lower antigen concentrations such a difference was not apparent and indeed, at a concentration of 1 μg/ml infected animals seemed to have responded better than control animals. On addition of M.E. to the culture medium a similar result was obtained (Fig. 4.6). Again control animals responded more vigorously to fluke L.F.E. than infected animals at concentrations of 6, 3 and 2 μg/ml with an equivocal result being observed at 1 μg/ml. It was noticeable that antigen responses in general were enhanced by the incorporation of M.E. into the culture medium.

The effect of reinfection on reactivity of ovine lymphocytes to fluke L.F.E. is illustrated diagrammatically in Figs. 4.7 and 4.8. Responses of reinfected animals are again compared with animals given a primary infection at the same time as the reinfecting doses were administered to the former group. From Fig. 4.7, which shows antigen responses in the absence of M.E. it can be seen that there was a dramatic difference in the responses

of the 2 groups of animals. Those animals undergoing primary F. hepatica infection responded much more vigorously to fluke L.F.E. than reinfected animals. This difference was apparent at the lower antigen concentrations used, (i.e. 3, 2 and 1 µg/ml) and the only concentration where it was not apparent was at 6 µg/ml where the responses of the two groups were of a similar order. A similar pattern of response emerged from the same comparison of antigenic responses which incorporated M.E. into the culture medium (Fig. 4.8). However, it was evident that although reinfected sheep had lower responses to fluke L.F.E. than once-infected animals the order of this difference was much lower than in Fig. 4.7. In this latter case the increased response of once infected animals was most marked at a concentration of 6 µg/ml.

Cattle

Figs. 4.9 - 4.16 illustrate the comparative responses of lymphocytes from cattle undergoing primary and secondary F. hepatica infection to mitogenic and antigenic stimulation in culture medium containing F.C.S. with and without the addition of M.E. with those of a non-infected control animal. Due to a shortage of cattle for experimental purposes, as explained previously, it was considered that a control animal uninfected throughout the duration of the experiment provided a better comparison with reinfected animals than one animal given a primary infection. For this reason the results of bovine L.T.T. must, by necessity, be presented in a slightly different form than those for ovine infections, and it must be remembered that fewer control responses were available compared with those of infected animals.

Mitogen responses

Fig. 4.9 illustrates the responses of lymphocytes from cattle

given a primary infection of F. hepatica with those of a non-infected control animal, to stimulation by the mitogens P.H.A., P.W.M. and Con A, in the absence of M.E., L.P.S. stimulation is again considered along with antigen responses. Only slight differences were apparent between the two groups of animals, the most marked of which was a greater response of the non-infected animal to Con A than the infected group. Incorporation of M.E. into the culture medium (Fig 4.10) made no difference to this picture, except that the responses of both groups to Con A was similar, thus, under the latter circumstances, there appeared to be no difference in response, of infected and non-infected cattle, to any of the mitogens used.

On reinfection (Figs. 4.11 and 4.12) it emerged that, in the absence of M.E. (Fg. 4.11), a similar result was obtained i.e. only marginal differences were observed between the two groups of animals, the most noticeable of which was a slightly reduced response to Con A in the reinfected group compared with the control. Addition of M.E. to the culture medium (Fig. 4.12) resulted in an overall increase in the absolute values of all responses recorded. However, relative responses to the various mitogens remained unchanged, apart from a slight decrease in response to P.W.M. by reinfected animals, when compared with the control.

Antigen responses

The responses of cattle given a primary and secondary F. hepatica infection to stimulation with L.P.S. and various dilutions of F. hepatica L.F.E. are illustrated in Figs. 4.13 - 4.16. During the first part of this experiment a considerable range of antigen concentrations were employed to determine optimal dilutions. It was found that antigen dilutions of 36 and 2 μg/ml both resulted in a degree of stimulation, and, for this reason, during the second

part of the experiment i.e. following reinfection, these dilutions were the only ones employed.

From Figs. 4.13 and 4.14, a number of points can be made concerning the effect of L.P.S. and L.F.E. on lymphocytes from bovines given a primary F. hepatica infection. Firstly, the results of L.P.S. stimulation gave rather contradictory results, in that, in cultures without M.E.(Fig. 4.13), apart from one isolated value, responses of infected cattle were generally lower than control values. However, in cultures containing M.E. responses of infected animals were generally higher than control values. On reinfection (Figs. 4.15 & 4.16) the same pattern emerged, i.e. infected animals gave lower responses to L.P.S. in cultures without M.E., and greater responses in cultures with M.E., compared with the control. Possible reasons for this discrepancy will be considered in the discussion section of this chapter.

As far as antigen responses are concerned following primary F. hepatica infection (Figs. 4.13 & 4.14) it was clear, especially in cultures containing M.E. that lymphocytes of infected animals responded better to fluke L.F.E., than those from the non-infected control, particularly at concentrations of 36 and 2 µg protein N/ml. Cultures without M.E. showed a similar trend although, in general, all responses were of a lower order. Following reinfection, as explained previously, only the antigen concentrations mentioned above were employed. It was apparent that reinfected animals responded much more vigorously to F. hepatica L.F.E. than the control (Figs. 4.15 & 4.16). It was again found that greater responses were obtained when M.E. was incorporated in the culture medium. The most dramatic difference between the two groups was obtained at an antigen concentration of 2 µg Protein N/ml when M.E. was incorporated in the culture medium (Fig. 4.16).

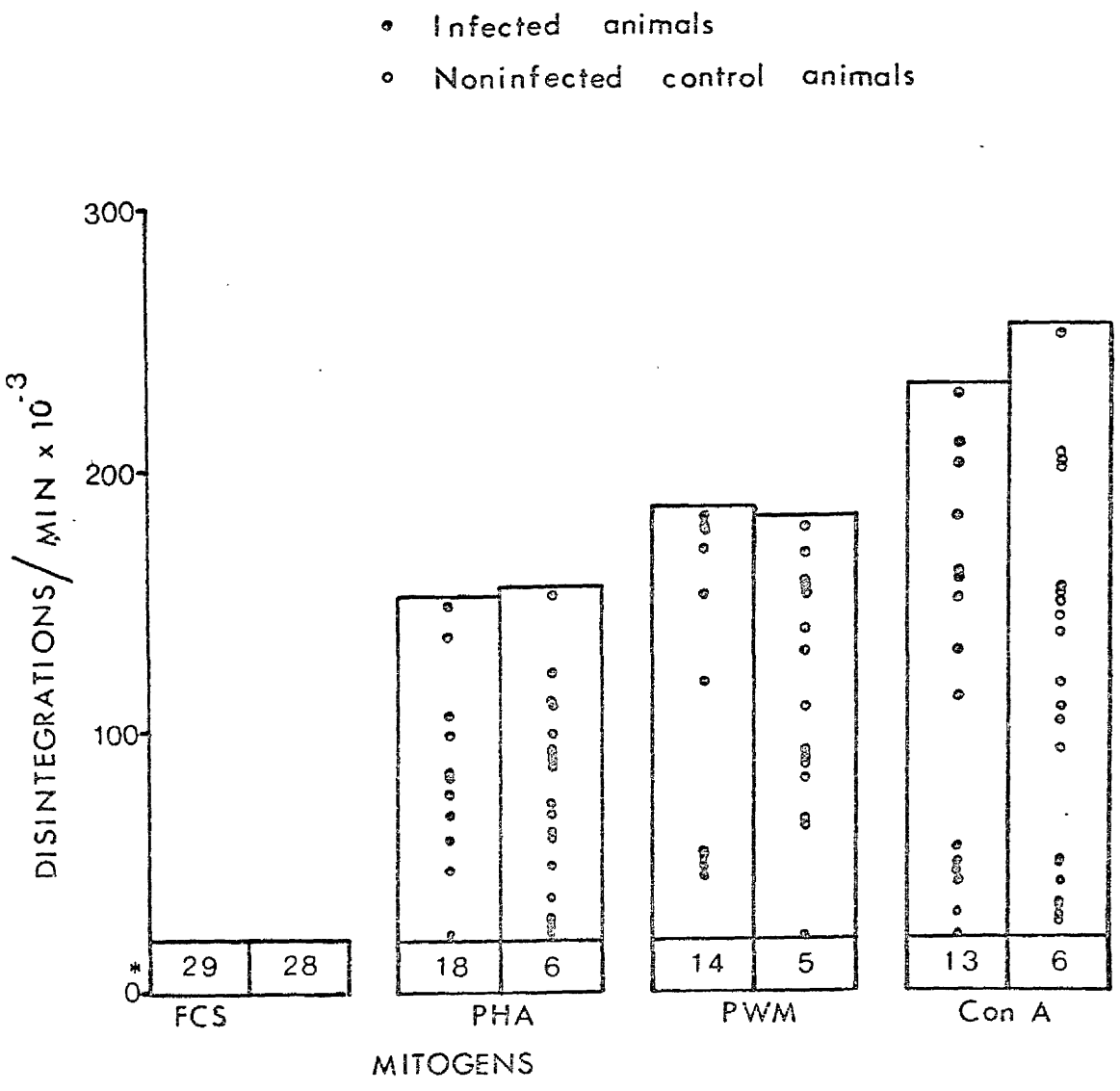


Fig. 4.1. Ovine primary F. hepatica infection. Mitogen responses without M.E.

* = No. of counts <20,000 dpm

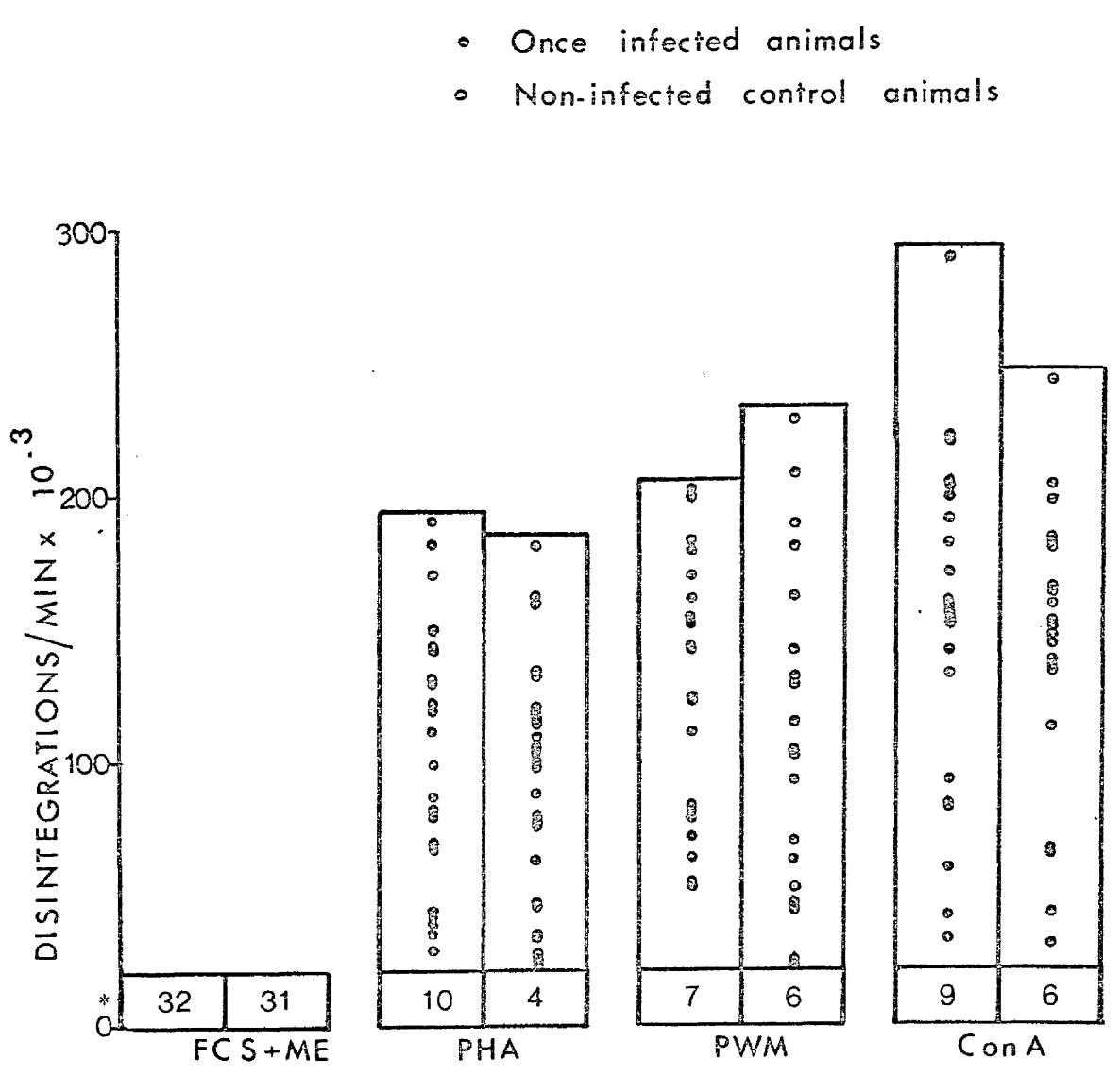


Fig. 4.2. Ovine primary F. hepatica infection. Mitogen responses with M.E.

* = No. of counts < 20,000 dpm

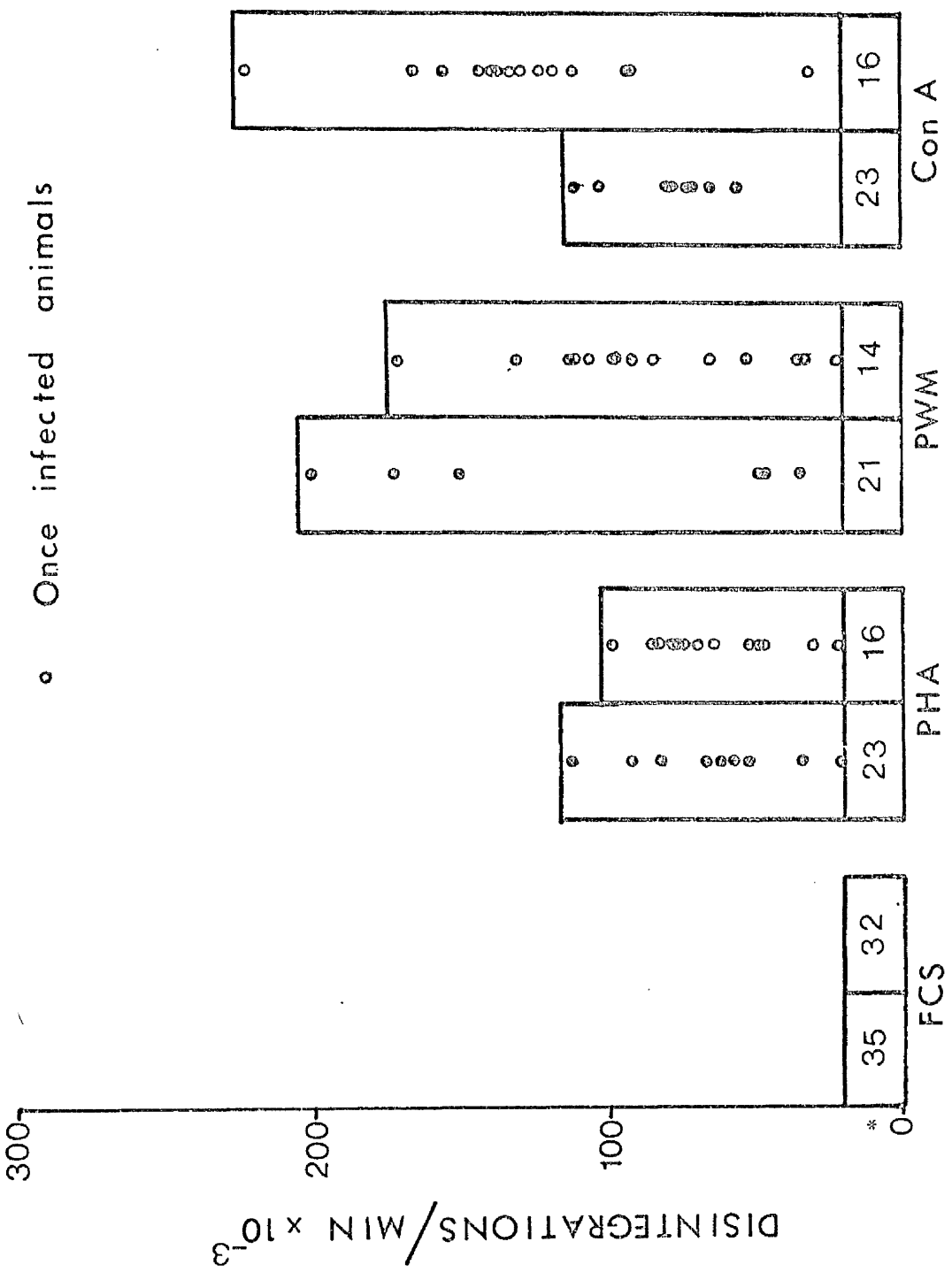


Fig. 4.3. Ovine secondary F. hepatica infection. Mitogen responses without M.E.
* = No. of counts < 20,000 dpm

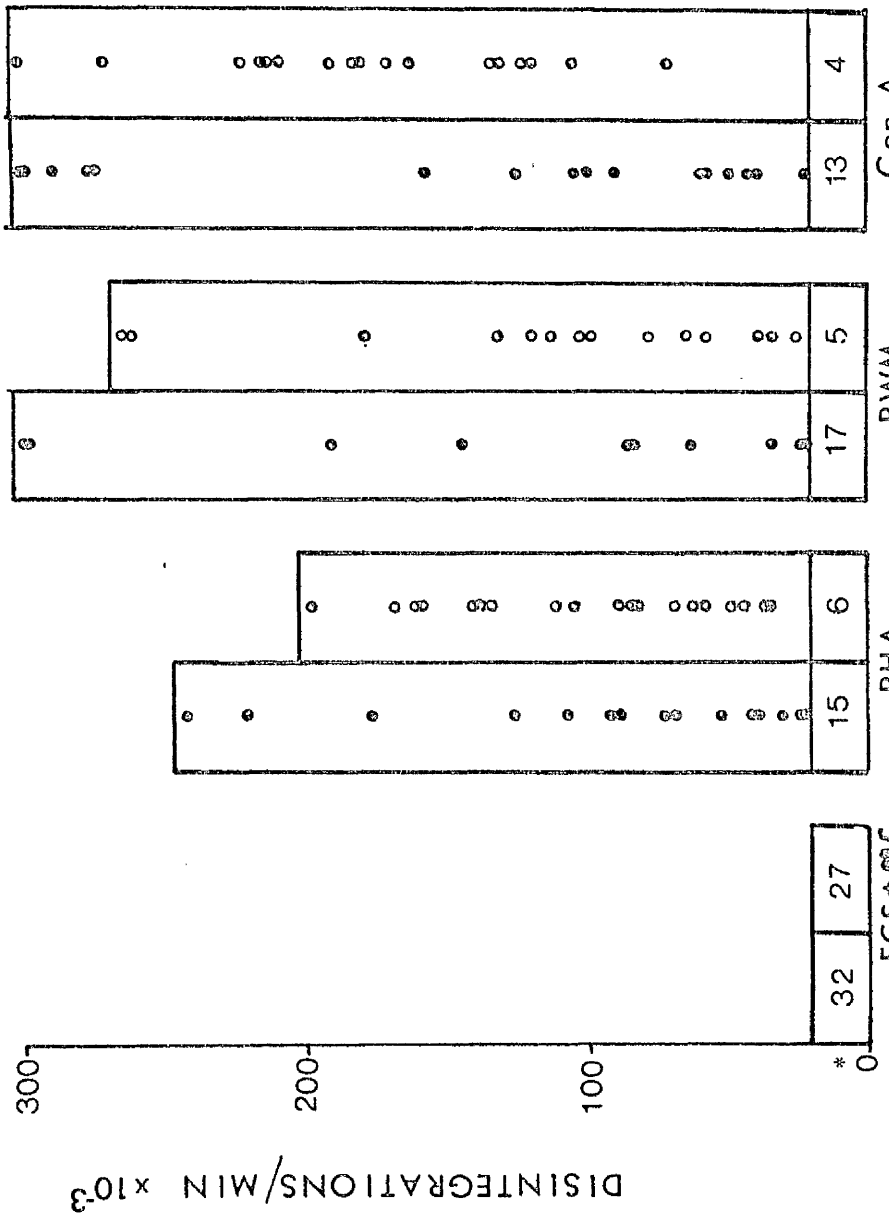


Fig. 4.4. Ovine secondary F. hepatica infection. Mitogen responses with M. E.

* = No. of counts <20,000 dpm

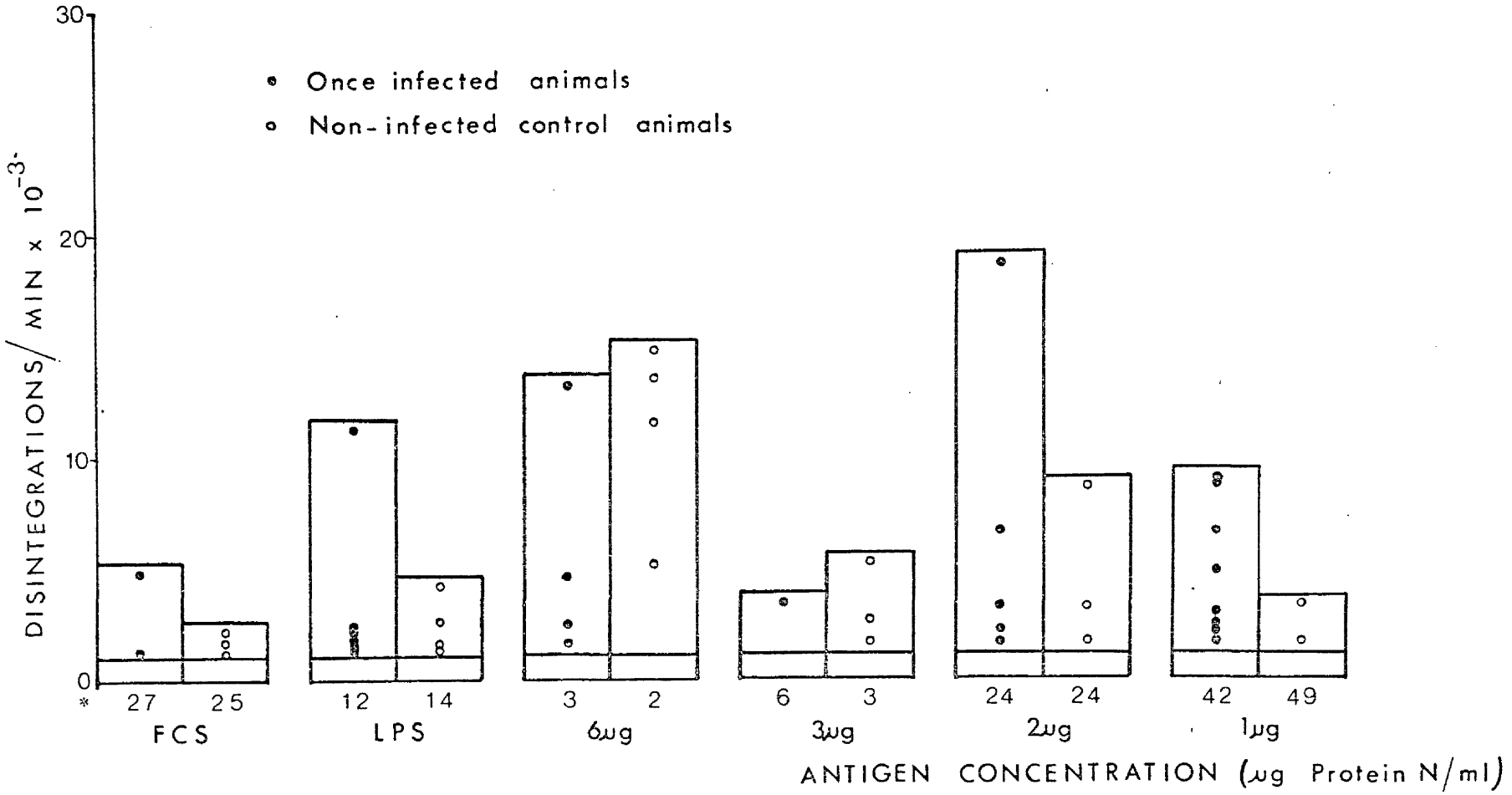


Fig. 4.5. Ovine primary F. hepatica infection. L.P.S. and antigen responses without M.E.

* = No. of counts < 1,000 dpm

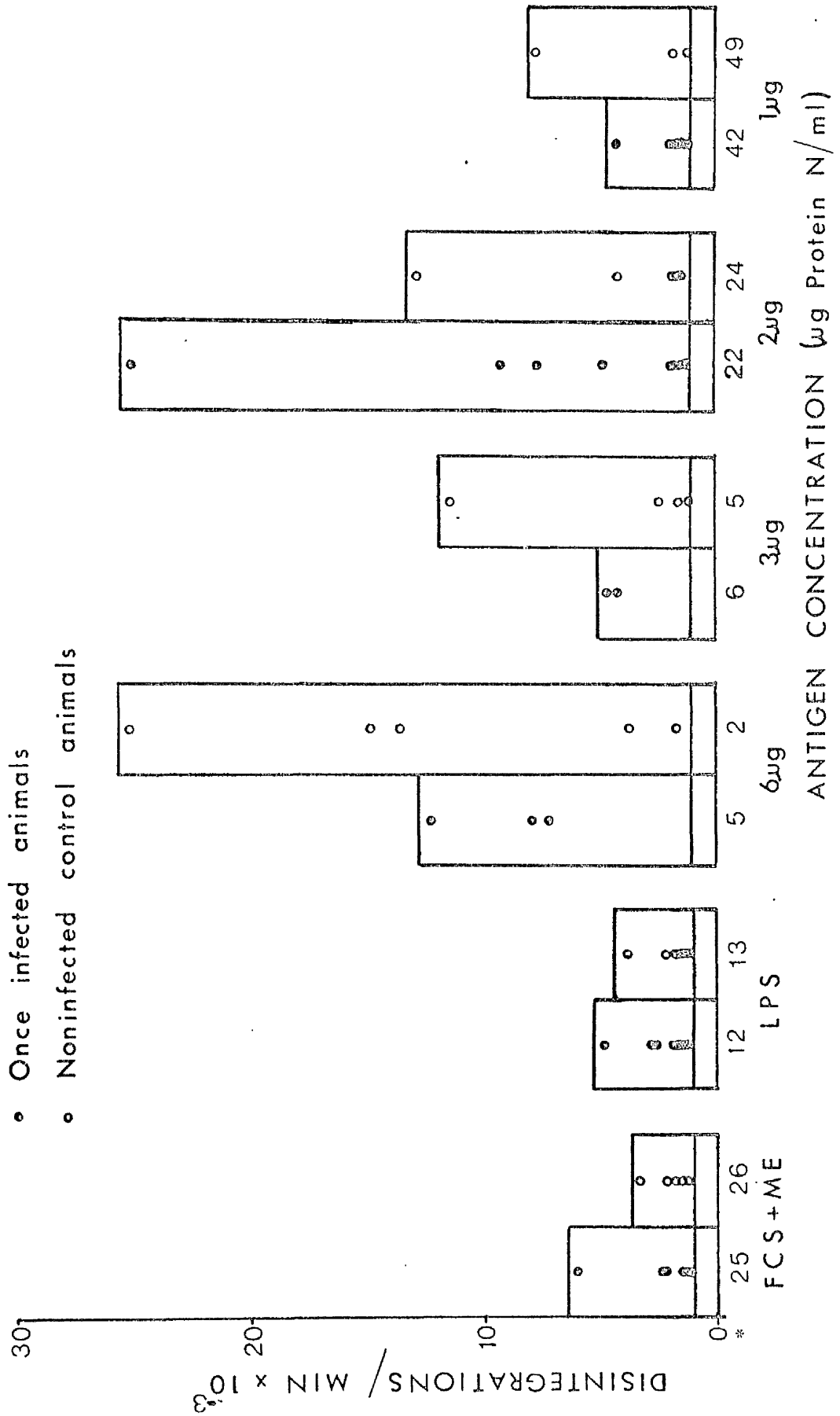


Fig. 4.6. Ovine primary F. hepatica infection. L.P.S. and antigen responses with M.E.

* = No. of counts <1,000 dpm

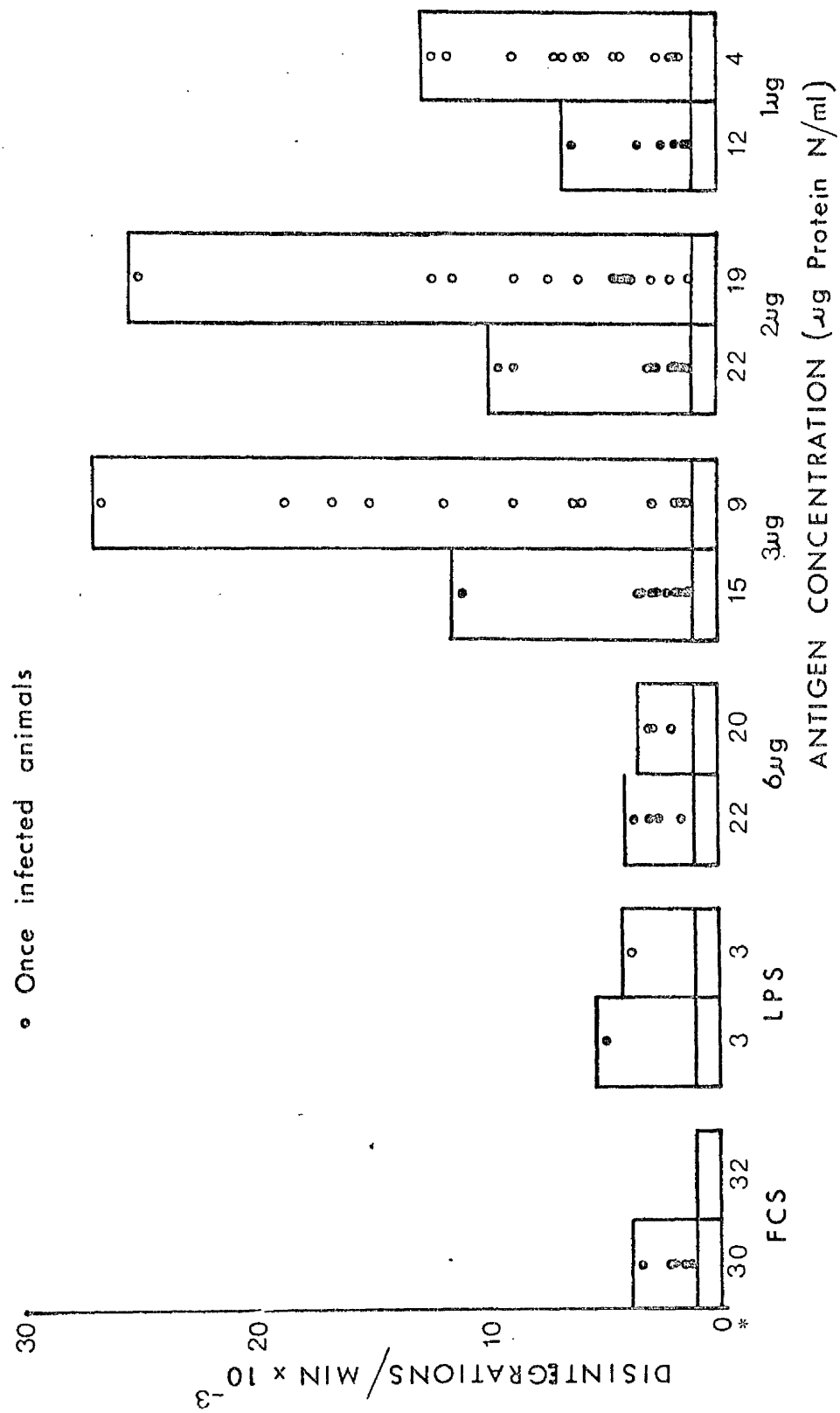


Fig. 4.7. Ovine secondary F. hepatica infection. L.P.S. and antigen responses without M.E.

* = No. of counts <1,000 dpm

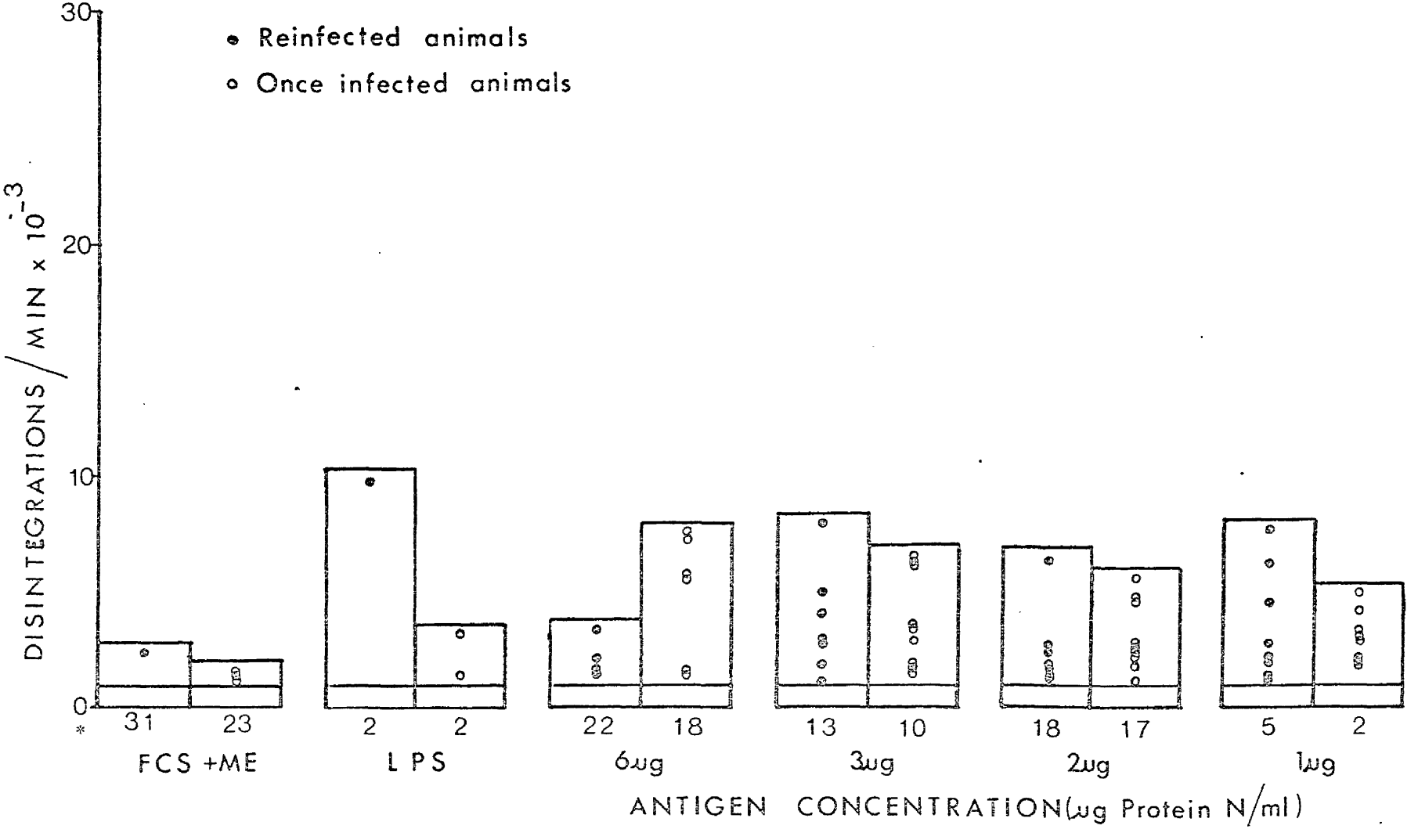


Fig. 4.8. Ovine secondary F. hepatica infection. L.P.S. and antigen responses with M.E.

* - No. of counts < 1,000 dpm

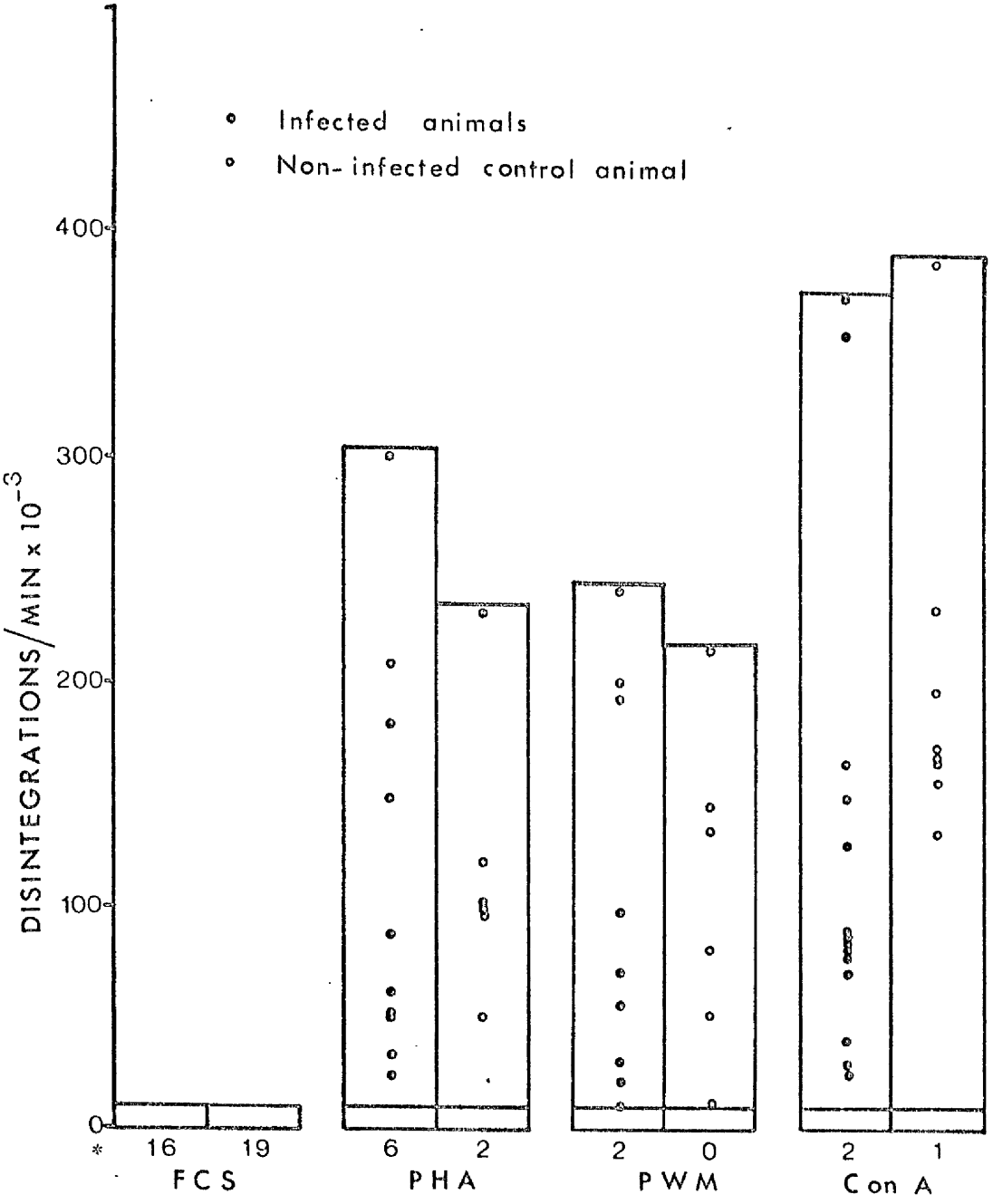


Fig. 4.9. Bovine primary F. hepatica infection
Mitogen responses without M.E.

* = No. of counts <10,000 dpm

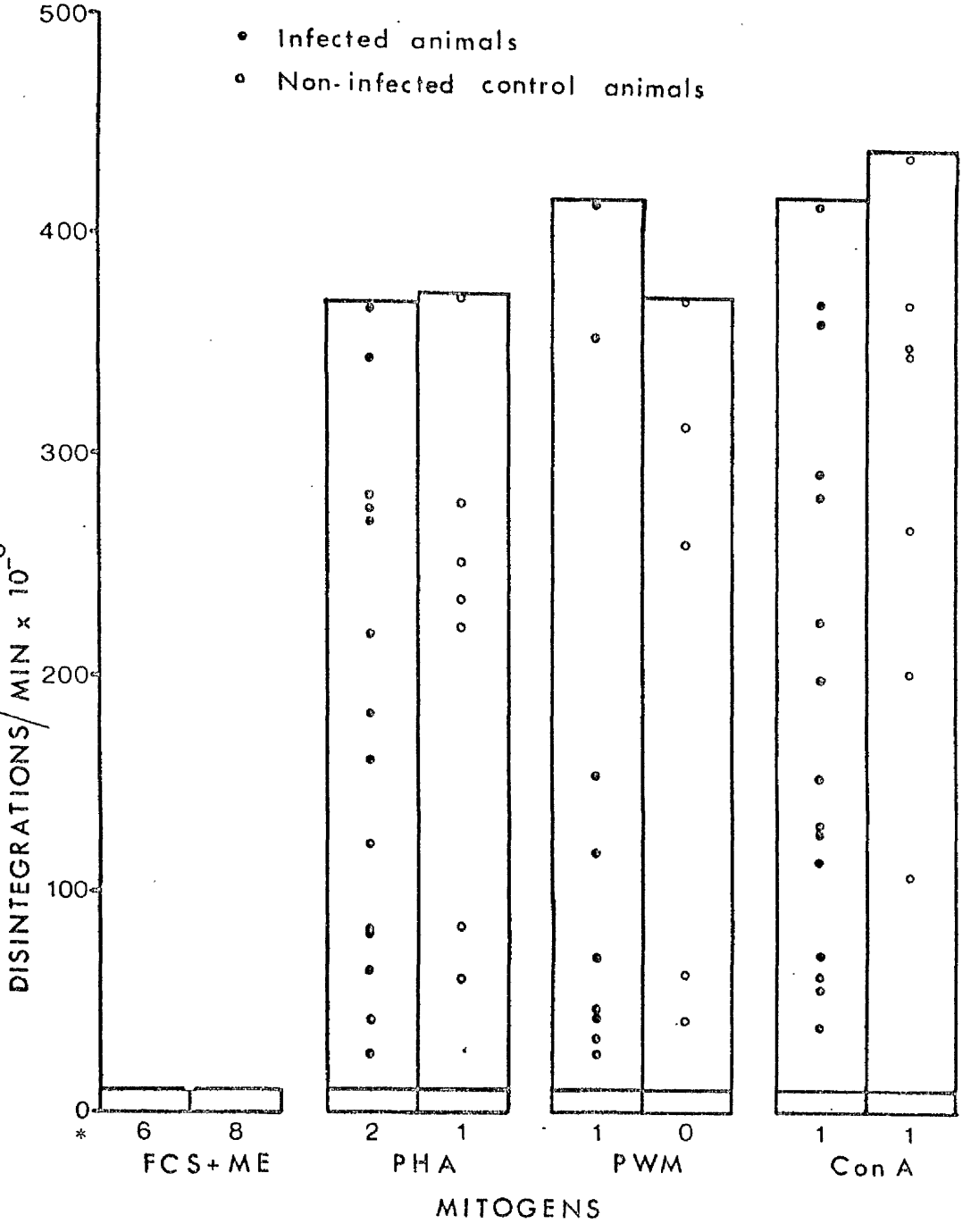


Fig. 4.10. Bovine primary F. hepatica infection. Mitogen responses with M.E.

* = No. of counts <10,000 dpm

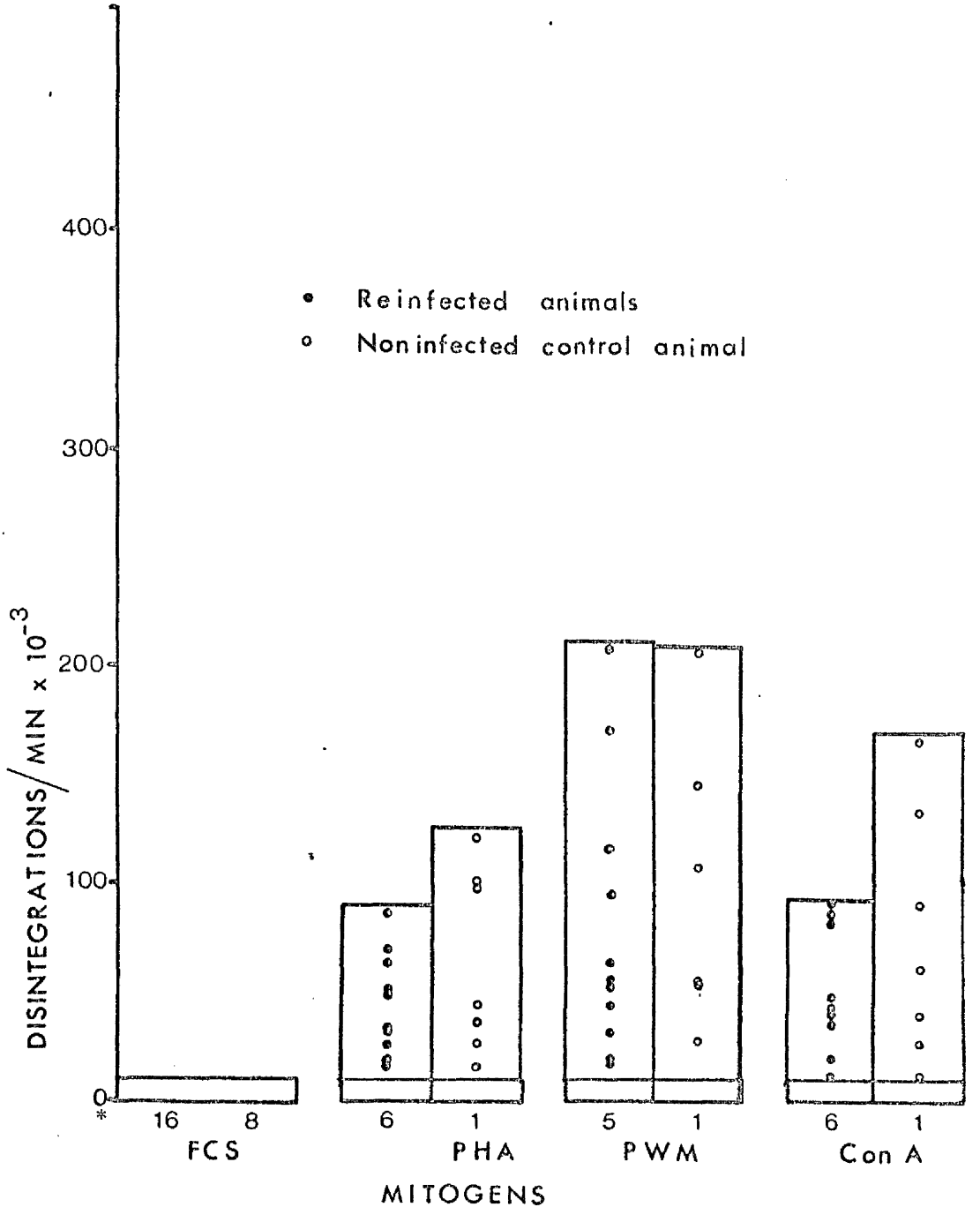


Fig. 4.11. Bovine secondary F. hepatica infection. Mitogen responses without M.E.

* = No. of counts <10,000 dpm

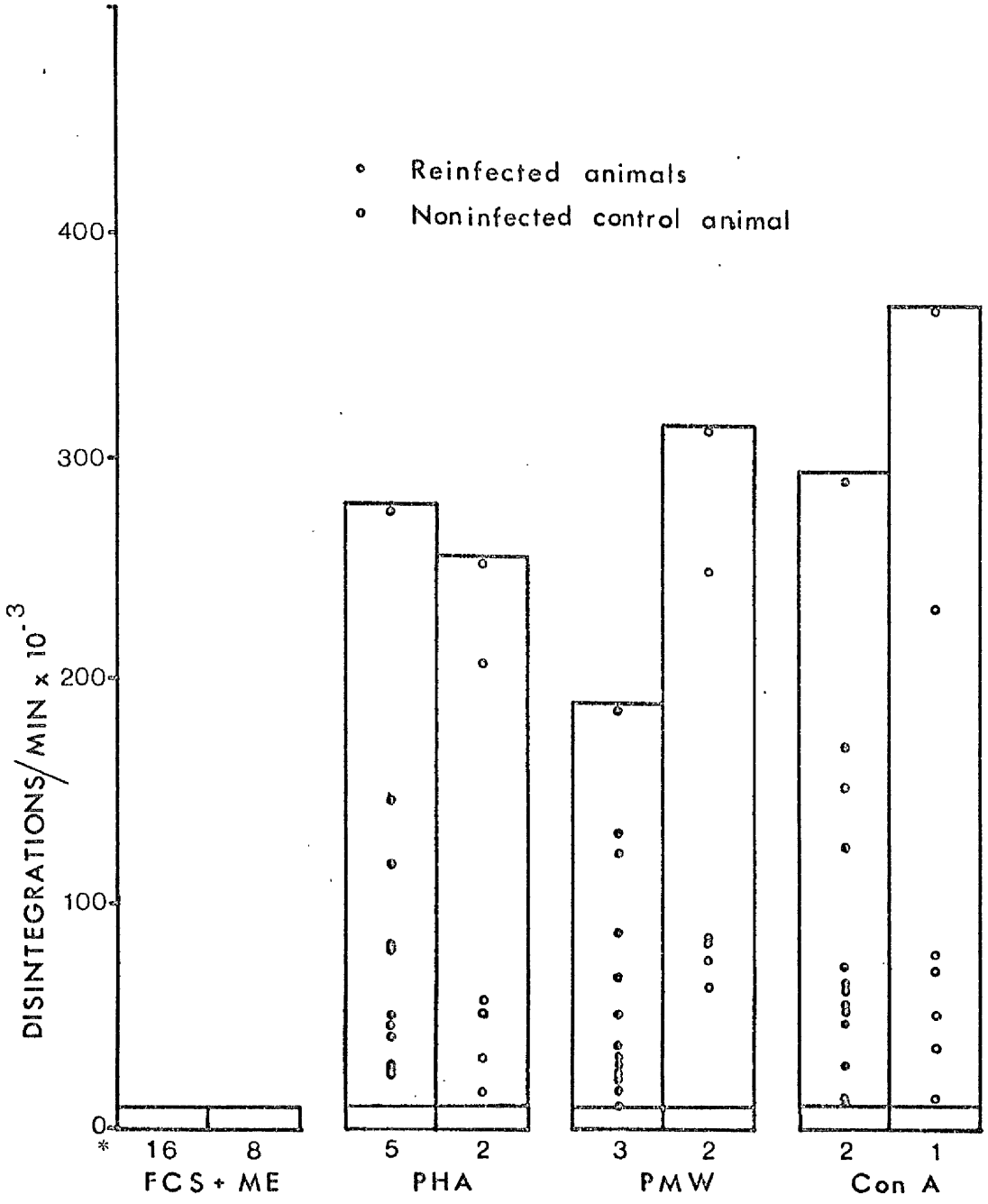


Fig. 4.12. Bovine secondary responses F. hepatica infection. Mitogen responses with M.E.

* = No. of counts <10,000 dpm

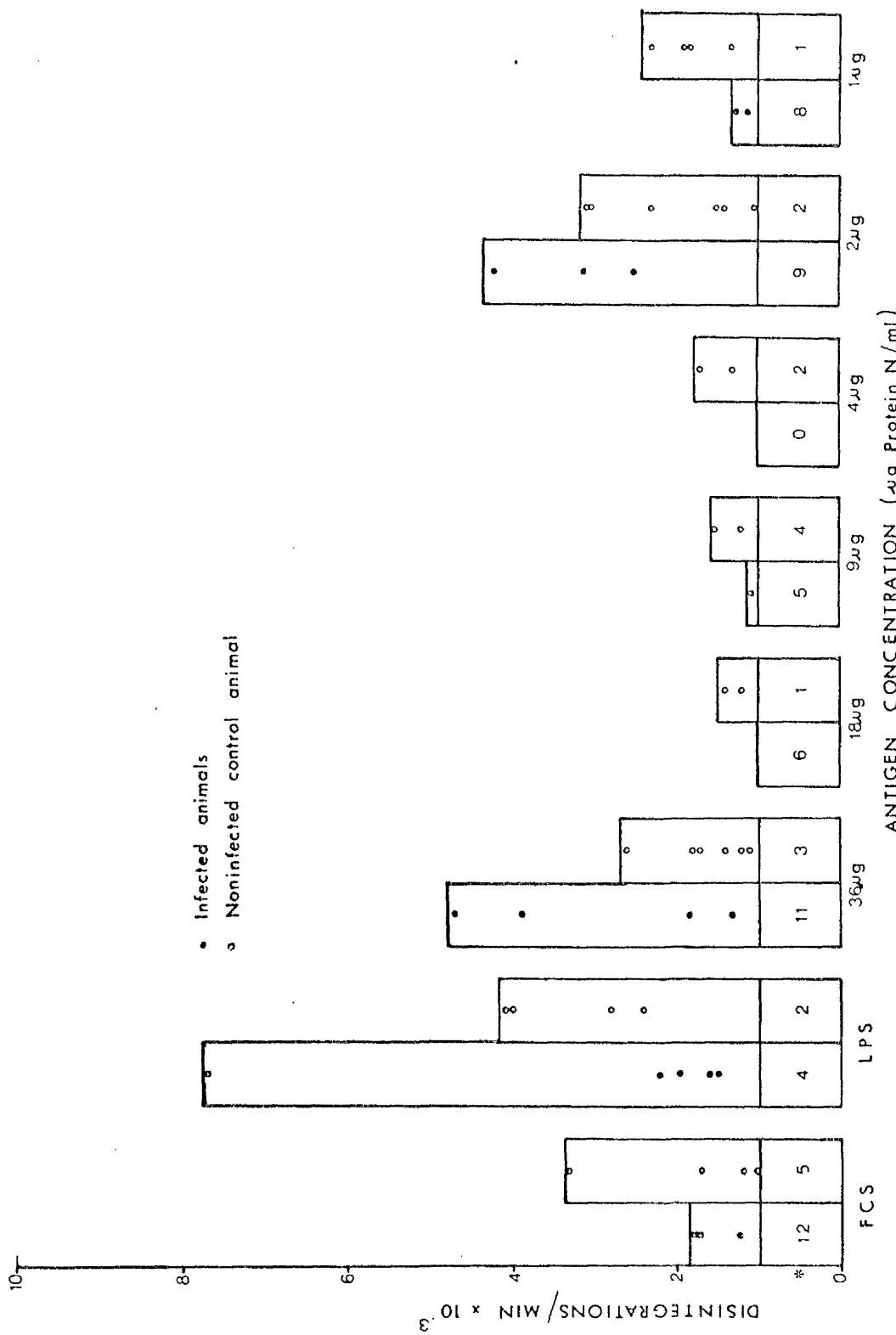


Fig. 4.13. Bovine primary F. hepatica infection. L.P.S. and antigen responses without M.E.

* = No. of counts <1,000 dpm

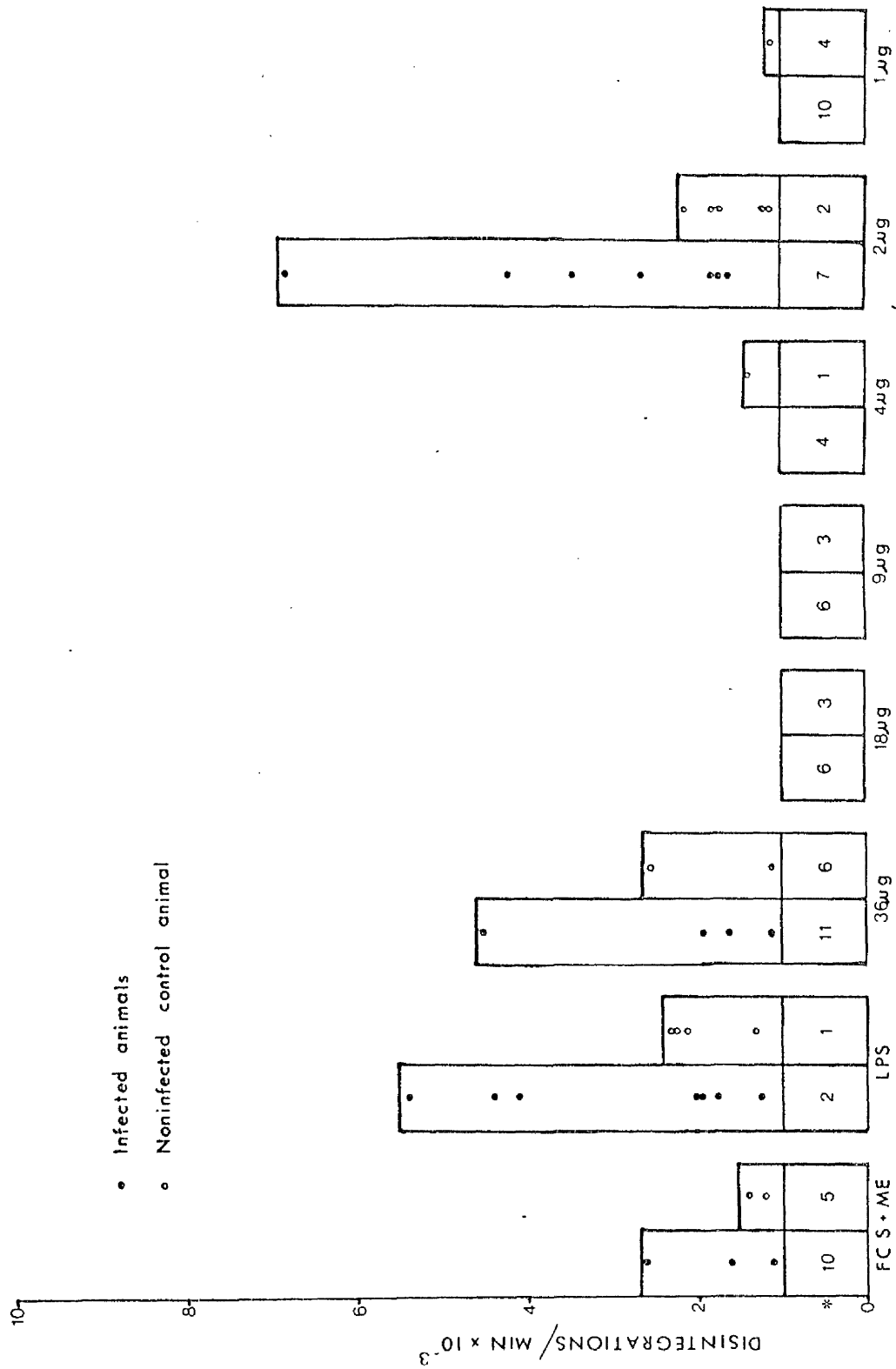


Fig. 4.14. Bovine primary F. hepatica infection. L.P.S. and antigen responses with M.E.

* = No. of counts <1,000 dpm

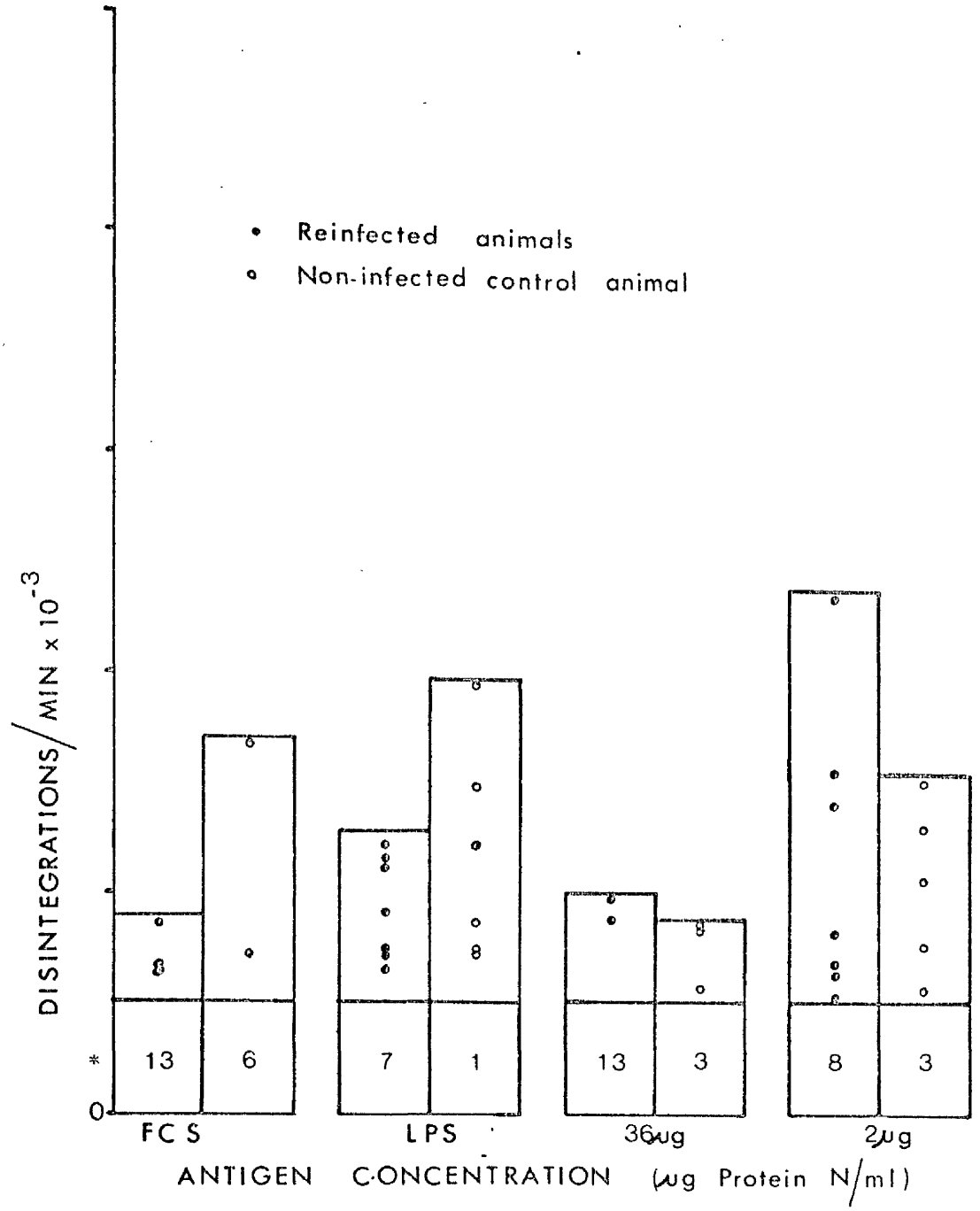


Fig. 4.15. Bovine secondary F. hepatica infection
L.P.S. and antigen responses without M.E.

* = No. of counts < 1,000 dpm

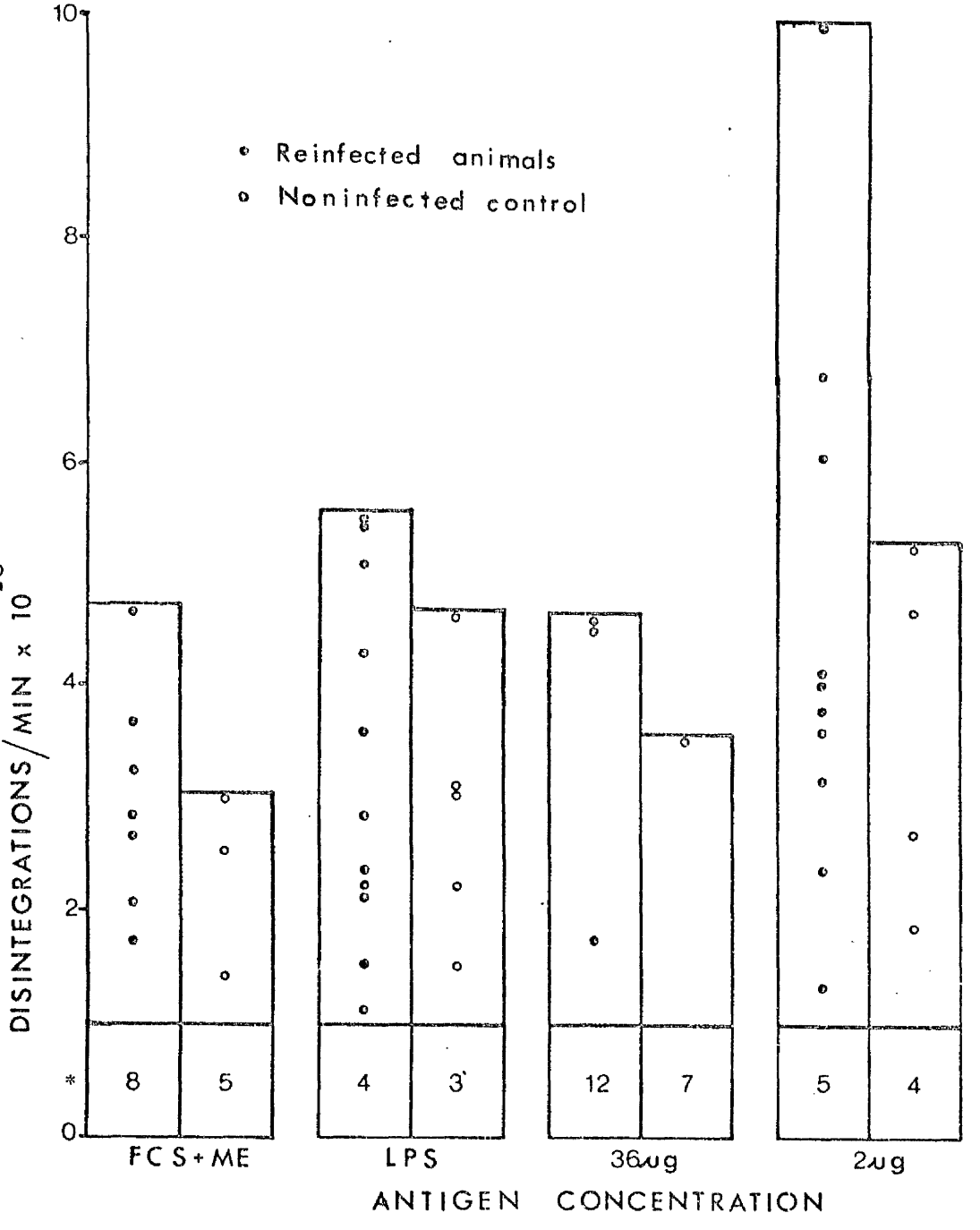


Fig. 4.16. Bovine secondary F. hepatica infection L.P.S. and antigen responses with M.E.

* = No. of counts <1,000 dpm

DISCUSSION

Sheep

Mitogen responses

It was apparent from the present study that the responses of sheep undergoing primary and secondary F. hepatica infection, to the mitogens P.H.A., P.W.M. and Con A, were depressed compared with the control animals. It was further demonstrated that reinfection exerted a relatively greater depressive effect than primary infection.

A similar effect to that described above has been previously reported by other workers in sheep infected with other parasites. Thus Adams and Cripps (1977) observed depressed responses to P.H.A. and Con A, following primary infection of sheep with the nematode Trichostrongylus colubriformis. Although recognising that the thymus-dependence of Con A and P.H.A. reactive lymphocytes has not been formally demonstrated in sheep, as in other species (Janossy et al., 1973; Greaves et al., 1974), the former authors (Adams and Cripps, 1977) were of the opinion that the number of thymus-dependent lymphocytes in both blood and intestinal lymph was reduced during the initial stages of T. colubriformis infection.

By contrast, the same author (Adams, 1978) reported that following primary infection of sheep with the abomasal nematode Haemonchus contortus peripheral blood lymphocyte responsiveness to P.H.A. and Con A was not diminished. The author interpreted this finding as evidence that H. contortus infection had not caused a polyclonal impairment in the transforming ability of peripheral blood lymphocytes or a reduction in the proportion of T cells in the lymphocyte population present in the blood, i.e. a generalised immunosuppression had not been induced.

Interestingly enough, a similarly impaired mitogenic response to that described by Adams & Cripps (1977), in sheep, was recently reported by Burrells et al. (1978), under completely different circumstances. These latter authors recorded a decreased response of ovine lymphocytes from sheep in late pregnancy and at parturition to stimulation by the mitogens PHA and PWM, and suggested that this may be due to some functional impairment of the lymphocytes involved. The possible involvement of plasma inhibitor(s) in this suppressive effect was proposed by the authors on the grounds that a greater suppressive effect to PWM was demonstrated in lymphocytes cultured in the presence of autologous plasma (AP) than foetal bovine serum (FBS).

Antigen responses

From the present work it was apparent that the response of peripheral lymphocytes from sheep undergoing infection with F. hepatica to fluke LFE was of a lower order than that of non-infected animals. It was further observed that lymphocytes from reinfected sheep had poorer responses to LFE of F. hepatica than once-infected animals.

These results are consistent with the findings of several recent studies on other sheep helminths including those by Chen and Soulsby (1976); Adams and Cripps (1977); Adams (1978); Kuppner (personal communication). The first named workers (Chen and Soulsby, 1976) examined the blastogenic responses of peripheral blood leucocytes to an antigen prepared from the third stage larval form of Haemonchus contortus, and demonstrated a reduction in antigen induced blastogenic response associated with an increase in faecal egg output during the peri-parturient period in infected ewes. It was purported that such a phenomenon may be due to changes in the population of antigen sensitive cells in the peripheral circulation, possibly due to antigen sensitive peripheral

leucocytes being removed from the circulation to the mammary glands for the purpose of local colostral antibody production. The possible involvement of humoral and/or serum factors was also noted, and the authors opined that these aspects merited further study.

Similar changes in lymphocyte populations were described by Adams and Cripps (1977) who reported cellular changes occurring in the intestinal lymph of sheep infected with the enteric nematode Trichostrongylus colubriformis. In agreement with Chen and Soulsby (1976) and the findings of the present study the latter authors (Adams and Cripps, 1977) recorded reduced numbers of lymphocytes reactive to T. colubriformis fourth larval stage antigen in both blood and intestinal lymph of sheep during the early stages of infection with the parasite. In the opinion of the authors lymphocytes inherently sensitive to nematode antigens were temporarily removed from the blood stream during the initial stages of T. colubriformis infection and the appearance of such lymphocytes in the intestinal lymph was delayed. Although the authors did not speculate upon possible reasons for these changes it was suggested that alterations of this nature in the population of antigen reactive lymphocytes in lymph and blood may act as an impediment to the dissemination of the immune response against T. colubriformis and may underlie the relatively slow onset of resistance to this parasite.

The same author later reported (Adams, 1978) the induction of selective immunological unresponsiveness in cells of blood and lymphatic tissue of sheep infected with the abomasal nematode Haemonchus contortus In this study the response to larval extracts of H. contortus (HcL3) was used to assay the nematode antigen reactive cells from blood and lymphatic tissue of sheep undergoing active infection with H. contortus. It was

demonstrated that in vitro reactivity to HcL3 was virtually absent from the blood of sheep during active H. contortus infection, but was present with increased intensity in sheep in which infection had abated naturally or which had been treated with an anthelmintic. These findings indicated to the author that active H. contortus infection had caused a depletion from peripheral blood or inactivation of those lymphocytes reactive to nematode antigens. As discussed previously, in this study, in vitro responses to the mitogens PHA and Con A were not diminished during the infection and therefore the authors proposed that the parasite had induced a state of selective rather than complete immunological unresponsiveness in an attempt to evade the immunological reactions of the sheep. Possible mechanisms for this observed depletion or inactivation of nematode antigen-reactive lymphocytes proposed by the author included the following hypothesis: firstly, that such lymphocytes had been retained in the abomasal wall, presumably to assist in a local immunological response or secondly that reactive cells had been blockaded by soluble antigen or antigen-antibody complexes in vivo or in vitro, in the latter case due to complexes formed by antigen combining with the products of antibody-secreting cells during the period of cultivation. Whichever mechanism was functional it appeared that the unresponsive state of sheep to the nematode may not only enhance the likelihood of survival of the parasite, but also hinder the development of an effective regime for immunisation against the parasite.

As far as the present studies are concerned there would appear to be considerable evidence, on the basis of reduced mitogenic and antigenic responses of sheep infected with F. hepatica compared with non-infected animals, for a similar state of unresponsiveness to that described by Adams (1978), for H. contortus infection. It would appear, however, unlike the

former situation, since non-specific mitogenic as well as specific antigen responsiveness was impaired, that F. hepatica may have been responsible for a polyclonal impairment in the transforming ability of peripheral blood lymphocytes or a reduction in total numbers or functional capacity of T and B lymphocytes. That suppressor cell activity is implicated in such a response is likely, and the possible involvement of such cells will be described in greater detail in Chapter VI. The fact that lymphocytes of reinfected sheep gave poorer responses to mitogens and fluke L.F.E. than once-infected animals indicated that the secondary fluke infection may have enhanced the impairment of the immune system previously described despite the fact that the primary infection had been removed by anthelmintic. If this were true then it may partly explain the observed acute susceptibility of sheep to repeated F. hepatica infection and the inability of sheep to mount a functional immune response to the fluke i.e. a response which would protect the animal from the pathogenic effects of the parasite.

Cattle

Mitogen responses

In contrast to the results obtained in ovine fascioliasis it was apparent that the response of lymphocytes from infected and non-infected cattle were of a similar order. The only significant points of difference between the two groups was a decreased response of lymphocytes from animals undergoing primary F. hepatica infection to stimulation with Con A, and a slight decrease in response to P.W.M. by reinfected animals compared with control values. As mentioned previously it has been demonstrated that Con A selectively stimulates T lymphocytes (Rouse and Babuik, 1974) in the bovine and that P.W.M. stimulates T and B lymphocytes (Peavy et al., 1974). There would therefore

appear to be some evidence to suggest that there is a slight impairment of function or reduction in numbers of primary T lymphocytes responsive to those mitogens, following F. hepatica infection in cattle. In addition responses of cattle to the mitogen L.P.S. are worthy of note. L.P.S. has been shown to stimulate B lymphocytes, almost exclusively, in the mouse (Doenhoff et al., 1974). Whether this is also true in cattle has not yet been fully demonstrated. In the opinion of Lazary et al. (1974) L.P.S. stimulation may represent a specific response of sensitised lymphocytes from animals exposed to a cross-reacting antigen from E. coli, and thus could be due to specific antigenic stimulation of the cells rather than non-specific mitogenic stimulation by virtue of the B cell stimulatory activity of L.P.S. per se. This picture is further complicated by the fact that M.E. has a weak mitogenic activity on B cells and this may partly explain the increased responses of once-infected and reinfected cattle to L.P.S. stimulation compared with control values, in the presence of M.E., observed in the present studies. It is considered by the present author that until such time as the exact nature of stimulation of bovine lymphocytes by L.P.S. has been clearly defined, interpretation of the results of L.P.S. stimulation in the present study, in view of the equivocal results obtained, would not be meaningful.

Observations similar to those of the present study have been reported by several groups of workers examining the response of the bovine to mitogenic stimulation under a variety of different circumstances. Weiland and Straub (1976), reported differences in the in vitro response of lymphocytes to Con A of lymphocytes from cattle infected with enzootic bovine leucosis (E.B.L.). It was recorded that lymphocytes from animals suffering from E.B.L. were significantly less responsive to Con A than those of non-infected animals.

The authors demonstrated that lymphocytes from animals with low leucocyte counts were more reactive than from animals with high leucocyte counts, and concluded that leukotic cells were not stimulated by Con A and hence the residual cell populations of normal reactive lymphocytes were alone responsible for the impaired response to Con A. The unresponsiveness of the leukotic cells to Con A was thought to be due to their B cell characteristics, since Con A is exclusively a T cell mitogen in cattle (Rouse and Babiuk, 1974).

A comparable study of responses of cattle under different circumstances was reported by Wells et al. (1977) who observed reduced mitogenic responses in lymphocyte cultures from newly calved cows. The proliferative responses to stimulation with P.H.A. of newly calved cows were shown to be considerably lower than non-pregnant animals. The authors considered that this reduction in immunological activity may be related to the elevated plasma concentrations of corticosteroids in parturient cattle (Guidry et al., 1976) which are known to be capable of inhibiting the manifestations of cell-mediated immunity. This observation has been discussed earlier in relation to the effects of corticosteroids on the course of F. hepatica infection (Corba and Spaldonova, 1974, 1975). The former authors (Wells et al., 1977) pointed out that the subpopulation of bovine peripheral blood lymphocytes associated with the in vitro proliferative response to P.H.A. has been shown to have characteristics of T. cells, and suggested that a reduction in the responsiveness of T cells around parturition may be of importance in the aetiology of disease in cattle during the immediate post-parturient period.

Antigen responses

From the results of the present studies it was apparent that peripheral lymphocytes of cattle infected with F. hepatica responded more vigorously to

fluke L.F.E. than those of non-infected animals. This was true during both primary and secondary infection although differences in response to L.F.E. when compared with non-infected control values were more marked on reinfection.

Comparison of the above findings with those of related studies in this field referred to elsewhere is instructive although, at the time of writing, L.T.T. responses to parasite antigen in cattle infected with helminths have not yet been recorded.

From studies in cattle Genchi et al. (1973); Genchi and Sartorelli (1974), reported, among other findings that the migration inhibition test (M.I.T.) was positive in 71 out of 80 cattle infected with F. hepatica and negative in all 40 uninfected cattle tested. The authors interpreted this result as implicating cellular factors responsible for a delayed type hypersensitive response. Other interesting findings of this work included the fact that significantly more histamine was released by cells of fluke-infected cattle after incubation with F. hepatica antigen, than by cells from uninfected animals. The authors also demonstrated that histamine released from leucocytes of non-infected cattle could be increased by exposure to plasma from fluke-infected animals. This observation is particularly stimulating bearing in mind the thoughts of Chen and Soulsby (1976) and Burrells et al. (1978) on the possible role of serum factor(s) in the blastogenic response to mitogenic and antigenic stimulation. As it may be remembered, the former authors (Chen and Soulsby, 1976) suggested that serum factors may in some way modify antigen-infected blastogenesis in the sheep, and the latter authors (Burrells et al., 1978) observed that suppression of sheep lymphocyte responses to P.H.A. and P.W.M. were enhanced in the presence of autologous plasma, and suggested that inhibitor(s) which may

affect normal responses of lymphocytes to antigenic and mitogenic stimulation may be involved. The work of Genchi and Sartorelli (1974) indicated that heterologous plasma from donor cattle sensitised to fluke antigen could in some way modify the response of leucocytes from non-infected, non-sensitised control animals. The significance of these observations in the light of the considerable literature on the transfer of resistance to a wide range of disease, parasitic and otherwise, by a serum "transfer factor" will be discussed at greater length in Chapter VI.

From the present studies it was apparent that there were marked differences, in terms of lymphocyte transformation, between the two ruminant species in response to F. hepatica infection. Sheep lymphocytes displayed a reduced ability to respond to both non-specific mitogenic and specific antigenic stimulation which would suggest that the liver fluke in this species exerts a non-specific immunosuppressive effect on polyclonal lymphocyte transformation as well as inducing a state of non-responsiveness to fluke antigen. In cattle, on the other hand, no such effects were demonstrable and although there did appear to be some slight impairment of T cell function, on the basis of mitogen responses, infected cattle responded much more vigorously than non-infected animals to fluke antigen at all stages of infection. This may be of great importance in the responses of the two species to reinfection. In sheep it was clearly demonstrated that mitogenic and antigenic responses were impaired when compared with once-infected animals, whereas reinfected cattle displayed no such debility, although within the present studies it must be pointed out that the comparison of reinfected with once-infected cattle was not possible.

In summary, on the basis of the present studies, insofar as

lymphocyte transformation in vitro reflects the cell-mediated immune response in vivo, immunosuppressive effects have been demonstrated in sheep which were not observed in cattle. In the opinion of this author these effects may, in part, explain the inability of sheep to mount an effective immune response against the parasite. Further knowledge concerning the mechanisms of this phenomenon in sheep could well be obtained from studies designed to examine the circulation and localisation of lymphocytes derived from local lymph nodes e.g. (hepatic, coeliac) with particular reference to the response of such cells to F. hepatica antigen. Studies of this nature involving cannulation of local lymph ducts would be particularly helpful in this respect.

CHAPTER V

CROSS-IMMUNISATION STUDIES OF FASCIOLA HEPATICA INFECTION IN SHEEP

INTRODUCTION

Over the past few years it has emerged from various studies that infection with other helminths, some closely related, others only distantly related as well as with some protozoan and bacterial genera may modify the response to infection with the liver fluke Fasciola hepatica, in ruminants including sheep.

The species involved include:

(a) Helminths: Fasciola gigantica

Taenia hydatigena (larval stage)

Nippostrongylus brasiliensis

Schistosoma mansoni

(b) Protozoans: Babesia divergens

Trypanosoma congolense

(c) Bacteria: Salmonella dublin

The influence of these parasites will now be discussed under the three broad headings outlined above.

(a) Helminths. Dorn (1974) in studies on guinea pigs experimentally infected with both F. hepatica and the closely related liver-fluke F. gigantica was able to demonstrate a reduction in fluke numbers of around 50% after homologous challenge with F. gigantica, but not after homologous challenge with F. hepatica, nor after heterologous challenge infections with either parasite.

However, Campbell et al. (1977) were able to demonstrate the stimulation of resistance to F. hepatica infection in sheep due to prior infection with the larval cestode Cysticercus tenuicollis, which is of course taxonomically remote from the trematode F. hepatica. Thus sheep previously

exposed to a period of 12 weeks infection with C. tenuicollis showed a reduction of approximately 95% in flukes recovered, from a challenge infection of 400 metacercariae, compared with previously uninfected control animals. This resistance was apparent whether challenge with F. hepatica metacercariae was superimposed upon the larval cestode infection or after removal of the latter with an anthelmintic. Resistant animals also had a significant reduction in numbers of F. hepatica eggs in their faeces and were apparently protected from the pathogenic effects of the liver fluke as measured by hepatic fibrosis and packed cell volume. Merino sheep infected with C. tenuicollis for 3 weeks only were not resistant to F. hepatica infections and the authors suggested that this may be due to the time required for development of the effector mechanism, the basis of which was believed to be immunological. The alternative theory that mechanical trauma caused by migration of the larval cestode had produced a physical barrier to F. hepatica invasion was dismissed by the authors on the grounds that fibrosis as evidenced by liver weights and microscopic observations, was less in responsive animals than in non-responsive animals.

The stage of development at which the challenge fluke infection was affected in resistant sheep was not clear in these studies, but it was thought that rejection occurred either before or shortly after penetration of the liver, resulting in fewer numbers of flukes developing to adulthood in the bile ducts.

It remained uncertain whether active infection with C. tenuicollis provided protection due to direct activation of a specific immune reaction by cross reacting antigens or whether indirect potentiation of non-specific tissue reactions by a specific immune mechanism was involved. However, since immunity to larval cestodes has been successfully stimulated using parasite-free material (Rickard and Bell, 1971), an optimistic view was expressed

for the possible employment of such methods to vaccinate sheep against F. hepatica.

Further evidence of cross reactions of F. hepatica with other helminths was provided in studies on the nematode Nippostrongylus brasiliensis infections in rats (Goose, 1977; Doy et al., 1978 a) & b)). The former study demonstrated that F. hepatica infected rats were more resistant than controls to N. brasiliensis. This resistance applied to intra-intestinally implanted N. brasiliensis adults, as well as subcutaneously injected larvae and was not serum-transferable. The reverse situation was studied by Doy et al. (1978a) whose results indicated that rats previously infected with 3,000 N. brasiliensis larvae had significantly lower fluke burdens than control rats, when challenged with 30 F. hepatica metacercariae. Immune serum from rats infected 4 weeks previously with 40 F. hepatica metacercariae did not significantly improve protection, nor did the administration of immune serum protect in its own right. The possible involvement of intestinal eosinophils, a feature of both infection with N. brasiliensis and F. hepatica was proposed by these authors (Doy et al., 1978b).

The relationship between F. hepatica and the trematode Schistosoma mansoni was investigated by Hillyer et al. (1975); Hillyer (1976); Hillyer et al. (1977).

In experimental infections in golden hamsters and mice it was clearly established (Hillyer et al., 1975) that immunisation with a crude saline extract, or a partially purified extract of adult F. hepatica conferred a significant degree of resistance to infection with cercariae of S. mansoni, compared with animals immunised with bovine serum albumin. Both extracts of F. hepatica were demonstrated to have antigens which cross reacted with those found in adult S. mansoni.

The same author (Hillyer, 1976) showed further that ~~an~~ antisera to S. japonicum adults cross reacted with a crude extract of F. hepatica in a precipitation reaction. Subsequent studies (Hillyer et al., 1977) indicated that mice and hamsters inoculated with whole worm extracts of F. hepatica developed antibodies which were reactive with extracts of adult S. mansoni. Animals immunised in this manner also produced 3 - 8 times more S. mansoni antibody in an indirect haemagglutination test than infected, but non-immunised animals. When hamsters were immunised with whole worm extracts of F. hepatica or with F. hepatica antigens cross-reacting with S. mansoni and subsequently challenged with schistosome cercariae, 29 -57% less adult S. mansoni were recovered than in non-immunised control animals. Similarly immunised mice had 39 - 65% less adult S. mansoni than non-immunised controls.

(b) Protozoans. Recent studies (Goose, 1977; Hughes et al., 1977b) have demonstrated possible relationships between F. hepatica infection and infection with the protozoans Trypanosoma congolense and Babesia divergens respectively.

Goose (1977) investigated T. congolense infections in rats experimentally infected with F. hepatica and obtained some degree of resistance to challenge with the protozoan following prior infection with the trematode.

Hughes et al. (1977b) examined the effect of initial F. hepatica infection on the pathogenicity of subsequent B. divergens infections in intact and splenectomised calves. The results of this study indicated that there was no more than a tendency for concurrent F. hepatica infection to exacerbate B. divergens infections of intact cattle. However, it was reported that intact animals infected 13 weeks previously with F. hepatica had higher and more persistent B. divergens parasitaemias than control animals, and in addition tended to have more severe febrile responses. There was no evidence that

splenectomised animals similarly infected with F. hepatica had more severe responses to B. divergens infection than control animals.

(c) Bacteria. An association between the occurrence of salmonellosis and fascioliasis in cattle was described by Frik (1969) and Dijkstra (1973) who maintained that S. dublin excretion was closely linked to concurrent F. hepatica infection. This view was opposed by Taylor and Kilpatrick (1975) who attributed the apparent correlation to the fact that both infections were influenced by similar climatic conditions and management practices. Since then it has been reported that cattle infected with fluke showed an increased susceptibility to experimental infections with S. dublin (Aitken et al., 1976; Aitken et al., 1978 a) & b)). Those authors investigated firstly the effects of experimental S. dublin infection in cattle infected 13 weeks previously with F. hepatica, and secondly the effects of intravenous S. dublin infection on cattle at different stages of F. hepatica infection. The former study (Aitken et al., 1978 a)) indicated that there was a reduction in the lethal dose of S. dublin (5×10^7 bacteria compared with 10^9) in cattle infected 13 weeks previously with F. hepatica compared with control fluke-free animals. It was further noticed that surviving animals infected with F. hepatica excreted larger numbers of S. dublin more frequently and for a longer period than fluke-free animals. The authors postulated that the enhanced susceptibility of F. hepatica infected animals to S. dublin may be in part due to impairment of liver function due to hepatocellular damage resulting in decreased phagocytosis by cells of the reticular-endothelial system and detoxification of endotoxin. It was also suggested that immunosuppression due to F. hepatica infection may be involved, which may include both cellular and humoral defence mechanisms, and that considerable importance may be attached to the fact that a low level of

fascioliasis, common in cattle in the field situation may predispose to clinical salmonellosis and for persistent excretion of S. dublin creating a carrier state and encouraging spread of infection.

Subsequently, a similar study by the same authors (Aitken et al., 1978 b)) was undertaken to determine how S. dublin infections were influenced by F. hepatica infections of differing duration. It was reported that a similar enhancement of pathogenicity as previously described in cattle with 13 week F. hepatica infections, occurred in animals infected with F. hepatica 25 weeks before S. dublin infection. However, when S. dublin infection was given to cattle 1 week after F. hepatica infection it produced no more severe clinical effects than in fluke-free cattle. Excretion of S. dublin was again greatly enhanced in cattle infected 25 weeks after F. hepatica infection, but not in animals with only week-old F. hepatica infection.

The authors attributed this difference to the fact that F. hepatica may produce factors which interfere with phagocytosis or antibody produced against F. hepatica may block interactions between S. dublin and phagocytic cells. It was presumed that an insufficient quantity of such factors had been produced after only one week of infection to affect the course of S. dublin infection.

It was also pointed out that cattle chronically affected with fluke in the field situation may also be more severely affected by S. dublin infection.

As well as interactions with other parasites and bacteria the course of F. hepatica infection, in common with many other helminth infections may be altered by a number of host factors including age, sex, breed, and in the case of sheep, haemoglobin type (Altaif and Dargie, 1978 a) & b)).

Effects of age

Descriptions of acute fascioliasis in cattle are very rare, in the field situation. Possibly the only description is provided by Ross (1966 a) in Northern Ireland, where severe clinical disease was described, significantly in animals only 5 - 8 months of age. There has been a great deal of controversy over the occurrence of clinical fascioliasis in adult cattle which in itself suggests that an age factor may be involved in resistance to fascioliasis in cattle, since at the date of writing, the occurrence of clinical fascioliasis in adult cattle in this country has not been documented.

In the laboratory model of experimental fascioliasis in the rat there have been a number of reports of age resistance. Goose and McGregor (1974) reported that susceptibility to F. hepatica infection in Wistar strain rats increased with age from weaning to puberty. Thereafter an age resistance developed and rats became less susceptible. These findings are in general agreement with those of Hayes et al. (1974b). The latter authors using male outbred Albino rats (C.D. rats) observed a reduction of 56% in number of flukes recovered from rats infected with 20 F.hepatica metacercariae when 54 weeks old, compared with rats similarly infected when 4 weeks of age. An incidental finding in this study was the effect of age on existing F. hepatica infection. It was recorded that some rats infected 52 weeks previously with 20 F. hepatica metacercariae had much enlarged but empty bile ducts, indicating that fluke loss had occurred. It was suggested that the effect of host age on an existing F. hepatica infection in the rat, merited further study.

Various features of the age resistance phenomenon in rats experimentally infected with F. hepatica were described by Rajasekariah and Howell (1977), using male Wistar rats. The authors summarised these features as follows:

1) Demonstration of age resistance depended on the infecting dose of metacercariae being greater than 1.

2) Age resistance was consistently observed in comparisons of fluke burdens from 15 and 25 week-old rats, with those of 5 week-old rats.

3) Once resistance was established it did not appear to increase with age, since no difference was observed between fluke recoveries from 15 and 25 week old rats.

4) 10 week old rats varied in their degree of expression of resistance and it was thought that resistance developed around this age.

Effects of sex and breed (strain)

Various reports have shown that sex and breed (strain) may modify the host's response to F. hepatica infection. The majority of these have been concerned with the laboratory model of F. hepatica infection in the rat, although Van Tiggele (PhD thesis 1978) in studies on experimental and natural F. hepatica infections in sheep also presented some evidence that sex may be an important factor in determining the response to the parasite.

Goose and McGregor (1974) recorded that male Wistar strain rats were more susceptible to experimental F. hepatica infection at puberty than female rats. This situation was reversed in male rats implanted with oestradiol and in female rats implanted with testosterone prior to infection. This sex difference was not demonstrated in weanling rats or in mature animals. These workers further reported that rats of both sexes were resistant to a challenge infection following an initial immunising infection, females being more resistant than males. In this study flukes recovered

from male rats tended to be larger than those recovered from females.

Hughes and Harness (1974); Hughes et al. (1976c), while conducting experiments attempting to immunise rats against F. hepatica, noticed significant differences in fluke burdens between male and female control rats. This led those workers to investigate sex, as well as strain differences as factors controlling responses to F. hepatica infection in the rat. The strains used were Piebald Virol Glaxo (PVG) and Sprague Dawley (SD). When male and female rats of both strains were killed 3 months after experimental infection with 20 metacercariae of F. hepatica, i.e. when they were 8 weeks of age, a highly significant difference was found between fluke burdens of the 2 strains, the PVG strain being more susceptible than the SD. There was also found to be a highly significant sex difference within the PVG strain; the male rats proving much more susceptible to F. hepatica infection than the females. In the SD strain sex did not influence fluke recovery significantly.

Differences were also observed in longevity of F. hepatica infection between the two strains. PVG rats lost their fluke burden 7 - 8 weeks after infection while SD rats did not rid themselves of flukes until 12 months of infection.

Further evidence of the effect of sex and strain of rat on the course of F. hepatica infection was provided by Dargie (personal communication) who noted that the Hooded Lister strain of rat appeared to be more susceptible than the Wistar strain to experimental infection with F. hepatica. Within the Hooded Lister strain female rats appeared to be more resistant than males.

Effects of haemoglobin type

Considerable evidence has accrued over the years to support the theory that the response of sheep to helminth parasites may be in part determined

by their haemoglobin type. Most of this work has been carried out with the abomasal nematode Haemonchus contortus.

Sheep, as well as goats, demonstrate clear polymorphism for haemoglobin in that 2 allelles (A and B) are normally found (Harris and Warren, 1955; Huisman et al., 1958 a) & b)). The expression of these allelles in haemoglobin types varies considerably between different breeds of sheep.

Several groups of workers including Evans and Whitlock (1964); Jilek and Bradley (1969); Radhakrishnan et al. (1972); Allonby and Urquhart (1976), in various parts of the world, have shown that grazing sheep which are more able to cope with H. contortus infection in terms of maintaining higher haematological parameters and lower faecal egg counts tended to be of haemoglobin type A (HbAA).

This influence of genetic factors on resistance to helminths was further studied by Altaif and Dargie (1978 a) & b) in studies of experimental infections with H. contortus on Scottish Blackface and Finn Dorset sheep. These authors were able to demonstrate, firstly that haemoglobin A (HbAA) sheep of both breeds suffered less severe clinical and pathophysiological effects in response to H. contortus infection than haemoglobin B (HbBB) sheep. It was further reported (Altaif and Dargie, 1978 b)) that similar advantages as outlined above were exhibited by haemoglobin AB(HbAB) sheep compared with Hb BB sheep. In both studies the importance of breed was underlined by the fact that Scottish Blackface sheep were less severely affected clinically and pathophysiologically by H. contortus infection than Finn Dorset sheep, regardless of haemoglobin type, the former breed also showed a "self-cure" response absent in the latter. While acknowledging the importance of haemoglobin type as a guide to genetic resistance of individual sheep to parasitism in general the authors were of the opinion that breed differences

were much more impressive, and of potentially greater practical value.

With a view to the evidence presented above Van Tiggele (PhD thesis 1978) examined the possible effects of sex and haemoglobin type on the response of Texel Sheep in the Netherlands to different levels of experimental infection with F. hepatica.

Interestingly enough, this work indicated that haemoglobin type B (HbBB) i.e., the reverse of that of Scottish Blackface and Finn Dorset sheep resistant to haemonchosis, were much more resistant to infection with 1,000 metacercariae than haemoglobin type A (HbAA) sheep. HbAA sheep survived as long as 13 weeks after infection with 1,200 metacercariae whereas HbBB sheep survived as long as 30 weeks after similar infection. In addition female AA sheep died from acute fascioliasis with relatively few flukes, 8 weeks after infection with either 400 or 600 metacercariae. The author pointed out that HbAA Texel sheep were relatively rare in the field population and suggested that this may be explained by a natural selection due to fascioliasis towards HbBB types, although acknowledging that according to other studies (Allonby and Urquhart, 1976; Altaif and Dargie, 1978 a) & b) such sheep may well be less resistant to H. contortus infection.

Bearing in mind all the possible effects of the factors described above, in the present cross immunity studies with F. hepatica care was taken to eliminate where possible the effects of age, sex and breed on individual responses to F. hepatica. In addition haemoglobin types of the sheep under experiment were determined before any studies began and an effort was made to standardise groups of animals with respect to haemoglobin types.

Possible immunomodulatory effects of L. tetramisole (Levamisole) on parasitic infections

Levamisole is a synthetic anthelmintic first described by Thienpont et al. (1966), with a broad spectrum of activity against most nematode species of man and domestic animals, which has been safely and extensively used in human and veterinary medicine.

Another property of this basic compound was demonstrated by Renoux and Renoux (1971), who reported that levamisole augmented the protective effect of a Brucella vaccine in mice. Since that time numerous studies have been carried out, (recently reviewed by Janssen (1976); Symoens and Rosenthal (1977), into both the immunodulatory effects of levamisole and in vitro under a wide range of conditions and disease states. These included the effects of the drug on micro-organisms, various cells, humoral and cell-mediated immunity, inflammation, neoplastic disease, chronic infectious disease and parasitic disease. Much conflicting evidence was produced regarding the action of levamisole. For example, in vivo levamisole increased the protective effect of bacterial, protozoal or tumour vaccines (Desowitz, 1975; Irwin et al., 1975; Ibrahim et al., 1977); increased resistance to infection with pyogenic bacteria, herpes viruses or tumour cell invasion in newborn rodents (Fischer et al., 1974, 1975; Thiry et al., 1975), but did not influence resistance to a primary invasion by pathogenic bacteria, viruses, protozoa or tumour cells (Renoux and Renoux, 1971; El Chemali and Vas, 1974; Benazet et al., 1973). In vitro levamisole increased phagocytosis by polymonphonuclear cells or macrophages when added to these cells or given to donor animals and humans (Ippen and Quadripur, 1975; Van Heule et al., 1976), increased nucleic acid or protein synthesis in antigen stimulated T. lymphocytes without affecting B cell responses (Hadden, 1975; Merluzzi et al., 1976;

Renoux et al., 1977), and restored to normal the number of T cells in humans in whom these numbers were reduced (Bensa et al., 1976; Ellegaard and Boesen 1976; Levo et al., 1975).

It emerged from many of the studies outlined above that levamisole influenced host defences by modulating cell-mediated immune responses by improving the responses of polymorphonuclear cells, macrophages and T. lymphocytes to antigens. This was generally not accompanied by an increase in serum antibody level and effects were most marked in cells from hosts "compromised" in some way, e.g. in terms of age or intercurrent disease. It was suggested (Symoens and Rosenthal, 1977), that levamisole, in disease states characterised by reduced cellular responsiveness, antigenic persistence and B cell hyperactivity, by improving cellular immune functions stimulated early inflammatory events, removing persistent antigen and controlling B cell reactivity.

In so far as the parasitised animal or human is a compromised host it is logical to presume that levamisole, by virtue of its immunomodulatory properties previously discussed, may alter the response of the host to infection with a protozoan or helminth parasite. This, in fact, has been demonstrated in the altered reaction of the host to the protozoans Toxoplasma gondii (Fegies and Guerrero, 1977); Plasmodium berghei (Richard-Lenoble et al., 1974; Desowitz, 1975), and the helminth Nippostrongylus brasiliensis (Liauw et al., 1977). Richard-Lenoble et al. (1974) investigated the effect of various immunostimulants in mice and rats experimentally infected with Plasmodium berghei berghei. These workers reported firstly that the optimum action of levamisole was obtained when antigen and the drug were presented simultaneously, and secondly that the stimulating action of

levamisole remained independent of the variation in specific circulating antibody. An ancillary finding of this study was that animals infected with the protozoan and levamisole had a markedly diminished mortality rate compared with infected, control animals.

The same protozoan P. berghei featured in an investigation by Desowitz (1975) into immunogenic enhancement of antigen by the addition of adjuvants, including levamisole, in experimental P. berghei infection in mice. This author recorded that levamisole when given either 24 hours before, or in combination with the soluble P. berghei antigen had a definite immuno-enhancing effect in that the mortality rate in treated mice was approximately one-third that of the control group. However, treated mice did not display a reduction in parasitaemia nor was the patent period of infection appreciably shorter, compared with control mice. Interestingly enough, in this investigation levamisole appeared to have enhanced the primary response to the parasite when given with or before P. berghei antigen. This observation in some way contradicts those of Renoux and Renoux (1971) who were unable to demonstrate any effects of levamisole on the course of primary invasion by bacteria, but found rather that levamisole was only effective in hosts previously sensitised to the antigen. The author (Desowitz, 1975) opined that the elucidation of the mechanism(s) by which levamisole exerted its immunoenhancing effect would be of value and merited further study, with a view to application to primate Plasmodium infections.

In the helminth field Liauw et al. (1977), reported stimulation by levamisole of a delayed type hypersensitive reaction in mice infected with Nippostrongylus brasiliensis. This reaction occurred when mice infected subcutaneously with the helminth were challenged intradermally

with adult N. brasiliensis whole worm antigen, and the authors reported that levamisole exerted an immunostimulant effect in the system outlined above. Such an effect resulted in enhancement of this delayed-type hypersensitive reaction, but was operative only within a well-defined regimen controlled by the dose rate of the drug and the time of application of the helminth antigen. The authors considered this system to be a promising laboratory tool in dectection of immunostimulant effects.

As far as the present experiments are concerned, provision was made in experimental design, for consideration of the possible role of levamisole, either alone, or in combination with other parasitic infections, in modulating the immune response of sheep to F. hepatica.

EXPERIMENTAL DESIGN

45 lambs were used in these studies, in 2 complementary experiments, 29 animals being employed in the first experiment (Experiment 'A'), and 16 in the second (Experiment 'B').

Experiment 'A'

Object. This experiment was designed to investigate the possible cross protective effect of prior infection with other helminths on the pathogenic effects of F. hepatica.

Two of the helminths used in this experiment, namely Ascaris suum and Taenia hydatigena have a migratory pathway which, like that of F. hepatica, involves, at some stage, invasion of liver tissue. It was hoped that the experiment may indicate whether this similarity in migratory pathway caused a specific or non-specific response which ~~may~~ might be responsible for cross protection against F. hepatica infection in sheep.

In addition, the first of a two-part study was undertaken into the

possible immunomodulatory effects of the anthelmintic L. tetramisole (Levamisole) either per se, or following infection with both abomasal and intestinal nematodes, on the course of fascioliasis in sheep. The second part of this study was incorporated into Experiment 'B'.

Experimental design

The schedule of the various infections and treatments of Experiment 'A' are shown in Table V.1. Lambs were allocated in the various groups on the basis of weight. Haemoglobin type was determined for all lambs prior to the commencement of the experiment and this was taken into account when lambs were being divided into groups. This information is summarised in Table V.2.

Group No.	Day 0	(Week 2) Day 14	(Week 6) Day 42	(Week 9) Day 63	(Week 12) Day 84	(Week 15) Day 111	(Week 27) Day 195
I (N)	Nematode infection*	Nematode infection	-	-	-	250 F. hepatica metacercariae	Kill
II (NL)	Nematode infection	Nematode infection	Levamisole 6 mg/kg	Levamisole 6 mg/kg	Levamisole 6 mg/kg	250 F. hepatica metacercariae	Kill
III (L)	-	-	Levamisole 6 mg/kg	Levamisole 6 mg/kg	Levamisole 6 mg/kg	250 F. hepatica metacercariae	Kill
IV (C)	-	-	-	-	-	250 F. hepatica metacercariae	Kill
V (R)	5,000 Ascaris suum ova	-	-	-	-	250 F. hepatica metacercariae	Kill
VI (T)	1,000 Taenia hydatigena ova	-	-	-	-	250 F. hepatica metacercariae	Kill
VII (NC)	Nematode infection	Nematode infection	-	-	-	Kill	Kill
VIII (TC)	1,000 Taenia hydatigena ova	-	-	-	-	Kill	Kill
IX (AC)	5,000 Ascaris suum ova	-	-	-	-	Kill	Kill

*Nematode infection = 5,000 L_3 of a mixture of Ostertagia circumcincta and Trichostrongylus vitrinus

Table V.1: Infection Schedule - Experiment 'A'

Group No.	Lamb No.	Hb type	Weight (kg)
I	N1	B	21
	N2	AB	13
	N3	B	17
	N4	B	17
II	NL1	B	13
	NL2	AB	17
	NL3	B	22
	NL4	B	20
III	L1	B	15
	L2	AB	19
	L3	B	19
	L4	B	21
IV	C1	AB	12
	C2	B	17
	C3	B	20
	C4	B	21
V	A1	B	16
	A2	AB	21
	A3	B	16
	A4	B	14
VI	T1	B	26
	T2	AB	11
	T3	B	15
	T4	B	14
VII	NC1	AB	20
	NC2	B	16
VIII	TC1	B	21
	TC2	AB	18
IX	AC1	AB	21

Table V.2: Weights and Haemoglobin Types

Experiment 'A'

All infections outlined in Table V.1 were given as oral doses and levamisole was injected subcutaneously. Faecal samples were collected weekly from animals challenged with F. hepatica metacercariae, commencing 9 - 10 weeks post challenge and continuing until slaughter, and examined for the presence of F. hepatica ova. All lambs were bled weekly, directly from the jugular vein and serum stored immediately at -20^{o}C, commencing 2 weeks before challenge with F. hepatica metacercariae, and continuing until slaughter.

Experiment 'B'

Object. The object of this experiment was to confirm and extend the findings of Experiment A, in that the effects of prior exposure to more than one helminth infection on F. hepatica infection in sheep was studied. Further studies on the role of L tetramisole (Levamisole) on immunity to ovine fluke infection were carried out. In these studies levamisole was given either before infection with the larval cestode C. tenuicollis or prior to infection with this parasite, following exposure to both abomasal and intestinal nematode species. It was hoped that these studies may demonstrate whether or not prior infection with other helminths rendered susceptible sheep immunologically "compromised" hosts, and if so whether levamisole exerted an immunostimulant effect sufficient to result in a functional immunity to F. hepatica infection in sheep.

Experimental design

16 lambs were used in this experiment. Lambs were again assigned to the various groups on a weight basis. As in Experiment 'A' haemoglobin typing was carried out before the commencement of this experiment and this information was also taken into account when groups were being allocated. The various infections and treatments of the groups are given in Table V.3. Lamb weights and haemoglobin types are shown in Table V.4. For reasons of economy it was decided in this experiment to use the same animals as in Experiment 'A' to control A. suum, T. hydatigena and nematode infections.

Group No.	Day 0	(Week 2) Day 14	(Week 6) Day 42	(Week 9) Day 63	(Week 12) Day 84	(Week 13) Day 91	(Week 28) Day 196	(Week 41) Day 291
1 (LT)	-	-	Levamisole 6 mg/kg	Levamisole 6 mg/kg	Levamisole 6 mg/kg	1000 Taenia hydatigena ova	250 F. hepatica metacercariae	Kill
2 (NT)	Nematode infection *	Nematode infection	-	-	-	1000 Taenia hydatigena ova	250 F. hepatica metacercariae	Kill
3 (F)	Nematode infection	Nematode infection	Levamisole 6 mg/kg	Levamisole 6 mg/kg	Levamisole 6 mg/kg	1000 Taenia hydatigena ova	250 F. hepatica metacercariae	Kill
4 (C)	-	-	-	-	-		250 F. hepatica metacercariae	Kill

*Nematode infection = 5,000 L_3 of a mixture of Ostertagia circumcincta and Trichostrongylus vitrinus

Table V.3: Infection Schedule - Experiment 'B'

Group No.	Lamb No.	Hb type	Weight (kg)
1	LT1	AB	23
	LT2	B	23
	LT3	B	19
	LT4	B	27
2	NT1	AB	23
	NT2	AB	40
	NT3	B	21
	NT4	B	20
3	F1	AB	21
	F2	B	26
	F3	B	30
	F4	AB	39
4	C1	AB	20
	C2	B	39
	C3	B	26
	C4	B	30

Table V.4: Weights and Haemoglobin Types

Experiment 'B'

All infections outlined in Table V.3 were administered as oral doses, and levamisole was injected subcutaneously. Lambs were bled weekly directly from the jugular vein commencing 2 weeks before F. hepatica infection, and continuing until slaughter. Faecal samples were collected weekly, commencing 9 - 10 weeks after F. hepatica infection, until slaughter.

Passive Transfer Experiment II

Object. In addition to the two cross-immunity experiments previously described an ancillary experiment was set up using the laboratory model of F. hepatica infection in the rat. In contrast to passive transfer Experiment I (see Chapter III) female rats of the Hooded Lister strain were used because it was felt that these may be immunologically more responsive than male rats used previously (Dargie, personal communication). This experiment was designed to determine the relative protection, in terms of reduction in fluke burdens, afforded by serum from fluke-infected sheep and cattle, when compared with serum from sheep infected with C. tenuicollis. It was hoped that this may clarify the role of the larval cestode in inducing immunity to F. hepatica in sheep.

Experimental design

The various groups used in this experiment are summarised in Table V.5.

Group No.	No. of rats	Type of serum	Challenge*
1	8	8 week ovine F. hepatica	+
2	8	8 week bovine F. hepatica	+
3	8	14 week ovine C.tenuicollis	+
4	8	Normal	+
5	10	-	+

Table V.5: Passive Transfer Experiment II:

Experimental Design

* 20 F. hepatica metacercariae orally

As outlined previously (see Chapter III), all rats were injected intraperitoneally with 2 doses of 10 mls of the appropriate serum, the first dose being given at the time of challenge and the second 18 - 20 hours later. All rats were sacrificed 9 weeks later for recovery of adult flukes from the bile ducts.

RESULTS

Post-mortem findings

An arbitrary classification on the basis of gross pathology of the livers of the sheep in the various treatment groups of Experiments 'A' and 'B' is illustrated in Tables V.6 and V.7 respectively. The numbers of flukes and their size distribution in the livers of experimental sheep in the two experiments are shown in Table V.8 and V.9, and numbers of nematodes and larval cestodes recovered from the appropriate groups are shown in Tables V.10 and V.11.

Group No.	Lamb No.	Liver wt. (gms)	Fibrosis	Calcification	Haemorrhage
I	N1	521	+	-	+
	N2	448	++	-	+
	N3	512	+	-	-
	N4	546	++	-	-
II	NL1	346	+	-	-
	NL2	525	++	-	+
	NL3	539	+	-	-
	NL4	443	+	-	+
III	L1	375	+	-	+
	L2	488	++	-	++
	L3	486	+	-	+
	L4	617	+	-	+
IV	C1	512	+	-	-
	C2	511	+	-	+
	C3	641	++	-	+
	C4	730	+	-	+
V	A1	580	+	-	+
	A2	584	+	-	+
	A3	560	+	-	+
	A4	499	+	-	+
VI	T1	500	+	-	+
	T2	419	+	-	+
	T3	624	+	-	+
	T4	624	++	-	+

Table V.6: Classification of liver lesions in fluke-infected sheep

Experiment 'A'

Group No.	Lamb No.	Liver wt.(gms)	Fibrosis	Calcification	Haemorrhage
1	LT1	842	+	-	+
	LT2	762	++	-	+
	LT3	690	+	-	+
	LT4	746	+	-	-
2	NT1	716	+	-	-
	NT2	669	+	-	-
	NT3	933	+	-	+
	NT4	522	+	-	+
3	F1	670	-	-	+
	F2	1025	+	-	-
	F3	586	+	-	-
	F4	938	+	-	-
4	C1	785	++	+	++
	C2	883	+	-	+
	C3	556	+	-	+
	C4	615	+	-	+

Table V.7: Classification of liver lesions in fluke-infected sheep

Experiment 'B'

Group No.	Lamb No.	Nos. of flukes >12 mm	6-12 mm	<6 mm	Total	Remarks
I	N1	70	30	1	101	1 C. tenuicollis
	N2	No measurements			100	
	N3	No measurements			67	
	N4	3	47	13	63	
II	NL1	20	20	7	47	
	NL2	No measurements			29	1 C. tenuicollis
	NL3	5	29	1	35	
	NL4	2	10	1	13	
III	L1	6	32	6	44	
	L2	24	62	1	87	
	L3	No measurements			53	
	L4	35	43	3	80	
IV	C1	1	13	13	27	
	C2	11	21	3	35	
	C3	32	32	1	47	
	C4	13	32	2	65	
V	A1	25	31	3	59	2 0.6 mm granulomas lung
	A2	17	9	4	30	
	A3	14	28	7	49	
	A4	No measurements			42	3 0.5 mm granulomas lung
VI	T1	41	18	3	68	≤ 100 C. tenuicollis
	T2	35	13	2	50	≤ 30 Degenerate C. tenuicollis
	T3	12	20	6	38	≤ 100 C. tenuicollis
	T4	15	33	6	54	≤ 50 C. tenuicollis

Table V.8: Fluke recoveries

Experiment 'A'

In 5 animals fluke measurements were found to be impossible due to overnight degeneration of fluke material. 2 animals (N1 and NL2) were found to have aberrant C. tenuicollis infection. This was presumably due to exposure to infection prior to purchase, since all animals had been maintained under helminth-free conditions since being obtained from a local farm, at 10 - 12 weeks of age.

Group No.	Lamb No.	Nos. of flukes >12 mm	6-12 mm	≤6 mm	Total	Remarks
1	LT1	52	32	2	86	≲ 20 Degenerate C.tenuicollis
	LT2	14	36	11	61	≲ 60 C.tenuicollis
	LT3	3	22	2	27	1 C.tenuicollis
	LT4	75	17	18	110	≲ 50 C.tenuicollis
2	NT1	30	43	2	75	≲ 20 Degenerate C.tenuicollis
	NT2	25	18	22	65	< 30 Degenerate C.tenuicollis
	NT3	15	38	1	54	3 C.tenuicollis
	NT4	8	48	2	58	< 30 Degenerate C.tenuicollis
3	F1	22	14	1	37	≲ 70 C.tenuicollis
	F2	8	27	1	36	10 C.tenuicollis
	F3	27	26	6	59	≲ 20 C.tenuicollis
	F4	39	22	13	74	≲ 20 Degenerate C.tenuicollis
4	C1	10	103	1	114	
	C2	14	70	10	94	
	C3	7	22	25	54	
	C4	6	115	4	125	

Table V.9 : Fluke recoveries

Experiment 'B'

Group No.	Lamb No.	C. tenuicollis	A. suum	O. circumcincta	T. colubriformis	Misc.
VII	NC1	-	-	100	2,300	1,500 Nematodirus sp.
	NC2	-	-	100	750	
VIII	TC1	Numerous degenerate	-	-	-	-
	TC2	Numerous degenerate	-	-	-	-
IX	AC1	-	Evidence of liver migration	-	-	-

Table V.10: Infectivity controls

Experiment 'A'

Group No.	Lamb No.	O. circumcincta	T. colubriformis	Miscellaneous
2	NT1	50	-	-
	NT2	-	1900	50 Cooperia sp.
	NT3	-	500	50 T. vitrinus
	NT4	-	2900	
3	F1	-	-	150 Cooperia sp.
	F2	-	-	50 Cooperia sp.
	F3	-		
	F4	-	-	-

Table V.11: Nematode recoveries

Experiment 'B'

By comparison with Table V.10 it can be seen that the numbers of T. colubriformis recovered from the small intestine of the above groups of sheep are of the same order as in Experiment 'A'. The scarcity of O. circumcincta recovery in both experiments is not surprising in view of the fact that the two experiments had lasted for a period of roughly 6 and 10 months respectively during which time spontaneous loss of the abomasal nematode would have occurred as described by Armour et al. (1966).

Cooperia species recovered from the small intestine of both groups were presumably due to contamination of the O. circumcincta culture with this parasite. It is also of incidental interest to note that a few Cooperia species recovered from Group 3 had survived 3 anthelmintic treatments with levamisole at a dose rate of 6 mg/kg. T. vitrinus recovered from NT3 in Group 2 was presumably due to contamination of the T. colubriformis larval culture.

Statistical analysis of the fluke recoveries from the two experiments 'A' and 'B' are illustrated in Tables V.12 and V.13. In Experiment 'A' (Table V.11) the parametric Student's 't' test was favoured for such analysis as there was a normal distribution of observations and the variance of the individual groups was of comparable order. However, in Experiment 'B' Table V.12) on comparison of the individual groups it was found that in one case (Group 2 and Group 4), there was a large difference in variance and for this reason the non-parametric Mann-Whitney U Test was preferred for analysis of the data of this experiment.

Group No.	Lamb No.	Total No. Flukes	Mean ± S.D.	Reduction % vs. control	Probability 'p'
I	N1	101	82 ± 21 S.E. 10	46.4 increase	p<.05
	N2	100			
	N3	67			
	N4	63			
II	NL1	47	31 ± 14 S.E. 14 S.E. 7	30.3	N.S.
	NL2	29			
	NL3	35			
	NL4	13			
III	L1	44	66 ± 21 S.E. 10	32.5 increase	N.S.
	L2	87			
	L3	53			
	L4	80			
IV	C1	27	45 ± 18 S.E. 8		
	C2	35			
	C3	47			
	C4	65			
V	A1	59	45 ± 12 S.E. 6	1.1 increase	N.S.
	A2	30			
	A3	49			
	A4	42			
VI	T1	68	53 ± 12 S.E. 6	16.8 increase	N.S.
	T2	50			
	T3	58			
	T4	34			

Table V.12: Statistical breakdown

Experiment 'A'

* 'p' value using Student's 't' test.

N.S. = Not significant

Group No.	Lamb No.	Total No. Flukes	Mean ± S.D.	Reduction % vs. control	Probability 'p'
1	LT1	86	71 ± 36 S.E. 18	27	N.S.
	LT2	61			
	LT3	27			
	LT4	110			
2	NT1	75	63 ± 9 S.E. 5	35	N.S.
	NT2	65			
	NT3	54			
	NT4	58			
3	F1	37	52 ± 19 S.E. 9	47	p<0.05
	F2	36			
	F3	59			
	F4	74			
4	C1	114	97 ± 31 S.E. 16		
	C2	94			
	C3	54			
	C4	125			

Table V.13: Statistical breakdown

Experiment 'B'

* 'p' value using Mann Whitney 'U' test

N.S. = Not significant

From consideration of Tables V.2; V.6 and V.8 the haemoglobin type of experimental sheep in Experiment 'A' made no difference to the pathogenicity of F. hepatica infection either in terms of gross-pathological changes or numbers of flukes recovered. The same was true of experimental sheep in Experiment 'B' from examination of Tables V.4; V.7 and V.9. This finding will be discussed later.

In the first experiment (Experiment 'A') several interesting points may be drawn from the statistical analysis of fluke recoveries. Firstly, prior to infection none of the other helminth species, namely, Ascaris suum Ostertagia circumcincta and Trichostrongylus colubriformis; and Taenia hydatigena (Groups V, I, and VI respectively) conferred any degree of immunity to F. hepatica infection in terms of numbers of flukes recovered. These infections in fact resulted in 1.1%; 46.4% and 16.8% increases in the number of flukes recovered compared with the control animals. It is most interesting to note that prior infections with nematode species O. circumcincta and T. colubriformis resulted in a 46.4% increase in fluke recovery, which constituted a statistically significant difference ($p < 0.05$) when compared with the control group, using Student's 't' test. This observation will be discussed later, together with the failure of C. tenuicollis to confer immunity to fluke infection (Plates 6 & 7).

Another interesting observation from this data (Table V.12) was that the group of sheep treated with levamisole alone and subsequently challenged with F. hepatica (Group III) showed no evidence of immunity and indeed rendered 32.5% more flukes than the control group.

In this experiment the only treatment group which appeared to exhibit any resistance to the fluke challenge was Group II, which were exposed to a

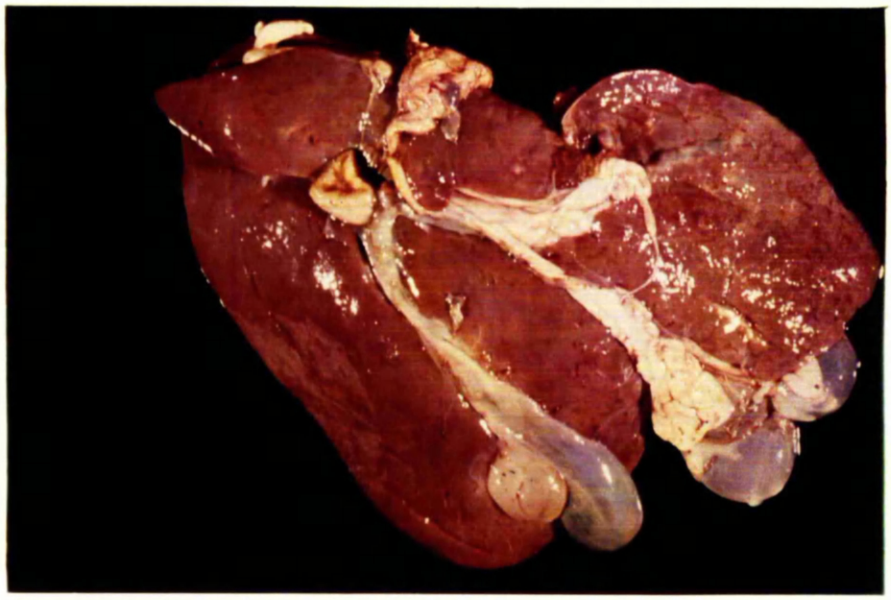


Plate 6. Liver from sheep NT3 Group 2, cross-immunity experiment B showing coexisting C. tenuicollis and F. hepatica infections.

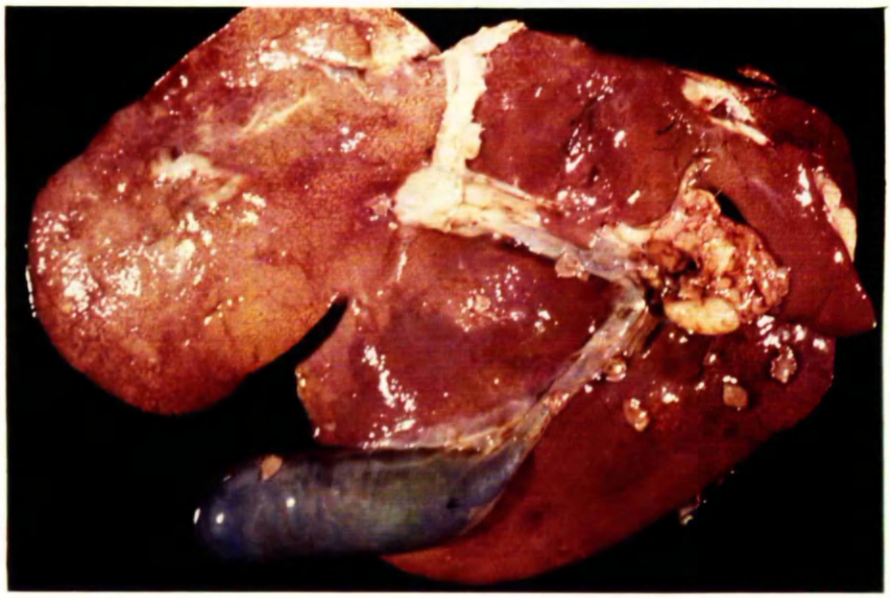


Plate 7. Liver from sheep C1 Group 4, cross-immunity experiment B showing typical F. hepatica lesions in challenge control animals.

nematode infection before treatment with levamisole and subsequent F. hepatica challenge. This group had a 30.3% reduction in numbers of flukes recovered compared with controls. This difference was not found to be statistically significant using Student's 't' test.

Finally, no conclusion could be drawn from Table V.8, regarding the breakdown in size of the different fluke burdens, as no particular trend or pattern appeared to be involved in the various treatment groups.

From Experiment 'B' by consideration of Tables V.9 and V.13 the following points emerged. Firstly, in all three treatment groups i.e. Groups 1, 2 and 3, there was a substantial decrease in the percentage of flukes recovered compared with control sheep (27%, 35% and 47% respectively). In Groups 1 and 2, which respectively received levamisole followed by T. hydatigena infection; and nematode infection, then T. hydatigena infection prior to challenge with F. hepatica observed reductions in fluke numbers were not statistically significant. However, sheep in Group 3, which received a nematode infection, levamisole and T. hydatigena infection prior to F. hepatica challenge rendered 47% fewer flukes than control animals which proved significantly different ($p < .05$) when compared with control burdens. It is pertinent to recognise at this stage that in both experiments the group of sheep which demonstrated the greater degree of resistance to F. hepatica infection in fluke recovery (Group II in Experiment 'A' and Group 3 in Experiment 'B'), had both been exposed to helminth infection prior to treatment with levamisole.

Again, as in Experiment 'A', no conclusions could be drawn from the information on measurements of the individual fluke burdens.

In view of the significant difference between the fluke burdens of Group 3 and Group 4 in Experiment 'B' it was decided to examine serum taken

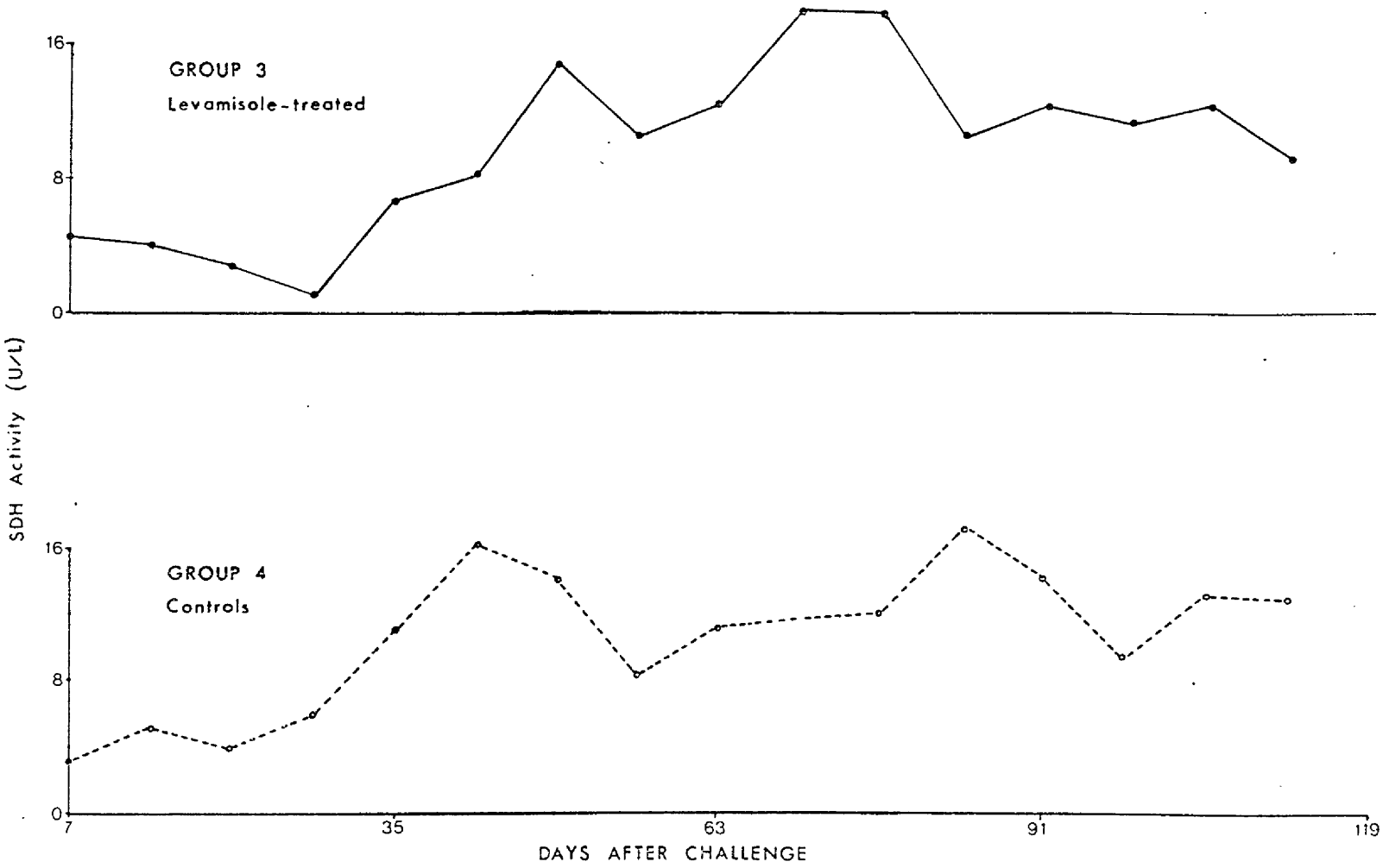


Fig. 5.1. Comparison of S.D.H. values following F. hepatica challenge in sheep primed with helminth infection before levamisole treatment (Group 3, Table V.3) and challenge control sheep (Group 4, Table V.3).

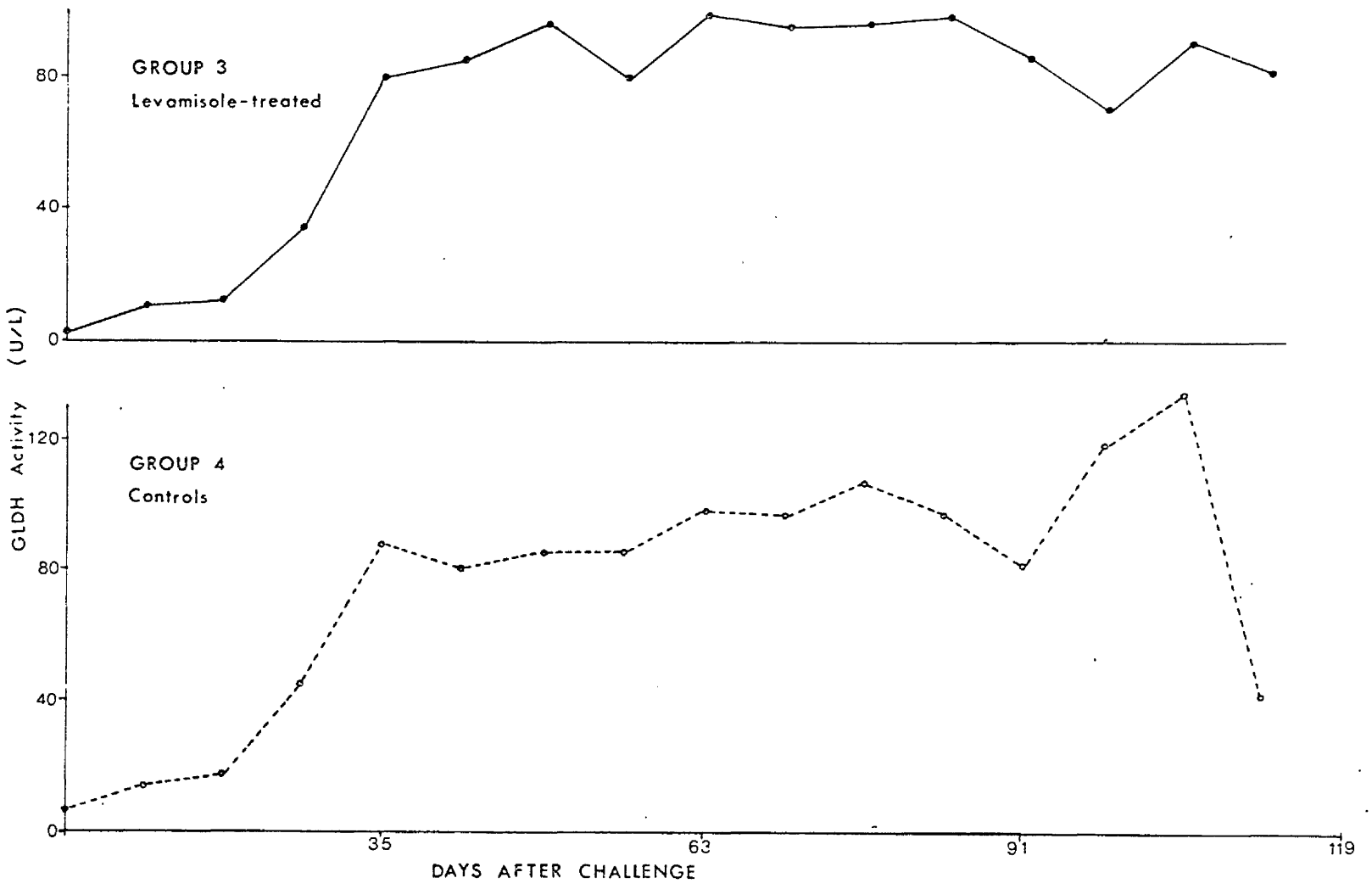


Fig. 5.2. Comparison of G.L.D.H. values following F. hepatica challenge in sheep primed with helminth infection before levamisole treatment (Group 3, Table V.3) and challenge control sheep (Group 4, Table V.3).

from these animals after F. hepatica challenge for the liver enzymes sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH). The results of this analysis are illustrated in Figs. 5.1 and 5.2. From this data it is apparent that the SDH levels rose slightly later and the maximum value reached was slightly lower in Group 3 sheep compared with Group 4 sheep, while GLDH levels were similar in both groups. Fuller results of these analyses are shown on pages 285-286 of the appendix. The significance of this will be discussed later.

Passive Transfer Experiment II

Group No.	No. of rats	Type of serum
1	8	8 week ovine F. hepatica
2	7	8 week bovine F. hepatica
3	8	14 week ovine C.tenuicollis
4	6	Normal
5	10	-

Table V.14: Passive Transfer Experiment II

Nos. of rats surviving experiment

2 rats from Group 4 and 1 rat from Group 2 died before the end of the experiment. Of these, flukes were recovered only from the rat from Group 2. Individual fluke recoveries from the bile ducts of infected rats are shown in Table V.15.

Group 1	Group 2	Group 3	Group 4	Group 5
2	2	3	2	1
2	2	3	3	2
2	2	3	3	3
2	2	4	3	3
2	4	4	4	3
4	4	6	4	4
5	4		6	5
5	4		8	5
				5
				7

Table V. 15: Passive Transfer Experiment II

Individual fluke burdens

A statistical breakdown of the fluke recoveries from this experiment is illustrated in Table V.16.

Group No.	Type of serum	Total No. Flukes	Mean ± S.D.	% Reduction vs control	Probability 'p'*
1	8 week ovine *F. hepatica*	24	3 ± 1.4 S.E. .5	21%	N.S.
2	8 week bovine *F. hepatica*	24	3 ± 1 S.E. .4	21%	N.S.
3	14 week ovine *C. tenuicollis*	22	3.7 ± 1.4 S.E. .6	2.6%	N.S.
4	Normal	32	4 ± 2.0 S.E. .7	+5.3%	N.S.
5	Control	38	3.8 ± 1.8 S.E. .6		

Table V.16: Passive Transfer Experiment II

Statistical breakdown of fluke recoveries

From this data it is apparent that although both batches of serum from fluke-infected ruminants (Groups 1 & 2) afforded some protection against *F. hepatica* infection in terms of a 21% reduction in fluke recoveries compared with control rats, neither these groups nor the group receiving serum from *C. tenuicollis*-infected sheep (Group 3) showed evidence of a statistically significant decrease in fluke numbers. Fluke recoveries from Group 1 were in fact closely similar to those from control Group 5.

DISCUSSION

The first interesting points to emerge, as an incidental finding in the present cross-immunisation studies, was the increase in fluke burden resulting from prior infection with nematodes *O. circumcincta* and *T. colubriformis*. This increase was in the order of 50% and proved statistically significant at

the 5% level. This result would appear to contradict the findings of Goose (1977) and Doy et al. (1978a). In these analogous studies in the laboratory model of F. hepatica infection in the rat both groups of workers reported that prior infection with Nippostrongylus brasiliensis resulted in a degree of immunity to F. hepatica infection. The latter study (Doy et al., 1978a) demonstrated a significant reduction in fluke burden of rats experimentally infected with N. brasiliensis alone or N. brasiliensis plus immune serum. The authors considered the nematode capable of inducing a heterologous protection against F. hepatica infection and although the mechanism of such protection remained obscure, it was felt that specific serum factors were not involved and that intestinal eosinophils might possibly be implicated. It must be stressed that the above conclusions drawn from the laboratory model of F. hepatica infection in the rat can only be applied in general terms to infection in sheep since not only a different species of host is involved, but also different nematode species. In view of the equivocal result from Experiment 'B', i.e. a 35% decrease in fluke burden in sheep infected with nematode species (Group 2) which was not statistically significant, in the opinion of the author, bearing in mind the small groups of animals used, although a trend or pattern was obvious, it would be dangerous to draw any conclusions from the present studies regarding the influence of prior nematode infection on F. hepatica infection in sheep.

Secondly, as mentioned previously in the present studies, haemoglobin type of experimental sheep had no bearing on resistance to fluke infection in terms of gross pathological changes or numbers of flukes recovered. This finding would appear to contradict the views of Van Tiggele (1978) who had reported that sheep of haemoglobin type BB were relatively resistant to fluke infection compared with AA sheep. This difference may well be due

to the fact that the latter author used Texel sheep while in the present studies experimental sheep were of the Finn-Dorset breed.

At this stage it will be informative to discuss the work of Campbell et al. (1977) and Dineen et al. (1978) in Australia on the stimulation of resistance in sheep to F. hepatica by infection with Cysticercus tenuicollis. The first part of this work (Campbell et al., 1977) reported that infection of sheep for a 12 week period with C. tenuicollis generated a high level of protection against F. hepatica, in terms of a 95% reduction in fluke burden compared with control animals. The authors also reported that liver fibrosis was much less extensive in resistant sheep than controls. The same authors (Dineen et al., 1978) recorded that sheep exposed to C. tenuicollis infection for 12 weeks were again protected when rechallenged with F. hepatica at 9 months. The authors further documented that sheep in which the initial C. tenuicollis infection were terminated by anthelmintic were resistant to primary infection with F. hepatica, but became fully susceptible to reinfection at 9 months. On the basis of these results it was suggested that the maintenance of resistance to F. hepatica required the persistence of active C. tenuicollis infection in the body cavity. In this experiment a significant reduction in fluke recovery was also obtained, surprisingly, in a group of animals which were intended to serve as infectivity controls for metacercariae of F. hepatica, and which had at no time been infected with C. tenuicollis. The response of these animals as monitored by fluke egg counts, P.C.V.'s and fluke recoveries was similar in every way to resistant sheep. This puzzling occurrence was explained by the authors as due to adventitious infection with the larval cestode during a period of 14 months grazing before housing, as C. tenuicollis cysts were found in the peritoneal cavities of the animals in question at post mortem. The authors favoured an immunological

explanation of this phenomenon of resistance, the mechanism of which was thought to be either by a specific stimulus provided by C.tenuicollis antigens cross reacting with F.hepatica, or by a non-specific tissue reaction involved by a specific immunological stimulus, or by a combination of both mechanisms.

In the present studies, in Experiment 'A' sheep infected for 15 weeks with C.tenuicollis, then challenged with F.hepatica, were found to be completely susceptible to infection (Table V.8), indeed many carcasses were found to harbour large numbers of both parasites (Plates 6 & 7). This finding has also been confirmed by the work of Hughes et al. (1978), who infected sheep, and other domestic species with C.tenuicollis prior to challenge with F.hepatica. These latter authors were unable to find any significant differences between the fluke burdens of animals previously infected with the larval cestode, and control animals. In these experiments, as in the Australian work, sheep were reared at pasture, and, apart from breed differences, the only other point of difference between the two studies was the use by the Australian workers of the anthelmintic levamisole at 3 weekly intervals from the age of 8 weeks until housing to control nematode infections. As discussed earlier in this chapter this compound is known to have immunomodulatory properties and Hughes et al. (1978) suggested that levamisole may be responsible for the different results obtained from the two similar studies.

In the present studies Experiment 'A' was designed primarily to test the effectiveness of C.tenuicollis per se and other helminths in protecting sheep against F.hepatica infection and Experiment 'B' was intended to recreate artificially the "Australian situation" indoors by infecting sheep experimentally with nematodes, then treating with levamisole prior to C.tenuicollis infection and F.hepatica challenge.

Significantly, as mentioned previously, the groups of sheep which demonstrated the largest reduction in fluke burdens had both been exposed to a prior helminth infection before treatment with levamisole, viz: Group II in Experiment 'A' and Group 3 in Experiment 'B'. Group II sheep were infected twice with abomasal and intestinal nematodes before 3 treatments with levamisole at 3 weekly intervals. These animals had therefore received exactly the same treatment as the control animals in the Australian work (Dineen et al., 1977) which had been sensitised to helminth antigen by continuous exposure to nematode infection at pasture for 14 months during which time they were regularly treated with levamisole. Group 3 of Experiment 'B' underwent exactly the same treatment regime, but were also infected with C. tenuicollis, i.e. this group of animals was entirely analogous to the resistant sheep of the Australian work, whose fluke burdens were reduced by up to 95%.

Further evidence for a degree of resistance in Group 3 sheep in Experiment 'B' is provided by examination of the GLDH and SDH levels of this group of animals compared with controls (see Figs. 5.1 and 5.2). In the control group SDH levels started to rise 3 - 4 weeks post-infection. This finding is in agreement with the work of El Harith (1977) and Van Tiggele (1978), who demonstrated a similar rise in SDH level in sheep experimentally infected with F. hepatica. In Group 4 animals SDH levels thereafter fluctuated at an abnormal level reaching peak values at 6 and 12 weeks after challenge. However, in Group 3 animals SDH levels did not rise appreciably until 5 - 6 weeks after infection, and the peak values reached were slightly lower than control animals. As mentioned previously GLDH levels (Fig.5.2) were found to be similar in both groups. These findings may indicate a delay in migration of invading flukes through the liver parenchyma, which may possibly reflect a degree of

immunity to infection, although it must be admitted that the relative G.L.D.H. activities of the relevant groups do not support this theory.

In the light of these findings, in the opinion of this author, the combination of levamisole treatement and prior infection with other helminth species, and not infection with C.tenuicollis resulted in a degree of resistance in sheep in the present studies, and possibly, in the work of Campbell et al., (1977) and Dineen et al. (1978). This opinion is supported by the results of passive transfer experiment II (Table V.16), in which administration of serum from sheep infected 14 weeks previously with C.tenuicollis simultaneous to F.hepatica infection was unable to confer any degree of resistance to F.hepatica in terms of a significant reduction in fluke recovery compared with control animals. Serum from fluke infected ruminants also failed to confer protection and possible reasons for this will be discussed in Chapter VI. Further support for the above view is provided by the work of Richard-Lenoble et al. (1974); Desowitz (1975) and Liauw et al. (1977). As discussed previously both the former groups of workers (Richard-Lenoble et al., 1974 and Desowitz, 1975) observed immunostimulation in rodents infected with the protozoan Plasmodium berghei following presentation of P.berghei antigen simultaneously with, or prior to levamisole treatment. Possibly, of more relevance to the helminth field was the work of Liauw et al. (1977), who described immunostimulation by levamisole of a delayed type hypersensitivity reaction following infection of rats with the helminth Nippostrongylus brasiliensis. The authors were able to demonstrate a relationship between the time of exposure to worm antigen and the time of levamisole treatment, in all cases prior exposure to helminth antigen enhanced the immunostimulatory effect of levamisole. These findings, as well as suggesting that immunostimulation may have occurred in the Australian work due to a prior exposure to helminth antigen over a long period before levamisole treatment, may also

explain the failure of levamisole to afford protection in some of the treatment groups in the present studies, e.g. (see Tables V.12 and V.13):- Groups III of Experiment 'A' which received levamisole treatment only, without prior exposure to helminth antigen and rendered 32.5% more flukes than the control group; and Group I in Experiment 'B' which was exposed to helminth antigen by virtue of infection with C. tenuicollis only after levamisole treatment, and which showed only a slight reduction in fluke burden compared with control animals, which was not statistically significant.

If the use of levamisole has indeed been responsible for a degree of resistance to F. hepatica in such a susceptible host as sheep it is interesting to speculate as to how this may have come about. From a large number of studies, recently reviewed by Symoens and Rosenthal (1977), it appears that the immunostimulatory properties of the drug are only expressed in the particular situation where the host is "compromised" in some way. From the work of Richard Lenoble et al. (1974) and Desowitz (1975), since levamisole was effective in immunostimulation in these studies, then by inference the animal previously exposed to parasitic infection or perhaps only parasitic antigen, must be in some way "compromised". As far as the mechanism of immunostimulation is concerned the work of Fegies and Guerrero (1977), on human Toxoplasma gondii provides some useful suggestions. These authors reported that the clinical improvement of patients suffering from toxoplasmosis when treated with levamisole, alone or in combination with sulpha drugs, was accompanied by a number of significant immunological events viz: a fall in anti-toxoplasma antibody titre with a marked increase in the percentage of T lymphocytes in the peripheral blood. The authors theorised that these events may be due to removal of a chronic antigenic

stimulus which had caused hyperactivity of B ymphocytes, possibly due to modification of the humoral response by T repressor lymphocytes. The authors pointed out that immunosuppression was a feature of this parasitic disease, and that patients suffering from the condition invariably had below average levels of T lymphocytes.

Immunosuppression has also been shown to be a feature of F. hepatica infection (Goose, 1977), which finding was substantiated in the present studies (see Chapter IV) and the comparison between toxoplasmosis in humans and ovine fascioliasis seems a justifiable one. Indeed a comparison of the two situations using sophisticated immunological methods would provide much valuable information concerning the exact immune status of experimental animals (see also Chapter IV). This could be done for example by monitoring the percentage of T and B lymphocytes and variations therein in response to the treatments described in the present studies. In this connection Larsh and Weatherly (1975) suggested that apart from the use of column chromatography pure suspensions of T and B cells may be obtained by the treatment of pools of mixed cells with antitheta antibody and complement to destroy T cells. Separation of T and B cells by similar means was recently carried out by Rich et al. (1977) in an immunological study on fractionation of lymphocyte sub-populations which regulate mixed lymphocyte reactions. Such techniques would undoubtedly prove fruitful if applied to F. hepatica infection in ruminants.

In view of the evidence presented above it would appear reasonable to assume that levamisole may well have a similar mechanism of action in the two situations described above and may well be of prophylactic value in a regime for prevention of ovine fascioliasis, using the compound with a suitable immunological trigger, possibly by prior exposure to somatic or metabolic antigens of F. hepatica.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

The findings of the present studies will now be discussed together with those of recent analogous work in the field of helminthology.

From Chapter III it was evident on the basis of the haematological parameters measured that cattle were better equipped to withstand experimental F. hepatica infection in that anaemia did not develop either following infection or reinfection to an extent in any way comparable with that observed in sheep, despite the fact that experimental cattle were infected and reinfected with approximately 4 times as many metacercariae as experimental sheep and reinfected cattle were found to harbour 3 times as many flukes as similarly infected sheep. This observation is in agreement with the studies of previous workers and may well reflect an inherent genetic difference in the response of the two ruminant species to infection.

An interesting feature of reinfection with F. hepatica in both ruminant species was the comparatively low liver enzyme values (G.L.D.H. and G.G.T.) recorded. Such a phenomenon was found to be more marked in cattle than in sheep. This finding does not support the view of Rushton (1977) who proposed a delayed migration of the secondary infection in reinfected sheep. This opinion was based partly on high S.G.O.T. activity in the serum of several reinfected animals. However, as discussed in Chapter I estimation of this particular enzyme has proved to be of limited value for diagnosis of F. hepatica infection in sheep in previous studies. It is this author's opinion therefore that the relatively low enzyme values recorded on reinfection was more likely to be a reflection of the self-limiting nature of the liver enzyme response than an indication of a host response causing a reduction in

or impairment of the metabolic activities of reinfecting flukes, since these parameters have been shown in the past to be of limited diagnostic value on reinfection.

As far as serological observations are concerned the findings of the present study have backed up the general opinions of previous authors, i.e. that I.H.T. is a reliable, sensitive test for early diagnosis of F. hepatica infection in cattle and sheep, and that D.I.D. is also a reliable test, but of less value due to the time involved and the relative insensitivity of the technique, since although few false positive results were obtained diagnosis of infection was made later and negative results were obtained during the course of infection in both ruminant species. The limitations of the E.l.i.s.a. technique for early diagnosis and quantitation of the antibody response have been discussed in some detail, and in the author's opinion, its unreliability and idiosyncratic nature are a cause for concern although, when functioning properly its sensitivity is undeniable. This was recently demonstrated by the work of Burden and Hammett (1978) who performed micro-E.l.i.s.a. successfully in cattle infected with F. hepatica. These authors, however, did not indicate how the problem of reading the test had been overcome and until such time as satisfactory standardisation of results is achieved it is the opinion of this author in agreement with contemporary workers in the field who have abandoned the technique in favour of more reliable techniques (Smith, personal communication; Van Tiggele, 1978), that I.H.T. remains a preferable alternative.

Of considerable interest from the quantitative study of antibody production was the observation that experimentally infected sheep, although producing considerable amounts of serum antibody, indeed more than similarly

infected cattle, and although such ovine antiserum removed at particular times after infection was able to protect rats from F. hepatica infection, themselves remained susceptible to reinfection. Possible explanations for this anomaly are numerous and it is stimulating to consider a few of the recent theories. Hanna (1978) in studies involving immunofluorescence reported that antibody titres against juvenile F. hepatica tegument peaked 6 - 7 weeks post-infection and declined steadily thereafter when flukes became established in the bile ducts. In the author's opinion this may have been due to replacement of the antigenic juvenile tegumental secretions with adult non-antigenic secretions during the course of infection. This evidence together with immunofluorescent studies on the loss of immune complex from the surface of antibody-labelled juvenile F. hepatica suggested to the author that the fluke protected itself from immunological attack by sheep antiserum by enveloping itself in a glycoprotein cocoon which masked underlying components from immune detection. Studies of a similar nature such as those carried out by Howard et al. (1979) utilising techniques involving radiolabelling of surface proteins of F. hepatica to differentiate host and parasite molecules will doubtless more accurately define the nature of those ~~protecitve~~ protective mechanisms referred to by Hanna (1978). An alternative theory (Goose, 1978) proposed that excreting/secretory products of the actively feeding flukes may protect the parasite, in the confined space of the bile duct, from host defences.

This latter study was carried out exclusively in the laboratory model of F. hepatica infection in the rat, and therefore may only be applied in very general terms, to F. hepatica infection in sheep. However the fluke may attempt to evade host responses, the fact remains that there would appear to be an immunological shortcoming peculiar to sheep, and associated with a

serum factor. In the past the failure of sheep antisera to protect rats from F. hepatica infection has been explained in terms of the time of infection at which antiserum was removed from sheep, on the grounds that insufficient antibody was being produced early in infection. However, it has been clearly demonstrated that antibody production per se does not provide functional immunity regardless of the amount produced and it is proposed rather that impairment of immunological function in sheep which is reflected in serum changes may be involved. This theory is supported indirectly by the work of previous workers including Chen and Soulsby (1976) and Burrells et al. (1978). Both groups of workers implicated serum factor(s) in modifying ovine immunological responses, and indeed the former authors suggested that inhibitor(s) which may impair normal immunological responses may be present in the serum of sheep undergoing infection with the abomasal nematode H. contortus.

The nature of the immunological defect in sheep infected with F. hepatica remains obscure, however, due to the large amounts of antibody produced to no avail it would seem reasonable to assume that such a defect is characterised by B cell hyperactivity and a reduction in the effective populations of T and B lymphocytes, i.e. a situation analogous not only to toxoplasmosis in humans as previously described (Fegies and Guerrera, 1977), but also perhaps to the situation recently reported by Ottesen et al. (1978) in individuals chronically infected with Schistosoma mansoni, where diminished responsiveness to schistosome antigens independent of humoral immune responses was recorded. The present theory regarding ovine F. hepatica is supported by the evidence presented in Chapter IV to suggest that F. hepatica infected sheep displayed a generalised immunosuppression characterised by a polyclonal impairment in the transforming ability of peripheral blood lymphocytes

or a reduction in total numbers or functional capacity of T and B cells, on the basis of reduced mitogenic responses of infected and reinfected animals. The mechanism by which such an immunosuppressive effect was exerted requires further investigation, but the involvement of suppressor cell activity is likely. Subpopulations of thymic suppressor cells were isolated in the rat by Ha et al. (1973), who considered that such cells represented less than 10% of the total thymocyte population. It was the author's opinion that such cells may not correspond perfectly to thymocyte subpopulations with conventional peripheral T lymphocyte activity and may in fact be present as distinct subpopulations within the thymus. Evidence of suppressor cell activity in the helminth field was recently reported by Colley et al. (1978) in human infection with Schistosoma mansoni. This informative piece of work demonstrated that peripheral lymphocytes from patients with chronic S. mansoni infection expressed suppressor activity after exposure to somatic S. mansoni antigen. Interestingly enough the authors also reported that such schistosome antigen induced suppressor activity was greatly increased when the cells were exposed to antigen in the presence of "suppressor" sera from patients with chronic S. mansoni infection. On the basis of these findings the authors proposed a synergistic relationship, involving antigen exposure of both cells and sera of chronically infected individuals, which assists in the induction of suppressor activity. Parallels between the above situation and chronic ovine fascioliasis are obvious and the conclusions of the former authors (Colley et al., 1978) that the immunoregulatory responses observed may be of great importance in the establishment of a stable chronic infection, in this author's opinion may well be applicable to chronic F. hepatica infection in sheep. Further evidence for the presence of a defect of an immunological nature in F. hepatica infected

sheep is provided by the stimulation of a partial immunity to F. hepatica infection in sheep treated with the immunomodulatory compound levamisole as described in Chapter V. Although it must be admitted that this compound alone did not alter the course of F. hepatica infection in sheep and only resulted in a degree of resistance when animals had been suitable primed or "compromised" by prior exposure to helminth antigen, the fact that modification of the response to infection did take place necessarily means, from the vast literature on the immunological properties of this compound, that the defence mechanisms of the host animal had been restored to normal, implying an initial abnormality. It would be of considerable interest to define the exact nature of the "compromise" or impairment of immunological function required for maximum efficacy of levamisole and whether by amplification of this initial embarrassment an effective prophylatic regime may be developed.

The situations in which levamisole proved to be of therapeutic value i.e. where an impaired immune system was a feature of the disease state, were strikingly similar to that where the leucocyte derived cell-free dialysate "transfer factor" was effective. The latter has been successfully used in a wide range of chronic disease states, including those due to helminth parasites (Maddison et al., 1976; Ross and Halliday, 1978, 1979) which would suggest that "transfer factor" may prove of value in protection against F. hepatica. The former authors (Maddison et al., 1976) reported that "transfer factor" prepared from leucocytes of infected immune donors when given in combination with hyperimmune serum reduced the clinical manifestations and worm burdens in monkeys infected with Schistosoma mansoni. Similarly promising results were reported concerning the transfer of resistance by "transfer factor" in sheep infected with the nematode Trichostrongylus axei, T. colubriformis

and O. circumcincta (Ross and Halliday, 1978, 1979). Such resistance was characterised by considerable reductions in worm burdens of treated animals and the authors noted (Ross and Halliday, 1979) that genetic similarity was not a prerequisite of successful transfer of resistance since sheep of different breeds had been used as donor and recipient. Thus it would appear that this compound, like levamisole may overcome problems of immunological unresponsiveness, which have been shown to be a feature of the helminth infections mentioned above and therefore may prove a useful tool in protecting sheep against F. hepatica infection. Indeed, preliminary studies of transfer factor in the rat (Halliday, personal communication) have indicated that this may well be the case.

From the various studies, immunological and otherwise described in Chapter III and IV it has been clearly demonstrated that the response of cattle to F. hepatica infection is strikingly different to that of sheep under conditions of adequate nutrition. No anaemia was observed, antibody titres were lower and there was no evidence of F. hepatica induced immunosuppression in terms of a decreased mitogenic or antigenic response of peripheral lymphocytes which was a feature of the disease in sheep. Several points however regarding the immune status of experimental calves are worthy of note from the present studies in comparison to those of previous workers. As mentioned previously (Chapter III) several times more flukes were recovered from reinfected calves than in the comparable studies carried out by Doyle (1971), and were in fact of an order similar to that reported by Van Tiggele (1978), in reinfection studies. The latter author, although agreeing with Doyle (1971) in demonstrating a degree of acquired resistance to F. hepatica infection in cattle was of the opinion that the relatively large number of flukes remaining after reinfection

may have originated to a considerable degree from the primary infection which may have been enhanced in development by the invading flukes of the secondary infection since, according to G.G.T. results, no flukes of the secondary infection had passed the parenchymal phase. Since G.G.T. levels on reinfection in the present studies also remained low, this theory would partly explain the reason for the relatively large numbers of flukes recovered on reinfection, i.e. many of this population were in fact from the primary infection. However, bearing in mind the dangers of drawing conclusions from liver enzyme results alone, and the small number of animals used, in this author's opinion such conclusions from the present studies, without back up evidence from serial parasitological findings could only be regarded as speculation.

Although from the present studies only limited information regarding the immune status of cattle to F. hepatica infection was obtained, from the evidence presented in Chapter V a degree of resistance to F. hepatica infection has been achieved in sheep, which is only the second time such a claim has been made in a species hitherto recognised as being ultra-susceptible to the parasite. The conclusions of the first demonstration of resistance to fluke infection in sheep by Campbell et al. (1977) and Dineen et al. (1978) have been discussed in some detail in Chapter V and further comment at this stage on any cross-immunising properties of the larval cestode C. tenuicollis proposed in these studies is deemed unnecessary as the same authors (Campbell et al., 1979) have very recently reported that challenge infections with T. hydatigena did not affect the number of flukes recovered from either primary or challenge F. hepatica infections casting further doubts on the protective value of infection with the larval cestode. It is worthy of note that in this latter study experimental sheep were reared from birth in concrete floored pens to prevent worm infection

and thus had no prior sensitisation with helminth antigen, nor were they treated with levamisole. In this author's view, supported by the findings of Hughes et al. (1978), this treatment regime was of paramount importance in the generation of resistance to F. hepatica infection, Since, in the present studies a degree of resistance to infection in sheep was noted only in animals given experimental helminth infections prior to levamisole treatment it is possible that suitable manipulation of the technique used to sensitise animals to helminth antigen may produce a greater degree of resistance, not only in sheep, as mentioned previously, but also in cattle where artifical immunisation has been carried out with some success (Armour et al., 1974; Nansen, 1975). These authors were able to generate a degree of resistance using irradiated metacercariae and the use of more sophisticated methods of immunisation e.g. using antigens from a period of in vitro culture of the parasite (E.S. antigens) may conceivably afford better protection. Such a technique was pioneered successfully in the cestode field by Rickard and Bell (1971), who vaccinated lambs against Taenia ovis using antigens obtained from a period of in vitro culture, before successfully applying similar methods to immunise cattle against infection with the larval form of the human tapeworm T. saginata, (Rickard and Adolph, 1976). Preliminary studies using a similar regime of immunisation against this parasite at our own laboratory have produced less optimistic results (Mitchell et al., in press) as indeed have the results of preliminary studies to apply E.S. antigens to immunisation against F. hepatica infection, and although initial studies using this technique by Lang (1976); Lang and Hall (1977) in the mouse gave promising results, the recent work of Davies et al. (1979) did not confirm this optimistic view. The latter authors Davies et al., 1979) attempted to immunise rats against infection with F. hepatica

using in vitro culture antigens from newly excysted metacercariae. It was reported that fluke burdens in rats which had received culture antigens did not differ significantly from control animals. In the opinion of the authors this may have been due to inherent species differences or to differences in the composition of the culture antigens used.

However disappointing these preliminary results may appear such immunisation techniques may well prove of value in protecting ruminants from F. hepatica infection in the future as our knowledge of the specific immunogenic material becomes more precise, in a regime employing an efficient immunogen in combination with an immunomodulatory agent such as levamisole or "transfer factor". To this end there is an undoubted need for detailed immunological studies to more accurately define the complex relationship which exists between the humoral and cell-mediated immune responses to F. hepatica infection in sheep and the shortcomings therein, by virtue of which immunomodulatory compounds may be of therapeutic value. A detailed examination not only of peripheral lympocyte populations, but also of hepatic lympocyte traffic and commitment of such cells to parasitic antigen would go a long way to clarifying this situation.

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APPENDIX

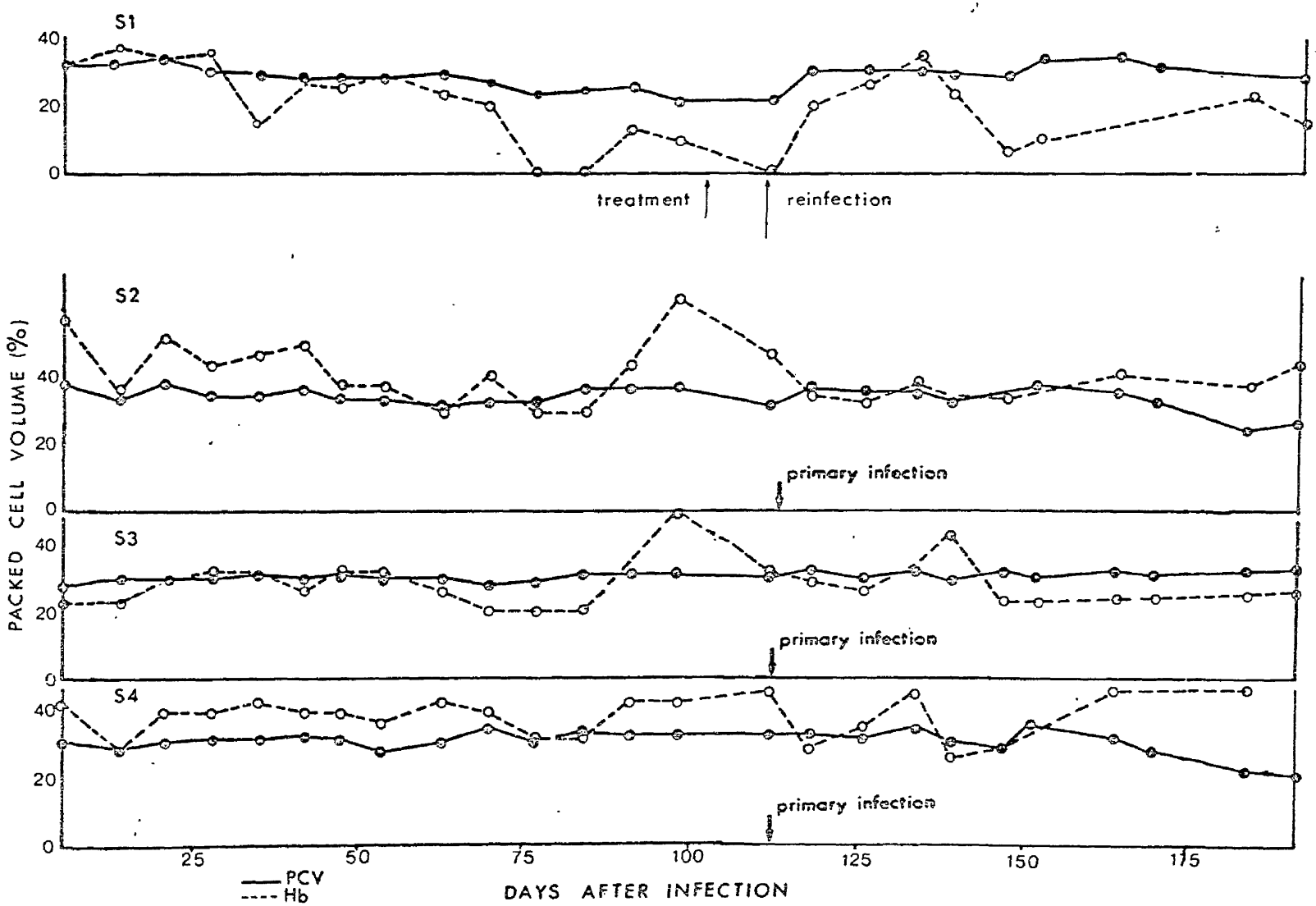

Fig. A.1 Individual values of P.C.V. and haemoglobin in sheep given primary and secondary F. hepatica infections and control sheep given a primary infection simultaneous with reinfection of the former.

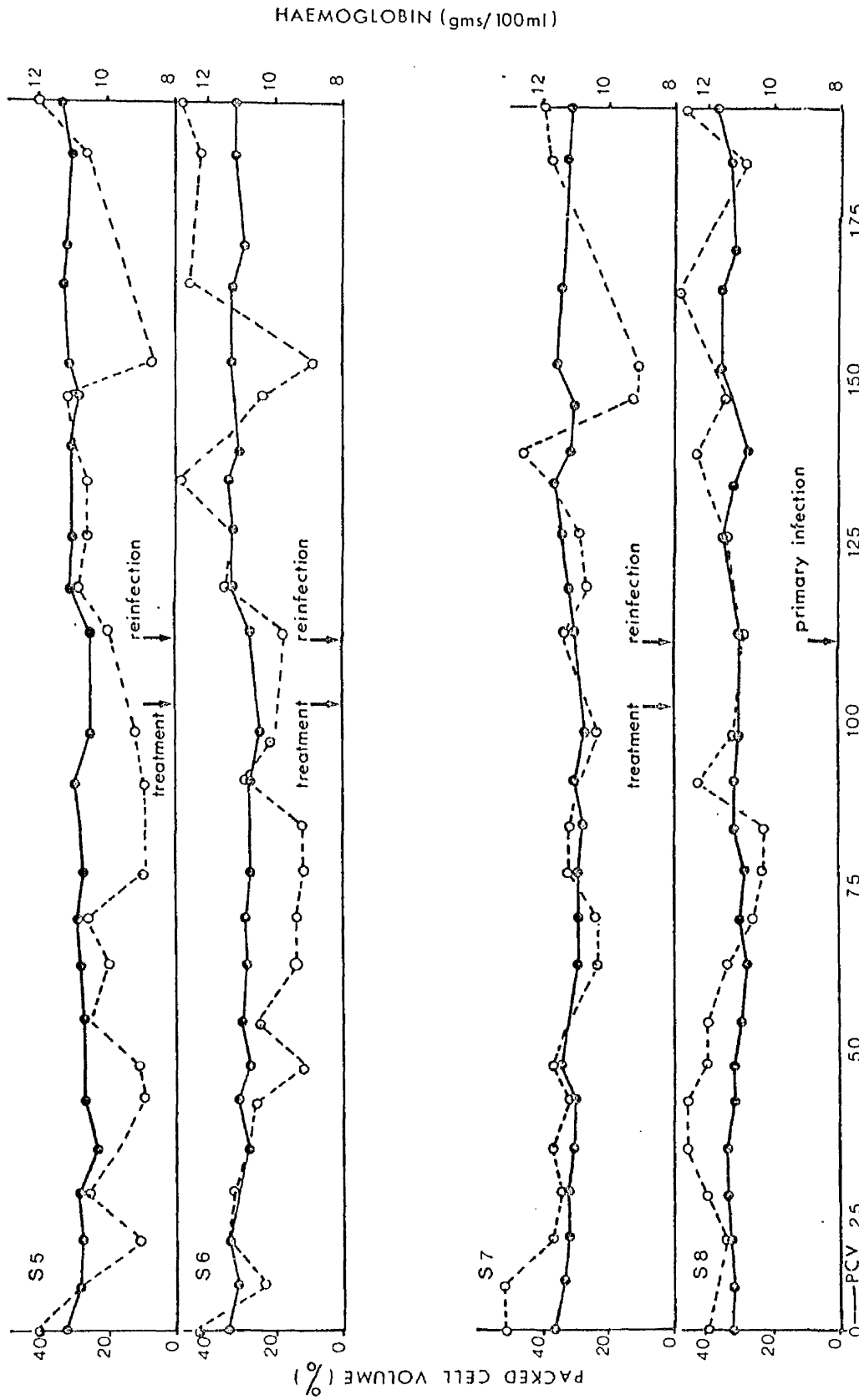

Fig. A.2 Individual values of P.C.V. and haemoglobin in sheep given primary and secondary F.hepatica infections and control sheep given a primary infection simultaneous with reinfection of the former.

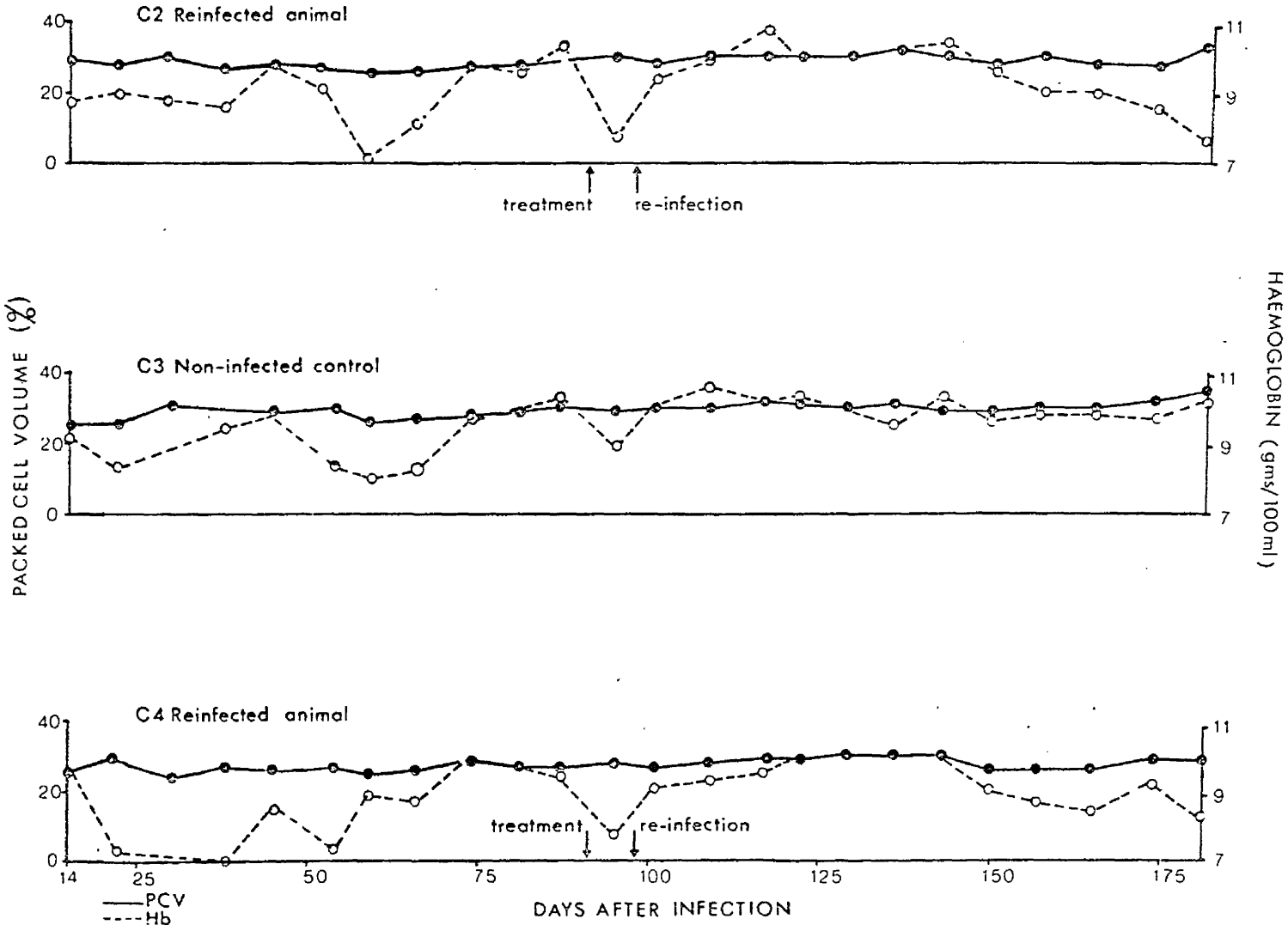

Fig. A.3 Individual values of P.C.V. and haemoglobin in cattle given primary and secondary F.hepatica infections and non-infected control.

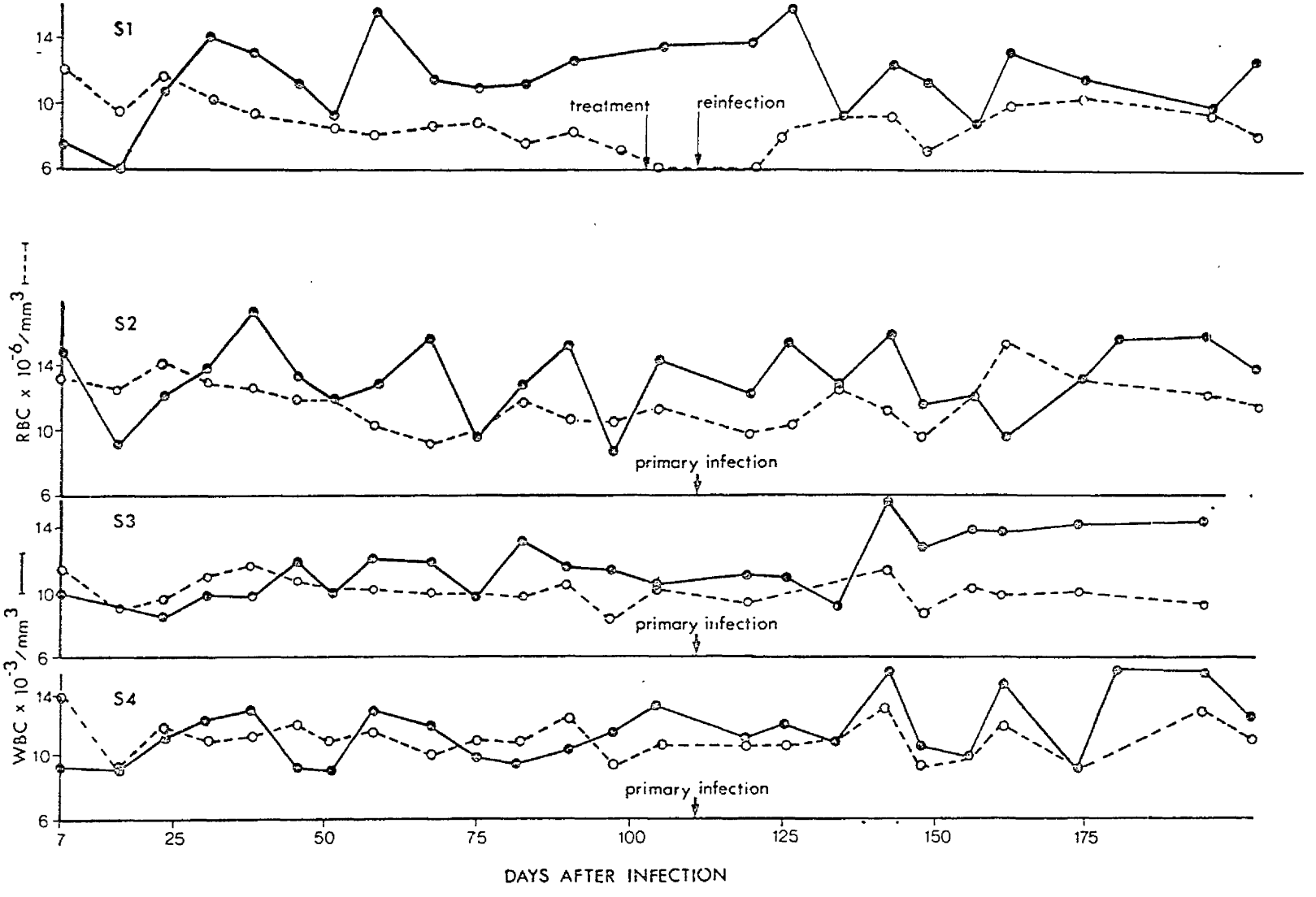

Fig. A.4 Individual values of wbc and rbc counts in sheep given primary and secondary F. hepatica infections and control sheep given a primary infection simultaneous with reinfection of the former.

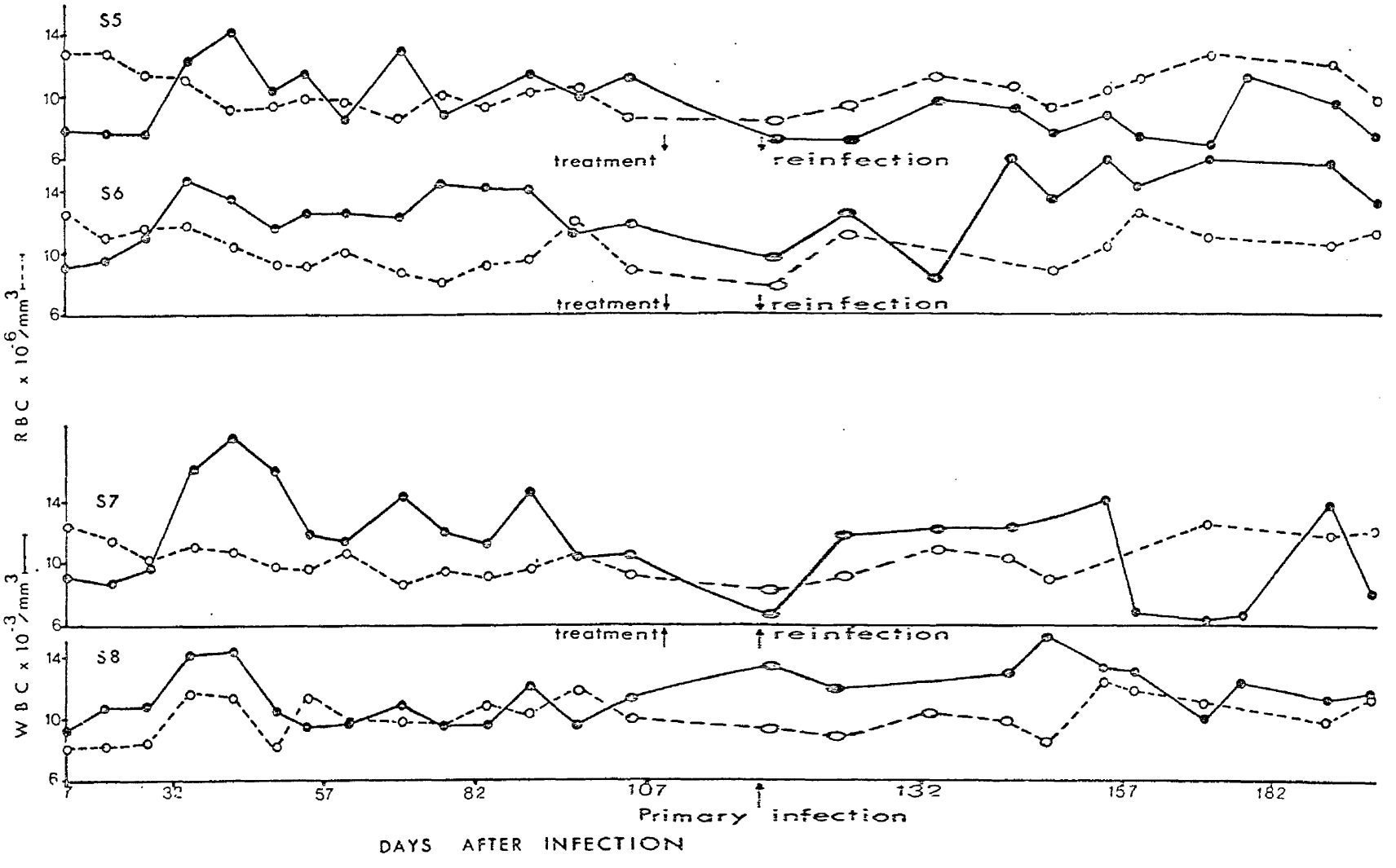

Fig. A.5 Individual values of wbc and rbc counts in sheep given primary and secondary F. hepatica infections and control sheep given a primary infection simultaneous with reinfection of the former.

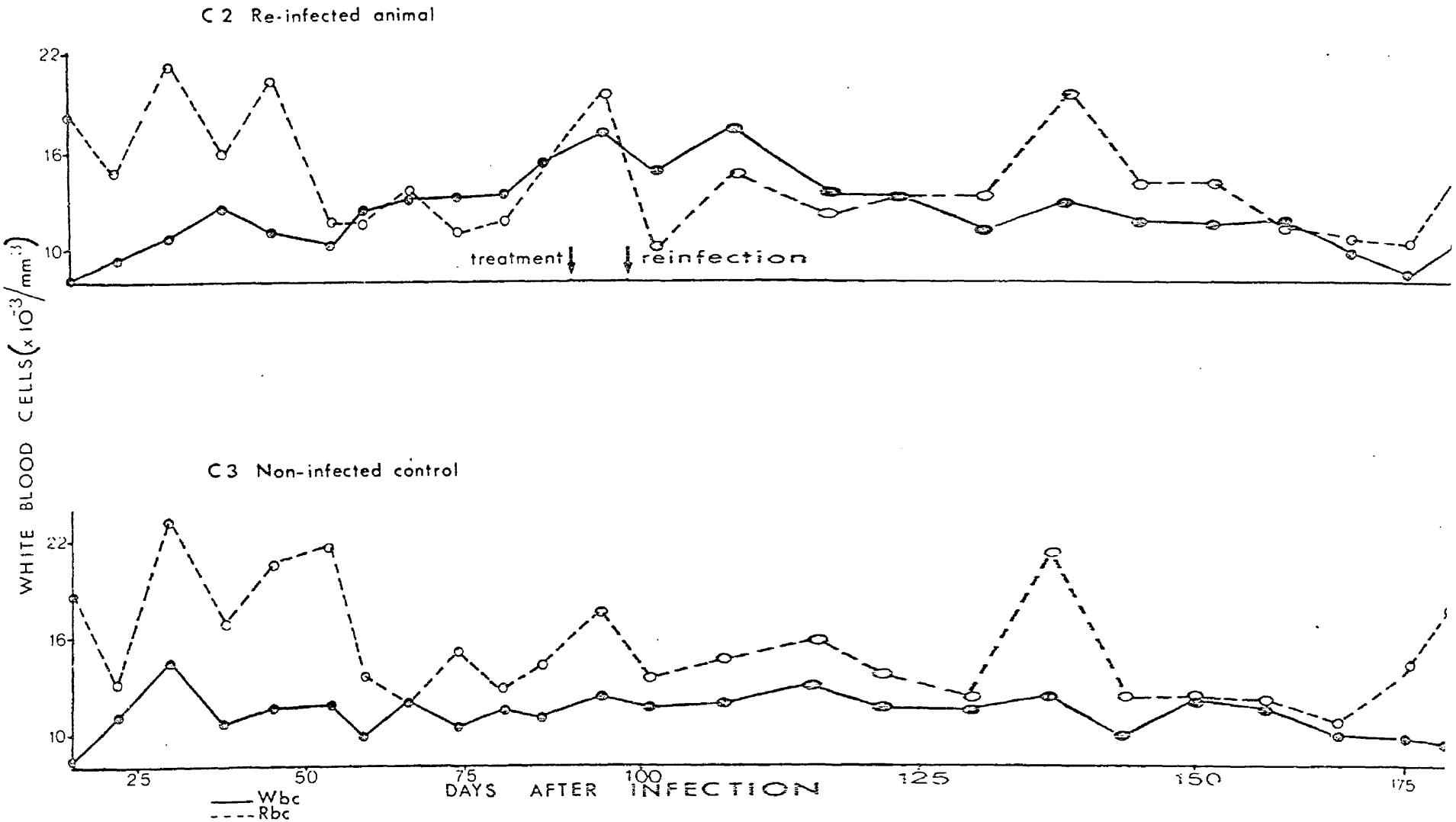

Fig. A.6 Wbc and Rbc counts in calf C2 given primary and secondary *F. hepatica* infections and non-inf control calf C3.

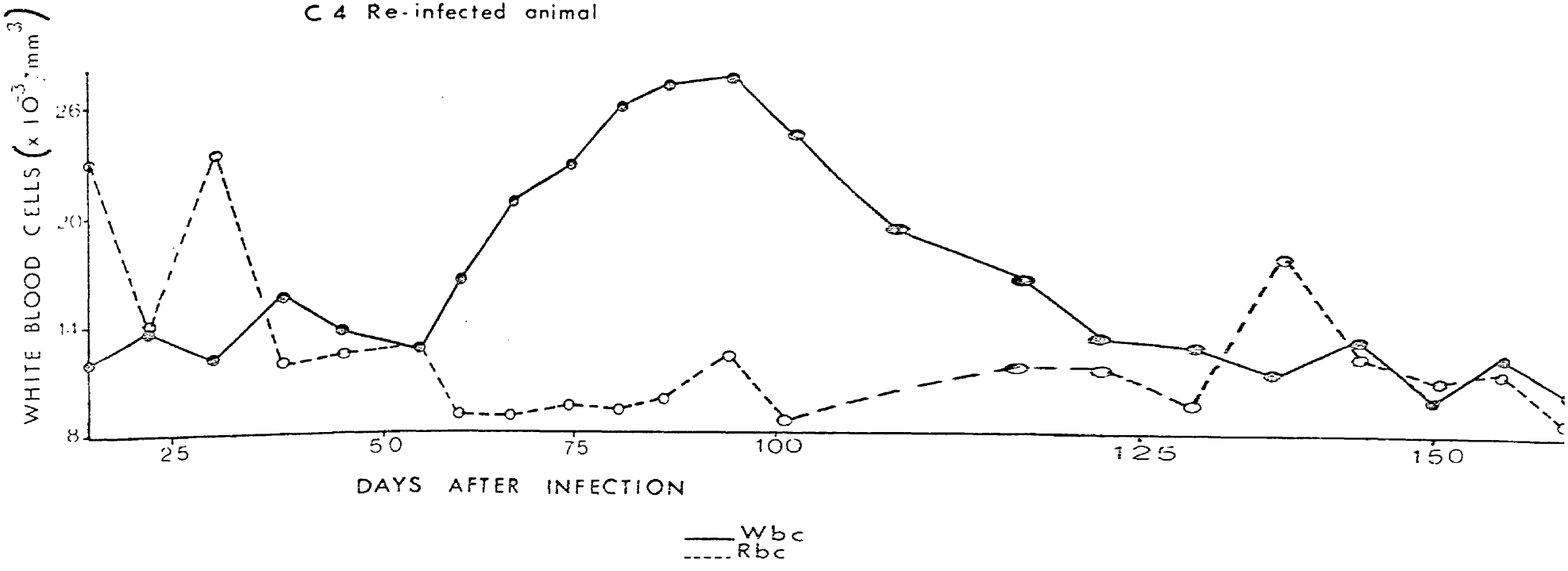

Fig. A.7 Wbc and Rbc counts in calf C4 given primary and secondary *F. hepatica* infections.

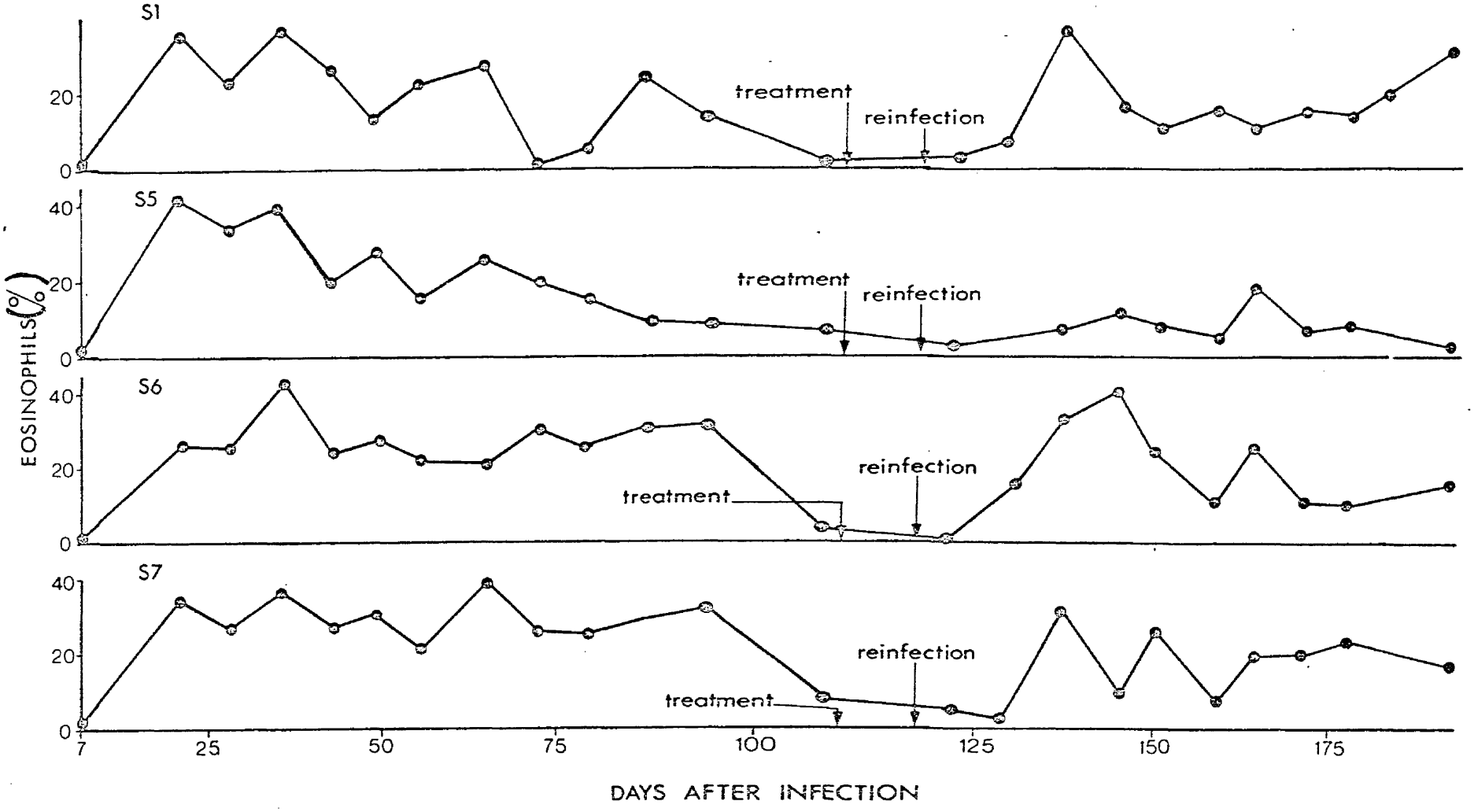

Fig. A.8 Individual values of eosinophil counts in sheep given primary and secondary F. hepatica infections

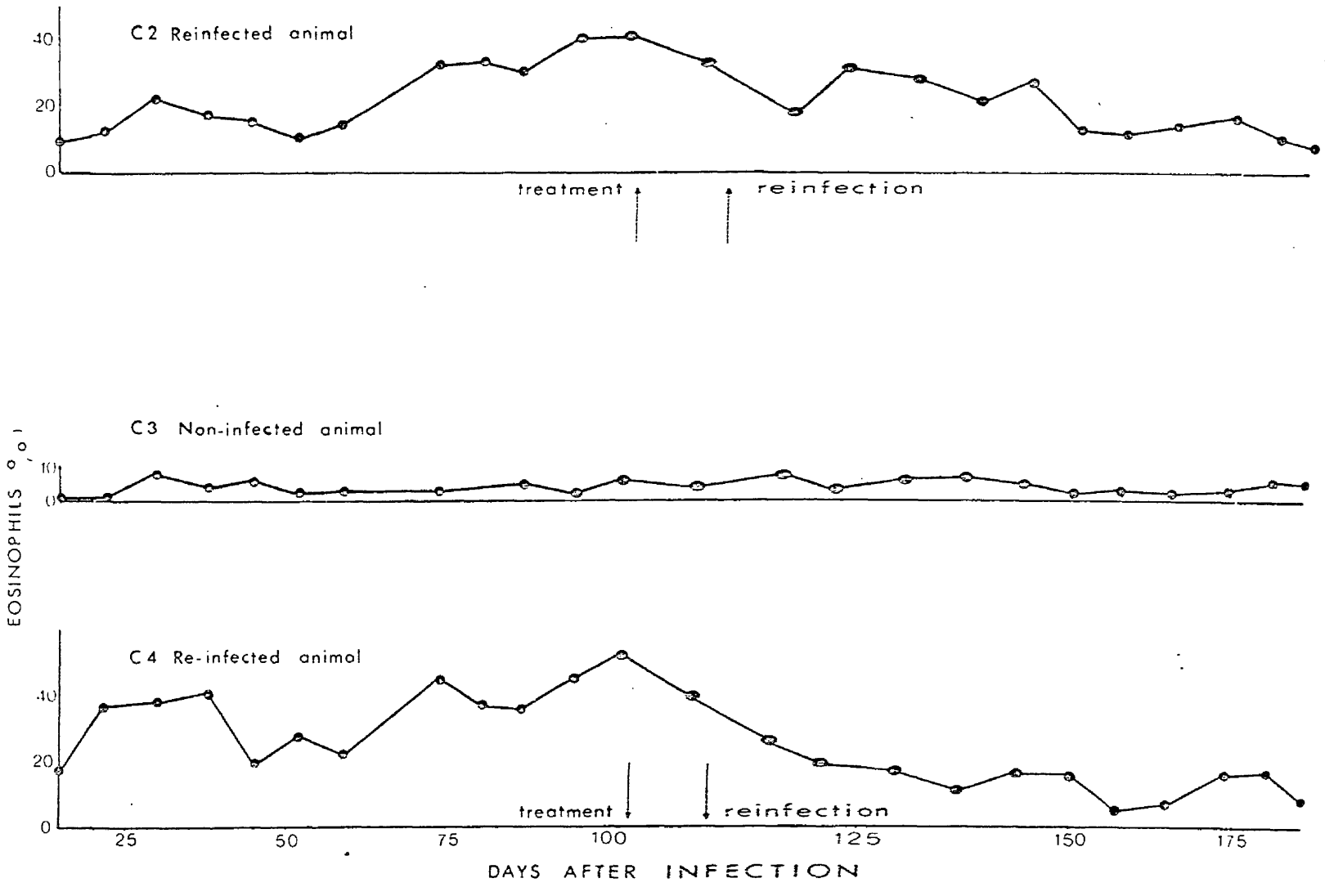

Fig. A.9 Individual values of eosinophil counts in cattle given primary and secondary F. hepatica infections and non-infected control.

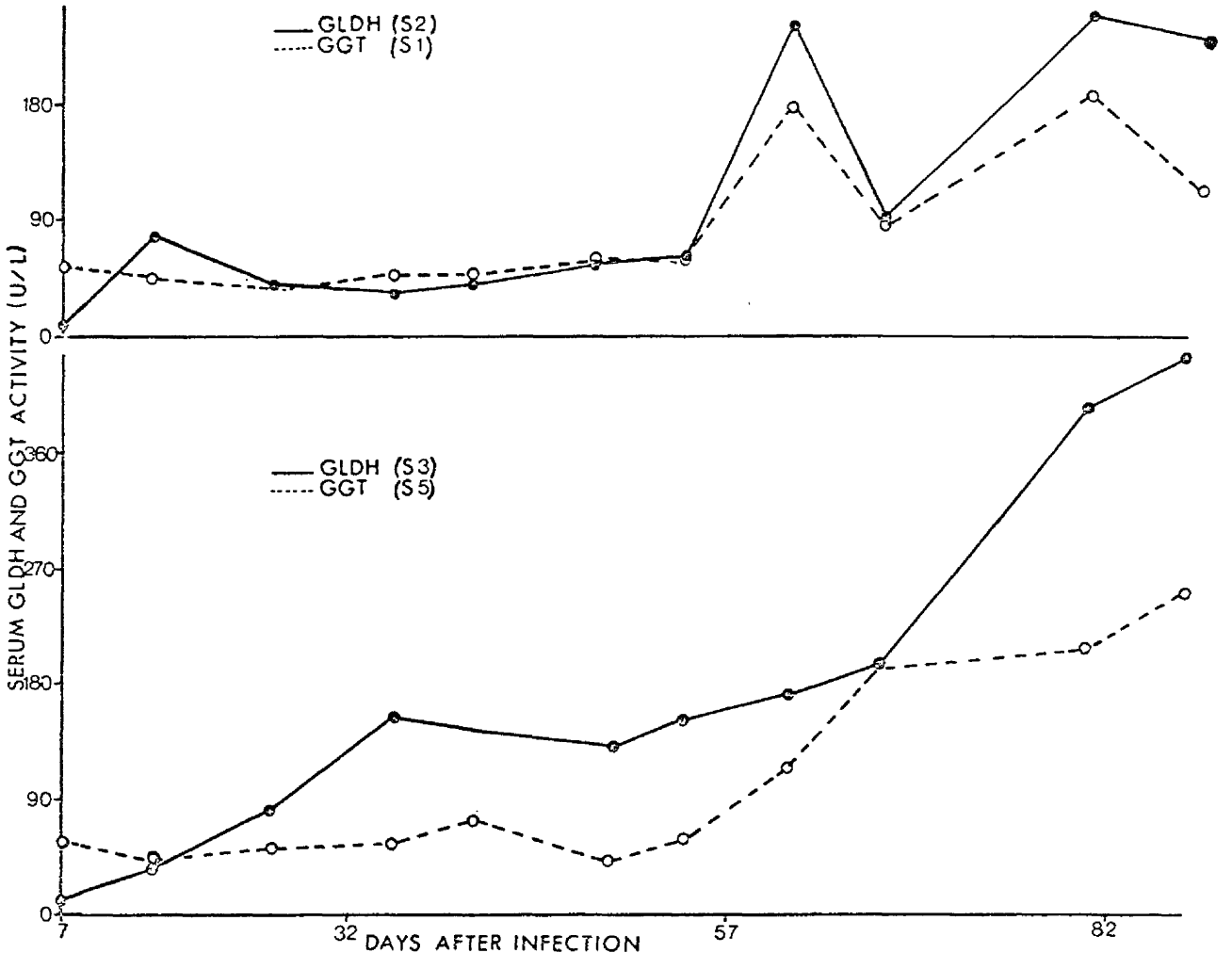

Fig. A.10 Individual values of liver enzyme activity in sheep undergoing primary F. hepatica infection.

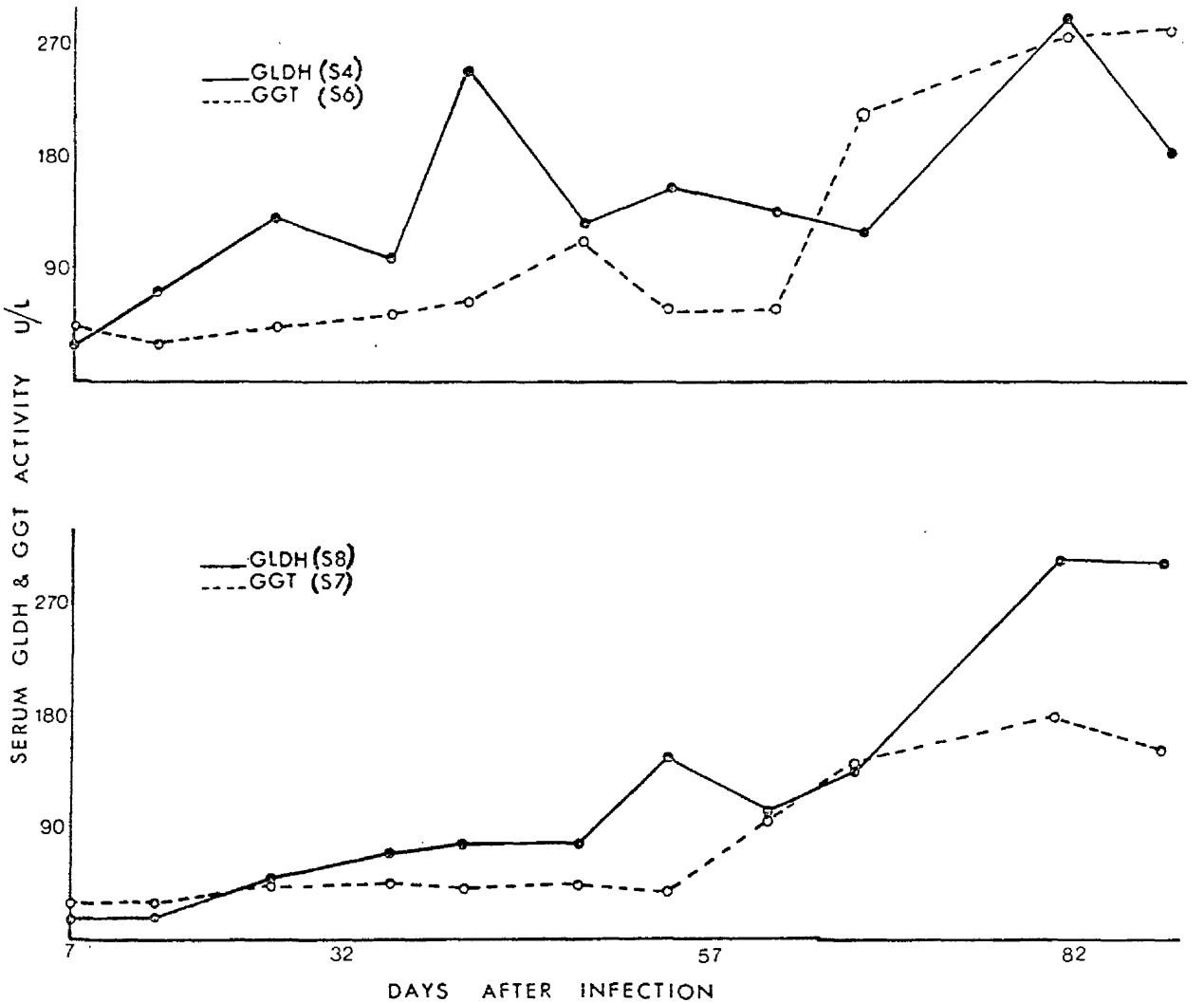

Fig. A.11 Individual values of liver enzyme activity in sheep undergoing primary F. hepatica infection.

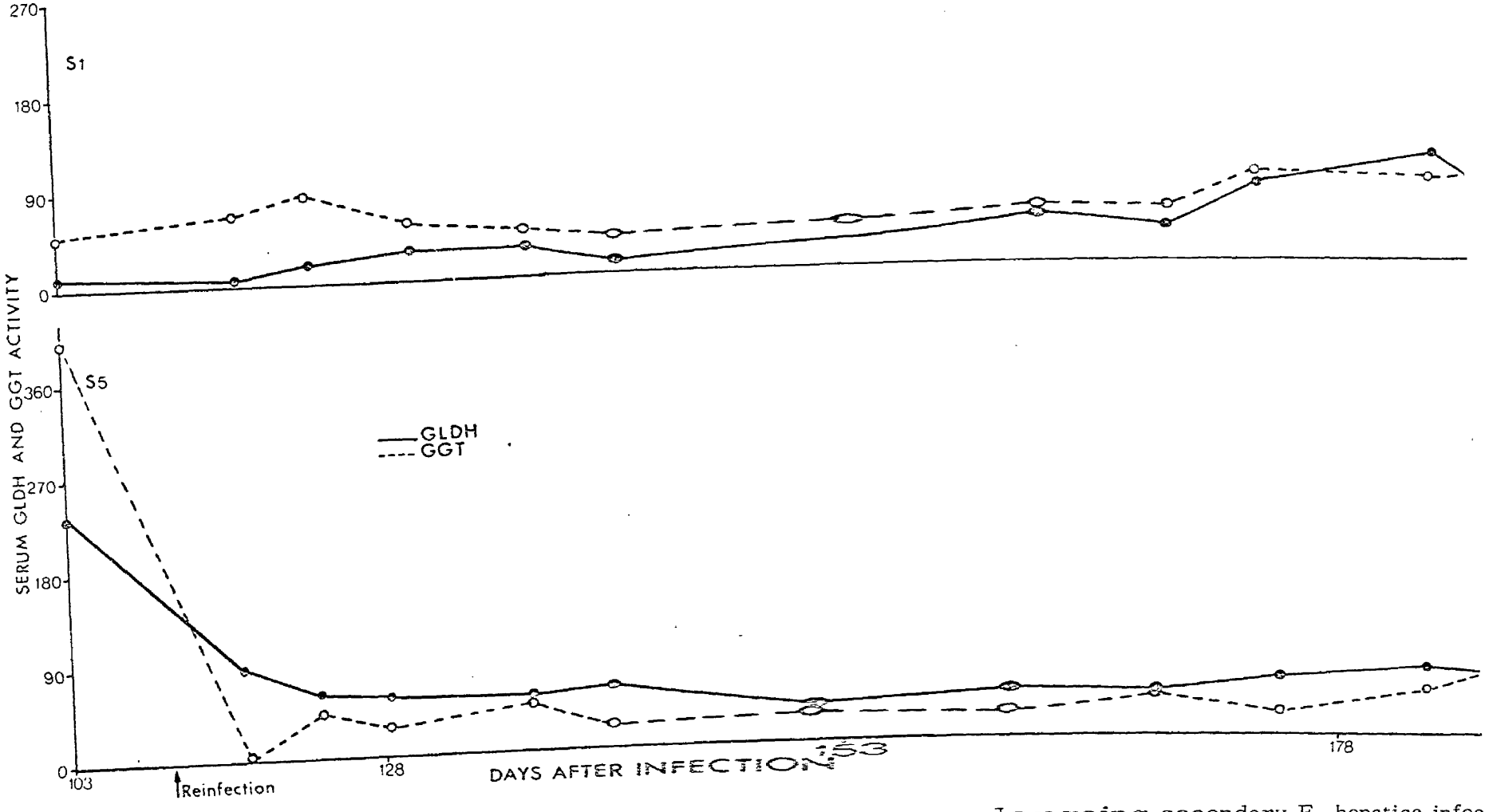

Fig. A.12 Individual values of liver enzyme activity in sheep undergoing secondary F. hepatica infec

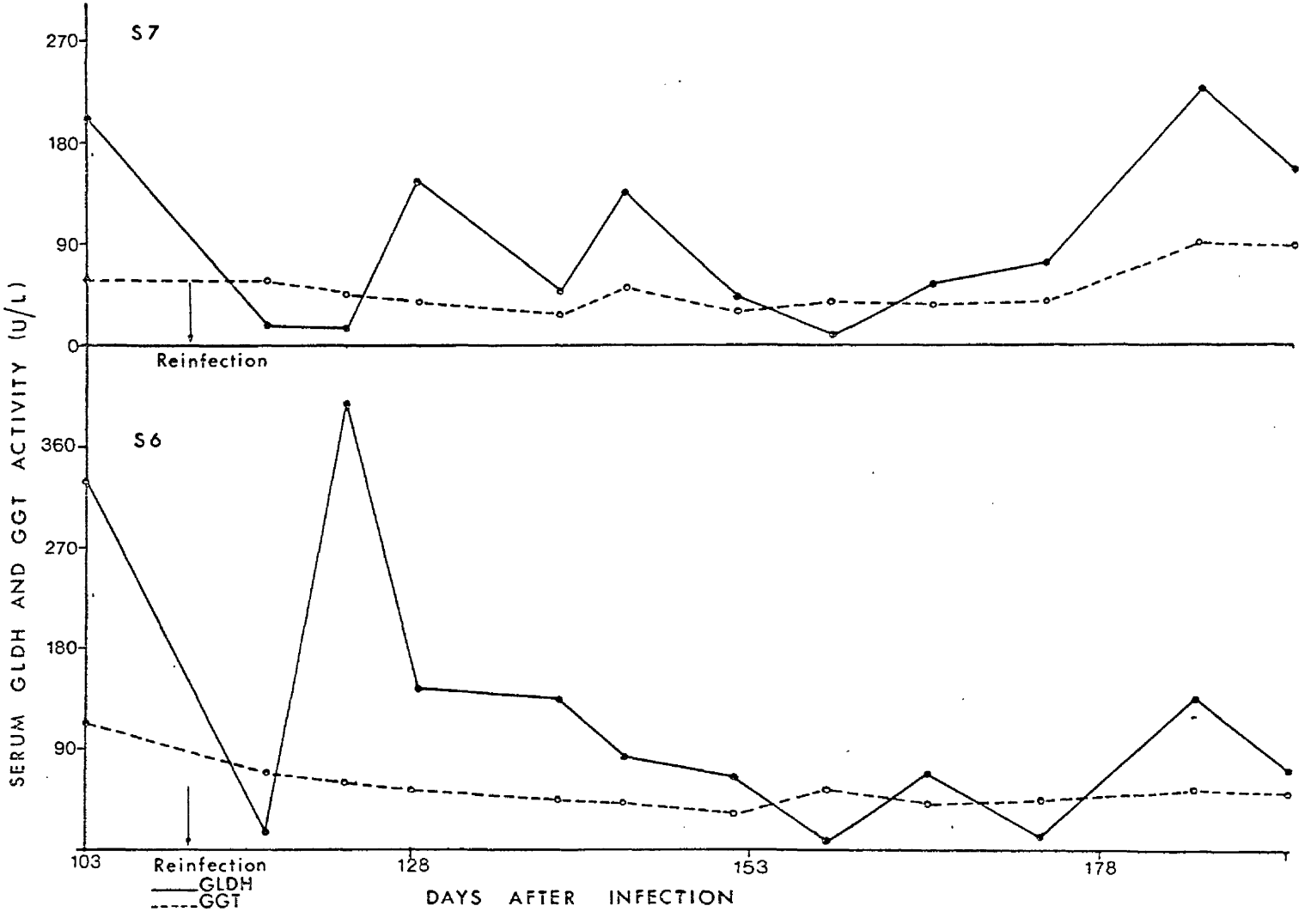

Fig. A.13 Individual values of liver enzyme activity in sheep undergoing secondary F. hepatica infection

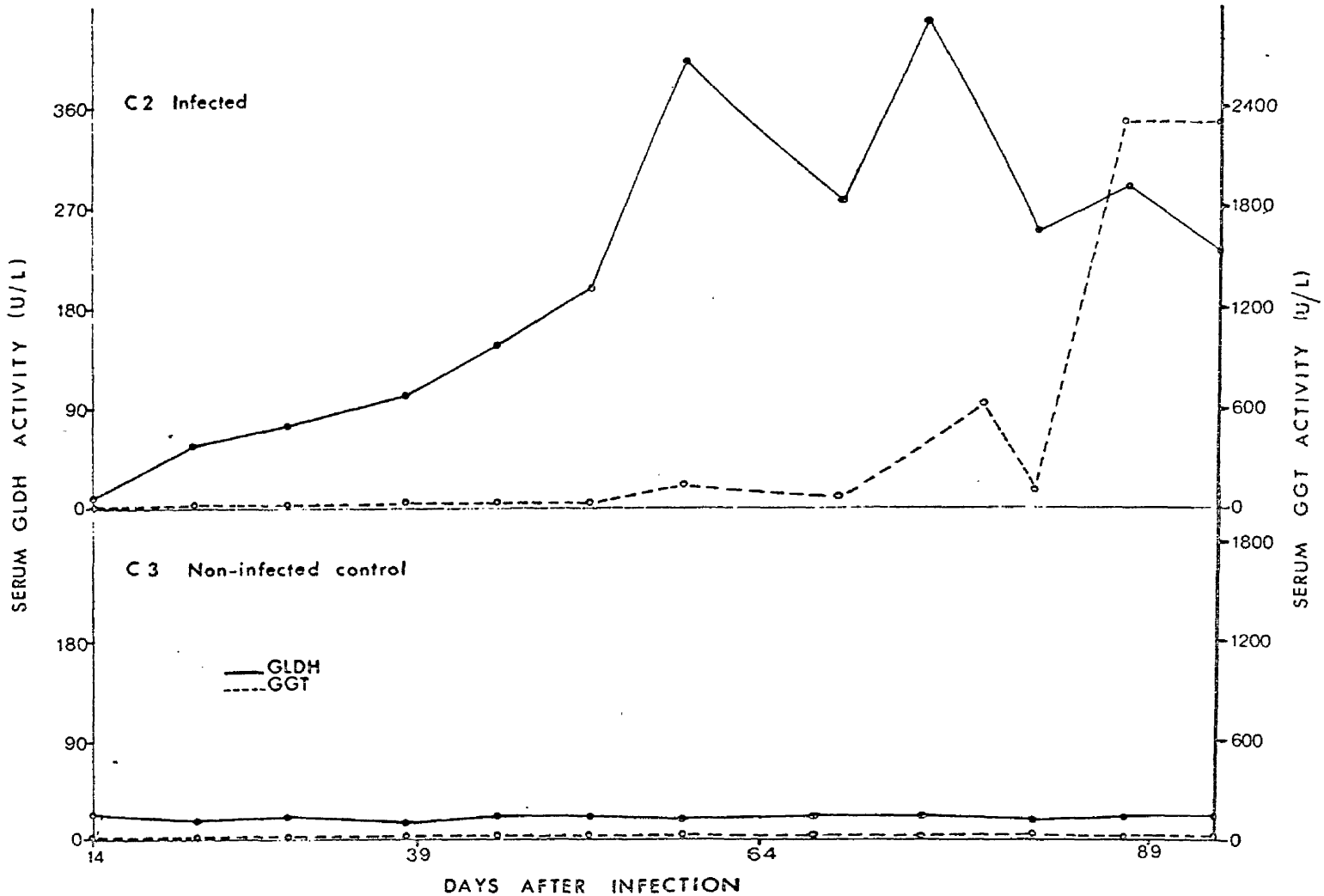

Fig. A.14 Liver enzyme activity in calf C2 undergoing primary F. hepatica infection and non-infected control calf C3.

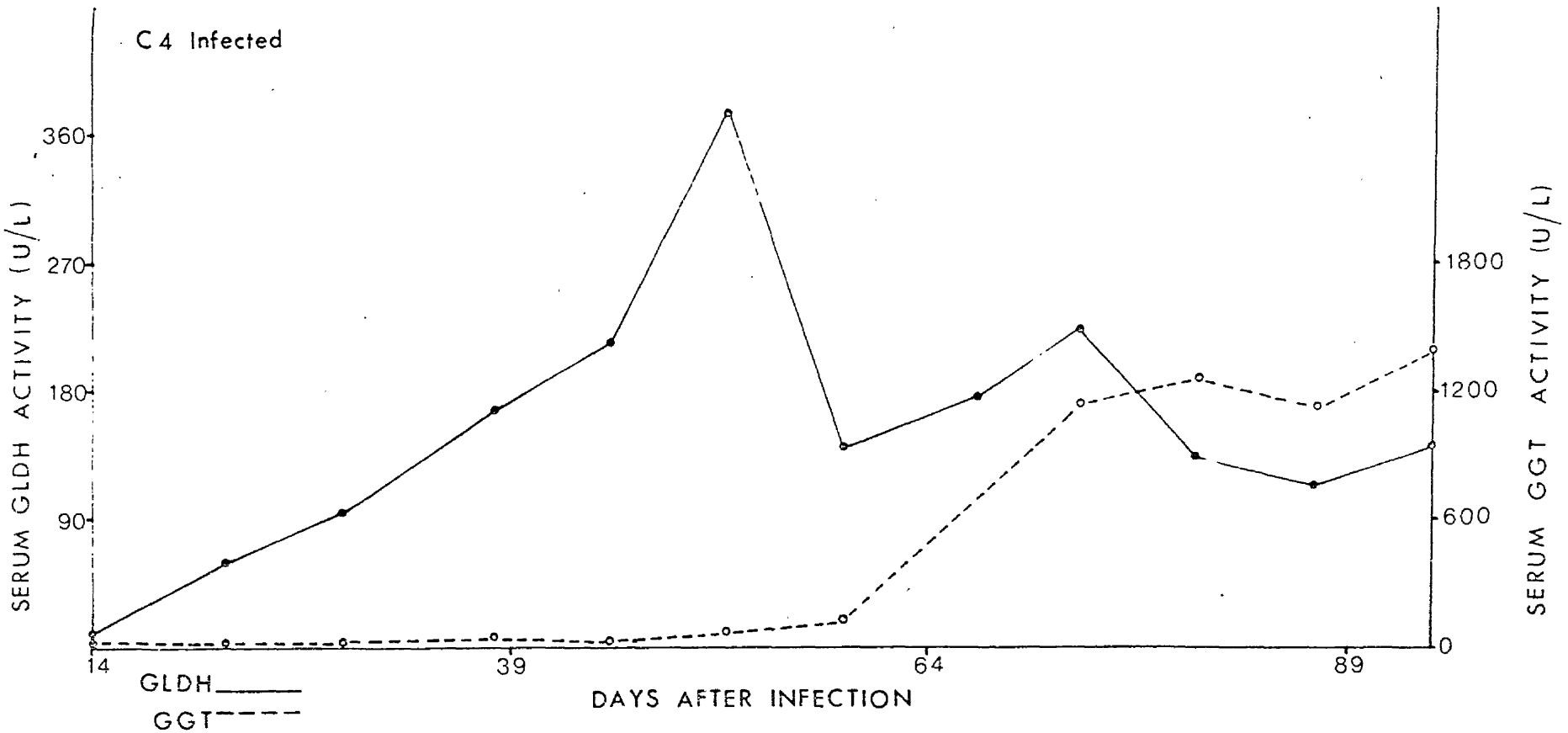

Fig. A.15 Liver enzyme activity in calf C4 undergoing primary F. hepatica infection.

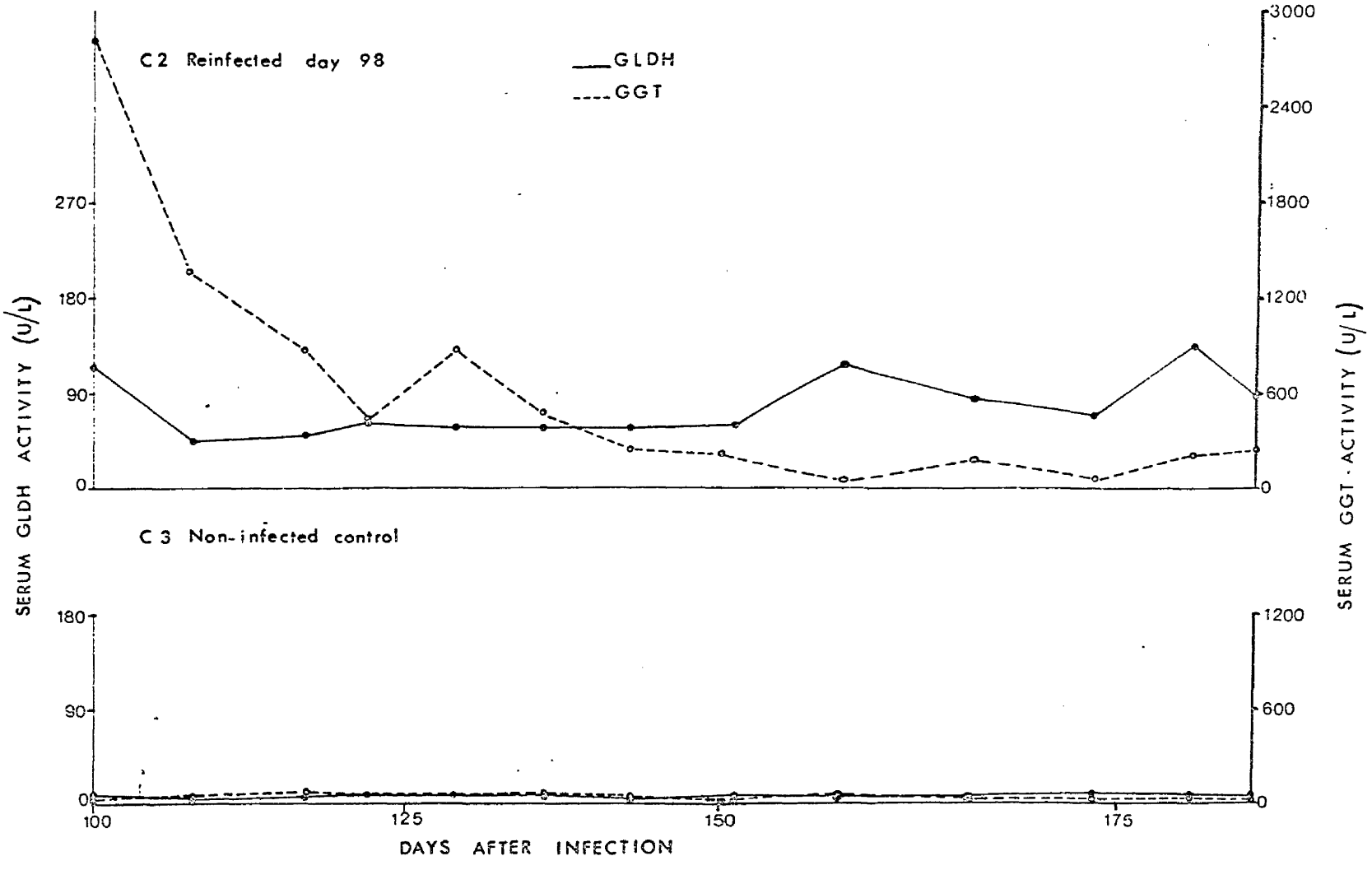

Fig. A.16 Liver enzyme activity in calf C2 undergoing secondary F. hepatica infection and non-infected control calf C3.

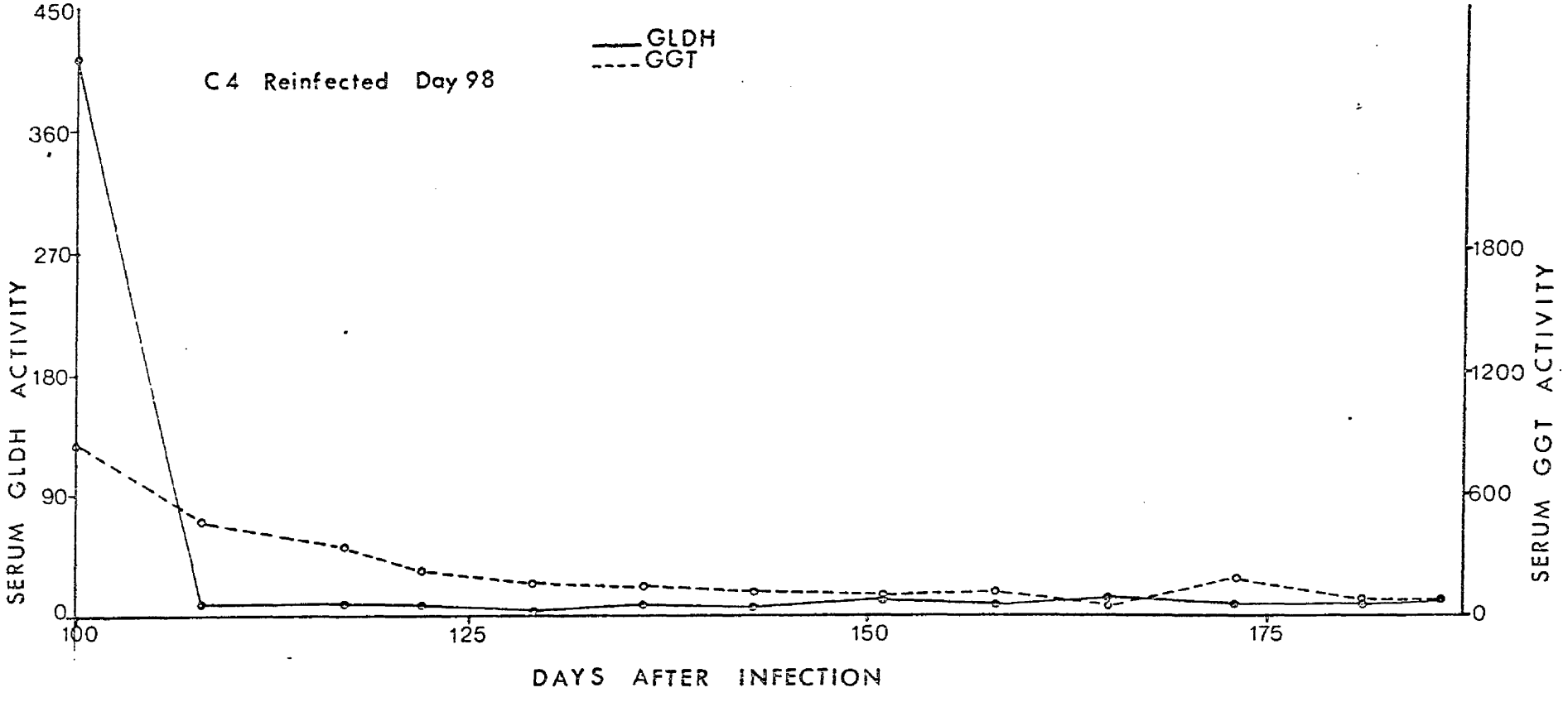

Fig. A.17 Liver enzyme activity in calf C4 undergoing secondary F. hepatica infection.

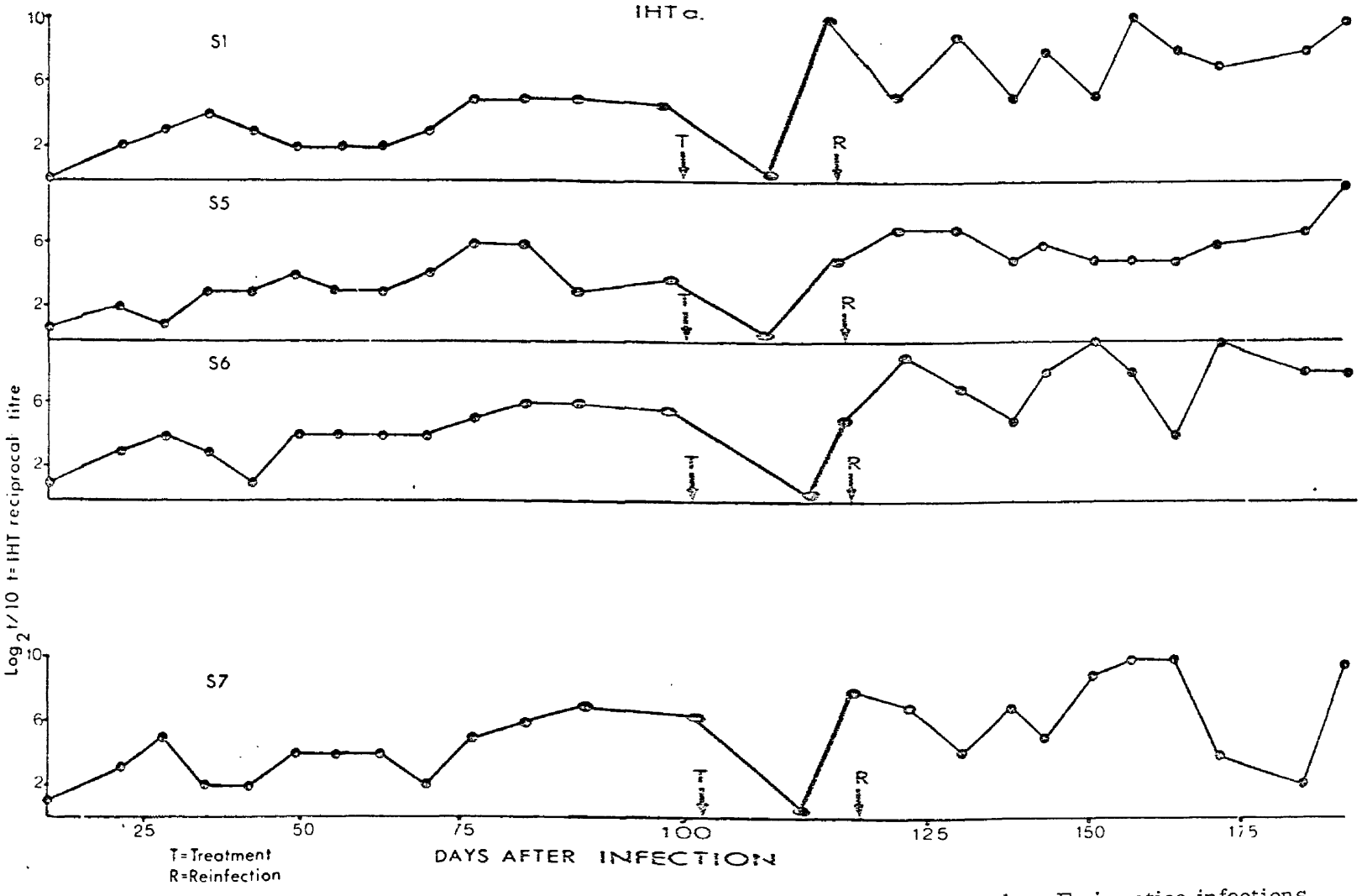

Fig. A.18 Individual I.H.T. titres in sheep given primary and secondary F. hepatica infections

a. Cells prepared in Glasgow

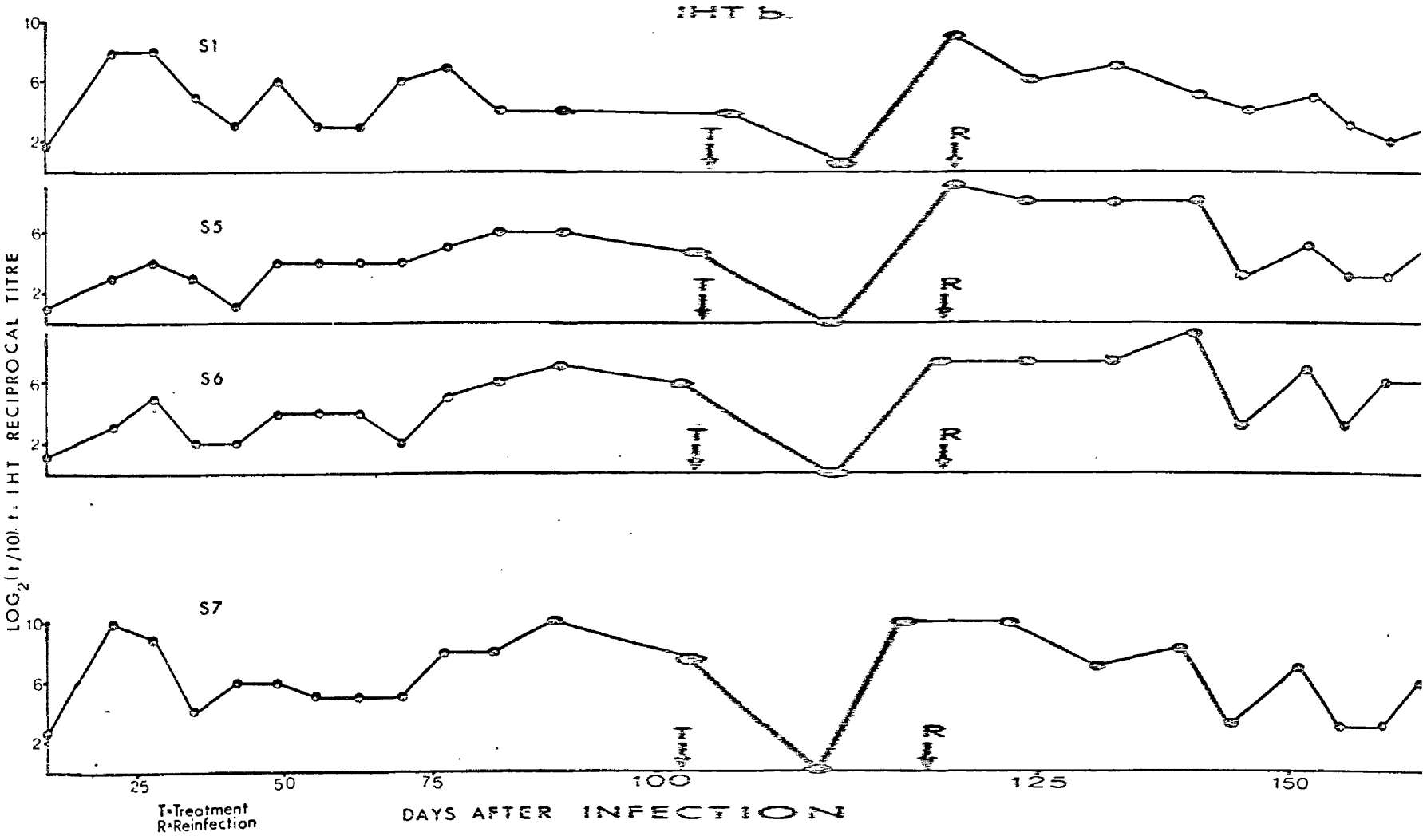

Fig. A.19 Individual IHT titres in sheep given primary and secondary F. hepatica infecti

b. Cells prepared in Rotterdam.

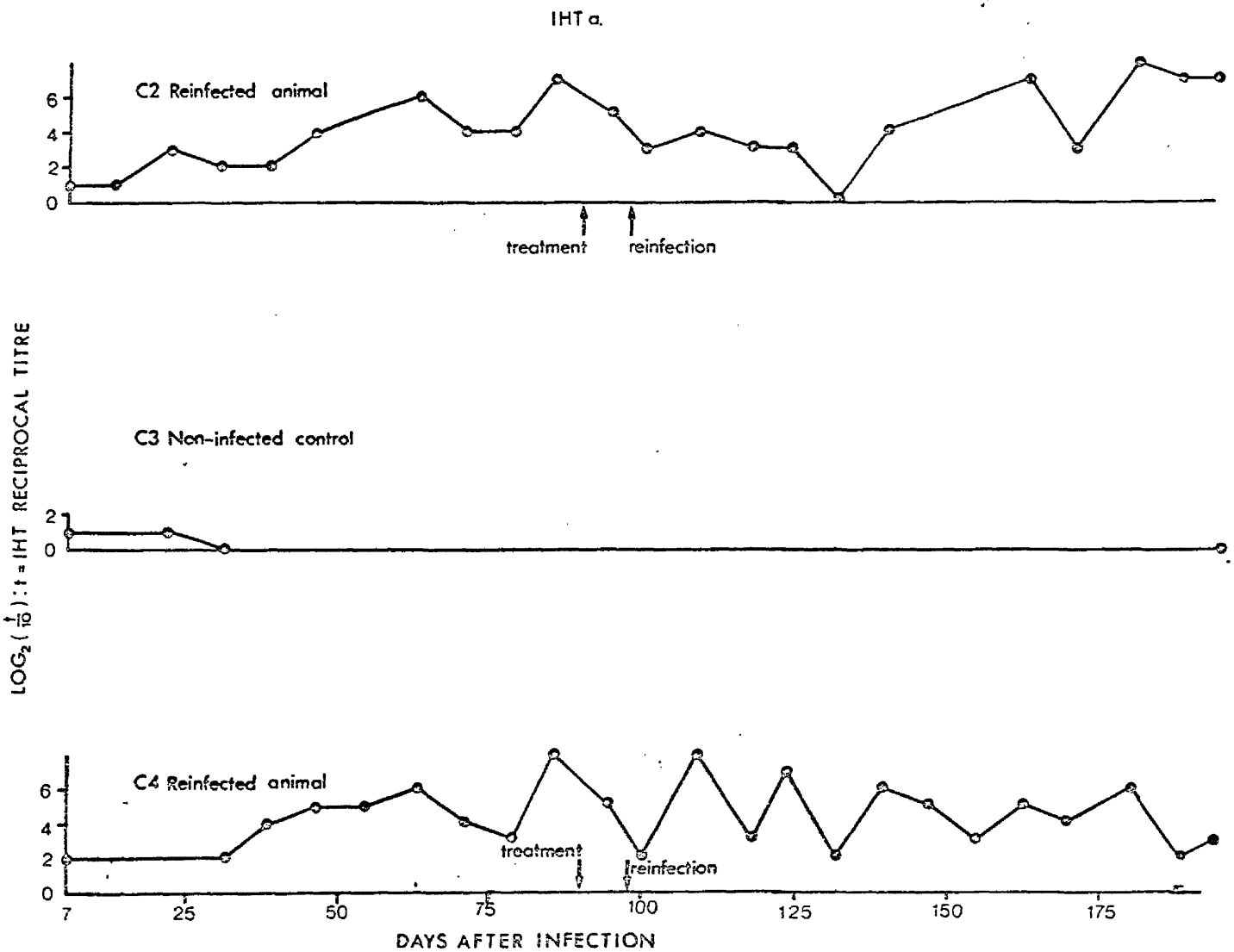

Fig. A.20 Individual IHT titres in cattle given primary and secondary F. hepatica infections and non-infected control. a. Cells prepared in Glasgow.

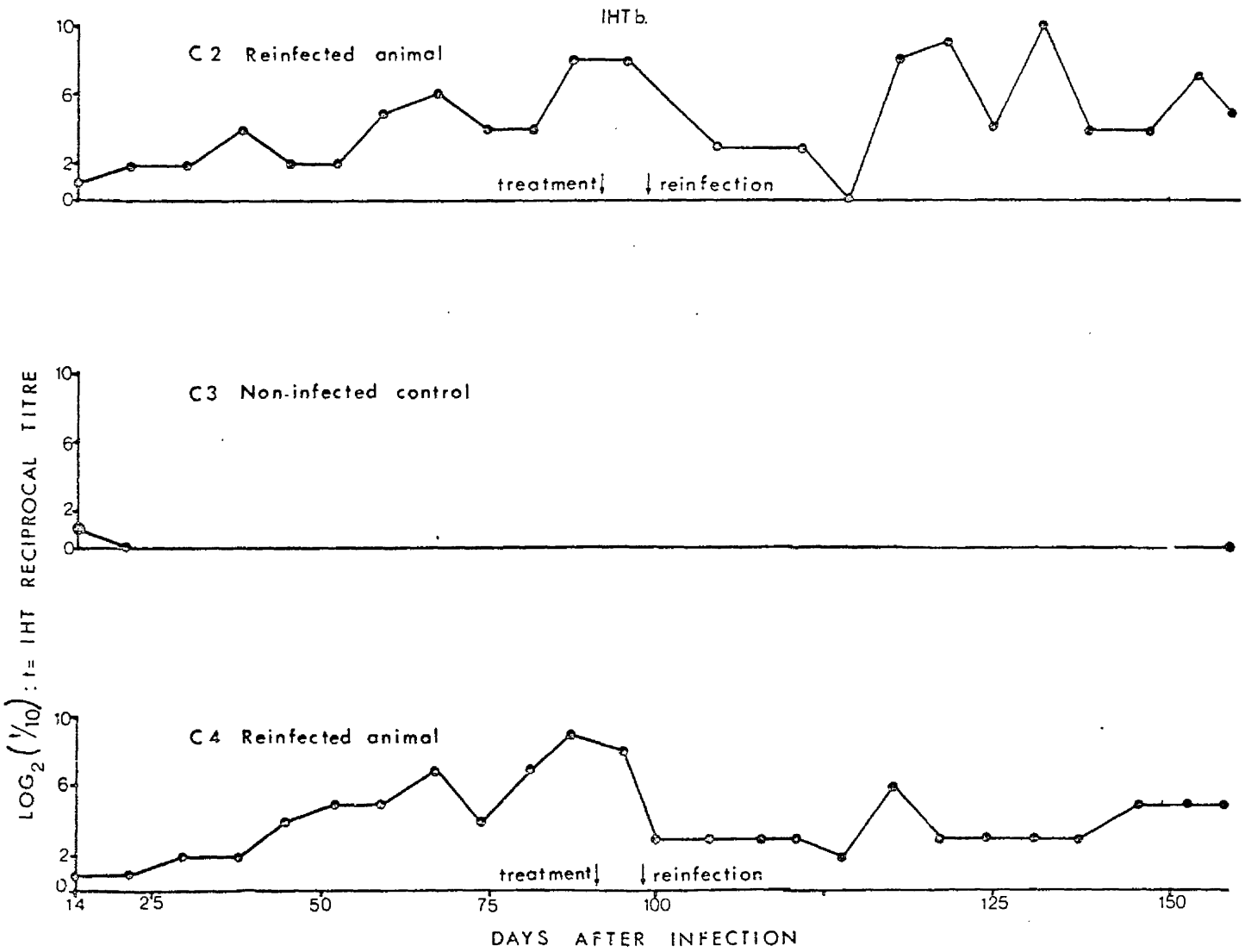

Fig. A.21 Individual IHT titres in cattle given primary and secondary F. hepatica infections and and non-infected control. b. Cells prepared in Rotterdam.

Group No.	Sheep No.	Weeks of infection 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
3	F1	3.9	4.3	3.2	5.7	30.5	24.6	50.8	13.6	70.5	88.6	72.9	108.3	115.2	41.2	110.3	134.2
	F2	1.8	11.8	23.8	76.6	113.7	130.0	144.8	94.3	81.4	92.9	117,2	101.2	152.5	130.2	103.4	no sample
	F3	3.2	13.6	5.5	17.2	84.1	109.9	101.4	122.0	65.4	79.8	75.8	89.2	45.5	21.5	no sample	82.3
	F4	3.9	10.4	15.8	39.3	90.0	71.3	87.4	87.6	104.0	118.9	124.6	96.5	130.4	88.5	60.5	30.9
	Mean	3.2	10.0	12.1	34.7	79.6	85.5	96.1	79.4	99.6	95.1	97.6	98.8	85.9	70.3	91.4	82.4
	S.D.	1.0	3.5	9.5	31.2	35.1	40.2	38.8	46.3	17.2	14.6	27.1	17.4	46.2	48.8	27.0	51.6
	S.E.	0.5	1.8	4.8	15.6	17.5	20.1	19.4	23.2	8.6	7.3	13.6	8.7	23.1	24.4	15.6	29.9
4	C1	4.3	4.1	39.4	106.2	131.9	96.5	88.6	42.2	72.8	145.2	110.0	102.2	89.1	146.6	145.6	no sample
	C2	11.0	9.7	5.5	11.2	84.1	108.9	101.4	106.4	104.8	79.8	81.7	89.2	45.5	no sample	no sample	82.3
	C3	4.7	2.2	8.5	27.2	54.4	71.3	116.0	126.9	112.3	147.4	131.8	117.2	131.8	125.1	124.3	23.4
	C4	4.5	no sample	14.8	37.4	82.3	44.1	39.8	71.7	104.0	137.7	105.6	78.0	55.4	83.4	103.4	20.1
	Mean	6.1	13.9	17.1	45.5	88.2	80.2	86.5	86.8	98.5	96.5	107.3	99.0	80.4	117.9	124.4	42.0
	S.D.	3.3	3.9	15.4	41.9	32.2	28.7	33.0	37.5	17.5	32.1	21.4	16.9	39.0	31.2	21.1	35.0
	S.E.	1.7	1.9	7.7	20.9	16.1	14.4	16.5	18.8	8.8	16.1	10.7	8.5	19.5	18.1	12.2	20.2

Table A.22 S.D.H. values following F. hepatica challenge in sheep primed with helminth infection before levamisole treatment (Group 3, Table V.3 and challenge control sheep Group 4, Table V.3), showing standard deviation and standard error.

Group No.	Sheep No.	Weeks of infection 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
3	F1	9.3	10.1	5.0	3.5	4.0	no sample	6.8	14.3	12.4	16.8	18.2	13.4	16.8	no sample	16.9	17.1
	F2	4.9	1.6	2.6	3.2	6.0	11.5	15.4	9.6	8.6	10.0	14.9	16.8	7.7	8.7	14.8	8.1
	F3	2.9	3.4	1.0	0.4	11.8	13.5	26.2	6.7	14.7	23.9	17.9	2.5	17.6	19.6	1.3	4.4
	F4	0.7	0.8	3.2	1.0	5.8	1.1	11.4	10.9	13.8	13.0	12.6	8.4	7.1	4.9	15.5	6.3
	Mean	4.5	4.0	2.9	2.0	6.9	8.7	15.0	10.4	12.4	15.9	15.9	10.3	12.3	11.1	12.1	9.0
	S.D.	3.7	4.2	1.6	1.6	3.4	6.6	8.3	3.2	2.7	6.0	2.6	6.2	5.7	7.6	7.2	5.6
	S.E.	1.8	2.1	0.8	0.8	1.7	3.8	4.1	1.6	1.3	3.0	1.3	3.1	2.8	4.4	3.6	2.8
4	C1	2.1	3.7	5.1	11.9	18.4	17.8	14.6	16.8	14.7	17.8	29.5	18.0	14.0	16.5	28.2	24.4
	C2	4.6	1.0	no sample	6.2	8.7	16.9	8.7	4.2	16.7	8.1	3.5	18.9	13.5	0.7	5.2	5.0
	C3	2.3	12.4	2.1	2.5	5.6	8.3	13.4	1.2	9.4	12.2	13.1	13.0	14.8	17.0	14.3	9.6
	C4	3.4	4.8	8.1	3.2	10.8	21.9	19.1	10.9	3.4	no sample	1.8	6.1	no sample	3.5	4.7	11.9
	Mean	3.1	5.5	3.9	5.9	10.9	16.2	14.0	8.3	11.1	12.6	12.0	16.6	14.1	9.4	13.2	12.8
	S.D.	1.1	4.9	3.5	4.3	6.7	5.7	4.3	7.0	6.0	6.8	12.6	3.2	0.7	8.6	11.0	8.3
	S.E.	0.6	2.5	2.1	2.2	3.9	2.9	2.2	3.5	3.0	4.0	6.3	1.9	0.4	4.3	5.5	4.2

Table A.23 G.L.D.H. values following F. hepatica challenge in sheep primed with helminth infection before levamisole treatment (Group 3, Table V.3 and challenge control sheep Group 4, Table V.3), showing standard deviation and standard error.