STUDIES ON THE PATHOLOGY OF EXPERIMENTAL OVINE FASCIOLIASIS

THESIS

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by

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GENERAL INTRODUCTION

Fascioliasis is a disease of national and international economic importance. Historical records show the enormous economic losses that have been sustained (reviewed by Reinhard, 1957). Greater awareness of the ecological and meteorological factors influencing the disease process, the introduction of efficient anthelmintics and selective use of molluscicides have enabled the disease to be controlled (reviewed by Boray, 1969). Despite this fascioliasis remains a serious financial problem to the farming community as is shown in the following extract:

> "Liver fluke endemic in UK.
>
> Cattle and sheep on half the farms in the UK have liver fluke, according to a survey made by ICI pharmaceuticals division. The survey, based on information collected by abattoir managers and meat inspectors, involved 600,000 cattle and sheep over a 15-month period, and was supervised by Mr. G. Froyd, FRCVS. As a result, says ICI, it is now known that fluke is endemic throughout the country - cattle are the major victims and, says the company, the loss to the farmer is at least £50 million a year. Of 37,338 cows surveyed, 40 per cent had fluke-infected livers while 17 per cent of heifers and steers (160,113 total) were positive. Known Irish cattle were 52 per cent positive. The average for all cattle (over 200,000) was 21 per cent - a figure close to Ministry of Agriculture estimates.
>
> Among ewes, 13 per cent of 77,951 animals surveyed were positive and of 299,228 lambs processed, 5 per cent were positive."
>
> Vet. Rec., (1974) 95, 75.

As part of a multidisciplinary approach, the work for this thesis was undertaken to reconsider the lesions produced in ovine fascioliasis in light of recent advances made in the fields of pathology and immunology.

INTRODUCTION

The Parasite

The liver fluke, Fasciola hepatica (Linneaus, 1758), belongs to the order digenia that contains all the parasitic trematodes found in man and animals (Soulsby, 1968). These parasites have characteristic migratory patterns in their definitive hosts. In the case of F. hepatica this involves migration of the immature flukes after cercarial excystment in the intestine through the intestinal wall, peritoneal cavity and liver substance to the biliary system where they grow to maturity. During this period the leaf-shaped parasite increases in size from a few millimeters in length in the intestine up to 30 mm in length by 13 mm in breadth in the biliary system (Soulsby, 1968). At all stages of growth the cuticle surrounding the parasite provides a very resistant but not impermeable covering (Bjorkman and Thorsell, 1964; Lee, 1966) with posteriorly directed spines. Situated on the blunter anterior end of the parasite and located slightly ventrally are large suckers and between the two suckers a genital sinus. A blind ending digestive tract connects via a muscular oesphagus to the anterior sucker and the hermaphrodite reproductive system terminates at the genital sinus through which eggs are discharged upon maturity (Soulsby, 1968).

The observations of Dawes (1961, 1963) showed that the suckers were instrumental in excavating a passage for the flukes from the intestine to the bile ducts. In the liver the suckers were used systematically to burst hepatocytes producing a cytoplasmic homogenate which was then aspirated into the oesphagus. The flukes progressed forwards in an arc-like course (Dawes, 1961) probably being helped in this process by the muscle fibre component of their body walls and the spines (Lee, 1966). The suckers and spines were possibly used in a similar capacity in the biliary system where they produced extensive erosion, ulceration and blood loss (Dawes and Hughes, 1970). In addition to the traumatic effects, it has been suggested that waste products and toxins (Dawes, 1961), fluke eggs (Urquhart, 1956; Dow, Ross and Todd, 1967, 1968) and hypersensitivity mechanisms (Dargie, Armour, Rushton and Murray, 1974) may contribute towards the disease process.

The pathology of fascioliasis

Although hepatic damage attributable to F. hepatica was recorded as early as 1379 (as quoted by Reinhard, 1957) it was not until 1968 (Dow et al., 1968) that a description of the sequen-

tial pathology of the experimental disease process became available in sheep. Previous to 1968, the studies on the pathology of ovine fascioliasis were confined to descriptions of naturally-infected sheep and comparisons of the lesions produced by natural infections and by other liver parasites and pathogenic agents (Bugge, 1927, 1928; Turner, 1930; Soyogan, 1955, 1956, 1958; Stenius, 1963).

Dow et al. (1968) provided a gross and histological account of lesions developing between weeks 1 and 40 following inoculation of sheep with 100, 200, 500 and 650 metacercariae supplemented with material from 'controlled natural infections'. They traced the progress of the flukes through the parenchyma noting the morphology of the acute and healing tracts and, finally, when the flukes had entered the bile ducts, the chronic cholangitis elicited. A range of vascular, parenchymal and biliary changes developed as a result of this migration. Direct involvement of the flukes with intrahepatic blood vessels produced thrombosis. Portal veins and less frequently arteries close to the acute tracts were infiltrated with eosinophils. Developing in the parenchyma in the later stages of infection were lympho-reticular nodules and lesions associated with fluke eggs. Biliary changes consisted of bile ductule hyperplasia, hyperplasia and erosion of the epithelium of the major bile ducts and peribiliary fibrosis.

More recently, clinicopathological studies have established three phases in ovine fascioliasis, acute, subacute and chronic. The gross pathological changes seen in the acute phase of the disease have been described by Boray (1967, 1969), Ross, Dow and Todd (1967a), Roberts (1968), Demidov (1969) and Pullan, Sewell and Hammond (1970). All these reports recorded the presence of at least 1000 immature flukes in friable haemorrhagic livers covered with extensive fibrinous deposits. Widespread peritonitis, copious quantities of blood-tinged ascitic fluid, anaemia and death within 12 weeks were also features of the acute disease. Boray (1967, 1969) and Ross et al. (1967a) extended their results with histopathological descriptions of necrosis involving all areas of the liver and numerous subcapsular haemorrhages. Ross et al. (1967a) further recorded healing tracts but only in the very early stages, rupture of blood vessels, infarction and oedema and eosinophil cell infiltration of portal vein and arterial walls. A few flukes were located in slightly distended and mildly hyperplastic bile ducts. Ross et al. (1967a) identified a second type of acute disease by the presence of slightly less numbers of flukes in the liver, reduction in the degree of ascites and the presence of large subcapsular haemorrhages.

Animals acquiring a fluke burden of about 800 flukes either all at once or over a longer period of time developed the subacute disease which terminated fatally in about 20 weeks (Boray, 1967, 1969; Ross et al., 1967a; Pullan et al., 1970). At necropsy, flukes were distributed between the parenchyma and bile ducts in variable numbers. Thus, many haemorrhagic acute tracts and distended and fibrosed bile ducts were seen grossly. Fibrous repair of the older tracts gave the livers a firm texture. Ascites was usually absent but localised fibrinous deposits were present over the ventral lobes.

Chronic fascioliasis developed when 200 or more flukes were established in the bile ducts (Ross et al., 1967a; Boray, 1967, 1969; Rubaj and Fumaga, 1969). Infected sheep were extremely emaciated and oedematous. Their livers were grossly, diffusely fibrosed with small contracted ventral lobes and thickened varicosed bile ducts. Irfan and Lee (1968) observed similar gross and histological lesions to those described above in naturally occurring acute, subacute and chronic fascioliasis.

In the above studies it was generally implied that hepatic damage in ovine fascioliasis was attributed to the direct traumatic effects of the flukes. However, it has been found that the hosts immunological status influences the course and development of the lesions. In sheep with a lowered plane of resistance to infection due to splenectomy (Sinclair, 1970) and the administration of immunosuppressive drugs (Sinclair, 1968) acute tracts were seen grossly to be very much more haemorrhagic and they healed more slowly than in infected controls. The flukes in these sheep had greater mean lengths than in the controls which was suggestive of a more rapid growth rate. On the other hand, in experiments (Sinclair, 1971, 1973) designed to increase the hosts immunological status by infecting then reinfecting, the lesions produced by the reinfection flukes were different to the infection controls and to the lesions seen in immunosuppressed sheep, and the growth rate of the reinfection flukes was retarded. In the first of these experiments sheep were infected, treated at week 4 and then reinfected 5 weeks later. Sinclair (1971) concluded from necropsies carried out at weeks 6, 8, 10, 12 and 14 after reinfection that compared to the infection controls, eosinophils were increased in numbers around the fluke tracts 8 weeks after reinfection and that there was earlier evidence of fibroblastic and lymphocyte infiltration in the livers. While haemorrhage was recorded as occurring around the reinfection tracts it was considered to be less than that described by Dow et al. (1968) in a primary

fluke infection. Later, Sinclair (1973) compared the effects of an F. hepatica infection in previously uninfected sheep, in sheep treated to terminate a week 9 primary infection and in sheep which had had five previous infections eliminated at week 1. Based only upon the gross findings from necropsies carried out at weeks 8, 10 and 12 after infection Sinclair (1973) concluded that 8 to 10 weeks after infection the livers of the two treated groups of sheep were "tougher, lighter-coloured showing a less severe hepatitis" than in the infection control sheep. Haemorrhagic fluke tracts were not mentioned.

The pathology of F. hepatica infections in the other naturally infected and economically important animals, cattle and to a lesser extent pigs, like sheep, have been repeatedly reported but only a limited number of publications provided any detailed sequential pathology (Dow et al., 1967; Ross, Dow and Todd, 1967b; Doyle, 1972; Nansen, Anderson, Harmer and Riising, 1972). Infected cattle and pig livers have many lesions in common with sheep but important differences are evident and lesions are present that have not been recorded in sheep.

Following an inoculation of calves with 200 to 1300 metacercariae the flukes that became established had a preferential migration for the bovine ventral and caudate lobes and some continued their migrations for up to 23 weeks after infection (Dow et al., 1967; Doyle, 1972). A minority of flukes in the 200 to 10000 metacercarial dose range used by Dow et al. (1967) and most when the dose was increased to 5000 (Dow et al., 1967) about 8 weeks after infection were seen to be inhibited in their movement and then became fibrously encapsulated and killed. Those flukes not inhibited and encapsulated during their progress through the parenchyma to the bile ducts produced extensive necrotic tracts, portal and hepatic vein phlebitis and arteritis with and without thrombosis. Also associated with the tracts was a vascular lesion identified by Dow et al. (1967) as an arteritis but considered by Morrill and Shaw (1942), Doyle (1972) and Flagsted, Anderson and Neilsen (1972) to be a phlebitis. Upon colonisation of the bile ducts the flukes induced epithelial hyperplasia and ulceration, heavy fibrosis and progressive calcification (Morrill and Shaw, 1942; Dow et al., 1967; Keck and Supperer, 1967; Jubb and Kennedy, 1970; Rahko, 1969, 1970; Doyle, 1972). Rahko (1969, 1970) working with naturally infected, and Doyle (1972) with experimentally infected cattle also recorded increased mast cell and globule leukocyte populations in infected bile ducts. As a

result of tract healing extensive fibrosis developed, including a distinctive type called perilobular fibrosis that divided the parenchyma into a series of lobules (Dow et al., 1967; Doyle, 1972). This type of fibrosis was seen to increase significantly upon reinfection (Doyle, 1972). Also developing in the parenchyma were many lymphoreticular nodules particularly near dead flukes and egg granulomata in the parenchyma (Dow et al., 1967).

In pigs, experimental (Ross et al., 1967b; Nansen et al., 1972) and natural infections (Sofrenović, Buljević and Stanojević, 1961; Ohshima, Ito and Sadao, 1971) have shown that flukes become rapidly restricted in their parenchymal migrations, surrounded by an intense eosinophil cell reaction and connective tissue and killed. This resistance to infection was, however, of a higher order than that seen in cattle as no flukes of a low experimental infection in eight week old pigs were able to reach the bile ducts (Ross et al., 1967b; Nansen et al., 1972). A few flukes have been detected in the bile ducts of natural infections (Bugge and Muller, 1928). During their limited parenchymal migrations the flukes produced short tracts which were quickly healed, thrombosis and an eosinophil cell-associated portal vein phlebitis (Ross et al., 1967b). Nansen et al. (1972) demonstrated that resistance to F. hepatica developed post-natally at the same time as the basic fibrous tissue component of the pig's liver. This fibrous tissue content was, therefore, assumed to play a major role in limiting the migration of the flukes (Nansen et al., 1972).

The differences in resistance of sheep, cattle and pigs to F. hepatica prompted Ross (1967) to catagorise the resistance expressed as high, medium and low. These differences were considered to be partly due to variations in the fibrous tissue content of the normal species liver; pigs with the most fibrous livers had a high resistance, cattle with moderately fibrous livers a medium resistance and sheep because of relatively non-fibrous livers a low resistance. The speed and effectiveness of collagen deposition during infections (Ross, 1967) and any established fibrosis and cholangitis (Boray, 1969) were also considered to be important factors in limiting a F. hepatica infection.

Sheep were not alone in their low resistance category as many other species were included, e.g., laboratory animals, horses, donkeys and man. Laboratory animals have been used extensively to study many aspects of fascioliasis including the pathology. Thus, comprehensive pathological descriptions have been provided by Urquhart

(1956) for the rabbit, by Dawes and Hughes (1964) and Lang (1966) for the white mouse and by Thorpe (1965a) for the rat. All these reports detailed the traumatic damage sustained during the intrahepatic phases of migration and when the flukes were established in the bile ducts. Urquhart (1956) paid particular attention to the different types of fibrotic lesions that developed and collectively termed them a coarse cirrhosis. He listed healing tracts, infarcts, chronic cholangitis, portal canal and connective tissue hyperplasia, and egg granuloma formation as being the fibrotic components of the coarse cirrhosis. While the interest of Dawes and Hughes (1964) was mainly focused upon the migratory and phagic activities of the flukes, they identified within the migratory period two phases, a 'free' migratory phase lasting for up to 6 weeks and a 'localised' phase developing after week 6 and before entry of the flukes to the bile ducts. During the localised phase hepatic damage was considered more extensive than could be accounted for by the direct trauma of the flukes. Mice were seen to die during this phase. Lang (1966) in a straightforward documentation of parenchymal and biliary lesions was in broad agreement with the results of Dawes and Hughes (1964). In a later experiment (Lang, 1967) a more rapid accumulation of lymphocytes upon reinfection was seen and this reaction was interpreted as being indicative of a delayed-type hypersensitivity response. Such a response was suggested as being involved in the resistance the mice offered to reinfection (Lang, 1967).

Thorpe (1965a) working with the rat traced the morphology of the acute fluke tracts from week 1 to week 10 post-infection. He noted the inducement of thrombosis and large infarcts, bile ductule formation from hepatocytes and eosinophil cell infiltration of portal vein walls. The fibrosis produced in rats was concluded (Thorpe, 1965a) as being due to healing of the tracts and infarcts and not to intrahepatic cholangitis as flukes were only found in the extra hepatic ducts. In another experiment (Thorpe, 1963) designed to test the effectiveness of anthelmintics and immunity induced by x-irradiated metacercariae, additional parenchymal fibrosis was produced by encapsulation of dead flukes. The flukes were killed by the treatment administered and as a result of x-irradiation when at the metacercarial stage.

Thus, up to the present time pathological investigations of F. hepatica infections have shown that flukes during migration to their predilection sites the bile ducts, are subject to fibrotic and immunological reactions which, depending upon the species, may or may not

affect the progress of migration of the flukes and their rate of growth. During the course of this migration in normal and immunosuppressed animals vascular reactions have been induced that cannot be accounted for by the direct traumatic effects of the flukes. In addition, a range of lesions have been recorded in intrahepatic blood vessels, the identity of which varies from report to report. Finally, as a result of infection various types of fibrosis have been identified developing in species other than the sheep.

The objective of this thesis was to investigate the pathological changes that occurred during an experimental primary infection in sheep (Section II) with a view to, first, reassessing the sequential development of the acute lesions produced during migration, and, second to identify the different types of fibrosis developing, their distributions and their causes. At the same time, because of divergencies recorded in the vascular lesions in different species, the identity of the different vascular lesions produced was examined (Section III). Since preliminary investigations in Glasgow have suggested that the hepatic damage in reinfected sheep was often more severe than could be accounted for by the straightforward traumatic effects of the flukes, Section IV of this thesis contains a study of the lesions produced in sheep reinfected with F. hepatica. Prior to the above investigations a study of the normal ovine liver was undertaken to establish the basic intrahepatic morphology and the identifying features of the intrahepatic vasculature (Section I).

In Section V an investigation was conducted into the immunological responses of sheep to the effects of F. hepatica migration because when tissue cells are damaged irreversibly, cell wall and subcellular constituents normally isolated from bodily defense mechanisms are released into the circulation. Some of these constituents are enzymes, one example of which is serum glutamic oxaloacetic transaminase (SGOT). This enzyme can be detected readily in the serum and its level provides evidence of cell damage and an index of the severity of damage (Wordsworth and Dykes, 1969). Other components can be shown to be antigenic; this is achieved by immunising animals with whole or fractionated homologous or heterologous cell components together with an adjuvant. The antisera produced are then incorporated in standard serological tests using the original immunising agent as antigen (reviewed by Weir, 1973). Human liver cell wall (Chordi, Lledias, Santamaria, Alvarez-Moreno, Oritz and Landazuri, 1969; Büschenfelde and Miescher, 1972) and rat liver (Asherton and Dumonde, 1962; Pinckard and

Weir, 1966) antigens have been identified by these methods. Similarly, constituents of myocardial cells (Grazenfeld, Rosenman, Davies and Laufer, 1966), kidney cells (Intorp and Milgrom, 1968), adrenal cells (Milgrom, Tuggae and Witebsky, 1963) and nervous tissue cells (Hatcher and Macpherson, 1969) can be demonstrated as being antigenic.

Since the antibodies produced by immunisation were capable of reacting with cell constituents they were termed auto-antibodies (Burnet, 1963). Besides the production of auto-antibodies, immunisation with tissue antigens and adjuvants has induced pathological changes in specific organs, usually those organs incorporated as antigen in the initial immunisation procedure. For example, immunisation of rabbits with (1) human liver cell wall antigens produced cirrhosis (Büschenfelde, Kossling and Miescher, 1972), (2) rabbit seminal plasma induced an orchitis (Yantorno, Debanne and Vottero-Cima, 1971) and (3) rat myocardial cells stimulated a focal myocarditis (Kaplan and Craig, 1963). In addition, organ damage induced by pre-treatment with cytotoxic drugs (Grazenfeld et al., 1966) or bacterial components (Kaplan, 1965) or radio-active gold (Wordsworth and Dykes, 1969) was enhanced by immunisation with tissue antigens.

In view of the fact that subcellular constituents exist which can stimulate antibody production, the release of such components during cell death provides an opportunity for an animal to produce auto-antibodies to its own tissues. There is also the possibility that these auto-antibodies may initiate or enhance organ damage. That the body does not normally react in this way to tissue damage and "self-antigens" is a fundamental characteristic of the immune system (Weir, 1967) which depends upon a recognition mechanism capable of distinguishing "self-antigens" from "non-self" antigens. In response to "non-self" antigens humoral and cell-mediated reactions are invoked that alone or in combination help to remove the offending antigen. With regard to "self-antigens" a state of unresponsiveness or tolerance exists (reviewed by Nossal, 1973). However, even although an animal is normally unresponsive to "self-antigens", responsiveness to "self-antigens" in the form of auto-antibody production can be induced spontaneously by inbreeding within the New Zealand Black (NZB) strain of mice and the obese strain of chickens (Playfair, 1973). Auto-antibodies are also a feature of a group of diseases called auto-immune diseases in man and animals. Before a disease can be classified as auto-immune it must conform to recognised clinical and serological criteria (Doniach, 1970). The pertinent criteria are: The disease is of unknown aetiology; Appropriate

auto-antibodies are detectable; Protracted course with exacerbations and remissions and not always progressive; Histological evidence of immune activity; More than one organ can be involved (Doniach, 1970). Diseases conforming to these criteria are well recognised in man (Roitt, 1970) and include such important diseases as systemic lupus erythematosus and rheumatoid arthritis and the liver disorders of primary biliary cirrhosis, active chronic hepatitis and cryptogenic cirrhosis (Doniach, 1970). In domestic animals systemic lupus erythematosus and rheumatoid arthritis have been recorded in a small number of dogs (Lewis, Schwartz and Gilmore, 1965; Halliwell, Lavelle and Butt, 1972) but, as yet, auto-immune liver disorders have not been recognised.

Besides being present in auto-immune diseases, auto-antibodies have been detected in a wide variety of disease states not conforming to the above criteria but involving cell degeneration in the human liver (Gajdusek, 1958), heart (Ehrenfeld, Gery and Davies, 1964), bladder (Jokinen, Alfthan and Oravisto, 1972), thyroid (Irvine, 1964) in dogs with induced myocardial damage (Pinckard, Olson, O'Rourke, Palmer, Kelley and Goldfein, 1971) and in rats with cytotoxic liver damage (Weir, 1961, 1963). Auto-antibodies have also been found in the serum of normal rats (Kidd and Friedewald, 1942; Weir, Pinckard, Elson and Suckling, 1966), pigs (Warr, Lascelles, Caline and Coombs, 1971) and rabbits (Mackenzie and Boreham, 1974). In addition, auto-antibodies have been detected in human (Shamina, Thewaini and El-Shawi, 1965) and lapine (Kurata and Noda, 1965) schistosomiasis and in rabbits infected with Emeria stiedae (Asherson and Rose, 1963), Trypanosoma congolense (Mansfield and Kreirer, 1972) and Trypanosoma brucei (Mackenzie and Boreham, 1974).

Thus the finding of auto-antibodies in a range of tissue-destructive and parasitic diseases in man and animals and the fact that flukes produce considerable hepatic cell death promoted a search for auto-antibodies in ovine fascioliasis.

GENERAL MATERIALS AND METHODS

Experimental animals

Scottish Blackface sheep of both sexes approximately four months old and 35 to 45 Kg. live body weight were used in all the experiments. The sheep had been reared indoors from birth. When one week old they were separated from their mothers and fed on whole milk for the first four weeks. During the third and fourth week hay, lamb weaner pellets (British Oil and Cake Mills Ltd., Renfrew, Scotland), were introduced and milk feeding was discontinued by the end of the fourth week. When approximately eight weeks old, all males were castrated and at the same time all the sheep were docked and inoculated with the combined clostridial vaccine 'Covexin' (Burroughs Wellcome and Co., Beckenham, Kent). All inoculations were repeated two weeks later.

Histological procedures

The sheep were stunned with a captive bolt pistol and then exanguinated by incising the carotid arteries and jugular veins. The abdomen was opened and the liver removed. Blocks of tissue were selected from the liver lobes, to include parenchymal tissue and all the blood vessels identified in Section I; the location of the blood vessels being taken from the acrylic resin casts prepared in Section I. Once selected, the blocks were fixed in mercuric chloride-formaldehyde or Carnoys fluid (Culling, 1963) for at least 48 hours then processed in a standard three-day phenol-amyl acetate-celloidin series and finally impregnated with wax. Sections were stained with haematoxylin and eosin, Martius scarlet blue, periodic acid-Schiff, Gordon and Sweets method for reticulin (Culling, 1963) and the Carnoys-fixed tissues with Astra blue-safranin at pH 0.3 (Enerbach, 1966).

Identification of branches of the intrahepatic systems

Branches of the portal and hepatic venous systems and the biliary and arterial systems involved with pathological changes in the infected sheep in Sections II, III and IV were identified using the nomenclature and criteria detailed in Section I.

Parasitological techniques

Sheep inoculations

Sheep were inoculated orally with F. hepatica metacercariae provided by Dr. J. Armour, Department of Veterinary Parasitology, Glasgow Veterinary School and by The Ministry of Agriculture, Central Veterinary Laboratory, Weybridge. The number of metacercariae used was based upon experience gained in Glasgow. An inoculation of 400

metacercariae was considered sufficient to produce hepatic damage for a study of the primary disease process and still allow a further 400 metacercariae to be given as a reinoculation without causing deaths.

Recovery of F. hepatica burdens.

Following the selection of blocks of tissue for histology the livers were sliced and squeezed to recover flukes. The liver slices were placed in buckets containing warm physiological saline for 1 hour and then finally squeezed. The debris at the bottom of the buckets was searched for whole and parts of flukes. The total number of flukes recovered from each liver was found by adding the number of whole to the number of part flukes containing the anterior sucker. All whole flukes were measured and the mean fluke length and standard error (SE) determined for each sheep. Flukes under 12 mm in length were counted and expressed as a percentage of the total number of whole flukes recovered to obtain the percentage of immature flukes in each sheep (Dow et al., 1968).

Biochemical investigations

Serum samples from the sheep were tested for their serum glutamic oxaloacetic transaminase (SGOT) activity on a Technican A.A. II System and the results expressed in Sigma - Frankel (S-F) units.

Statistical analysis

The statistical methods employed were those described by Bishop (1966). Throughout this thesis all deviations of the means are expressed as the standard errors (SE) of the means.

SECTION I

THE MORPHOLOGY OF THE OVINE LIVER

INTRODUCTION

References to ovine liver anatomy are confined to gross descriptions (Montane and Bourdelle, 1917; Ellenberger-Baum, 1943; May, 1953; Sisson and Grossman, 1959; Nickle, Schummer and Seiferle, 1973), a comparative and developmental study of the microcirculation in the foetal, neonate and adult liver (Wendelin, 1972) and to ultrastructural examinations of the perisinusoidal space (Grubb and Jones, 1971; Gemmell and Heath, 1972), and bile ducts (Gemmell and Heath, 1974).

Recognition of important areas of species divergence in liver anatomy, the paucity of information pertaining specifically to sheep and the fact that present day intrahepatic vascular nomenclatures are inappropriate for sheep led to a study of the normal sheep hepatic architecture and vascular systems and the formulation of a new vascular classification. Such a study is a prerequisite before investigating disease processes in the liver.

In this study, the techniques employed were histological sections in combination with acrylic resin casts. The latter method has been used widely in the study of the intrahepatic vasculature of many other species (Puckett and Neuman, 1940; Logan and De Ome, 1949; Elias and Petty, 1953; Elias and Popper, 1955).

MATERIALS AND METHODS

Livers from nine normal Blackface sheep were used.

Acrylic resin casts

Neat Tensol (I.C.I. Ltd., mixed in a ratio of 25 parts of Tensol to 1 part hardener) was injected from a syringe via a plastic tube inserted and tied into the portal vein or the hepatic vein or the biliary system. Tensol diluted with an equal volume of acetone to facilitate perfusion was injected into the hepatic artery. Light pressure on the syringe plunger was found to be sufficient to fill adequately the various systems. After hardening which generally took about three hours, the livers were placed in acid baths for three days, removed and washed thoroughly in water. Six livers were examined by this technique.

On the whole, the portal and hepatic systems perfused well and produced similar casts from different livers. Small pieces were removed as necessary for closer examination and photography. Slight

variations in the depth of perfusion were encountered; this did, however, allow viewing of deeper lying structures, avoiding the laborious trimming advocated by Tompsett (1970).

Histological procedures

Three livers were used for histological study. Blocks of tissue were selected from areas of the liver that were known to contain specific blood vessels; the location of the vessels being determined from the casts. The blocks were then processed as described in the general materials and methods.

To demonstrate the possible existence of stellate cells (Wake, 1971), blocks of tissue were fixed in Baker's calcium formalin (Culling, 1963) for 24 hours and snap frozen in a dry-ice isopentane mixture. Frozen sections cut at 20μ were stained in 0.02% gold chloride for 16 hours in the dark then quickly immersed in 5% sodium thiosulphate and counter-stained with Sudan IV and haematoxylin. All sections were mounted in an aqueous mountant.

RESULTS

Intrahepatic systems

The branches of the portal, hepatic and arterial systems identified are illustrated in Figures 1 and 2 and a new portal vein nomenclature is proposed.

Portal system

Tensol casts

The outline of the four liver lobes ventral, central, dorsal and caudate as described by Ellenberger-Baum (1943) was readily appreciated from the casts made of the portal system (Fig. 3).

In the liver hilus, the portal vein divided into two veins of large calibre termed distributing portal veins (Fig. 3). A short right distributing branch supplied the caudate, dorsal and half of the central lobe. A longer, left distributing vein supplied the other half of the central and the whole ventral lobe (Fig. 3). Each distributing vein branched and gave rise to portal veins called primary portal veins then entered the lobes. The ventral, central and dorsal lobes each received three primary portal veins. The caudate lobe received one which bifurcated within the lobe. Near the lobe edge each primary vein underwent repeated division to form terminal portal veins (Fig. 3).

Between terminal portal veins of adjacent primary portal veins occasional anastomoses were identified (Fig. 4).

Arising perpendicularly from primary and terminal portal veins were numerous veins termed secondary portal veins. Two types were identified. One was long and straight with frequent side branches; these veins were called long secondary portal veins (Figs. 1, 5 and 6). The other secondary veins were shorter and were either perpendicular or turned and ran parallel to a primary or terminal vein; these veins were termed short secondary portal veins (Figs. 1, 5 and 6). The long secondary veins were much more numerous than the short secondary veins. Both types of secondary veins gave rise to many small or tertiary portal vein branches which connected with the sinusoids (Figs. 5 and 6). Long secondary portal veins supplied sinusoids deep within the parenchyma while short secondary portal veins connected with sinusoids adjacent to primary portal veins (Figs. 1, 5 and 6). Tertiary portal veins traversed a narrow, clearly demarcated, empty zone (identified in histological sections as containing portal canal connective tissue and the limiting plate of hepatic cells - see later and Fig. 18) adjacent to the walls of long and short secondary portal veins before connecting with the sinusoids. Sinusoids, when filled with Tensol, formed conical or spherical masses which freely interconnected with sinusoidal masses supplied by tertiary veins arising from the same or adjacent secondary veins and with sinusoids entering the central hepatic veins (Fig. 7). Distributing portal veins gave rise only to long secondary portal veins.

Histological findings

Distributing portal veins were readily identified by their very large size and position next to the main bile duct just underneath the visceral surface of the central lobe (Fig. 8). Primary portal veins were smaller than distributing veins and were contained within the lobar masses (Fig. 9). One identifying feature of primary veins was that when they were sectioned transversely, secondary portal veins of a very much smaller calibre were found radiating from them (Fig. 9). While terminal portal veins also gave rise to secondary portal veins, they were readily identified by their small calibre and their location at the edge of lobes. Secondary portal veins were the veins encountered most frequently in portal canals in histological sections. They were very much smaller in diameter than primary and terminal portal veins and were the only portal veins to give rise to tertiary veins

(Fig. 10). Tertiary portal veins traversed the short distance between secondary portal veins and the sinusoids (Fig. 10).

The walls of distributing, primary, terminal and secondary veins were composed of connective tissue and muscle fibres. The distributing veins had the thickest walls, thereafter, the amounts of connective tissue and muscle fibres became progressively less with each division (Figs. 8 and 9). The walls of tertiary portal veins were composed of a single layer of endothelial cells, supported by portal canal connective tissue (Fig. 10).

Hepatic system

Tensol casts

Size, pattern of branching and presence or absence of sinusoidal connections permitted identification of three hepatic vein branches central, sublobular and hepatic. Central hepatic veins were the smallest veins and received most of the sinusoidal connections and then entered larger sublobular veins at the right angles (Fig. 11). Individual sinusoids were also found draining into the sublobular veins (Fig. 11). The sublobular veins joined to form large hepatic veins before entering the posterior vena cava (Fig. 12). Sublobular veins also connected with the posterior vena cava directly (Fig. 12).

Histological findings

Central hepatic veins were identified by their thin connective tissue wall of collagen, numerous sinusoidal connections (Fig. 13) and their position within the parenchyma equidistant from the portal canals. On the other hand, sublobular hepatic veins were larger in diameter, had thicker walls composed of collagen and smooth muscle, only a few sinusoidal connections (Fig. 14) and were irregularly distributed throughout the parenchyma. Hepatic veins were the largest vessels encountered. They had thick walls of collagen and smooth muscle and no sinusoidal connections.

Arterial system

Tensol casts

In the liver hilus the hepatic artery provided branches that paralleled the primary (Fig. 15), long and short secondary portal veins and supplied an extensive arterial network around the main bile duct and gall bladder. Side branches from arteries adjacent to portal veinsformed an elaborate arterial plexus around the whole intrahepatic

biliary tree (Fig. 16). Multiple, short side branches from this plexus and a lesser number of short branches arising directly from an artery connected with the para-portal sinusoids (Fig. 16). Extremely narrow arterial branches coursed over the surfaces of primary and secondary portal veins before discharging into these veins where a mixing of arterial and portal vein Tensol resulted (Fig. 17).

Histological findings

Branches of the hepatic artery were found in all portal canals with two or more arteries of differing calibre frequently being present. These vessels corresponded to typical muscular arteries and were readily distinguished from accompanying portal veins by their smaller calibre and thicker muscular walls (Figs. 8 and 9).

Biliary system

Tensol casts

While casts of the biliary system proved useful for demonstration of the peribiliary arterial plexus, they were of little value in identifying divisions of this system.

Histological findings

Diameter, morphological characteristics and location permitted identification of three types of ducts. Large bile ducts were found paralleling distributing and primary portal veins (Figs. 8 and 9) were lined by columnar cells with a brush border. The walls of the ducts next to the distributing portal veins were folded (Fig. 8) and glands were present in the sub-mucosa. Ducts adjacent to secondary portal veins were smaller and lined by cuboidal cells lacking a brush border. In many portal canals and also throughout the parenchyma were small bile ducts or ductules composed only of a few cuboidal cells.

Parenchyma

The sheets of liver cells forming the main hepatic mass were one cell thick and formed an anastomosing network enclosing lacunae lined by Kuppfer cells. A continuous layer of hepatic cells termed the hepatic limiting plate by Elias (1953) was present immediately surrounding all portal canals (Figs. 10 and 18), hepatic veins and underneath the capsule of Glisson. The hepatocytes of the limiting plate showed a range of morphological features. Some were similar to surroun-

ding hepatocytes, others had slightly more eosinophilic cytoplasm while others were smaller, irregular in outline and had deeply eosinophilic cytoplasm (Fig. 18); these latter cells were most strongly PAS positive. Isolated cells with similar morpholical features were scattered throughout the parenchyma. The limiting plate surrounding the portal canal was crossed by tertiary portal veins, reticular fibres and branches of the hepatic artery; these branches of the hepatic artery were visualised crossing the limiting plate only by the cast technique. The limiting plate surrounding central and sublobular veins was traversed by sinusoids and, in common with the capsule, reticular fibres. Reticular fibres lined the lacunae where they formed a fine, continuous network arranged radially between apposing portal canals and central veins (Fig. 19). The fibres interlinking adjacent portal canals were not so regularly arranged; these were shorter and appeared to be fragmented (Fig. 19).

In some portal canals, single or isolated groups of cells with morphological features in common with bile ductule epithelium and limiting plate hepatocytes were present. These were usually located between the bile duct or ductule and the portal canal limiting plate, frequently distorting the letters outline but never breaching it. These cells had an oval, oblong or kidney-shaped nucleus with a small amount of slightly eosinophilic cytoplasm. Similar cells were present in association with intraparenchymatous bile ductules.

The portal canals were lacking in inflammatory and immunological cell types. Small lymphocytes, plasma cells and macrophages were found only occasionally while mast cells were commonly present in the vicinity of the bile ducts.

Using gold chloride-Sudan IV staining, stellate cells were demonstrated in the ovine liver as cells with deep purple cytoplasm and fat globules (Fig. 20). They were distributed singly along the sides of the sinusoids or within hepatic cell plates where they gave the impression of spanning the liver plate.

DISCUSSION

The present study shows that a number of distinct divisions of the ovine portal system which conforms to the lobar pattern proposed by Ellenberger-Baum (1943) can be identified by size, location and sequence of branching. A new nomenclature based upon these findings is proposed in order that the branches of the portal system may be precise-

ly identified in normal and diseased states. The names distributing, primary, secondary, terminal and tertiary veins were chosen to identify the portal vein branches in histological sections. A further division of the secondary veins into long and short secondary veins was identified with Tensol casts.

Classifications of the intrahepatic portal system are available in other species but these are not applicable to the ovine liver. Mall (1906) studying the dog's liver, identified by measurement six portal vein branches but did not describe their distribution or morphological characteristics. Using the cast technique, Logan and De Ome (1949) enumerated at least ten divisions in the bovine portal vein in the caudate lobe without giving further detail. A classification which identified portal vein branches in human, rabbit, rat and dogs has been proposed by Elias and Sherrick (1969). These workers described conducting portal veins, axial distributing portal veins, marginal distributing portal veins, inlet venules and terminal twigs. While the portal vein system of these species had anatomical features in common with sheep, their proposed classification did not allow the identification of many of the ovine portal vein branches. In sheep, primary portal veins were readily distinguishable from distributing portal veins by their position and size whereas Elias and Sherrick (1969) did not make this distinction and referred to both veins as conducting portal veins. The nearest equivalent to axial distributing veins in sheep were the long secondary veins. Both types of veins gave rise to branches which connected with the sinusoids but the long secondary veins of the ovine liver were longer and more numerous. Short secondary and marginal distributing veins supplied similar sinusoidal regions, namely sinusoids adjacent to primary veins. The marginal distributing veins were described and illustrated as running parallel to a conducting portal vein within the portal canal containing the conducting vein. On the other hand, short secondary veins in the ovine liver were located outside the portal canal containing the primary portal vein. Tertiary and terminal portal veins were equivalent to inlet venules and terminal twigs. In this study terminal portal veins arose from primary portal veins and gave rise to long and short secondary portal veins, a situation not present apparently in human, dog, rat, or rabbit livers (Elias and Sherrick, 1969). The porto-portal anastomoses found between adjacent portal veins in the ovine liver have also been found in equivalent locations in human livers (Elias and Sherrick, 1969). Similarly, Logan and De Ome (1949) reported anastomoses between

their number 10 vessels in the bovine liver. One important species variation in the intrahepatic portal system is present in the rat liver (Gershbein and Elias, 1954) as marginal distributing veins (short secondary veins) are absent. Thus, for blood to reach the sinusoids adjacent to conducting veins (primary veins) it has to flow in via a circuitous route from axial distributing veins (long secondary veins).

In the ovine liver, blood drainage from the sinusoids was confined to the central and sublobular hepatic veins; this confirmed the observations of Wendelin (1972) in the sheep and was similar to that described in the rabbit (Sousa and Cruz, 1957). Drainage in the rat (Gershbein and Elias, 1954) and mouse (Lee, Elias and Davidson, 1958) is via sinusoidal connections to all levels of the hepatic vascular system including the posterior vena cava. By contrast, the central hepatic vein is the only receptor in human and dog livers (Elias, 1953; Elias and Popper, 1955).

The peribiliary arterial plexus demonstrated in this study by the casts was also recognised in sheep by Wendelin (1972) using the micro-angiographic technique. It has also been described in dogs, rabbits (Mall, 1906), cattle (Julian and De Ome, 1949) and humans (Elias, 1949; Elias and Petty, 1953). In the ovine liver, the para-portal sinusoids were filled mainly by arterial tensol from this plexus suggesting that this was the major source of arterial supply to the sinusoids. While direct connections between the hepatic artery and the para-portal sinusoids were found, their contribution to sinusoidal arterialisation was difficult to evaluate because of the profusion of sinusoids filled from the peribiliary arterial plexus. Wendelin (1972) concluded that the peribiliary arterial plexus was largely responsible for the sinusoidal arterial supply in the ovine liver. On the other hand, in man and in the dog, a direct supply via arteries arising from the hepatic artery and discharging into the sinusoids was considered to be the main source of sinusoidal arterialisation (Mall, 1906; Elias and Petty, 1953).

The very fine arterial branches coursing over and eventually discharging into the ovine portal veins clearly demonstrated by the casts were interpreted as being portal vein vasa vasorum. Rappaport and Knoblauch (1967) have stated that the portal and hepatic vein vasa vasorum are supplied by the hepatic artery.

The casts produced by injecting a blood vessel with Tensol, while providing a useful model for further anatomical study, do not

necessarily represent physiological routes of blood flow prevalent *in vivo*. Manual injection plus the relatively high viscosity of Tensol possibly resulted in vessels being filled in an indiscriminate pattern. Nevertheless, it was apparent from this study that blood could flow along a number of alternative pathways within the liver as is shown in Fig. 2. The calibre of the distributing and primary veins and the prolificacy of long secondary veins indicated that these vessels were probably the major route of blood flow to the sinusoids. The major sinusoidal drainage route was via the multiple sinusoidal connections with the central vein and from there to the sublobular and hepatic veins. Minor routes of supply and drainage of hepatic blood flow were provided by short secondary veins to the sinusoids and by sinusoidal-sublobular vein connections by-passing central veins. In the vicinity of the posterior vena cava, sublobular veins by-passed hepatic veins to enter the posterior vena cava directly and probably acted as minor routes of drainage. Exchange of blood between parallel routes of major status was possible because of the existence of porto-portal anastomosis and the freely anastomotic network of sinusoids (see Fig. 2).

The ovine biliary system appeared similar to that described for other mammalian species (Elias and Sherrick, 1969). It was, however, not possible to define branches of the biliary system using the cast technique and it is, therefore, proposed that the portal vein nomenclature adopted in this study should be used for the biliary system, e.g., a bile duct contained in a portal canal with a primary portal vein can be called a primary bile duct. Similarly, arteries adjacent to primary portal veins can be called primary arteries.

In the ovine liver the organisation of hepatocytes into freely anastomosing sheets one cell thick enclosing fine reticular fibres and sinusoids was typical of mammalian livers (Elias, 1949; Elias and Sherrick, 1969; Ham, 1969). Moreover, a limiting plate of hepatocytes was identified surrounding the portal canals, the hepatic veins and lining the capsule of Glisson. In the limiting plate of the ovine liver, hepatocytes with a shrunken irregular outline and deeply-staining cytoplasm were occasionally found. The limiting plate hepatocytes of man show similar distinguishing features (Elias, 1953) and the term "dark liver cells" has been proposed to describe them (Aterman, 1963). The nature of these cells is controversial. While their cytological features would suggest that they are in a state of degeneration, Gonote and Mosses (1968) considered that they were the result of fixation artefact, and Bhagwat, Ross and Currie (1972) could find no

electronmicroscopical features in these cells that could distinguish them from other hepatocytes. In man, the limiting plate is considered important because of its inter-position between the sinusoids and the portal canal connective tissue and because strong morphological evidence exists from studies of the embryo and of hepatic pathology that hepatocytes are capable of giving rise to bile ductule epithelium (Elias, 1967; Elias and Sherrick, 1969). The cells found in association with the limiting plate and bile ductules in this study with morphological features common to both possibly represented a transitional cell-type.

One important species variation seen in the parenchyma is in the number, thickness and arrangement of the fibres composing the reticular fibre network. By contrast with the adult pig (Ham, 1969), the fibres composing the reticular network in the sheep were much fewer and thinner and they were not arranged into septa interlinking adjacent portal canals to form a lobular pattern as they do in the adult pig. In fact, the reticular fibres between adjacent portal canals in the sheep were fewer and more fragmented than the remainder of the fibre network. Since Elias and Sherrick (1969) considered the reticular fibre network to play an integral supportive role in the liver, the areas of fragmented fibres in the sheep may indicate areas of relative structural weakness which may predispose these areas to pathological changes as occurs in the development of monolobular fibrosis in fascioliasis in sheep (Section II).

The affinity of specific cells within the ovine liver for gold chloride and fat stains indicated the presence of stellate cells. Such cells were described in the rabbit first by Von Kuppfer (1876) and later by Wake (1971). With the electron microscope Wake (1971) found that these stellate cells were located in the perisinusoidal space. Similarly, Gemmell and Heath (1971) described a cell containing fat globules, possibly a stellate cell, within the perisinusoidal space of the ovine liver. Wake (1971) showed in the rabbit that these cells store Vitamin A. Another potential function of these cells is that they are involved in hepatic fibrogenesis; following hepatic damage in the mouse these cells proliferate and hypertrophy and ultrastructurally assume a classical fibroblast appearance with concomitant deposition of collagen (McGee and Patrick, 1972).

While the morphology of the ovine liver was not markedly different from other mammalian livers, variations in the portal and hepatic vascular systems were demonstrable by the use of tensol casts

and histological sections. A knowledge of these differences and the availability of a classification which allows accurate identification of all the various branches of intrahepatic blood vessels is essential for the study of disease processes in the ovine liver particularly those involving vascular damage such as in fascioliasis (Dargie et al., 1974).

SUMMARY

Tensol casts of the portal, hepatic and arterial systems and histological sections were used in a study of normal ovine hepatic morphology. The morphology of the ovine liver was not markedly different from that of other mammalian species; differences were encountered in the portal and hepatic vasculature and perisinusoidal reticular fibre structure. Based upon the location, sequence of branching and histological characteristics a nomenclature for the division of the portal vascular system was proposed. The names of distributing, primary, secondary (long and short), tertiary and terminal veins were adopted to identify all the portal vein branches. These names can also be applied to branches of the arterial and biliary systems. The same criteria allowed the identification of three hepatic vein branches, central, sublobular and hepatic; the central hepatic veins and to a much lesser extent the sublobular hepatic veins drained the sinusoids. Casts of the arterial system demonstrated an arterial blood supply to the biliary system and to the sinusoids and to the portal veins as vasa vasorum.

The ovine hepatic parenchyma was composed of anastomosing sheets of hepatocytes one cell thick, a fine reticular fibre network and a continuous limiting plate of hepatocytes surrounding portal canals, hepatic cells and lining the capsule of Glisson. Stellate cells were identified.

Fig. 1. Diagram of intrahepatic portal vein branching.

DPV : distributing portal vein; IP : primary portal vein; L2P : long secondary portal vein; S2P : short secondary portal vein; 3P : tertiary portal vein; S : sinusoids; TPV : terminal portal vein; LP : limiting plate.

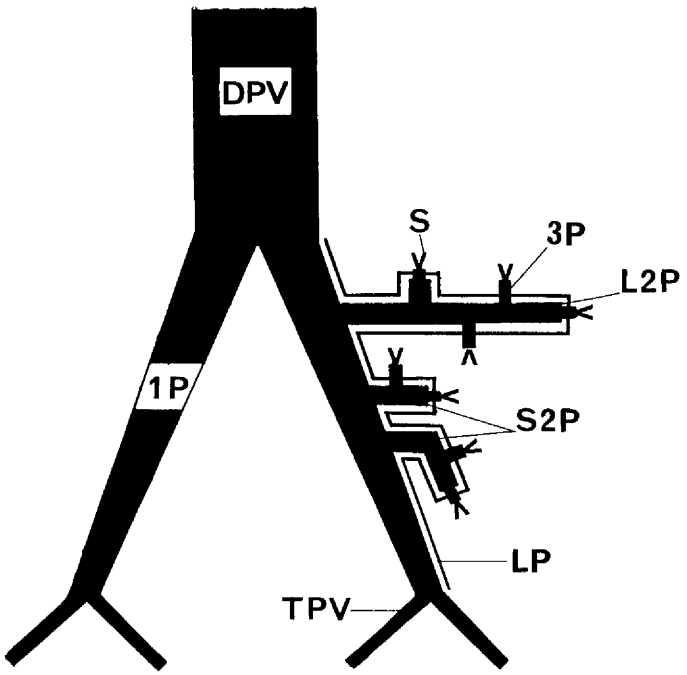
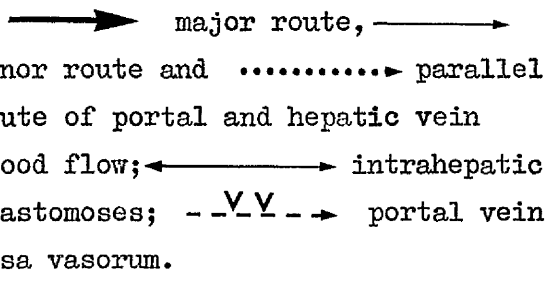

DPV
S
3P
L2P
1P
S2P
LP
TPV

Fig. 2. Routes of intrahepatic blood flow:



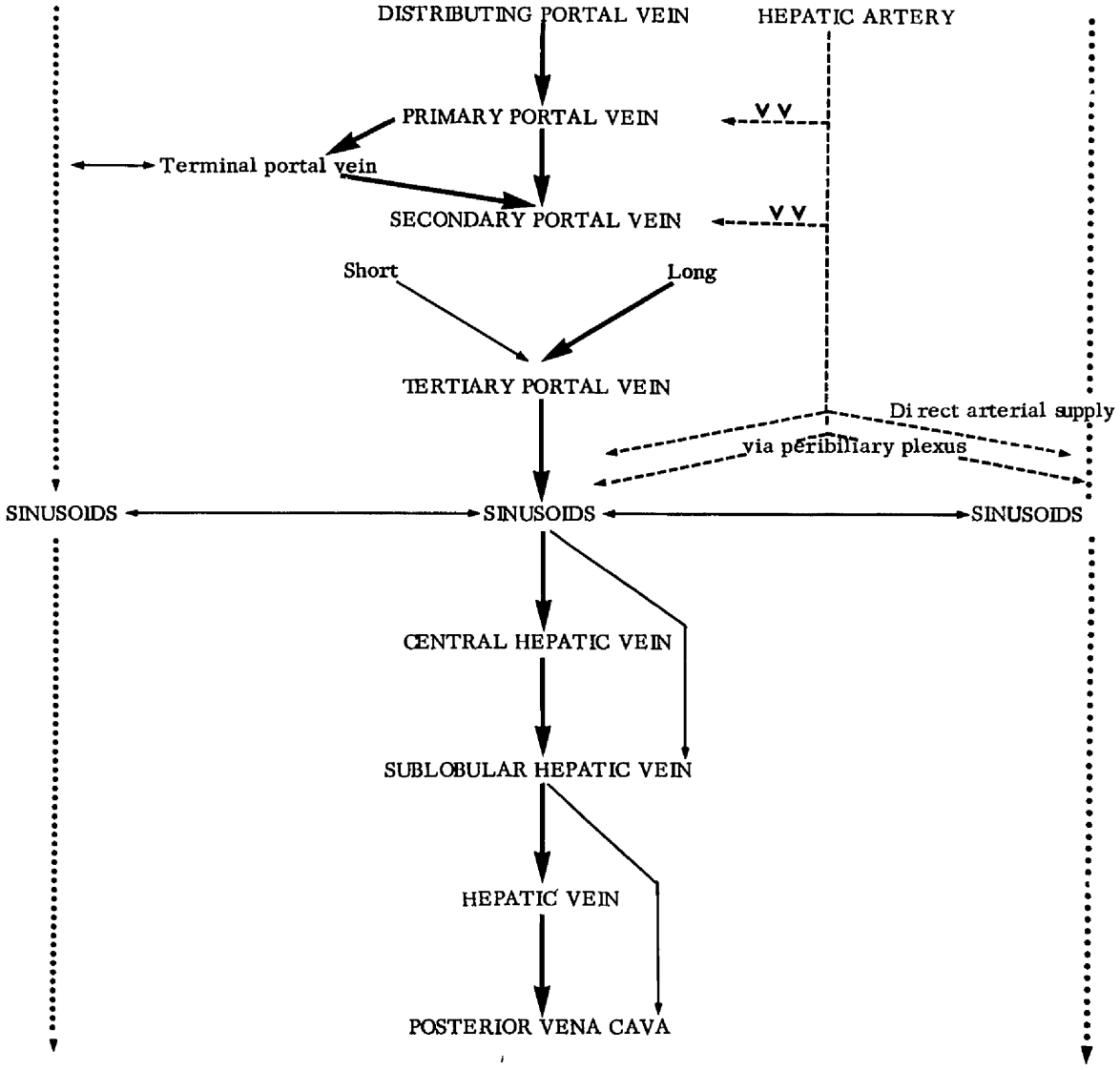
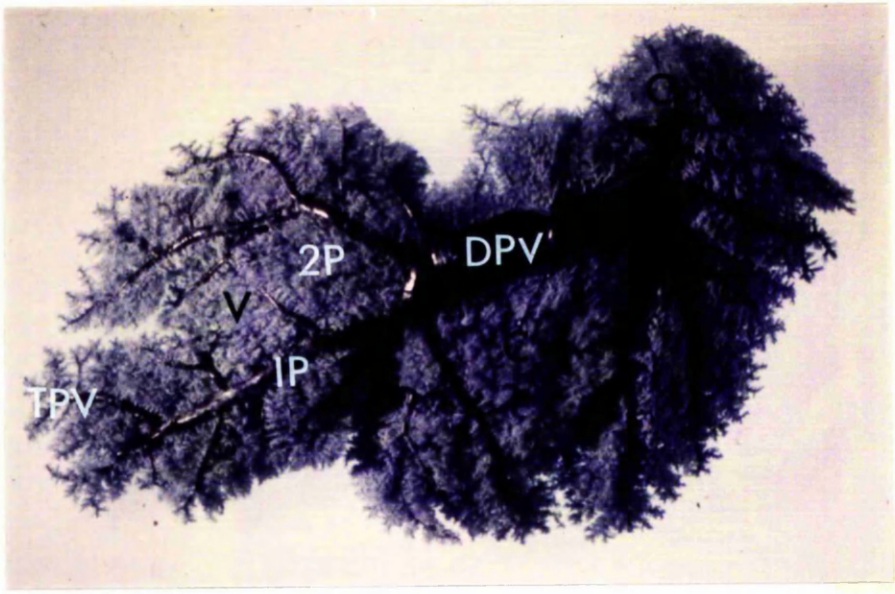

DISTRIBUTING PORTAL VEIN
HEPATIC ARTERY
PRIMARY PORTAL VEIN
Terminal portal vein
SECONDARY PORTAL VEIN
Short
Long
TERTIARY PORTAL VEIN
Direct arterial supply
via peribiliary plexus
SINUSOIDS
SINUSOIDS
SINUSOIDS
CENTRAL HEPATIC VEIN
SUBLOBULAR HEPATIC VEIN
HEPATIC VEIN
POSTERIOR VENA CAVA

Fig. 3. Cast of the portal system showing the vascular distribution to the ventral (V), central (C), dorsal (D) and caudate (Ca) lobes. The distributing (DPV), primary (1P), secondary (2P) and terminal (TPV) portal veins can be identified.

Fig. 4. Cast of a porto-portal anastomoses (⟶) between adjacent terminal portal veins (TPV).


2P
DPV
V
IP
TPV

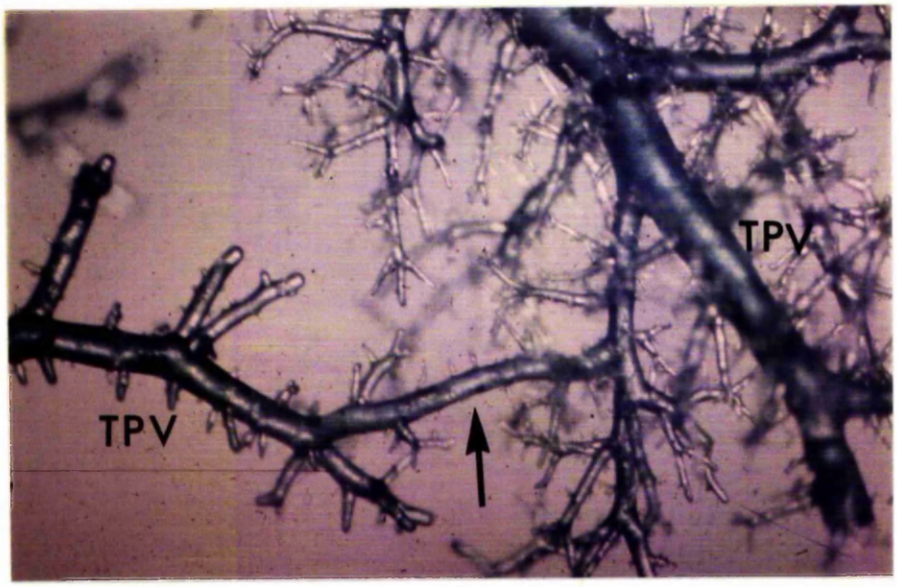
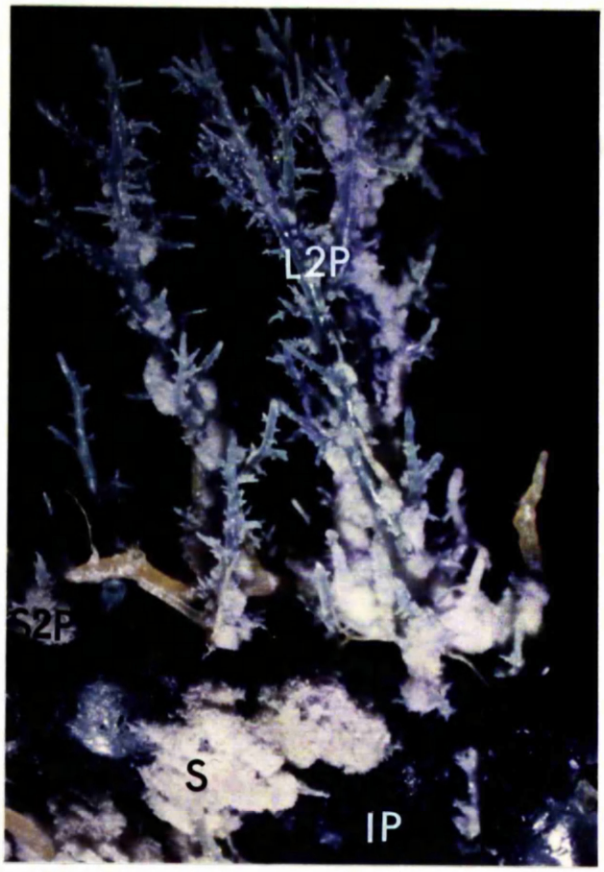

TPV
TPV

Fig. 5. Cast of the portal system showing primary (1P), long (L2P) and short (S2P) secondary portal veins and sinusoids (S).

Fig. 6. Cast of the portal system showing primary (1P), long (L2P) and short (S2P) secondary and tertiary (3P) portal veins and sinusoids (S). Note the short secondary portal vein (⟶) running parallel to the primary portal vein and the space (X) that in histological sections is filled by the limiting plate and portal canal connective tissue.


L2P
S2P
S
IP

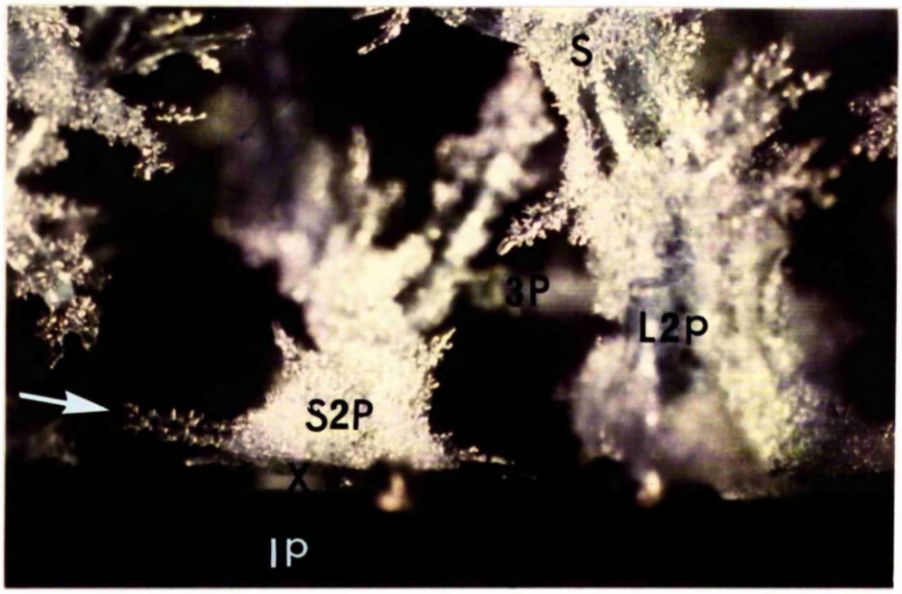

S
3P
L2P
S2P
X
IP

Fig. 7. Cast showing sinusoids (blue) perfused from the portal veins freely interconnecting with sinusoids (red) perfused from the hepatic veins.

Fig. 8. Distributing portal vein (DPV) located in the hilus of the central lobe adjacent to the distributing bile duct (D) and arteries (A).

HE x 12

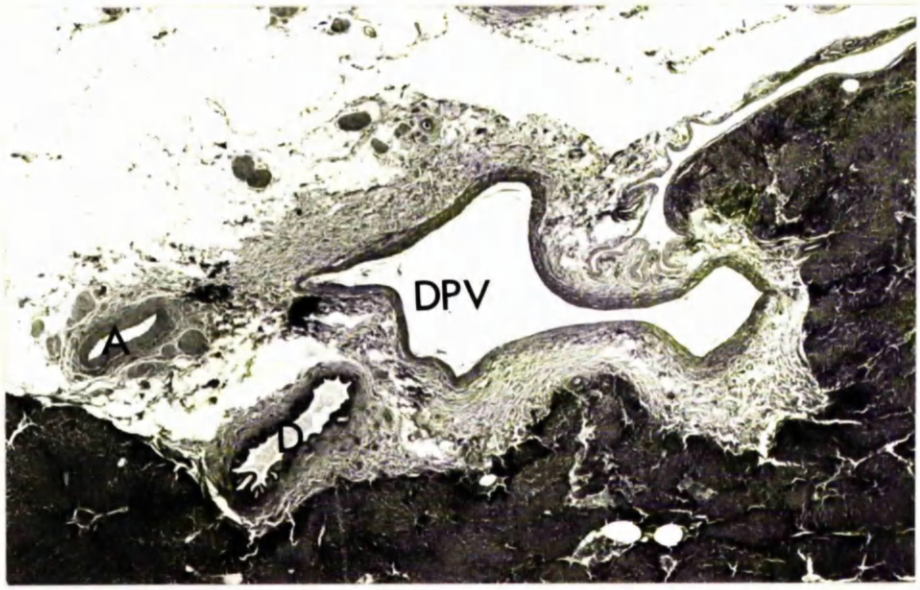
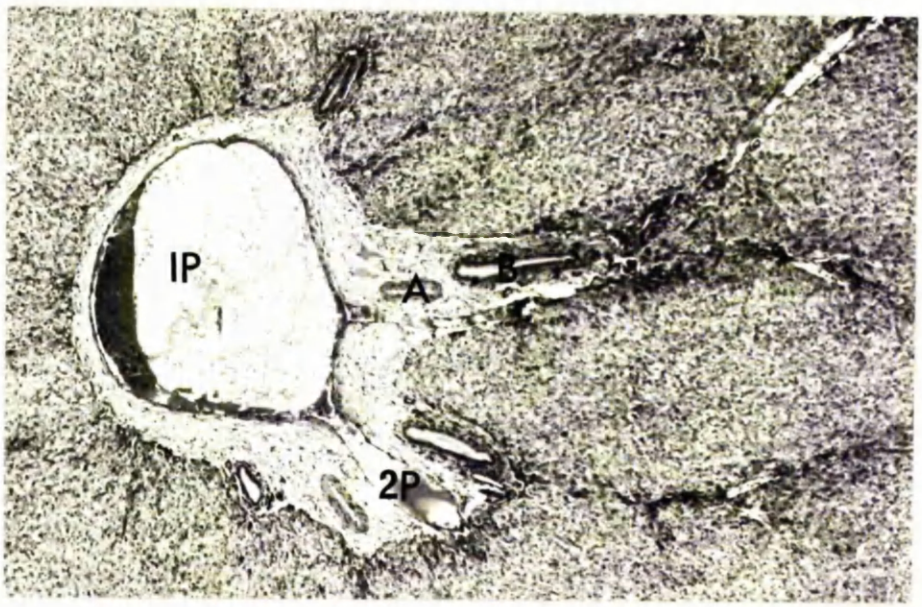

DPV
A
D

Fig. 9. A primary portal vein (1P) located in the lobar mass giving rise to secondary portal veins (2P). Primary bile ducts (B) and arteries (A) are also present.

HE x 35

Fig. 10. A portal canal containing a secondary (2P) and tertiary (3P) portal vein and bile ducts (B). The tertiary portal vein is traversing the limiting plate (arrows) and connecting with the sinusoids (S).

HE x 110


IP
A
2P

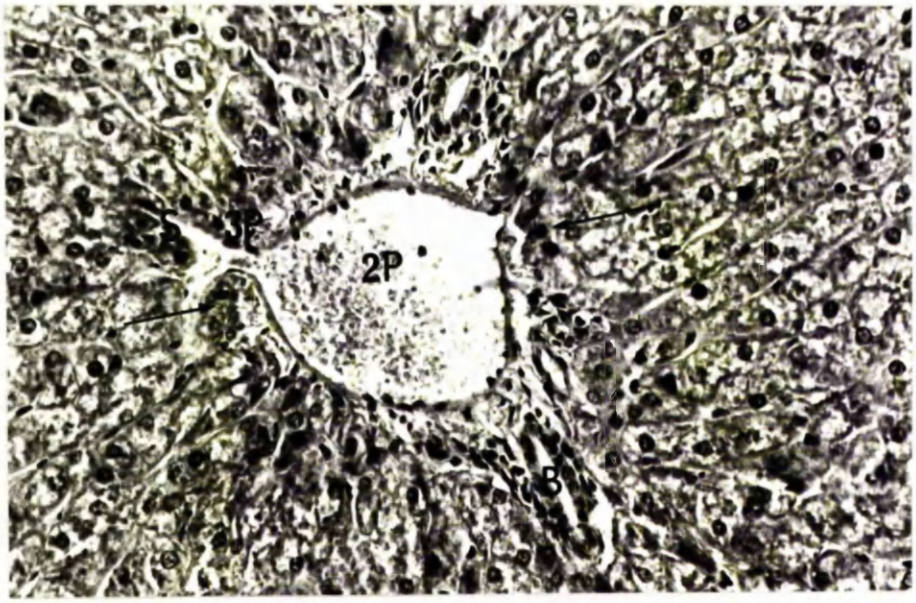
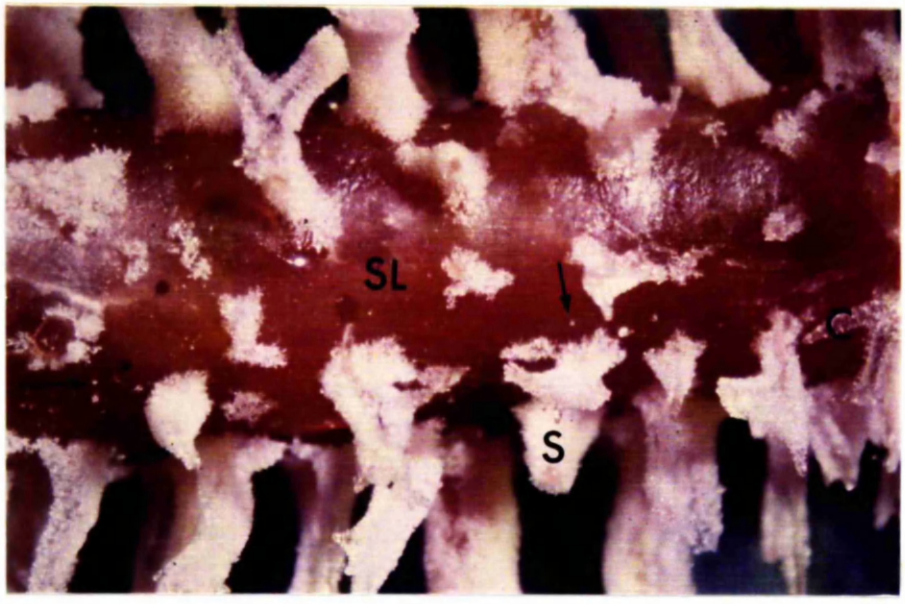

2P
B

Fig. 11. Cast of the hepatic system showing many sinusoids (S) connecting with central veins (C) and a few (arrows) with sublobular veins (SL).

Fig. 12. Cast of the hepatic system showing hepatic veins (H) and sublobular veins (SL) connecting with the posterior vena cava (PVC).


SL
S

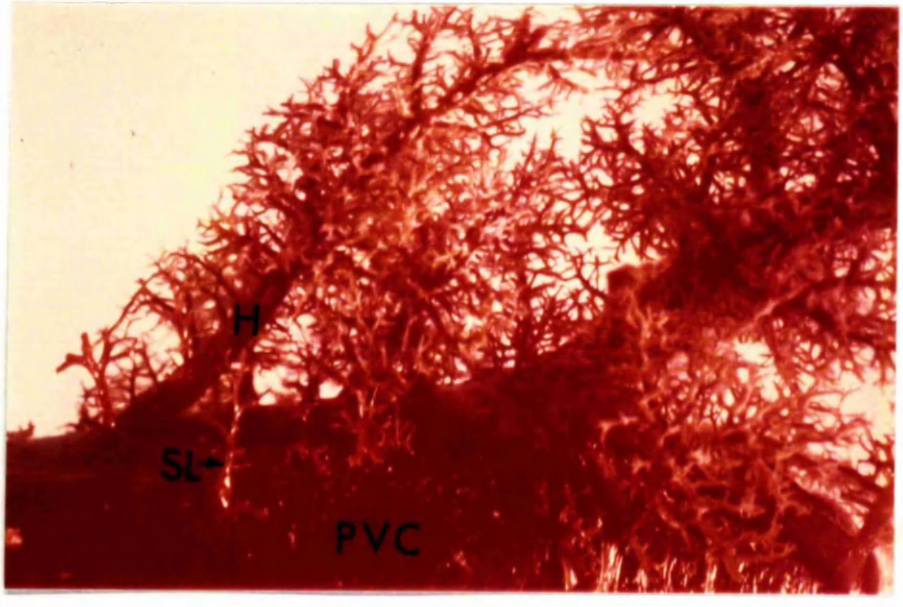
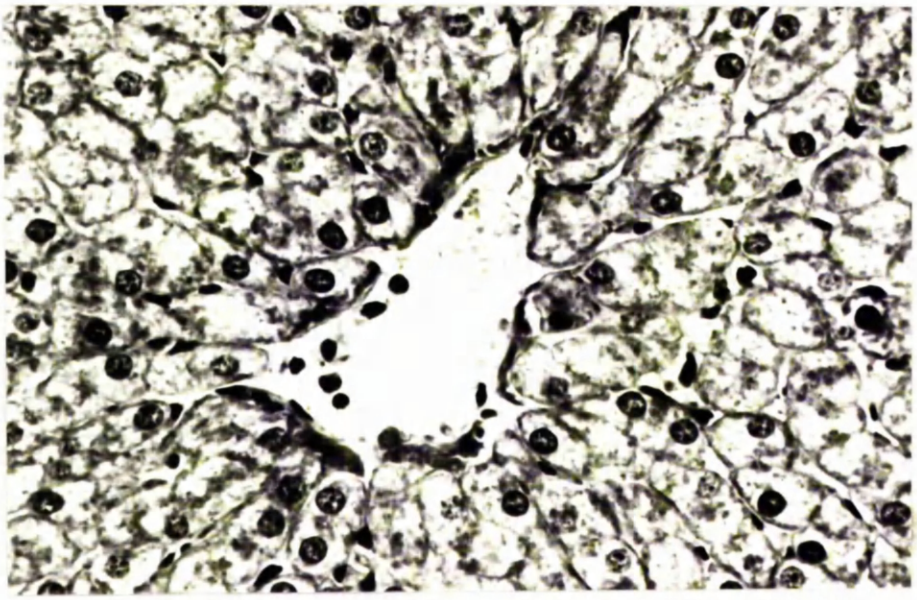

H
SL
PVC

Fig. 13. A thin-walled central vein with many sinusoidal inlets.

HE x 110

Fig. 14. A thicker-walled sublobular vein with a few sinusoidal inlets.

MSB x 40



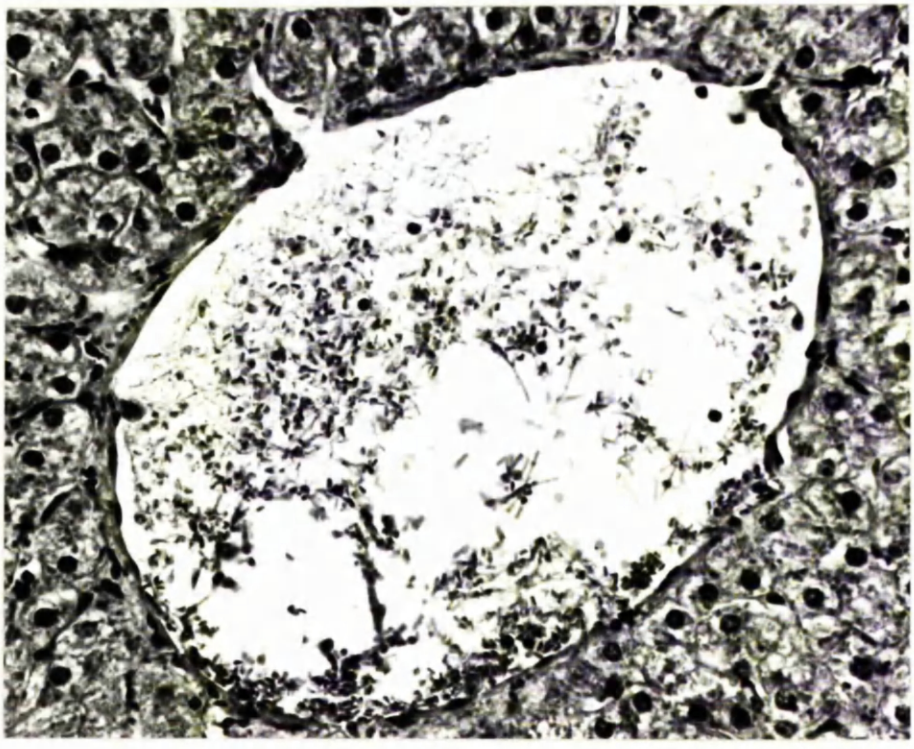


Fig. 15. Cast of an artery (A) paralleling a primary portal vein (1P). Numerous secondary portal veins (2P) surround the primary portal vein.

Fig. 16. Cast of the arterial plexus (yellow) surrounding a bile duct (red) and connecting with the sinusoids (⟶). Sinusoids are also connecting directly with the hepatic artery (⟶). Sinusoids filled with blue Tensol from the portal vein are in the background.


A
1P
2P

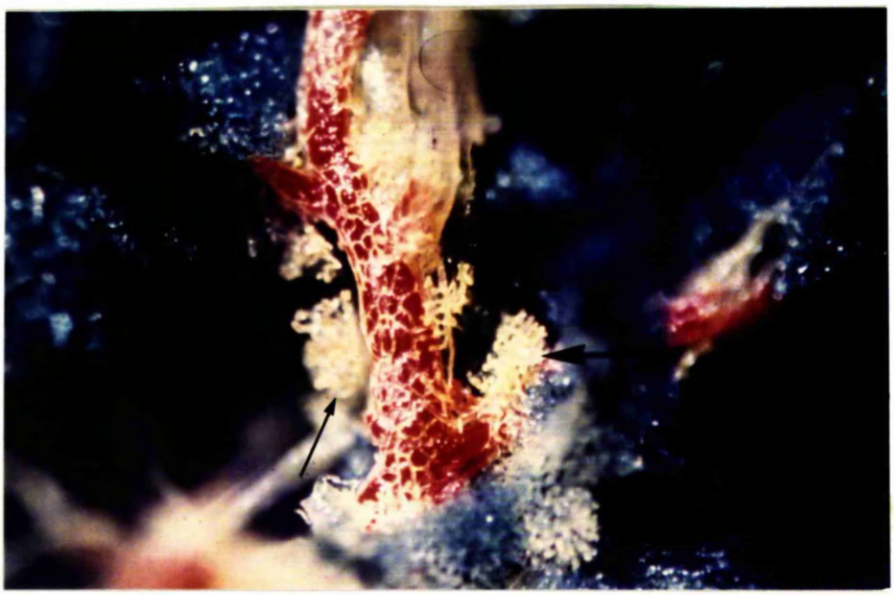


Fig. 17. Cast of the arterial system showing vasa vasorum arteries (arrows) coursing over the surface of a secondary portal vein (2P) and then joining with the secondary portal vein where mixing of the yellow arterial and blue portal vein Tensol has occurred.

Fig. 18. Portal canal containing a secondary portal vein surrounded by a limiting plate composed of cells with eosinophilic cytoplasm and shrunken nuclei (arrows).

HE x 110


2P

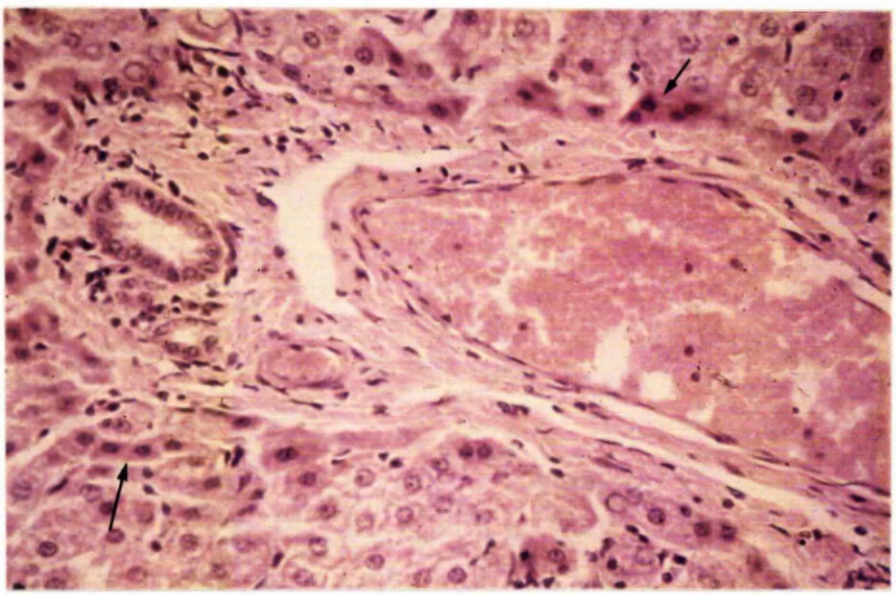
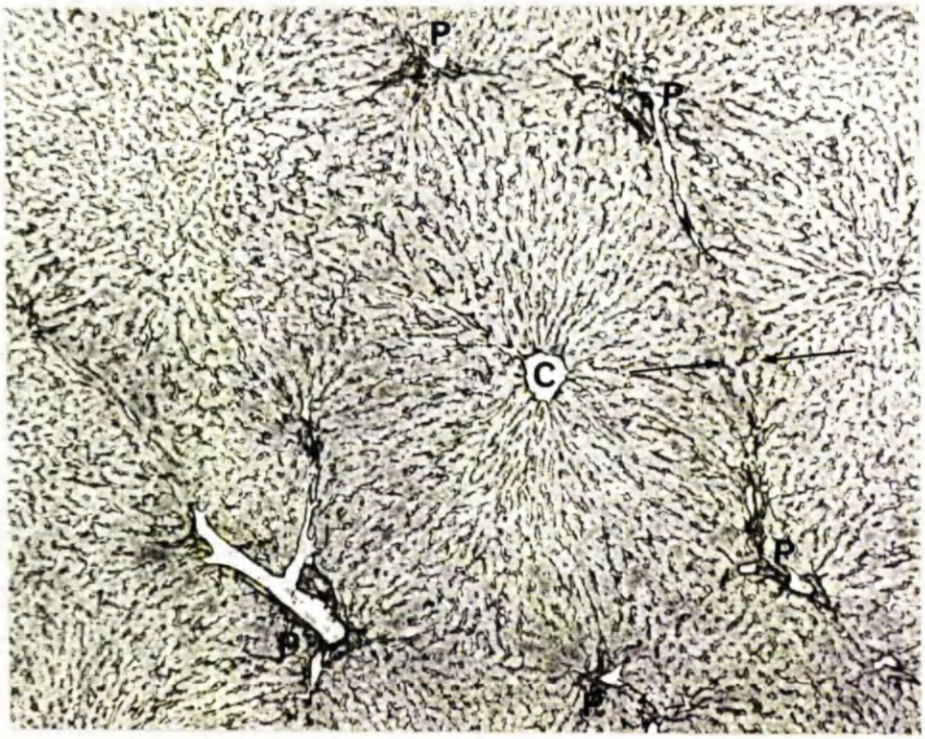


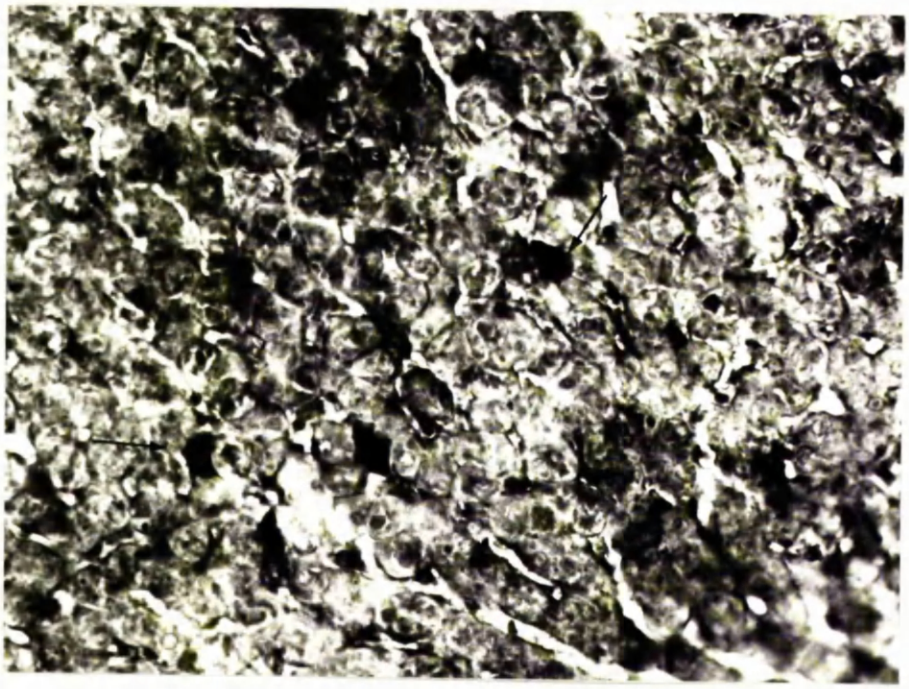
Fig. 19. Reticular fibres between opposing portal canals (P) and central vein (C) are arranged radially while the fibres between adjacent portal canals are shorter and fragmented (——→ ←——).

Gordon and Sweet x 35

Fig. 20. Stellate cells (arrows) containing large intracytoplasmic fat globules.

Gold-chloride Sudan IV x 250


P
P
C
P
P



SECTION II

PATHOLOGY OF AN EXPERIMENTAL PRIMARY *F. HEPATICA* INFECTION IN SHEEP.

INTRODUCTION

Fascioliasis is still one of the major causes of financial loss to the farming community. Despite this the hepatic changes produced in the experimentally induced disease in sheep have been described only once (Dow et al., 1968). Although this study recorded many of the lesions developing in ovine fascioliasis a reassessment was considered necessary to establish the range of lesions produced, their distributions, causes and consequences, and to provide a basis for comparison with reinfection lesions.

MATERIALS AND METHODS

Experimental design

Thirteen sheep were inoculated orally with 400 F. hepatica metacercariae. One sheep was to have been slaughtered at weeks 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30 and 42 and one sheep was to have been kept to replace any sheep that died. In the event, two sheep died from urolithiasis, one at week 10 and the sheep that was to have been slaughtered at week 42, at week 32. At necropsy, post-mortem degeneration was too advanced in both of these sheep to allow material to be collected.

Parasitological procedures

These were as described in the general materials and methods.

Pathological procedures

These were as described in the general materials and methods.

Biochemical estimations

All sheep were bled at weekly intervals from 2 weeks after infection to week 10, then at weeks 12, 16, 20 and 24. Serum samples were tested for their SGOT activity as described in the general material and methods.

RESULTS

Parasitology

The total number of flukes recovered from each sheep, their mean lengths, S.E's, ranges and percentage recoveries are given in Table 1. The total number of flukes recovered varied from a minimum of 86 at week 6 to a maximum of 197 at week 30 representing a range of percentage infectivity from 21 to 49%. During the bile duct stage of infection (weeks 12 to 30) the percentage recovery was between 33% and 49% suggesting that the lower recoveries at weeks 6 and 8 were due to the flukes being too small to recover.

The mean length of the flukes increased from 4.5 $\pm$ 0.2 mm at week 6 to 21.3 $\pm$ 0.3 mm at week 20. This increase in length took place primarily between weeks 6 and 12 with a particularly rapid increase occurring between weeks 10 and 12 when the flukes nearly doubled their length (9.4 $\pm$ 0.2 mm to 18.4 $\pm$ 0.3 mm). After week 12, the mean lengths remained relatively constant.

Pathology

Based upon the migratory pattern of the flukes, the hepatic lesions that developed were considered as occurring in two phases; the first phase covered weeks 1 to 12 when the flukes were migrating through the parenchyma and the second from week 12 when the flukes were established in the bile ducts.

Phase 1 - Migratory phase

Gross findings

This phase was characterised by the formation and progressive increase in size, length and tortuosity of fluke tracts. In and around these tracts, necrosis and haemorrhage developed followed by fibrosis.

As early as one week after infection, 1 mm in diameter cream-white foci were found sparsely distributed over the visceral and parietal surfaces of the liver (Fig. 21). Following this, tortuous and linear tracts up to 1 cm in length developed and by week 4 had expanded into a maze of tracts protruding superficially and extending deep into the parenchyma (Figs. 22 and 23). While tracts were found in the posterior areas of the central, dorsal and the whole of the caudate lobe, the

TABLE 1.

F. HEPATICA BURDENS OF SHEEP

INOCULATED ORALLY WITH 400 METACERCARIAE

Sheep Number	Weeks after Inoculation	Total Number Recovered	Percentage Recovery	Mean Length in mm	SE	Range in mm
G96	6	86	21	4.5	0.2	2-8
G92	8	87	21	8.7	0.2	5-13
G97	10	118	30	9.4	0.2	7-15
G90	12	133	33	18.4	0.3	10-23
G94	16	122	31	17.8	0.3	12-30
G89	20	139	35	21.3	0.3	12-30
G93	24	145	38	19.4	0.3	11-25
G100	30	197	49	17.7	0.2	10-25

majority were located ventral to the falciform ligament (Fig. 22). The tracts consisted initially of a central yellow-coloured zone of necrosis with or without haemorrhage (Fig. 23). Between weeks 6 and 12 larger necrotic and haemorrhagic tracts continued to be formed but in decreasing numbers; such tracts were extremely large and haemorrhagic and relatively evenly distributed throughout all lobes (Figs. 24 and 25). Associated with the above tract changes were many small white fibrino-fibrous tags scattered over the visceral and parietal surfaces of the liver with a preponderance over the ventral lobe. In addition, attached to the capsule were 1 cm diameter, raised, irregular, green-brown lesions suspected initially as being dead flukes (Fig. 26) but subsequently shown histologically to be organising haematoma

From week 6 and onwards organisation of the tracts was recognised as taking place by the white zone of collagen deposited around the tracts. Accompanying tract organisation, the ventral lobe became progressively reduced in size and all the lobes developed deep irregular surface fissuring (Fig. 25).

Histological findings

During this phase, the major lesions were located within or in the close vicinity of fluke tracts. The tract-system seen grossly consisted when first formed of a central zone of liquifactive necrosis containing cellular debris, fibrin and red blood cells. Surrounding the tract centre was an area of coagulative necrosis containing numerous microthrombi in intact sinusoids (Fig. 27). At week 1 the tracts were small in diameter and extended for short distances down from the capsular surface. In subsequent weeks the tracts became larger and extremely tortuous involving most of the ventral lobe and irregularly distributed areas in the other lobes. Once formed the tract centres become packed with viable, dead and polymorphonuclear leukocytes and eosinophils and red blood corpuscles (RBC's). While haemorrhage into fluke tracts was evident at all stages, it was most common and severe in the larger fluke tracts which were encountered in the later migratory stages, i.e., between 6 and 12 weeks after infection.

Between weeks 2 and 4 after infection, organisation and healing of the fluke tracts commenced; the area surrounding the necrotic tracts became infiltrated with macrophages and multinucleated giant cells, fibroblasts, lymphocytes and plasma cells. The macrophages and giant cells formed a palisade layer of actively phagocytosing

cells around the tract centres while the fibroblasts were located outside the macrophages and were involved with the deposition of strands of collagen (post-necrotic fibrosis) (Fig. 28). As organisation progressed, the tracts became smaller with granular eosinophilic centres surrounded by a broad band of relatively acellular collagen. After week 12 many tracts were reduced to broad irregular scars often enclosing haemosiderin-laden macrophages (Fig. 29).

Developing in association with tract healing were multiple septa of collagen of variable length and width (Fig. 29). They interlinked areas of post-necrotic fibrosis in a very irregular pattern to adjacent portal canals, to hepatic veins and to the capsule of Glisson, i.e., joining with the basic hepatic fibrous tissue framework. Contained within the septa were eosinophils, lymphocytes, haemosiderin-laden macrophages and later in the infection lympho-reticular nodules. In addition, the septa were often associated with proliferating bile ductules and sometimes contained narrow vascular channels (see Fig. 52).

Where fluke tracts traversed hepatic veins, portal veins and hepatic arteries, a traumatic phlebitis developed in the veins and nacrosis in the arteries. The vein walls were infiltrated by a mixed cell population consisting of polymorphonuclear leukocytes, plasma cells, lymphocytes and eosinophils. The hepatic veins were by far the most frequently affected and only in these veins were mural and occlusive thrombi seen to develop (Fig. 30). Although evidence of infarction was lacking (see Section IV), quite large variable-sized foci of hepatocytes undergoing coagulative probably ischaemic necrosis were often seen in the vicinity of acute tracts and thrombosed hepatic veins.

Paralleling the production of fluke tracts, a series of changes occurred in the portal canals adjacent to the tracts that were similar at all stages of infection apart from week 1. At this time the portal canals were infiltrated mainly by large actively-dividing pyraninophilic lymphoid cells with only an occasional polymorphonuclear leukocyte, eosinophil, plasma cell and macrophage (Fig. 31). As a result of this influx of cells the portal canals were enlarged and the surrounding limiting plate of hepatocytes was disrupted (Fig. 32). At the edge of the cellular infiltrate hepatocytes were found either undergoing degeneration or becoming segregated from the parenchymal mass by the infiltrate. This cellular infiltrate was also found subendothelially and within the walls of terminal and secondary portal

veins in affected portal canals (Fig. 32). As a result the walls were increased in thickness and the endothelium was displaced into the vein lumen. Thin-walled tertiary portal veins arising from infiltrated secondary portal veins were occluded by cells located subendothelially and compressed by cells within the surrounding portal canal (Fig. 32).

At all other stages of infection, portal canals adjacent to acute fluke tracts became progressively infiltrated and distended by eosinophils and markedly oedematous with distended lymphatics. The cellular infiltrate continued to advance into the parenchyma accompanied by hepatocyte degeneration (Fig. 33) and segregation of clumps of hepatocytes. In addition in these areas, hepatocytes were seen apparently transforming into bile ductules (Fig. 34), which, once formed, became surrounded by connective tissue. By week 8, bile ductules were extremely numerous, completely enveloping most portal canals. They also invested healing tracts and associated fibrous septa. After week 8, the number of bile ductules became less frequent.

Portal veins in the above portal canals were oedematous and infiltrated by predominately eosinophils (Fig. 35). Eosinophils and oedema were located intramurally and subendothelially with the result that the veins had thickened walls and their endothelium was thrown into numerous folds producing varying degrees of lumenal occlusion (Fig. 35). The smaller calibre terminal and secondary portal veins closest to the tracts were the most severely affected with many being totally occluded by endothelial folding. Ventral lobe primary veins were also involved but to a lesser degree and often only in a segmental pattern so occlusion was usually minimal. Tertiary portal veins traversing the expanded portal canals continued to be occluded and compressed by subendothelially-located cells and by portal canal reactions.

With the onset of tract organisation, the cellular composition of the portal canals changed whereby eosinophils declined in number while mononuclear cell-types including lymphocytes and plasma cells became more prominent. Latterly small lympho-reticular nodules were formed. Accompanying these cellular changes was a reduction in the amount of oedema although portal canal lymphatics continued to be distended. Similar changes were encountered in the associated portal vein wall.

Although collagen deposition was obvious around fluke tracts and in the vicinity of proliferating bile ductules as early as week 2,

it was not until week 6 that the collagen started to be deposited in portal canals to any degree. At the same time there was subendothelial and intramural deposition of collagen in the portal veins. As a result of collagen deposition, varying degrees of portal vein occlusion developed. Sporadic terminal and secondary portal veins, particularly in the ventral lobes, displayed the most severe degrees of lumenal occlusion; in some hardly any lumen remained (Fig. 36). Ventral lobe primary portal veins were fibrosed (Fig. 37) and as a consequence their lumenal diameters were reduced but never to a marked degree. Tertiary portal veins, besides having to traverse portal canals that were increased in diameter, were compressed and constricted by portal canal reactions and fibrosis (Fig. 35). Where acute tracts in the later stages of the migratory phase had passed close to fibrosed portal veins, the walls of these veins were reinfiltrated by eosinophils and oedema. As a result the thickness of the vein walls was increased and their lumenal diameters reduced further (Fig. 38).

From week 8 and onwards, notable changes occurred in the arterial system. Primary and secondary arteries in the ventral lobe became dilated and tortuous and a few primary arteries had thickened hyperplastic walls (Fig. 39). Arteritis was not seen, only areas of necrosis where an arterial wall had been damaged by fluke migration as stated earlier.

Marked changes also occurred in the hepatic venous drainage system. As a result of organisation of the traumatic vasculitis and thrombosis described previously, collagen deposition occurred in the vessel wall and organised thrombi were found occasionally encroaching on the vessel lumen. Where central hepatic veins were involved, sinusoidal inlets were often occluded (Fig. 40). Also affecting the hepatic veins was a lesion which was segmental in distribution in that it occurred only in that part of the vessel wall in the close vicinity of and adjacent to fluke tracts. This lesion involved first cellular infiltration followed by the deposition of collagen. In common with the portal canal changes, the cellular infiltrated in these hepatic veins at week 1 was composed mainly of large lymphoid cells. However, by week 2, and at all stages thereafter, a mixed cell population of eosinophils, polymorphonuclear leukocytes, lymphocytes and plasma cells was present. Fibrosis of this lesion resulted in thickening of hepatic vein walls and where central hepatic veins were involved severence of sinusoidal connections. In addition, central hepatic veins next to organising fluke tracts were often involved with the tract organisation

process which resulted in thickened vein walls and severed sinusoidal connections.

Marked changes were found in the capsule of Glisson, particularly in association with fluke tracts and therefore mainly over the ventral lobe. The timing and nature of the lesions closely coincided with that found in the hepatic veins. Initially, close to fluke tracts there was a lymphoid cell infiltrate that was followed by the mixed cellular response. The capsule became oedematous and covered with numerous superficial fibrinous deposits that soon became organised resulting in irregular fibrous thickening. Haemosiderin-laden macrophages, evidence of previous haemorrhage, were found in such fibrous thickened areas. Over these areas the mesothelium of the capsule was hyperplastic and thrown into numerous folds.

Phase 2 - Post-migratory or biliary phase

Gross findings

During this phase parenchymal alterations continued to take place in addition to prominent changes in the biliary system. At week 12 the bile ducts were only slightly dilated but with persistence of the fluke population the ventral lobe ducts became progressively more dilated, varicosed and heavily fibrosed (Fig. 41). Adult flukes bathed in an evil-smelling fluid squirmed their way out of the cut ends of the fibrosed ducts. The larger portal canals containing the fibrosed bile ducts and the much smaller canals joining with them were also enlarged and fibrosed and formed a distinct reticulated pattern in the parenchyma (Fig. 42). Accompanying the bile duct changes were gradual alterations in the size, shape and degree of fibrosis of the lobes. The ventral lobes became shrunken, pale, diffusely fibrous and very irregular in outline while the central and dorsal lobes, particularly at week 20, were enlarged due to the presence of large rather indistinct nodules of regeneration. All lobar surfaces were deeply and irregularly fissured and covered with many small fibrous tags.

Histological findings

Throughout this phase of infection parenchymal, portal canal, including portal vein, hepatic vein and capsular changes, continued to take place but the major changes were confined to the biliary system.

Even although one fluke was sectioned in the biliary system as early as week 4, it was not until week 12 that large numbers became

established. During this interval, flukes were not found in the biliary system but there was evidence of infestation in the form of mild epithelial hyperplasia with the mucosa being thrown into folds, focal epithelial erosion and the presence of numerous but unevenly distributed globule leukocytes. From week 12 and onwards, mature flukes were located in distributing primary and a few terminal bile ducts. The changes present in these ducts varied according to the time of infection and upon whether flukes were present or absent. At week 12 the bile ducts containing flukes were distended, minimally fibrosed and lined by hyperplastic alternating with extensively ulcerated epithelium (Fig. 43). Within the portal canal connective tissue and lamina propria surrounding these ducts, particularly underneath the ulcerated areas, were numerous eosinophils and lesser numbers of plasma cells and lymphocytes. Ducts not containing flukes were lined by a hyperplastic and folded epithelium supported by a lamina propria containing a mixture of eosinophils, plasma cells and lymphocytes. Eventually the epithelium in these non-infected ducts became markedly hyperplastic and folded, and often arranged into acinar-like structures some of which were cystic. This epithelium was composed of many mucin-secreting and columnar cells. With the persistence of the flukes (see Table 1), their continued feeding and migration, many ducts became very distended, heavily fibrosed and extensively ulcerated. Intact areas of epithelium in these ducts contained numerous globule leukocytes and their lamina propria was populated by predominantly plasma cells and lymphocytes. Ulceration in the later stages of infection frequently exposed the broad bands of collagen surrounding the ducts. Within this collagen were numerous small often tortuous and fibrosed peribiliary plexus arteries (Fig. 44 and see Fig. 64, Section III). A few heavily fibrosed ducts not containing flukes in the later stages of infection were lined by a single regenerative layer of columnar epithelium.

During this period of infection secondary bile ducts rarely harboured flukes but they frequently contained fluke eggs (Fig. 45), sporadic globule leukocytes, and were often surrounded by laminations of collagen. Very sparsely distributed throughout the collagen and lamina propria around these ducts were plasma cells and lymphocytes. Between weeks 24 and 30 prominent cellular reactions apparently related to the presence of fluke eggs developed in the secondary bile ducts, particularly in the ventral lobes. These reactions conformed to a recognisable sequence of events resulting in the destruction of the bile duct and development of a granuloma-like lesion (Fig. 46). The

first visible change in this sequence was the infiltration of the laminated peribiliary collagen by plasma cells, lymphocytes, eosinophils and oedema accompanied by a considerable increase in the numbers of globule leukocytes (Fig. 47). The next stage appeared to be disintegration of this epithelial lining followed by a continued accumulation of cells within and around the duct (Fig. 48). Eventually the duct was completely replaced by sheets of cells in which fluke eggs were frequently sectioned in a central position (Fig. 49). The cells surrounding the eggs and on occasions penetrating their shells consisted of eosinophils, clusters of macrophages and occasional multinucleated giant cells; outside this layer lymphocytes and plasma cells predominated. Thin strands of collagen were present around this granuloma-like reaction.

Within the parenchyma during the post-migratory period the few remaining partially healed tracts continued to organise accompanied by the formation of irregularly-orientated septa of collagen. Nearly the whole of the parenchyma in the ventral lobes and isolated areas in the other lobes were dominated by these changes. Also present in the parenchyma but to a very much lesser degree and only in specific areas were two types of fibrosis involving collagen deposition in a perisinusoidal location. Pericellular fibrosis consisting of a fine reticulated network of collagen (Fig. 50) was found radiating from healed fluke tracts, fibrosed portal canals, hepatic veins, the capsule of Glisson and groups of intra-parenchymatous bile ductules. This type of fibrosis was seen mainly in the ventral lobes. By contrast, monolobular fibrosis while identified at all times in the post-migratory period, was most prominent at week 20 in areas not associated apparently with fluke damage in the dorsal and to a lesser extent central lobes. As a result of this fibrosis, adjacent portal canals became interlinked by thin relatively acellular septa of collagen to form a distinct series of lobules (Fig. 51). Once formed the septa appeared to act as an impenetrable division between sinusoids on either side. Septa formation appeared to be preceded by a straightening, stretching and often degeneration of hepatocytes adjoining two portal canals followed by collagen deposition in a perisinusoidal location. Centrally-placed sinusoids appeared to be surrounded by the collagen to form vascular channels (Fig. 52). Similar changes took place in hepatic cell plates connecting portal canals and central veins in the ventral lobe but without collagen deposition so that a freely anastomotic system of sinusoids remained. In such areas the sinusoids were often dilated,

sometimes to the extent of having a varicosed appearance.

In portal canals, besides the biliary changes between weeks 12 to 30, many small lympho-reticular nodules developed and bile ductules continued to form but in lesser numbers. In addition, arterial hyperplasia and tortuosity were more prominent and collagen was progressively deposited within portal canals (portal fibrosis) (Fig. 45). Fibrosed terminal and secondary portal veins mainly in the ventral lobe became progressively more fibrosed during this period so that by week 30 venous-occlusion was more severe than at week 12. Collagen was also identified within hypertrophied arterial walls from week 20 and onwards.

As with the portal system in the later stages of infection, fibrosis of central, sublobular and hepatic veins appeared to be progressive with the result that many hepatic veins of all types developed thicker fibrous walls. In some instances, especially in the smaller central veins, fibrosis was severe and as a result the veins were markedly reduced in diameter and sinusoidal inlets were severed. This resulted in the surrounding sinusoids becoming distended with red blood corpuscles but only during weeks 12 to 20.

The capsule of Glisson during this phase of infection, apart from containing lesser numbers of haemosiderin-laden macrophages, lymphocytes, plasma cells and eosinophils, was as described for the pre-bile duct phase.

In the first phase of infection, areas of regeneration were identified confined mainly to the tip of the ventral lobes. Later they were seen most frequently underneath the capsule, next to the healing tracts in all lobes and forming islands of cells within septa and post-necrotic scars of the central and dorsal lobes. The hepatocytes involved had variable cytoplasmic and nuclear sizes and formed groups bunched together without intervening sinusoids (Fig. 53).

Biochemical results

The individual SGOT levels for the sheep are recorded in Table 2 together with the weekly mean values and S.E's. An increase in mean value occurred from less than 100 S-F units at week 2 to a maximum of 237 $\pm$ 31.8 S-F units at week 9 followed by a decline to less than 100 S-F units at week 20.

TABLE 2.

SERUM GLUTAMIC OXALOACETIC TRANSAMINASE (SGOT)
LEVELS OF SHEEP INOCULATED ORALLY
WITH 400 F. HEPATICA METACERCARIAE.

Sheep Number	Weeks after Inoculation												
	2	3	4	5	6	7	8	9	10	12	16	20	24
G89	66	80	87	94	87	95	156	240	241	148	88	76	-
G90	78	95	116	119	82	84	109	133	101	58	-	-	-
G91	126	175	179	169	135	141	184	340	Died				
G92	78	109	141	132	118	139	206	-	-	-	-	-	-
G93	76	108	104	94	83	81	169	210	163	95	103	94	80
G94	88	138	209	196	134	133	246	295	300	161	178	-	-
G95	104	149	270	307	280	297	296	362	201	131	83	90	92
G96	76	109	114	128	111	-	-	-	-	-	-	-	-
G97	97	158	191	186	148	118	108	117	103	-	-	-	-
G98	86	142	190	-	-	-	-	-	-	-	-	-	-
G99	79	-	-	-	-	-	-	-	-	-	-	-	-
G100	60	113	137	148	144	167	154	202	131	107	137	105	84
Mean	84.5	125	158	157	132	139	180	237	177	117	118	91	85
SE	5.1	8.8	16.5	19.9	18.2	21.9	20.4	31.8	28.2	15.4	17.8	5.9	3.5

DISCUSSION

Following inoculation, the number of flukes established and their migratory progress through the liver as judged by the percentage recovery of flukes and the SGOT levels were in agreement with previous results on artificial primary F. hepatica infections in sheep (Dow et al., 1968; Boray, 1969). The consequences of this infection was the production of necrosis, haemorrhage, cholangitis and a wide range of parenchymal, vascular and capsular lesions. The outcome of infection was hepatic fibrosis and a chronic hyperplastic cholangitis. Many of these lesions have been recorded in cattle, sheep, pigs and laboratory animals infected with F. hepatica (Morrill and Shaw, 1942; Urquhart, 1956; Thorpe, 1965a; Ross et al., 1967a; Dow et al., 1967, 1968; Rakho, 1969, 1972; Doyle, 1972). In view of this, this discussion will be confined to an assessment of the causes and mechanisms involved in the induction of acute liver damage and hepatic fibrosis in addition to an appraisal of lesions seen in this study but not described previously. Except where relevant, the vascular lesions will be discussed in Section III.

Developing as a result of fluke migration and feeding were hepatic necrosis, haemorrhage, inflammatory and necrotising vascular lesions and a cholangitis. Whilst these lesions can be ascribed to trauma, other factors may have been operative, especially during weeks 10 to 12 when extremely large haemorrhagic tracts were formed. Since it was during this period that the flukes nearly doubled their length, thus indicating avid feeding and rapid growth, most of the haemorrhage can be ascribed to trauma. However, cuticular excretion of metabolic products (Dawes, 1961; Thorpe, 1965a) and immunological mechanisms may also have been contributory factors. The latter because large haemorrhagic tracts have been seen in splenectomised and immunosuppressed sheep (Sinclair, 1968, 1970), in rabbits given anti-lymphocytic serum (Dodd and Nuillain, 1969) and in treated and reinfected sheep (Section IV).

In addition, in primary infections in mice (Dawes, 1963) and sheep (Dow et al., 1968) haemorrhagic tracts larger than accountable for by direct trauma from the flukes were seen prior to entry of the flukes to the bile ducts. Dawes (1963) attributed these tracts to the prolonged migration of larger flukes.

Additional contributory causes of cell degeneration in and

around the fluke tracts must have been ischaemia, induced secondary to microthrombosis of the sinusoids. Whilst areas of infarction similar to those seen frequently in infected rabbits (Urquhart, 1956) and rats (Thorpe, 1965a) and in Section IV were not observed, the smaller areas of coagulative necrosis near acute tracts and thrombosed hepatic veins were probably due to ischaemia. One explanation for the difference in incidence of infarction between laboratory animals and sheep is that for ischaemic necrosis to occur in the liver both the arterial and portal vein flow have to be impaired (Robbins, 1967). This event is more likely to occur following simultaneous thrombosis of the hepatic artery and portal vein in the infected smaller laboratory animals' liver than in the larger sheep liver. Furthermore, in the rat, the absence of marginal distributing portal veins (Gershbein and Elias, 1953) (termed short secondary portal veins in the sheep - see Section I) means that portal blood has to flow in via a circuitous route from adjacent axial distributing veins (long secondary portal veins). As a consequence the areas of parenchyma next to the conducting veins were considered to be susceptible to infarction (Gershbein and Elias, 1954).

In addition to the above changes, hepatocytes located singly and in small groups around portal canals, healing fluke tracts and free within the parenchyma were also seen to be degenerate. These cells were similar in location and morphology to degenerate hepatocytes present in normal sheep livers where they were found in lesser numbers (Section I). Their increased numbers in infected livers indicated an elevated turnover of cells possibly influenced by reactions often seen in their vicinity such as portal vein occlusion producing a reduced portal blood flow and dense cellular infiltrates which included immunological cells. One further reaction prominent during the migratory phase of infection was the apparent transformation of hepatocytes into bile ductules. This reaction has been recorded in rabbits (Urquhart, 1956), rats (Thorpe, 1965a), cattle (Ross et al., 1967a; Doyle, 1972) and sheep (Dow et al., 1968) infected with F. hepatica but the stimulus for their formation and their significance have not been considered. In this study, bile ductule formation was found around infiltrated portal canals, underneath the capsule and adjacent to healing fluke tracts, i.e., in areas where there was no limiting plate or the limiting plate had been disrupted. It has been suggested that the stimulus for transformation could be contact between hepatocytes and bile duct epithelium (Elias, 1967) or collagen (Doljanski and Roulet, 1934) or as a result of chemical stimulation

such as occurs in ethionine poisoning in the rat (Popper and Hutterer, 1969). Contact of hepatocytes with bile ducts or collagen were possibilities for stimulation in this study but ductules were also seen being formed from hepatocytes in areas not containing collagen and ducts but only lymphocytes, plasma cells and eosinophils. Also, the number of ductules appeared to increase to a maximum at week 8 after infection then decline which suggested that ductules were being formed then destroyed. This observation was consistent with the results of Rubin, Hutterer and Popper (1963) who demonstrated in the rat that bile ductules had a half-life of eight days and as a consequence there was a rapid turnover of ductules.

Bile ductules can be considered to be of significance for two reasons; firstly, whilst transformation of hepatocytes into ductular cells was not a degenerative change, it had a comparable effect to degeneration in so far as functional hepatocytes were apparently removed from the parenchyma. Secondly, bile ductules act as a nidus for collagen deposition (Carruthers, Kalifat and Steiner, 1962). This was occurring in this study as collagen was deposited around ductules once fully formed.

A number of different types of fibrotic lesions varying in cause, time of onset, severity and distribution were found. Post-necrotic fibrosis was the first type of fibrosis to develop during the organisation of the lobar and regionally deployed necrotic fluke tracts. With time these tracts were reduced to small uneven scars which were initially the predominant fibrotic lesions in the parenchyma. Accompanying the formation of these scars was the development of numerous irregularly-orientated fibrous septa of varying widths of unknown cause and function interlinking post-necrotic scars with fibrosed portal canals, hepatic veins and capsule. Portal canal fibrosis was due to the combined effects of collagen deposition around bile ducts and ductules and within portal vein walls, the canals themselves and egg granulomatas (see later). Fibrosis of the canals appeared to result from a slow accumulation of collagen replacing hepatocytes lost at their peripheries. Peribiliary fibrosis developed secondary to the traumatic cholangitis produced by flukes upon colonisation of the ducts. Portal vein fibrosis was possibly the sequellae to an immune-complex phlebitis (see Section III) and hepatic vein fibrosis the outcome of organisation of thrombi and a traumatic phlebitis (see Section III). Also developing during the bile duct stage of infection

in certain locations in the parenchyma were pericellular and monolobular fibrosis. Pericellular fibrosis developed in areas around fibrosed portal canals, post-necrotic scars and fibrous septa and underneath fibrosed areas of the capsule. The cause of this type of fibrosis was not immediately obvious but because of its location metabolite exchange between hepatocytes and the blood may be impaired leading to cell death (Popper and Hutterer, 1969).

Monolobular fibrosis was found focally primarily after week 16 mainly in areas containing minimal fluke damage, i.e., the dorsal lobes. The thin septa characteristic of monolobular fibrosis in this study were similar in morphology and location to the septa forming the classical lobule of the pig's liver (Johnston, 1917; Ham, 1969). Such septa in pigs developed post-natally in response to expansion of the parenchyma during cell division (Elias, Bond and Larzarowitz, 1954). In this study monolobular fibrosis and nodular regeneration developed in equivalent areas at approximately similar times, i.e., the dorsal lobes at about week 20. Expansion of the parenchyma during regeneration may, therefore, have produced the straighten and stretching of the cell plates in areas identified as being structurally weak (Section I) and the degeneration of the hepatocytes seen to precede collagen deposition and septum formation. A similar sequence of events also preceded septum formation in the pig (Elias et al., 1954). Alternatively, an increase in parenchymal volume sufficient to stimulate septum formation could also have occurred as a result of arterial hypertension (Section III). As the morphological evidence available implicates largely the ventral and not the dorsal lobe in hypertensive changes (Section III) this appears unlikely; but such changes in haemodynamics may well account for the sinusoidal dilation and varicosity observed between opposing central veins and portal canals.

Although the majority of the different types of fibrosis reported here were the outcome of hepatic repair they may have been playing an important role in opposing distortions produced during tract healing. In doing so, intrahepatic morphological relationships particularly the respective positions of portal, arterial and hepatic vessels were preserved. In the normal liver, the relative positions of these intrahepatic structures were considered (Elias and Sherrick, 1969) to be dependent upon internal support provided by the portal canals, hepatic veins, capsule and perisinusoidal system of reticular fibres. Hence, fibrosis of all these structures will, conceivably,

produce a more rigid infrastructure.

Only Urquhart (1956), Dow et al. (1967) and Doyle (1972) have given an appraisal of the causes of fibrosis in fascioliasis. Urquhart (1956) studying F. hepatica in the rabbit described trauma, ischaemia, portal canal lesions, egg granulomatas and infestation of the bile ducts as being responsible for hepatic fibrosis which he termed a 'coarse cirrhosis'. Dow et al. (1967) and Doyle (1972) identified post-necrotic and peribiliary fibrosis in cattle infected with F. hepatica and their causes. They also reported a monolobular-like fibrosis which Doyle (1972) observed in areas of maximum fluke damage, the ventral and caudate lobes, and which increased upon reinfection. It is unlikely that the lesions reported by Doyle (1972) were equivalent to the monolobular fibrosis seen in this study because of the different locations and the finding that monolobular fibrosis did not increase to any extent upon reinfection (Section IV).

The underlying mechanisms of hepatic fibrogenesis were considered by Popper and Hutterer (1969) to be dependent upon the presence of suitable stimulating agents, transformation of cells previously thought to be Vitamin A storage cells to fibroblasts, the half-life of the collagen deposited and disturbances in hepatic microcirculation. These fibrogenetic mechanisms have not been examined in cases of fascioliasis. In this study the identifiable potential stimuli for fibrogenesis were the dead and degenerating hepatocytes around acute tracts and portal canals (Popper and Hutterer, 1969), iron-laden macrophages within healed tracts and capsule (Orfli, Volini, Madera-Orsini, Minick and Kent, 1968), the changes in portal canals (Popper, Paronetto, Schnaffner and Perez, 1961), e.g., cholangitis, and bile ductule formation (Popper, Schnaffner, Hutterer, Paronetto and Barka, 1960). It was the opinion of Popper and Hutterer (1969) that all these stimuli provided an inert surface capable of facilitating collagen deposition. Recent ultrastructural studies on hepatic perisinusoidal cells, otherwise called fat storage cells (Ito and Nemoto, 1952; Elias and Sherrick, 1969) or stellate cells (Wake, 1971), in mice given carbon tetrachloride have shown that the cells become morphologically identical to fibroblasts and appear to deposit collagen (McGee and Patrick, 1974). Perisinusoidal cells with histological (Section I) and ultrastructural (Gemmel and Heath, 1972) features of stellate cells have been identified in the ovine liver. These cells may, therefore, have been producing the collagen deposited within the

parenchyma while fibroblasts were responsible for extra-parenchymal fibrosis (Popper and Hutterer, 1969).

Auto-radiographic studies in the rat have shown that septal collagen had a long half-life and as such was considered permanent (Rubin and Hutterer, 1967). By contrast, other types of collagen, e.g., that deposited around acute liver damage, had a relatively short half-life (Popper and Hutterer, 1969). Catabolism of this latter type of collagen took place if the initial fibrogenetic stimulus was removed (Popper and Hutterer, 1969). These differences in half-life were also reflected in the cellularity of the two types of fibrosis: short half-life collagen contained many fibroblasts while long half-life collagen contained only a few (Popper and Hutterer, 1969). Extrapolation of this data to the sheep infected with F. hepatica would indicate that post-necrotic fibrosis should have a short half-life and monolobular septal collagen a long half-life. Evidence in support of the short half-life of post-necrotic fibrosis is presented in Section IV. In this section the post-necrotic fibrosis in the sheep that had had a 10 week infection terminated by anthelmintic treatment was reduced compared to sheep in which the fluke burden was allowed to persist which suggested that the post-necrotic fibrosis in the former sheep was undergoing catabolism. Evidence on the longevity of septal collagen in fascioliasis is not yet available.

Disturbances in hepatic microcirculation was considered by Popper and Hutterer (1969) to produce a self-perpetuating cycle of collagen deposition in the parenchyma. This cycle involved, first, collagen deposition in the sinusoid as in, for example, pericellular fibrosis. This impeded blood flow and nutrient exchange leading, terminally, to cell death, collagen replacement of the dead cells and, thus, impeding further blood flow and nutrient exchange. In this study such a cycle may have been initiated and/or perpetuated by parenchymal collagen deposition and possibly by the reduced portal blood supply (Section III), since total experimental portal vein ligation in the rat results in rapid and widespread hepatocyte cell death (Steiner and Martinez, 1961). Furthermore, as the reduction in portal vein supply appeared to be progressive due possibly to portal hypertension (Section III), the presence and persistence of portal hypertension may have exacerbated an already established cycle of parenchymal collagen deposition.

Biliary distension, fibrosis, epithelial hyperplasia, erosion

and ulceration as seen in this study are lesions common to fluke-infected livers in all species. One important feature that has not been appreciated previously in the ovine liver is the expansion and hypertrophy of the normal rich peribiliary arterial plexus during infection (see Section III). Ulceration of the biliary epithelium frequently exposed the underlying collagen containing this plexus and, therefore, provided an opportunity for the plexus to be damaged by the flukes. Such damage, if present, was not apparently too extensive as flukes were seen to favour ducts lined by hyperplastic epithelium in preference to ducts without any epithelium.

Secondary bile ducts even although they did not harbour flukes became fibrosed and contained fluke eggs. The latter observation in the rabbit infected with F. hepatica led Urquhart (1956) to speculate upon a retrograde movement of eggs from the larger fluke-infested ducts where the eggs were discharged to the smaller non-infested ducts. The secondary ducts in this study were also involved with a series of changes leading to the destruction of the duct and formation of a lesion conforming to the pattern of an infective egg granuloma (Warren, Domingo and Cowan, 1967). Morphologically similar lesions have been recorded in the portal veins in schistosomiasis (Lichenberg, 1969), where it has been established that this reaction represented a cell-mediated hypersensitivity directed against eggs lodging in the vein walls (Warren et al., 1967). As a result of portal vein occlusion in schistosomiasis portal hypertension develops (Lichenberg, 1969) whereas the effect of obstruction of the bile ducts in fascioliasis has yet to be assessed.

Thus, as a result of infection, trauma inflicted by the flukes was responsible for widespread hepatocyte cell death and necrosis and thrombosis of blood vessels which led to infarction. In addition a cholangitis was induced. Accompanying the development of these changes were vascular occlusive lesions and egg granulomata and bile ductule formation.

The outcome of these reactions was the development of hepatic fibrosis, chronic hyperplastic cholangitis, venous-occlusion and bile duct obstruction. The hepatic fibrosis consisted of several components varying in severity and distribution.

SUMMARY

The flukes established from a primary F. hepatica infection in sheep produced a system of necrotic tracts in the parenchyma during their migratory and feeding activities that expanded between weeks 1 and 12 from a few localised areas in the ventral lobe to include most of the ventral lobe and focal areas of the other lobes. In addition, trauma, stress, waste products, ischaema and possibly immunological mechanisms may have contributed towards liver damage in and around these tracts especially during weeks 10 to 12 after infection. Accompanying the formation of these tracts was the development of bile ductules apparently from hepatocytes, portal and hepatic vein occlusion, and capsular thickening and eventually fibrosis.

Organisation of the fluke tracts and accompanying lesions produced post-necrotic, pericellular, monolobular, portal canal and portal and hepatic vein fibrosis. The identifiable stimuli for these fibrotic reactions were dead and dying hepatocytes, iron-laden macrophages, portal canal reactions and possibly in the case of monolobular fibrosis parenchymal expension due to regeneration.

Following colonisation of the bile ducts a chronic hyperplastic cholangitis ensued. Fluke eggs discharged into the bile ducts may have stimulated cell-mediated egg granuloma which resulted in the destruction of secondary bile ducts.

Fig. 21. Week 1: Tiny cream-white tracts (arrows) are scattered over the parietal surface of the ventral lobe.

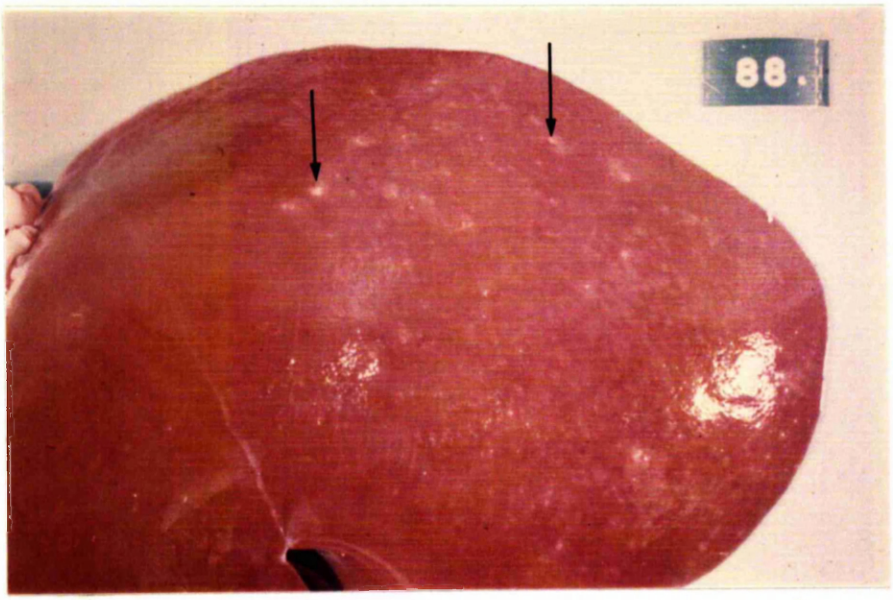

88.

Fig. 22. Week 4: A maze of necrotic and haemorrhagic tracts located mainly ventral to the falciform ligament.

Fig. 23. Week 4: Closer view of the necrotic and haemorrhagic tracts.

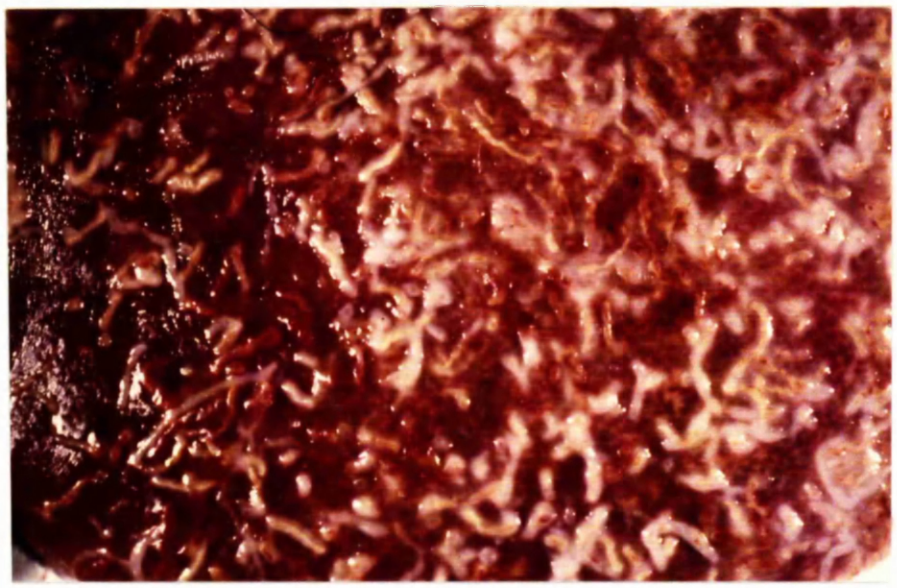


Fig. 24. Week 10: A relatively small number of large haemorrhagic tracts (arrows) are distributed throughout all liver lobes. Note the slight reduction in ventral lobe size and the irregular liver surface.

Fig. 25. Week 12: The ventral lobe is becoming more contracted and the distributing bile ducts are beginning to distend. A few haemorrhagic tracts can be seen in the ventral, central and dorsal lobes. Fibrin-fibrous tags overlie the ventral lobe.

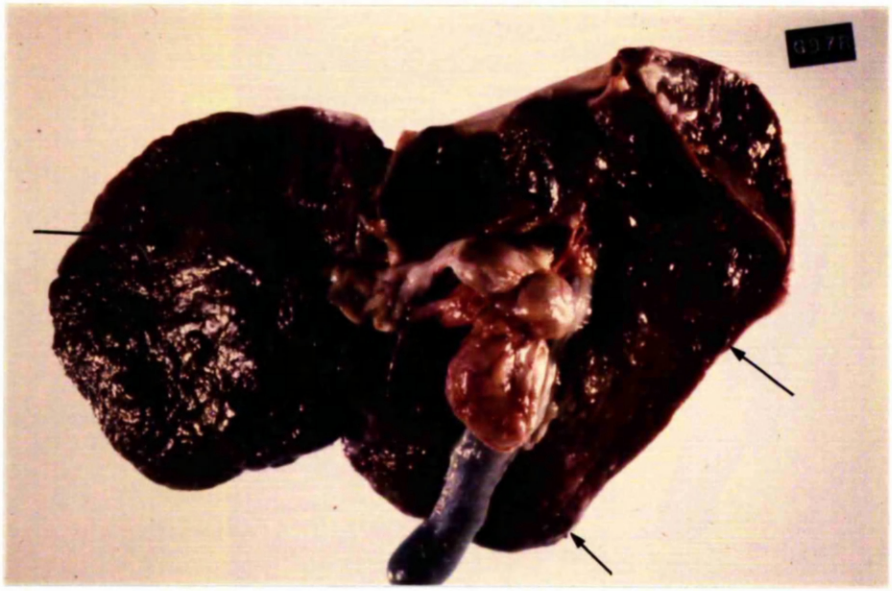


Fig. 26. Week 12: Subcapsular haematomatas.

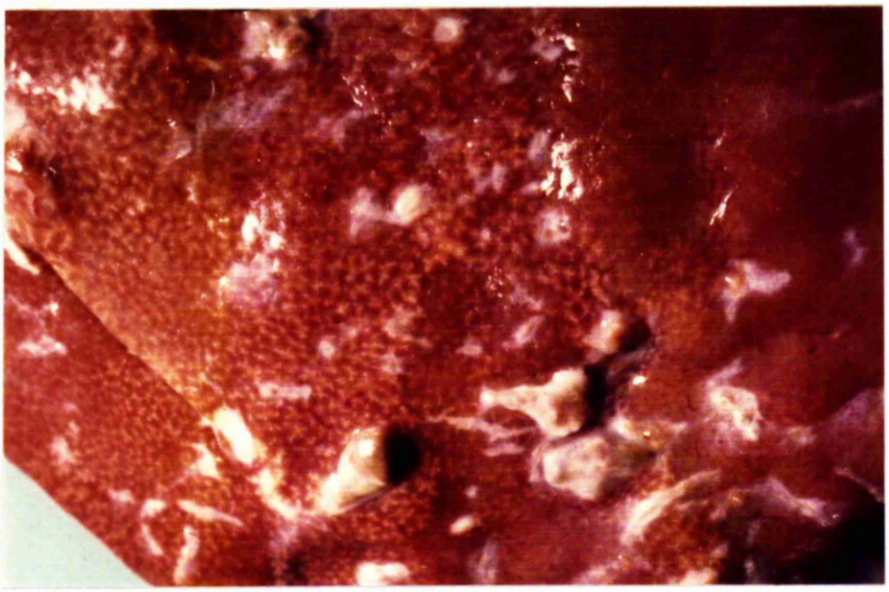
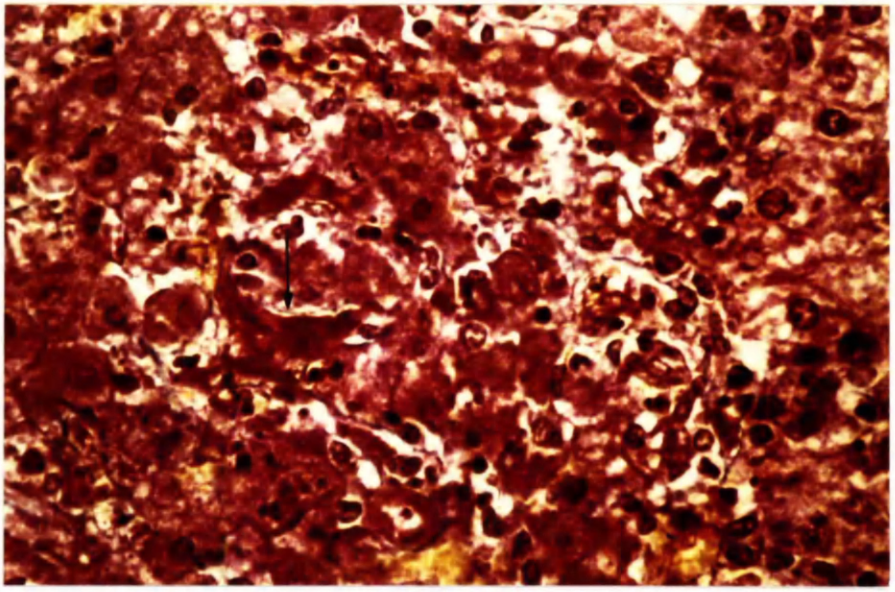


Fig. 27. A Microthrombus (arrow) plugging a sinusoids in an area of necrosis adjacent to a fluke tract.

MSB X 120

Fig. 28. Centre of an organising fluke tract containing macrophages and giant cells surrounded by post-necrotic collagen.

MSB X 40.



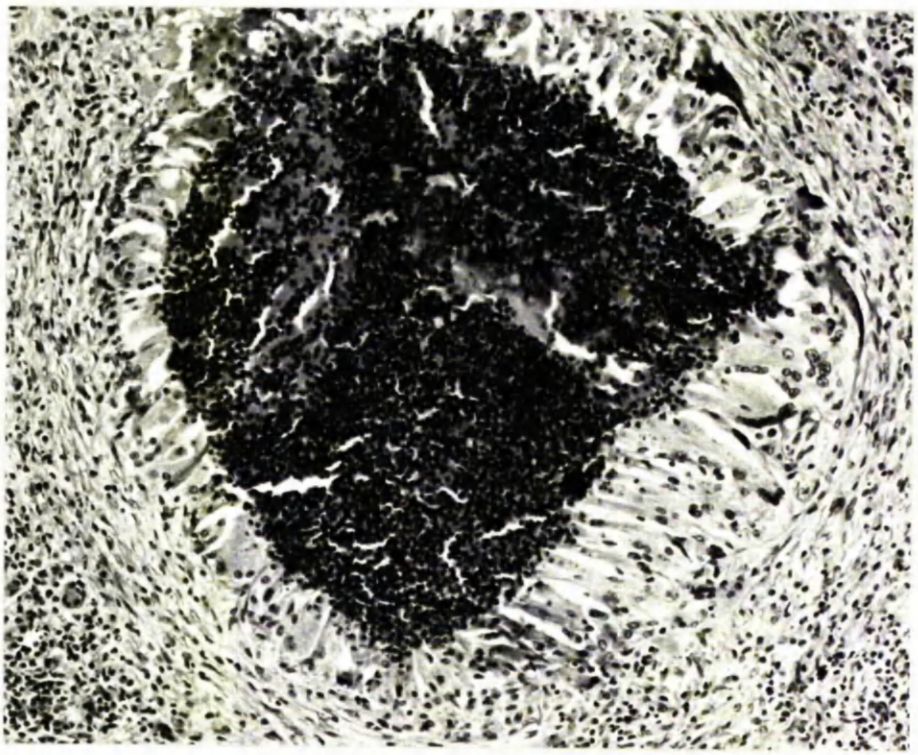
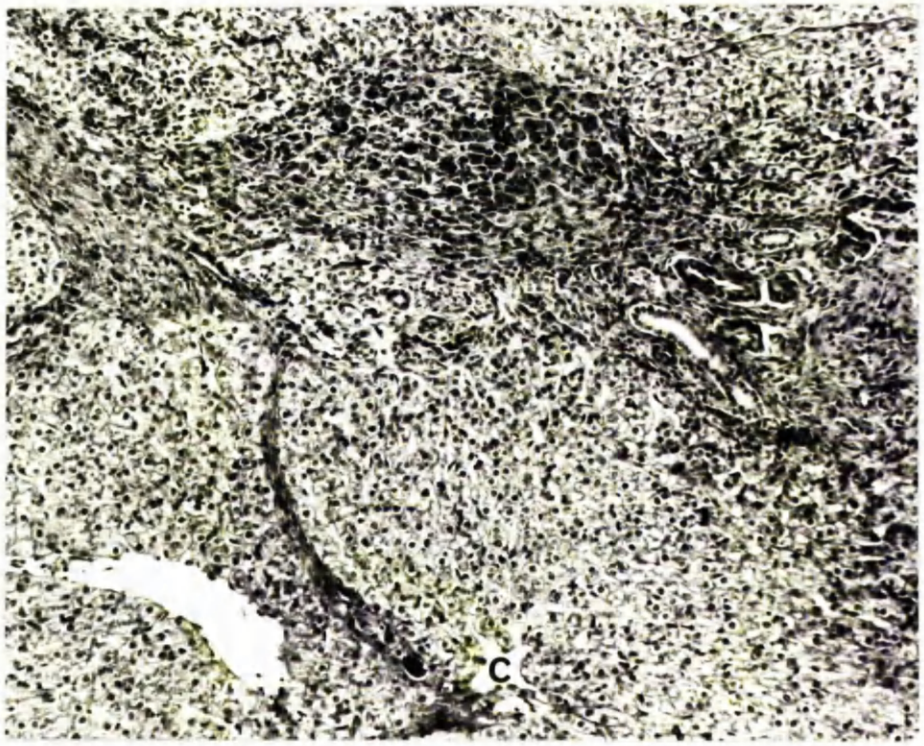


Fig. 29. Post-necrotic scar containing haemosiderin laden macrophages. Note the band of collagen radiating to a central hepatic vein (C).

HE x 40.

Fig. 30. A thrombus nearly occluding a sublobular hepatic vein.

M.S.B. x 12


C

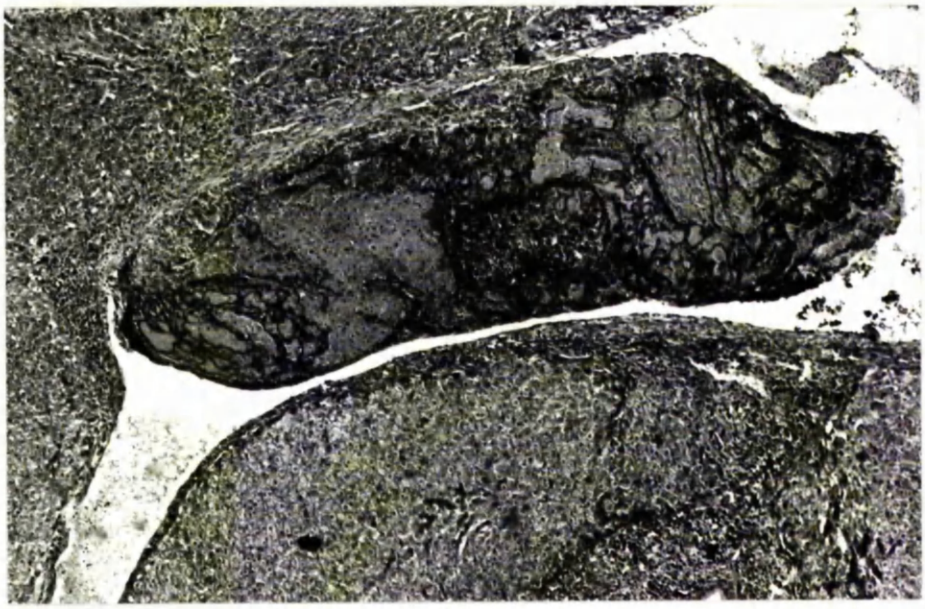
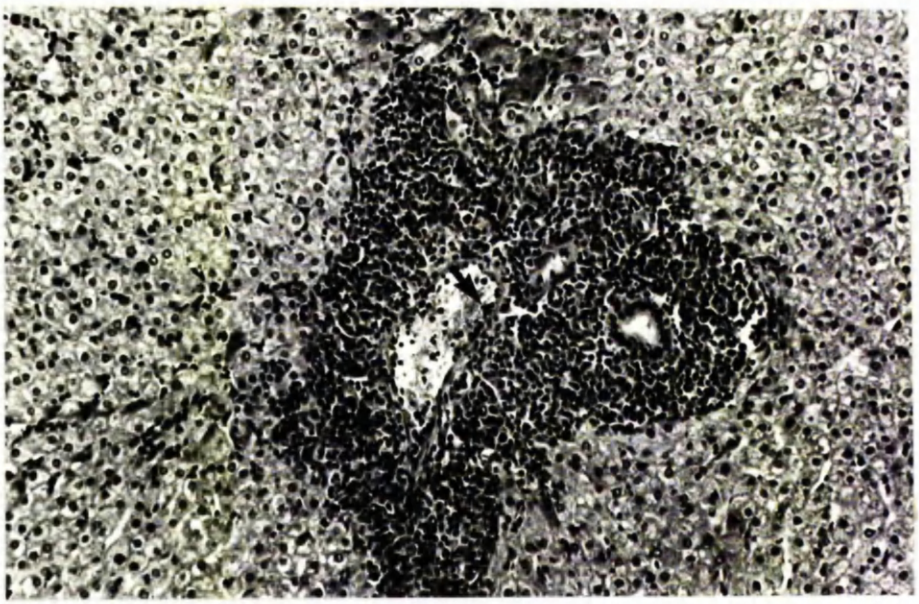


Fig. 31. A portal canal located near a fluke tract at the Week 1 stage of infection. The canal is considerably enlarged due to an influx of lymphoid cells which can also be seen underneath (arrow) the portal vein endothelium.

HE X 40.

Fig. 32. Lymphoid cells located in a portal canal at Week 1 are spilling out into the surrounding parenchyma destroying the limiting plate. The cells are also located intramurally and subendothelially of a secondary portal vein (2) which has been sectioned longitudinally and a tertiary portal vein (3). This has resulted in an increase in wall thickness and a decrease in lumenal diameter of both veins.

HE X 75.



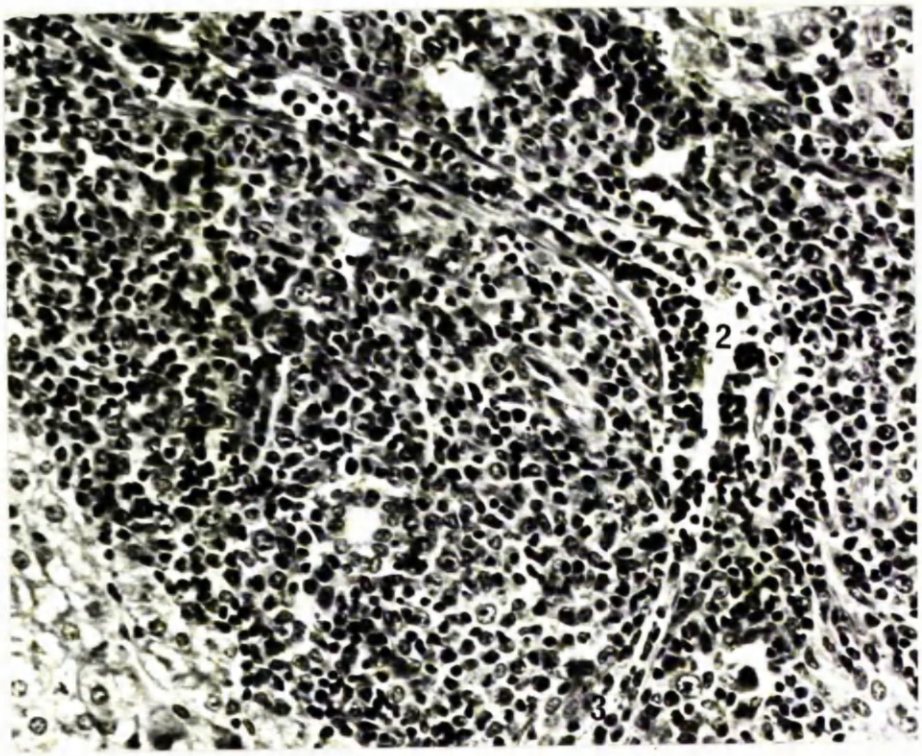
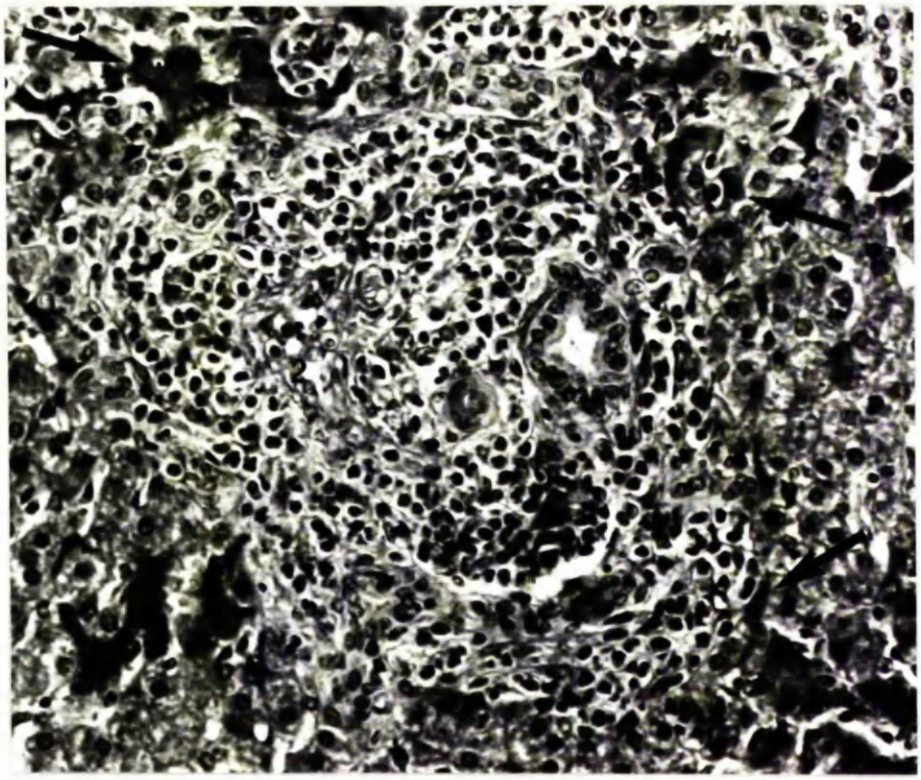

2
3

Fig. 33. Eosinophils, plasma cells, lymphocytes and polymorphonuclear leukocytes advancing into the parenchyma around a portal canal accompanied by hepatocyte degeneration (arrows). The secondary portal vein (2) in this canal is totally occluded by oedema and eosinophil cells making it barely distinguishable amongst the portal canal reaction.

HE X 75.

Fig. 34. Bile ductule formation from hepatocytes (⟶).

HE X 120.



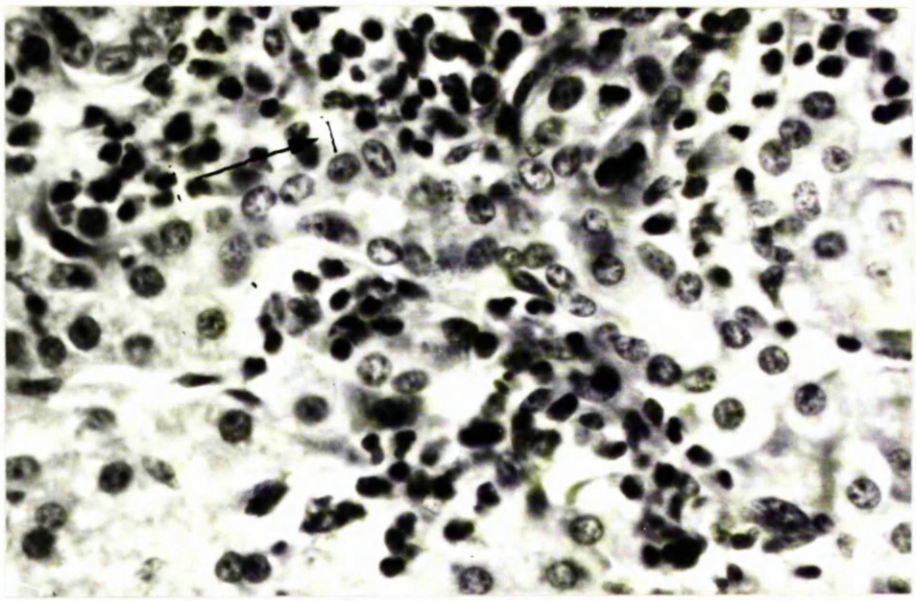
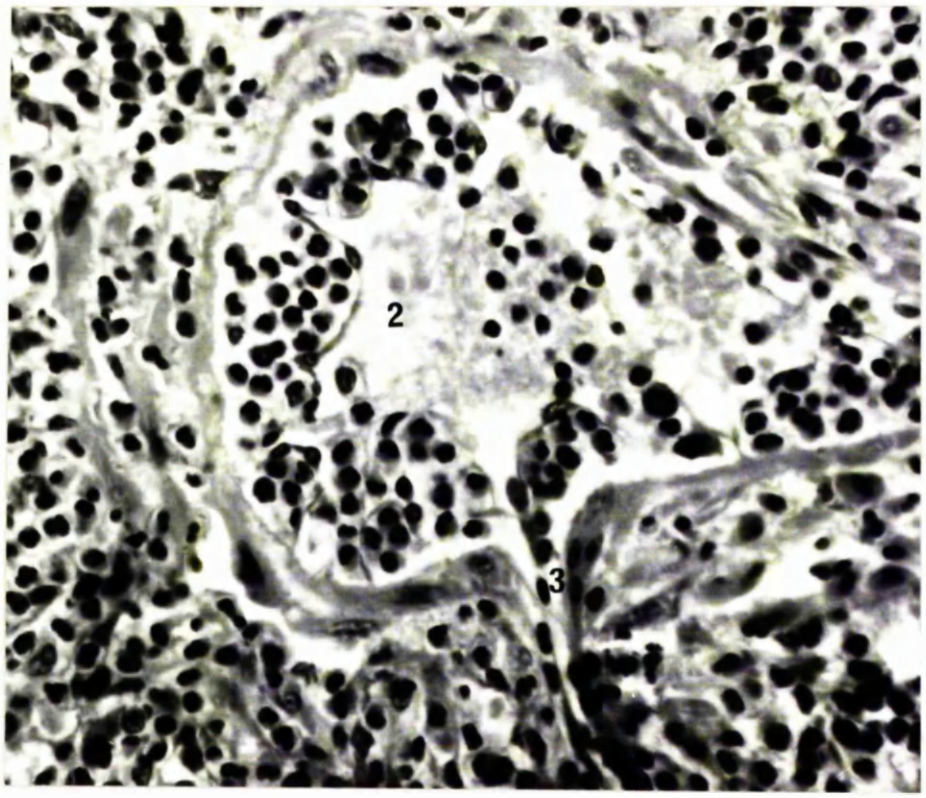


Fig. 35. Marked eosinophil cell infiltration and oedema of a secondary (2) and a tertiary (3) portal vein and also the surrounding portal canal. The wall of the secondary portal vein is thickened and its endothelial lining is folded resulting in a reduction of lumenal diameter. The tertiary portal vein is occluded due to subendothelial cell infiltration and pressure from the surrounding portal canal reaction.

HE X 120

Fig. 36. Fibrous thickening of the walls of a secondary (2) and tertiary (3) portal vein with a considerable reduction in lumenal diameter of the secondary vein. Adjacent to the fibrosed portal vein is the hepatic artery (A).

HE X 75.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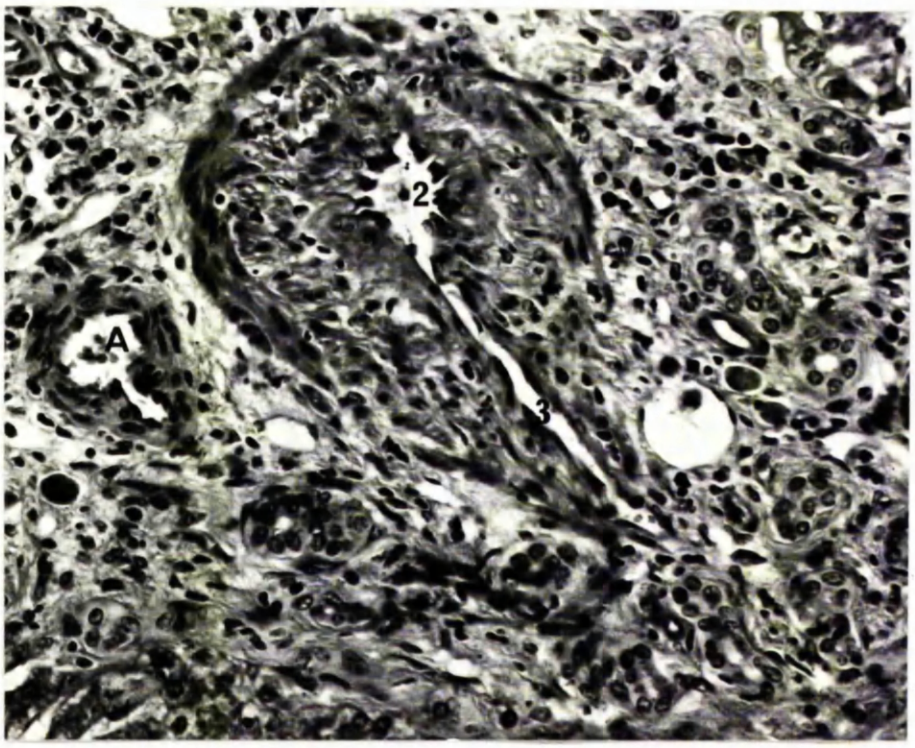
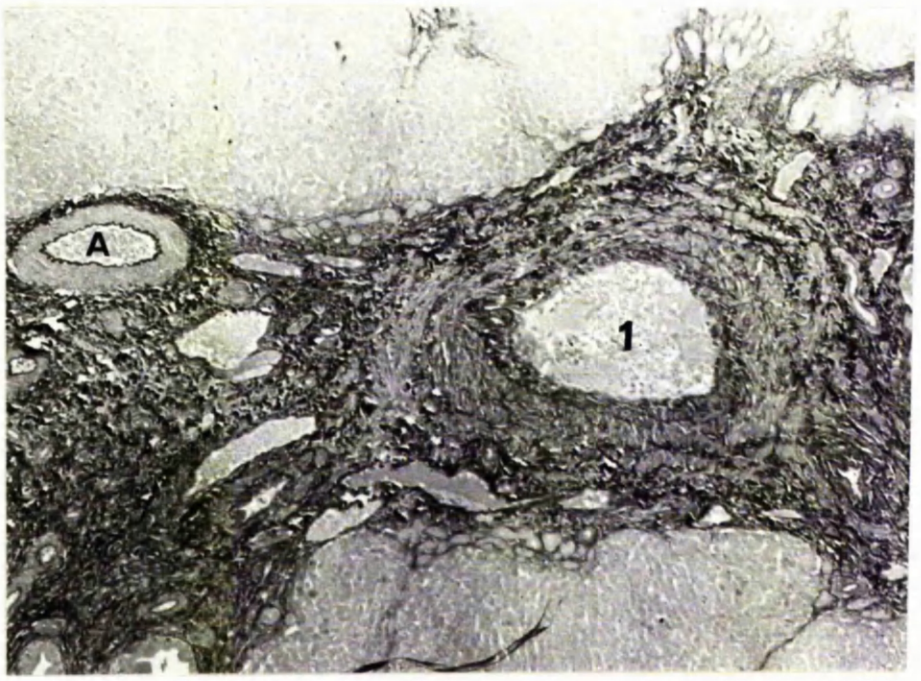

2
A
3

Fig. 37. Fibrous thickening of the wall of a primary portal vein (1). The vein is surrounded by a considerably enlarged portal canal containing distended lymphatics and a dilated artery (A).

HE x 12.

Fig. 38. Infiltration of a fibrosed secondary portal vein (2) by eosinophils producing more severe venous-occlusion. Two occluded tertiary portal veins (3) can be seen traversing the thickened secondary vein wall.

HE x 120.


A
1

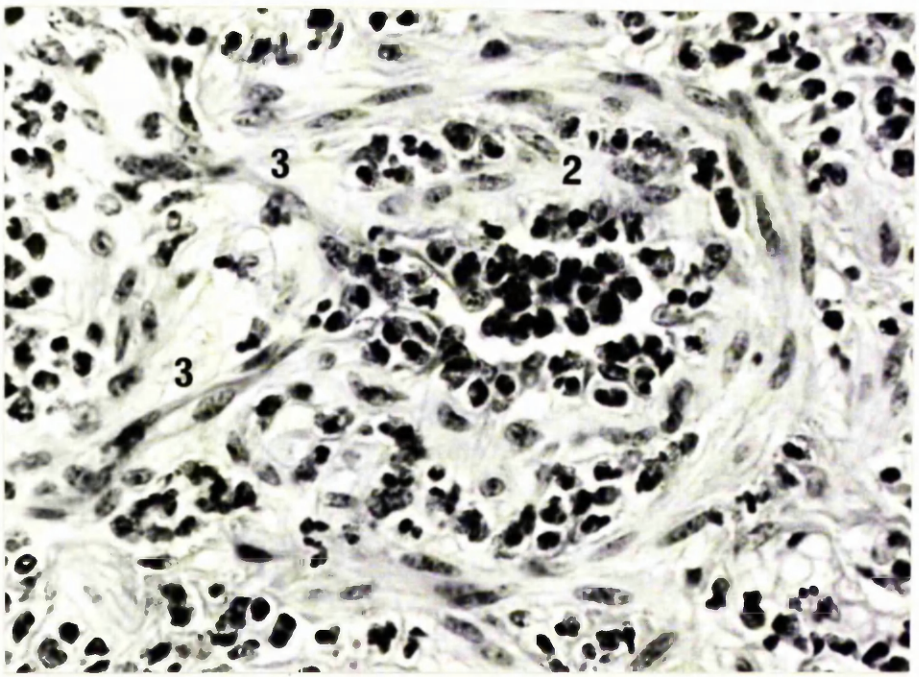
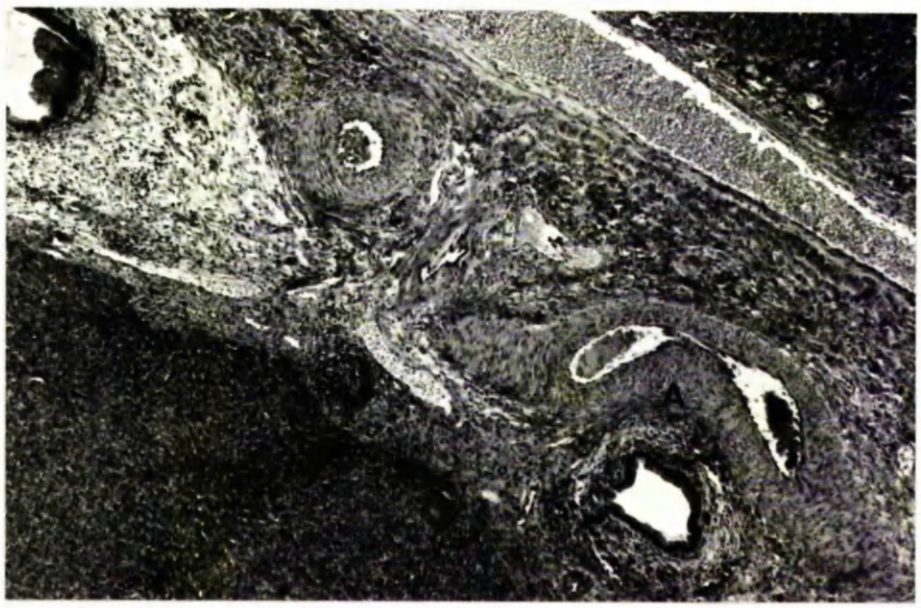

3
2
3

Fig. 39. A hyperplastic, tortuous primary artery (A) sectioned longitudinally and transversally.

MSB X 40.

Fig. 40. Organisation of a central hepatic vein resulting in severance of sinusoidal connections (arrows) and congestion of surrounding sinusoids.

MSB X 75.



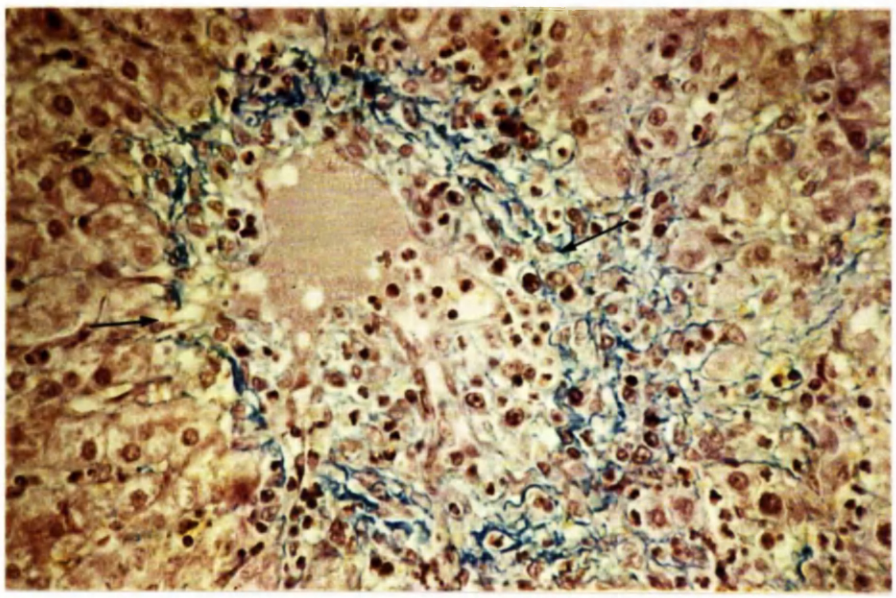
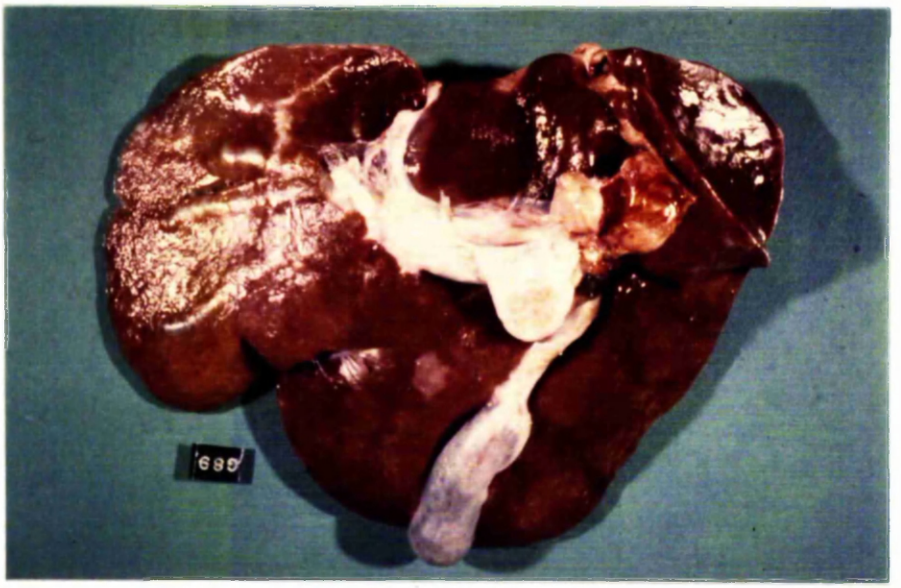


Fig. 41. Week 20: The liver has a contracted ventral lobe, distended primary and distributing bile ducts and nodules of regeneration in the central and dorsal lobes.

Fig. 42. Week 16: Transverse section through the ventral lobe to show thickened portal canals containing distended and fibrosed bile ducts.



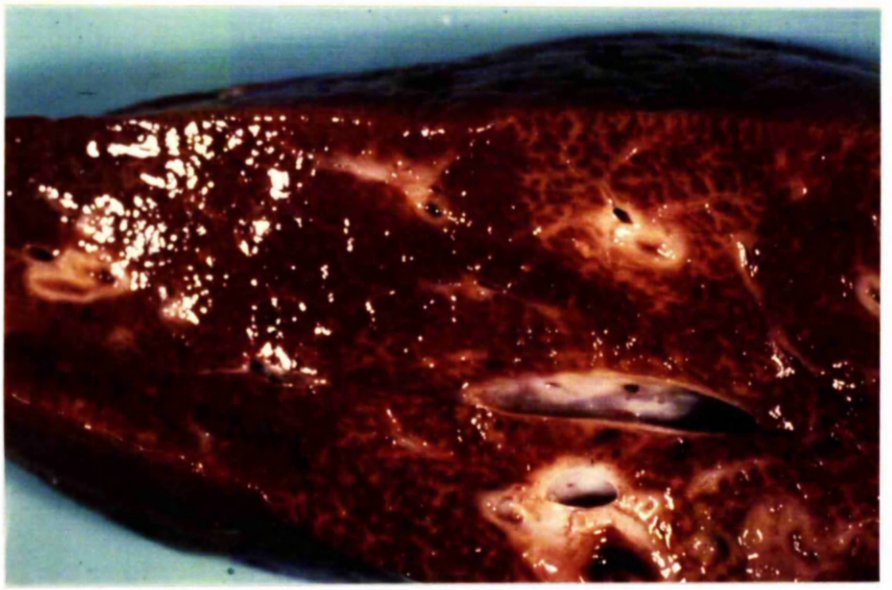


Fig. 43. A primary bile duct containing adult flukes. The epithelial lining is almost totally eroded and peribiliary fibrosis has yet to develop.

HE X 12.

Fig. 44. A markedly fibrosed and thickened bile duct containing numerous small often tortuous peribiliary plexus arteries (arrows).

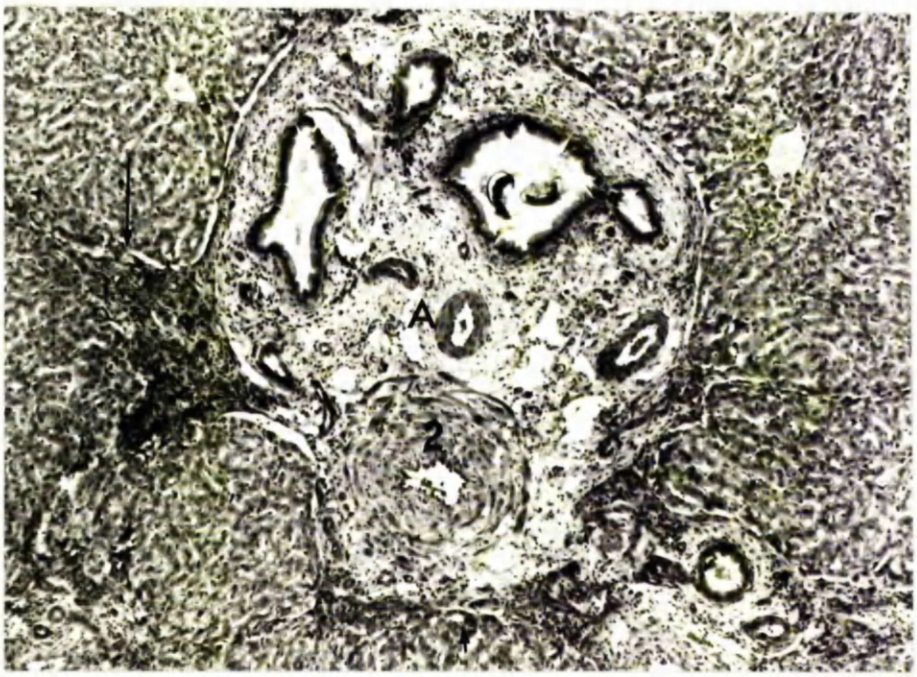
HE X 75.

Fig. 45. A portal canal at the Week 20 stage of infection. The canal is considerably enlarged due to portal fibrosis. Within the canal is a fibrosed secondary portal vein (2), a secondary bile duct containing fluke eggs, a dilated artery (A) and bile ductules (arrows).

HE X 40.

Fig. 46. A portal canal at the Week 30 stage of infection. The canal contains a fluke egg (arrow) surrounded by sheets of lymphocytes, plasma cells, eosinophils and macrophages. Also present is a fibrosed secondary portal vein (2) and a dilated artery (A). Note the absence of a bile duct.

HE X 40.


A
2


A
2

Stages in the Formation of Egg Granulomata

Fig. 47. Initial stage: The wall and surrounding connective tissue of a secondary bile duct have become oedematous. There is an increase in the number of globule leukocytes(➔) within the duct wall and lymphoid cells are beginning to accumulate within the oedematous connective tissue.

HE X 75.

Fig. 48. Intermediate stage: The oedematous bile duct wall has disintegrated. Note the presence of a fluke egg (arrow).

HE X 75.

Fig. 49. Final stage: With the destruction of a bile duct wall fluke eggs have become the centre of a granuloma reaction. Occasional bile duct epithelial cells can still be recognised (arrow).

HE X 40.

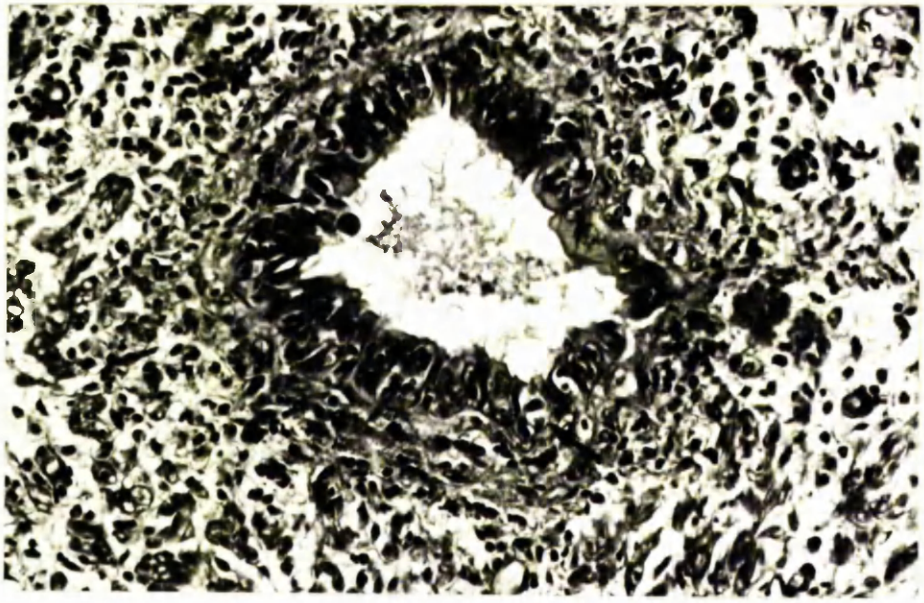
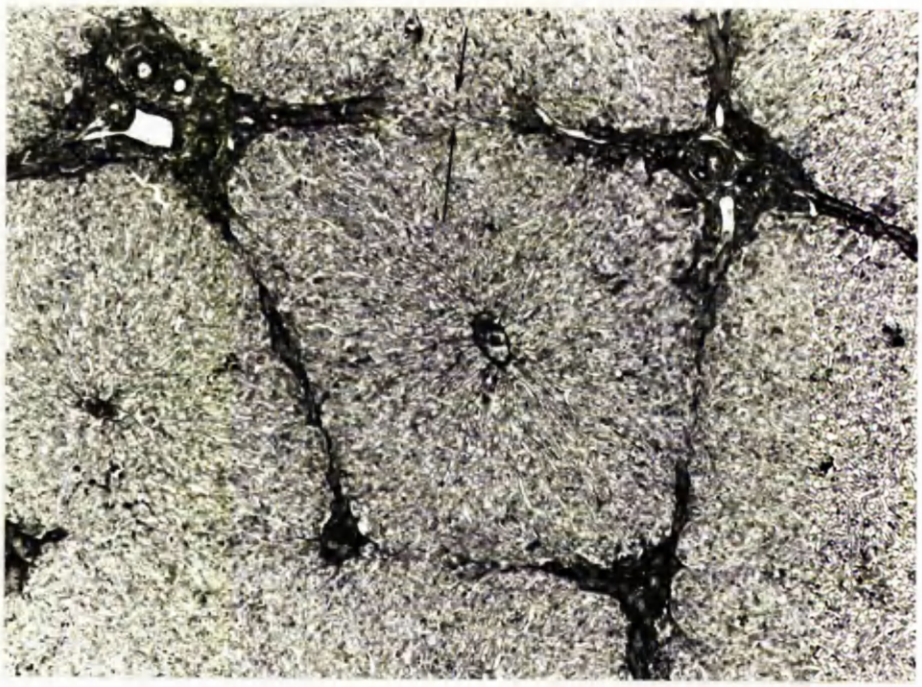


Fig. 50. Thin strands of pericellular fibrosis surrounding individual and small groups of hepatocytes.

MSB X 75.

Fig. 51. Monolobular fibrosis from the dorsal lobe at the Week 20 stage of infection. The portal canals are interconnected by thin septa. Note the straightening of the reticular fibres in areas (⟶ ⟵) where the septa are forming.

Gordon and Sweet X 40.



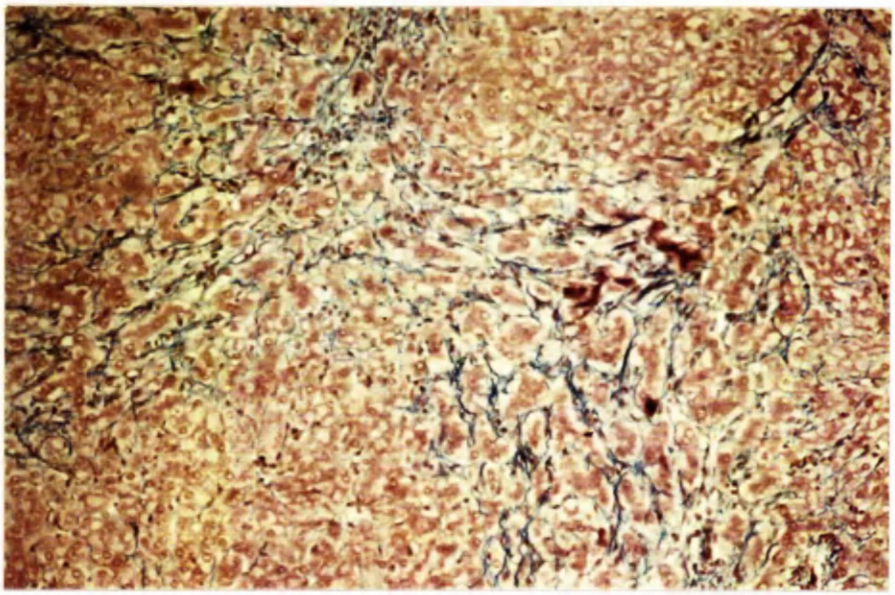
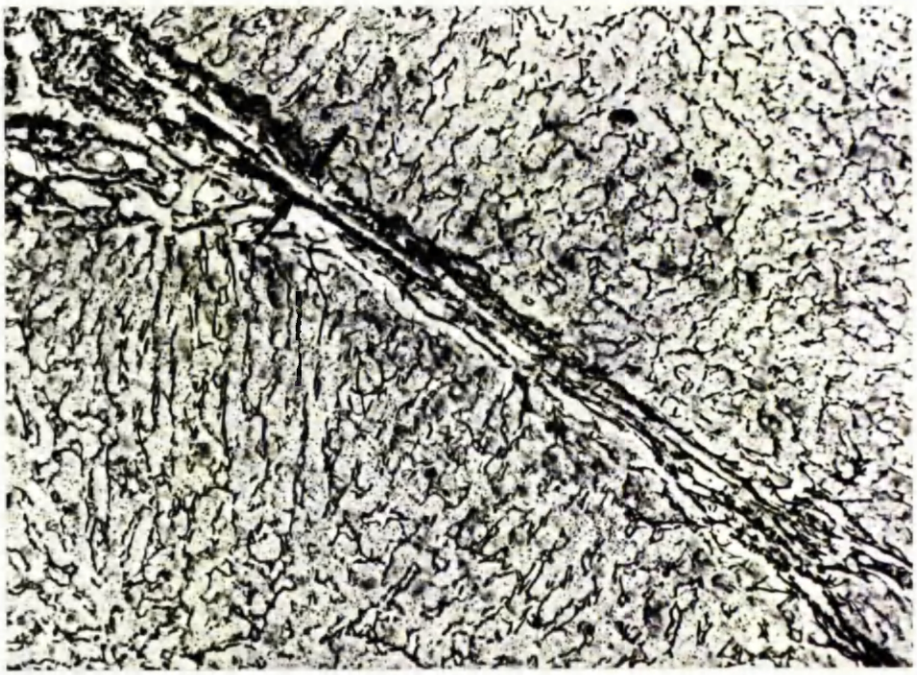


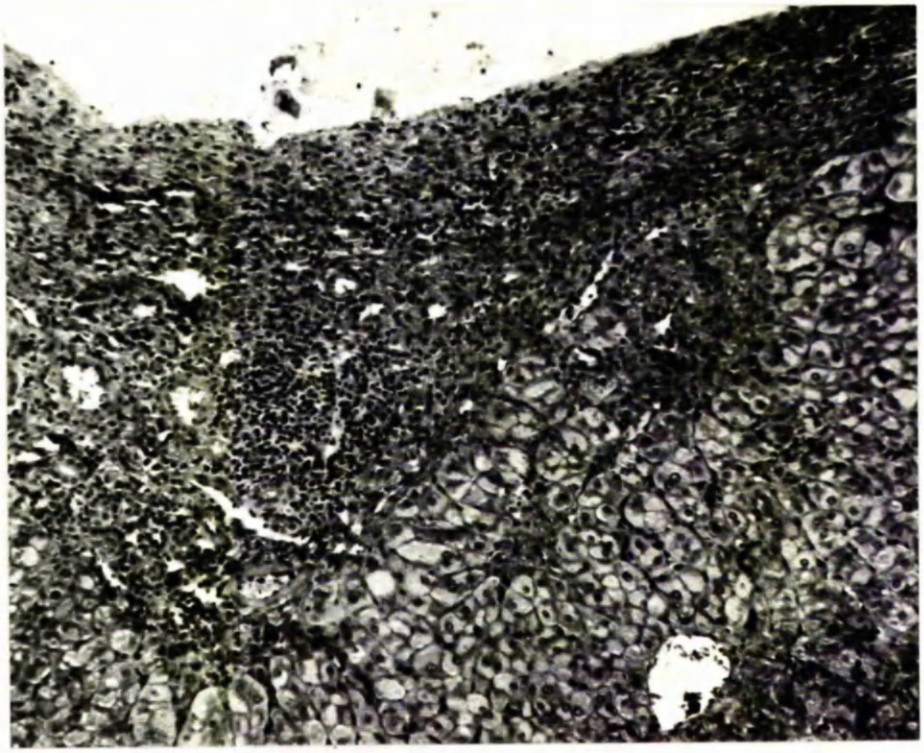
Fig. 52. A narrow cascular channel interconnecting a portal canal (top left) and a central hepatic vein (bottom right).

Gordon & Sweet x 120.

Fig. 53. An area of regeneration located underneath a fibrously thickened capsule of Glisson. This area consists of groups of hepatocytes with clear cytoplasm without intervening sinusoids.

MSB x 40.





SECTION III

THE VASCULAR SYSTEM IN OVINE FASCIOLIASIS

INTRODUCTION

One of the most significant findings in the pathology of ovine fascioliasis is the development of extensive changes in the intrahepatic vasculature (Section II). While vascular lesions have been described in several species including the sheep infected with F. hepatica (Morrill and Shaw, 1942; Urquhart, 1956; Thorpe, 1965a; Dow et al., 1967; Neiberle and Cohrs, 1967; Ross et al., 1967a; Rahko, 1969; Flagsted et al., 1972; Doyle, 1972), these reports were at variance with each other as to the type of lesion developing and the vessels involved. In this section, a study was made of the sequential development of the vascular lesions and the vessels and branches involved; the possible consequences of such lesions were considered. In addition to histological examination, acrylic resin casts were employed to study the effects of infection on the intrahepatic vasculature.

MATERIALS AND METHODS

In Section II a description was given of the range, development and distribution of the vascular lesions produced in a primary F. hepatica infection in sheep. To provide a basis for comparison with acrylic resin casts of infected livers these results are repeated in this section in a summarised and tabular form.

Histological procedures

These were as described for Section II and in the general materials and methods.

Preparation of acrylic resin casts

Three sheep were inoculated orally with 400 F. hepatica metacercariae. Two were treated to terminate their infections with rafoxanide at 15 mg per kg live body weight (Merck, Sharp and Dohme, Hoddesdon, Herts.) at week 12 and the remaining sheep was treated at week 24. Two weeks after anthelmintic treatment the sheep were slaughtered and their livers removed with care. Tensol was injected as described in Section I into the portal, biliary and arterial systems of one sheep slaughtered at week 14 and the sheep slaughtered at week 26 and into the portal and hepatic systems of the other sheep slaughtered at week 14.

RESULTS

During the course of the infection with F. hepatica the portal, hepatic, sinusoidal and arterial systems became involved in a series of changes which led to the development of portal and hepatic vein occlusion and alterations in sinusoidal and arterial blood flow. The branches of the portal and hepatic systems involved most frequently with occlusive lesions are shown in Fig. 54. Fig. 55 details the effects venous-occlusion was considered to have on the major, minor and anastomotic routes of intrahepatic blood flow identified in the normal liver in Section I (see Fig. 2) together with the alterations in the blood flow occurring in the sinusoidal and arterial systems.

Portal Venous System

Acrylic resin casts

These demonstrated both a lobar and regional reduction in portal venous volume.

Distributing and primary veins

The distributing veins and the primary veins supplying the central, dorsal and caudate lobes were of normal length and diameter while primary veins within the ventral lobes were shorter and slightly reduced in diameter (Fig. 56).

Terminal, secondary and tertiary portal veins

Few terminal and secondary portal veins were perfused in the ventral lobes of the week 12 and 24 casts (Fig. 56). Those that had were reduced in length and diameter and had in the case of secondary veins infrequent tertiary vein connections (Fig. 57). Differentiation of secondary veins into long and short veins was not possible because of their stunted appearance and lack of side branches (Fig. 57). In the central, dorsal and caudate lobes most secondary and terminal portal veins had perfused normally but dispersed amongst these veins were isolated groups of short stunted secondary veins (Fig. 58). Throughout the livers but particularly in the vicinity of the distributing portal veins and the primary portal veins of the ventral and central lobe were multiple, narrow, irregularly-arranged vessels interlinking veins the size of tertiary portal veins. These vessels were

considered to be porto-portal anastomoses (Fig. 59).

Histological findings

Portal veins directly involved with acute fluke tracts were necrosed but not thrombosed and veins in close proximity to fluke tracts underwent a sequence of changes that terminated in the development of venous occlusion. The sequence of events leading to the development of this latter lesion, the portal vein branches involved, their distribution and the effects of involvement are summarised in Table 3. Basically, the vessel walls were infiltrated initially with lymphoid cells, later the walls became oedematous and eosinophils predominated and finally the lesion organised with the deposition of collagen producing a narrowing of the vessel lumen. Where a recent fluke tract passed close to a fibrosed vessel its wall was oedematous and reinfiltrated with eosinophils. Once established, the fibrotic component of many veins continued to increase producing more severe venous occlusion even although the fibrosed veins were not involved with any more fluke tracts (see Figs. 32, 35 and 36, Section II).

Hepatic System

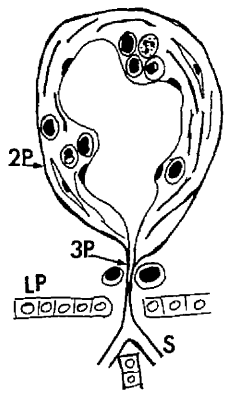
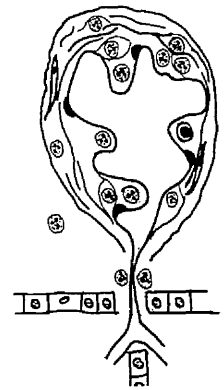
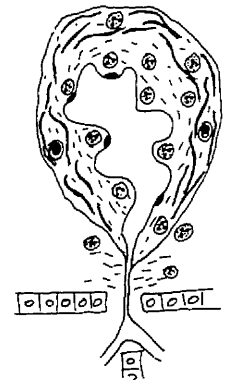
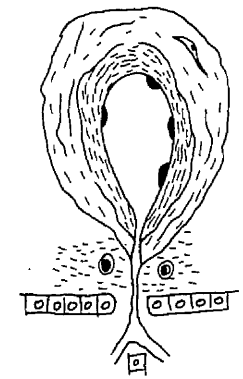
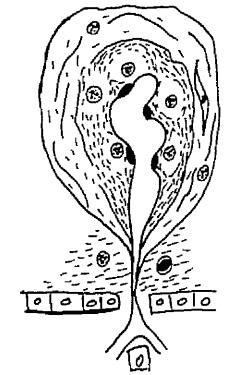
Acrylic resin casts

Very few hepatic veins in the ventral lobe were perfused; those that had were large, flattened, irregular-shaped veins with no sinusoidal connections, hence identification of these vessels was not possible. In the central, dorsal and caudate lobes many normal, central, sublobular and hepatic veins had been preserved together with groups of central veins that were involved with an irregular network of inter-connecting veins (hepato-hepatic anastomoses) (Fig. 60). Occasionally, in the central lobe, veins the size of tertiary portal and central hepatic veins were found inter-connected (porto-hepatic anastomoses) (Fig. 61).

Histological findings

The lesions seen histologically involving the hepatic system, their distribution and effects are summarised in Table 4. Where fluke tracts cut across hepatic veins a necrotising vasculitis developed that often led to thrombus formation. Hepatic veins were much more frequently involved with these changes than were portal veins.

TABLE 3. SUMMARY OF LESIONS AFFECTING THE PORTAL VEINS IN A PRIMARY F. HEPATICA INFECTION IN SHEEP.

Drawings of lesions affecting portal veins (explanation of symbols at the bottom of page).					
TIME	Week 1.	Weeks 2 - 12.	Weeks 6 - 16.	Weeks 6 - 30.	Weeks 8 - 12.
CHANGE	Intra mural and subendothelial infiltration by lymphoid cells and lesser numbers of eosinophils, plasma cells and polymorphonuclear leucocytes.	Intramural and subendothelial eosinophil cell infiltration and oedema formation. Endothelial folding, thickening of vein wall.	Reduction in oedema and number of eosinophils, appearance of fibroblasts initiation of collagen deposition in vein walls and subendothelially.	Progressive intimal and subendothelial fibrosis.	Reinfiltration of fibrosed vein walls by eosinophils and reappearance of moderate amounts of oedema.
VEINS INVOLVED	Terminal, secondary and tertiary portal veins.	Primary, secondary, terminal and tertiary.	As in previous column.	As in previous column.	Any fibrosed portal vein.
LOBAR DISTRIBUTION	Focally mainly ventral lobe, a few in the dorsal lobe.	Initially focally all lobes, subsequently most of ventral lobe and focally central, dorsal and caudate lobes. Only ventral lobe primary veins affected.	As in previous column.	As in previous column.	All lobes.
EFFECTS	Endothelial cell displacement producing narrowing of vein lumen. Compression of tertiary veins.	Terminal and secondary veins nearest tracts often severely occluded by endothelial folding, veins further away less severely occluded. Primary veins moderately occluded. Tertiary veins consistently and severely occluded.	Beginning of fibrosis. Degree of occlusion slightly less due to reduction in oedema.	Fibrous thickening of vein wall producing varying degrees of occlusion. Ventral lobe terminal and secondary veins most severely affected; occasional veins in other lobes also severely involved. Ventral lobe primary veins moderately fibrosed. Tertiary veins frequently occluded.	Exacerbation of any previous occlusion.

2P SECONDARY PORTAL VEIN	LYMPHOID CELL	ENDOTHELIAL CELL NUCLEUS
3P TERTIARY PORTAL VEIN	EOSINOPHIL	FIBROUS TISSUE COMPONENTS OF VEIN WALL
LP LIMITING PLATE	PLASMA CELL	ABNORMAL FIBROUS TISSUE
S SINUSOIDS	SMOOTH MUSCLE CELL	

TABLE 4

LESIONS AFFECTING THE HEPATIC VEINS IN A PRIMARY *F. HEPATICA* INFECTION OF SHEEP.

Time after Infection	Week 1	Weeks 2 - 12	Weeks 6 - 30
Histological changes	Intramural and subendothelial cellular infiltration identical to that described for the portal system.	Intramural and subendothelial cell infiltration by a mixed cell population of lymphocytes, plasma cells, eosinophils and polymorphonuclear leukocytes in veins close to fluke tracts. Necrosis and lumenal and mural thrombosis of veins in the path of tracts.	Collagen deposition intramurally and subendothelially in veins infiltrated by mixed cell population. Veins also involved with fluke tract organisation and organisation of thrombi.
Veins involved	Occasional central and sublobular.	Central and a few sublobular and hepatic veins.	As in previous column.
Distribution	Focally mainly ventral lobe.	Initially focally all lobes, subsequently most of ventral lobe and focally central, dorsal and caudate lobes.	As in previous column.
Effects	Narrowing of vein lumen; occlusion of sinusoidal connections to central veins.	Moderate occlusion of most veins except where large thrombi had formed when whole lumen could be occluded. Many sinusoidal inlets occluded.	Varying degrees of wall thickening with comparable reductions in lumenal diameter. Severence of sinusoidal connections. Temporary phase of sinusoidal congestion. Progressive fibrosis of vein walls.

Hepatic veins close to recent fluke tracts were infiltrated first by lymphoid cells and then by a mixture of cells which included eosinophils, plasma cells, lymphocytes and polymorphonuclear leukocytes. Organisation of this lesion led to fibrosis of the vein walls, narrowing of the lumen and, in the case of central veins, severence of sinusoidal connections. Central hepatic veins were also involved with fluke tract organisation that produced irregular fibrous thickening of the vein wall and severence of sinusoidal connections (see Figs. 30 and 40, Section II).

Sinusoidal System

Acrylic resin casts

Tensol did not perfuse into many sinusoids in those areas of the livers with abnormal secondary portal veins (Figs. 57 and 58). Where it had the sinusoids were arranged into acinar-like groups (Fig. 57) with few or no inter-connections between similarly arranged sinusoids from adjacent portal veins. Sinusoids supplied by normal secondary veins formed a freely inter-connecting system of vessels.

Histological findings

During the migratory phase of infection considerable destruction of sinusoids took place accompanied by haemorrhage into the tracts vacated by the flukes and the formation of micro-thrombi in intact sinusoids around the tracts. In the latter stages of infection the sinusoids were involved in a series of changes that led to the formation of fibrous septa containing vascular channels. These changes consisted of straightening of the sinusoids and flattening of the cell plates on either side of the sinusoids between, for example, adjacent portal canals. This was followed by degeneration of some of the cell plate hepatocytes and progressive collagen deposition in a perisinusoidal location enveloping apparently a sinusoid. Frequently, sinusoids between adjacent portal canals and hepatic veins were straighter than normal and dilated often to the extent of being varicosed. Fibrous septa developed only infrequently in these sinusoids (see Fig. 52, Section II).

Arterial System

Acrylic resin casts

Both arterial casts showed that in the ventral lobe primary and many secondary arteries were dilated and tortuous (Fig. 62) and ended near the lobe tips in an inter-connecting system of small arteries (Fig. 63). The peribiliary arterial plexus in ventral lobes around the primary bile ducts and in the central lobes around the distributing ducts (Fig. 64) was composed of dilated tortuous arteries. In addition, in the ventral lobes very few sinusoids had been perfused either directly from the tortuous secondary arteries or indirectly via the peribiliary plexus. Portal vein vasa vasorum, normally supplied by the hepatic artery, were not found.

In the central lobes the primary and secondary arteries were much thinner and straighter compared to those in the ventral lobes and were without any terminal inter-connections. Many sinusoids had been perfused from these secondary arteries and from the peribiliary arterial plexuses (Fig. 56). Portal vein vasa vasorum were also present.

Many arteries in the dorsal and caudate lobes were not perfused. This was considered to be due to insufficient Tensol being used. Those arteries that were perfused were similar to the arteries in the central lobe.

Histological findings

Apart from occasional areas of necrosis where fluke tracts impinged upon arterial walls, the most significant changes consisted of progressive intimal and medial hyperplasia and the development of tortuosity and intimal fibrosis. Hyperplasia was first evident at week 8 in the ventral lobe primary arteries. By week 30 many secondary arteries in the ventral lobes and occasional secondary arteries in the central lobe had also developed hyperplasia. Tortuosity was most marked in the ventral lobe primary arteries from week 12 and onwards. Intimal fibrosis was first seen at week 20 but only in occasional primary and secondary arteries in the ventral lobes. During weeks 16 to 30 progressive hyperplasia and the development of tortuosity and intimal fibrosis took place in the small arteries composing the peribiliary plexus around the distended and fibrosed parasitised primary and distributing bile ducts (see Figs. 39 and 44, Section II).

DISCUSSION

The present study has shown that in the ovine liver infected with F. hepatica extensive changes occurred in the intrahepatic vasculature with the development of portal and hepatic vein veno-occlusive lesions, anastomoses formation and distortion of the hepatic arterial system. Many of these changes appeared progressive, had a distinct lobar and regional distribution and affected specific branches of the intrahepatic vasculature.

In this study we found that portal veins near to acute tracts were infiltrated initially by large pyraninophilic lymphoid cells and then by eosinophils and oedema. Later collagen was deposited in the vein walls producing progressively more severe veno-occlusion. There are a number of studies recording portal vein changes in several species infected with F. hepatica. Lymphoid cell infiltration was reported in mice (Lang, 1966) and sheep (Ross et al., 1967a). Eosinophil cell and oedema involvement was described in rabbits (Urquhart, 1956), rats (Thorpe, 1965), cattle (Morrill and Shaw, 1942; Doyle, 1972; Flagsted et al., 1972), pigs (Ross et al., 1967b) as well as sheep (Neiberle and Cohrs, 1967; Dow et al., 1968). Fibrosis was recorded in portal veins in cattle (Doyle, 1972; Flagsted et al., 1972) and was attributed to recannulation of the vessel. While this is a possible sequellae to portal vein thrombosis (Parker and Seal, 1955; Brown and Britton, 1962) the portal vein fibrosis encountered in this study was the outcome of organisation of the vessel wall lesion.

The exacerbation of venous-occlusion by reinfiltration of eosinophils and oedema into fibrosed vein walls near acute tracts in the later stages of infection has not been reported previously; but similar changes were seen in fibrosed portal veins near acute reinfection tracts in Section IV.

The casts of the portal system demonstrated areas where the portal veins had perfused normally, areas where only a few terminal, secondary and tertiary portal veins had been perfused and the presence of porto-portal anastomoses. The areas of reduced portal vein perfusion corresponded in distribution to those areas of the liver where veins were identified histologically as being occluded; namely, most of the ventral lobe and focal isolated areas around fluke tracts in the other lobes. The casts of the portal veins in these areas may

not be a true reflection of portal vein patency *in vivo* because of the distortions resulting from the lack of control over the manual method of injection in addition to the effects of the high viscosity of Tensol and the variability in patency of the veins. However, taken together, the histological results and Tensol casts indicated that infection with *F. hepatica* produced lobar and regional reductions in portal venous supply. This was due largely to fibrosis and occlusion of long and short secondary, terminal and tertiary portal veins. Apart from Doyle (1972), an assessment of the branches of the portal venous system involved with occlusion in fascioliasis has not been undertaken. Doyle (1972), however, used a classification based upon the portal vein in human livers (Elias, 1953) to identify the veins involved in the livers of cattle infected with *F. hepatica*. Our studies (Section I) have shown that such a classification is not applicable to the sheep and possibly also to cattle. Nevertheless, the smaller calibre portal veins in the bovine ventral and caudate lobes were apparently the most severely affected; a situation comparable to that demonstrated in this study.

Two portal vein occlusive lesions similar to the lesion seen in this study have been described in schistosomiasis in man and animals. The most widespread lesion was due to occlusion of portal vein radicles following localisation of schistosome eggs within the vein wall (Lichenberg, 1955, 1969) stimulating the development of a delayed-type hypersensitivity granuloma (Warren *et al.*, 1967). Hussein (1972) working with schistosome-infections in the calf demonstrated a portal vein phlebitis apparently independent of egg reactions. This phlebitis involved infiltration of portal vein walls by eosinophils and oedema followed by fibrosis. Lichenberg (1955) was, however, of the opinion that such changes were stimulated by eggs.

Other portal vein occlusive lesions have been recognised in man but these were due to thrombosis developing secondary to localised infections, e.g., peritonitis and cholangitis, and as a complication of tumour involvement, polycythema vera and cirrhosis (Parker and Seal, 1955; Hales, Allan and Hall, 1959; ten Hove and Leevy, 1973).

The casting technique has been used to investigate portal vein patency in cirrhotic conditions in man. Fibrous tissue had a diffuse constrictive effect on portal vessels in portal (Hales *et al.*, 1959) and fatty cirrhosis (Popper, Elias and Petty, 1952) producing flattened distorted veins. Thrombi in biliary cirrhosis (Hales *et al.*,

1959) and egg granulomatasinschistosomiasis (Cheever, 1969 as quoted by Lichenberg, 1969) produced more focal obstruction. In all these studies porto-portal anastomoses were frequent findings. In schistosomiasis, as with fascioliasis, it is the smaller portal veins which become occluded but details of the types of veins involved and their distributions were not given (Cheever, 1969 as quoted by Lichenberg, 1970).

Since eosinophils have been shown to be attracted to and capable of phagocytosing immune complexes (Litt, 1961, 1964; Archer and Hirsch, 1963) and Parish (1972) has observed the presence of mononuclear cells preceding the accumulation of eosinophils within the peritoneum of the guinea-pig sensitised to attract eosinophils, it is possible that the portal vein lesion is caused by immune-complex deposition in the vein wall. Doyle (1972) and Flagsted et al. (1972) were of a similar opinion; alternatively, Flagsted et al. (1972) suggested that an unspecified toxin or metabolic product may also be involved. However, there is the possibility that the portal vein changes may have developed from an extension of the surrounding portal canal reaction rather than from a specific phlebitis, as the reactions present within the portal canal and portal vein walls were similar at all times.

Central, sublobular and hepatic veins were extensively involved with a number of reactions which led to fibrosis of the vein wall and the development of varying degrees of venous-occlusion. When central hepatic veins were affected sinusoidal inlets were severed and the surrounding sinusoids became temporarily packed with red blood cells. Fibrosis resulted from organisation of the phlebitis and the thrombi induced in veins in the path of the migrating flukes, from infiltration of the vein walls adjacent to acute tracts by a mixed cell population and from involvement of the veins in tract healing. In common with the portal veins, collagen continued to increase in the later stages of infection. Hepatic vein lesions have been reported in several species infected with F. hepatica. Thorpe (1965a), Dow et al. (1967) and Neiberle and Cohrs (1967) recorded eosinophil cells within hepatic vein walls and Urquhart (1956), Thorpe (1965), Dow et al. (1967, 1968) and Doyle (1972) described thrombosis.

Perfusion of the hepatic system with Tensol, while showing flattening of veins and the presence of many hepato-hepatic anastomoses, did not preserve a sufficient number of veins to permit any correlation

to be made with histologically abnormal veins. The poor perfusion of the hepatic system was probably due to the presence of thrombi, venous-occlusion and to the reasons outlined for the portal system.

Although portal and hepatic veins were in the same vicinity of an acute fluke tract the reactions that developed in these veins differed mainly in the composition of their cellular infiltrates and in the lack of thrombi in portal veins. These differences may have been due to the presence of the portal canal connective tissue and any accumulated reactions within the portal canal shielding the portal vein to a large extent from the direct effects of the flukes; whereas, in the case of the hepatic veins the absence of an investing layer of connective tissue exposed the veins to the direct effects of the flukes with the result that phlebitis and thrombosis developed frequently. Similarly, organising fluke tracts were able to involve adjacent hepatic veins directly and produce distortions in the vein walls. In other hepatic diseases, hepatic veins have become involved in the disease processes because of their vulnerability to external reactions. Pressure from tumours, abscesses and nodules of regeneration and constriction due to hepatic fibrosis have all been implicated in producing hepatic vein occlusion (Parker, 1959; Gould, 1968). Using acrylic resin casts the effects of these external lesions have been shown to produce flattened distorted veins and multiple hepato-hepatic anastomoses (Hales *et al.*, 1959).

Unlike in this study, hepatic veins can be involved in primary occlusive disease processes. Fibrosis of hepatic veins leading to venous-occlusion is a feature of the hepatic changes in animals and humans that have ingested plants or products of the plants belonging to the species *Senecio*, *Crotolaria* and *Heliotropium* (Craig, Kearney and Timoney, 1930; Bras and Hill, 1956; Parker, 1959; Hill, 1963; Thorpe and Ford, 1968; Jubb and Kennedy, 1970). Jubb and Kennedy (1970) were of the opinion that occlusion developed as a result of degeneration and collapse of perivenous hepatocytes followed by fibrous replacement. However, Bras and Hill (1956) and Hill (1963) described subendothelial oedema and endothelial cell proliferation preceding the deposition of collagen, and Thorpe and Ford (1968) demonstrated perivenous collagen deposition without any hepatocyte collapse.

Irrespective of the cause in man, hepatic vein occlusion *per se*, if extensive enough, results in rapid liver enlargement, ascites and jaundice (Robbins, 1967). In this study the only visible effects

of hepatic vein occlusion were a temporary phase of centrilobular congestion and an apparent reduction in hepatic venous bed as demonstrated by the cast. In areas of congestion, adequate alternative venous outlets may have been provided eventually by the hepato-hepatic anastomoses that developed in addition to any remaining patent sinusoidal connections to central and sublobular veins (see Fig. 55).

Although sinusoids were extensively destroyed and many were thrombosed during the migratory phase of infection, organisation of the fluke tracts and parenchymal regeneration restored to a large extent the normal parenchymal architecture. Even so, the parenchyma became subdivided by fibrous septa interlinking portal canals and hepatic veins which were seen to contain narrow vascular channels. It was possible that these channels were the histological counterparts of the numerous intervascular connections preserved in the casts. Gelatin injection studies have shown this to be so for the vascular channels present in fibrous septa in cirrhotic human livers (Popper et al., 1952). More recently, Schaffner and Popper (1963) have demonstrated that the sequence of events leading to the formation of these anastomoses involved in the first place deposition of a perisinusoidal basement membrane followed by formation of a collagen wall; a process they called capillarization and one which may have been occurring in ovine fascioliasis. The apparent straightening of the cell plates between adjacent portal canals accompanied by the deposition of perisinusoidal collagen suggests that the channels visible histologically in the infected sheeps' livers were probably derived from sinusoids. Functionally, anastomoses particularly porto-hepatic anastomoses (Rubbin and Popper, 1967; Iber, 1969) were considered to be of significance because they permitted blood to circumvent hepatocytes depriving them of nutrition (Popper and Hutterer, 1969). In the infected ovine liver anastomoses may also have been acting as by-passes around vascular occlusive and parenchymal fibrotic lesions.

Perfusion of sinusoids via abnormal secondary and tertiary portal veins preserved small acinar-like groups of sinusoids and not the freely inter-connecting sinusoidal system seen in the normal liver (Section I). While the outlines of similar acinar-like units have been observed in portal vein perfusion studies of normal rat and human livers (Rappaport, 1958; Rappaport et al., 1966) they occured in even greater numbers in rats given carbon tetrachloride (Rappaport et al., 1966) where they were more distinct due to cell degeneration and death at their peripheries. It is possible that the acinar groups of

sinusoids preserved in the casts were delineated *in vivo* first by the degenerating hepatocytes seen to precede septa formation (Section II) and then by the septa themselves.

Previous workers (Urquhart, 1956; Thorpe, 1965a; Dow *et al.*, 1967; Rahko, 1969; Flagsted *et al.*, 1972; Doyle, 1972) have recorded eosinophil cell infiltration and oedema of hepatic arteries. Such changes were not seen in this study although they were found in sheep reinfected with *F. hepatica* (Section IV). The most significant changes in the ovine hepatic arterial system were dilatation, hyperplasia and tortuosity. Such changes were indicative of increased arterial blood flow (Gould, 1968). The increased blood flow developed possibly to compensate for any reductions in portal supply and/or to overcome the effects of portal canal and parenchymal fibrosis. If the arterial system had been compensating in this way for a reduced portal supply then arterial perfusion of the sinusoids with Tensol should have been increased in those areas of maximum portal vein occlusion, namely the ventral lobe; this was not so. In fact, compared to arterial perfusion of the sinusoids in the relatively normal areas of the liver, i.e., the central lobes, the arterial perfusion of the sinusoids in the ventral lobes was reduced. The most likely explanation for this apparent discrepancy was that the pressure used in perfusing the arterial system was insufficient to overcome the sphincters (terminal sphincters regulate arterial discharge into the sinusoids in the normal liver, Rappaport *et al.*, 1966) of the hyperplastic arteries where as in the normal arteries of the central lobe it was more than adequate. Such *in vitro* differences may not have precluded compensation by the hepatic artery for the portal vein *in vivo*. Sphincter control and any portal vein fibrosis may have prevented perfusion of portal vein vasa vasorum in the ventral lobe. It is possible that the multiple intrahepatic arterial inter-connections preserved in the casts were also aiding intrahepatic blood flow by circumventing fibrotic areas to supply blood to regions of reduced portal flow.

The apparent outcome of portal and hepatic vein occlusion was a reduction in the major and minor routes of portal blood supply and drainage identified in the normal liver (Section I). In addition, as a result of decreased portal supply and increased arterial flow most sinusoidal blood supply was probably transferred from the portal vein to the hepatic artery. Within the sinusoids blood flow was restricted to anastomotic routes and acinar groups of sinusoids.

Finally, since portal and hepatic vein occlusion, anastomoses and increased arterial flow (ten Hove and Leevy, 1967; Gould, 1968; Iber, 1969) can contribute towards elevating portal blood pressure sufficiently to produce portal hypertension, a degree of portal hypertension may be present in cases of ovine fascioliasis. It is possible that the progressive fibrosis of the portal and hepatic veins in the later stages of infection was due to the development of portal hypertension.

SUMMARY

F. hepatica during its migratory phase in the ovine liver produced extensive changes in the intrahepatic vasculature. Portal and hepatic veins and hepatic arteries directly involved with fluke tracts were necrosed and in the case of hepatic veins thrombosed. Sinusoids were extensively destroyed as a result of fluke migration and many became thrombosed.

Portal veins in close proximity to fluke tracts were infiltrated first by lymphoid cells then by eosinophils and oedema and finally collagen was deposited in the vein wall producing venous-occlusion. Terminal, secondary and tertiary portal veins were the most severely affected particularly in the ventral lobe. Lymphoid cells also infiltrated into hepatic veins near acute tracts but were followed by a mixed cell population consisting of lymphocytes, eosinophils, plasma cells and polymorphonuclear leukocytes. Venous-occlusion developed as a result of organisation of this lesion and thrombi and due to the involvement of hepatic veins with healing tracts. Where central veins were involved sinusoidal connections were severed.

The effects of portal and hepatic vein occlusion was to reduce primarily the major routes of sinusoidal blood supply and drainage to the ventral lobes.

Paralleling the development of the above lesions the hepatic artery became hyperplastic, tortuous and intimal fibrosis developed. These morphological changes were indicative of increased arterial flow which may have been compensating for the reduced portal supply. A restructuring of the sinusoids also occurred producing intrahepatic anastomoses which may have been acting as by-pass routes around occlusive lesions. The vascular occlusive lesions, the anastomoses and the arterial changes were suggestive of a degree of portal hypertension developing.

Fig. 54. Effects of F. hepatica infection on the major (➡), minor (→) and anastomotic blood flow routes identified in Section I (see Fig. 2), demonstrating the blood vessels most severely occluded (▶◀).

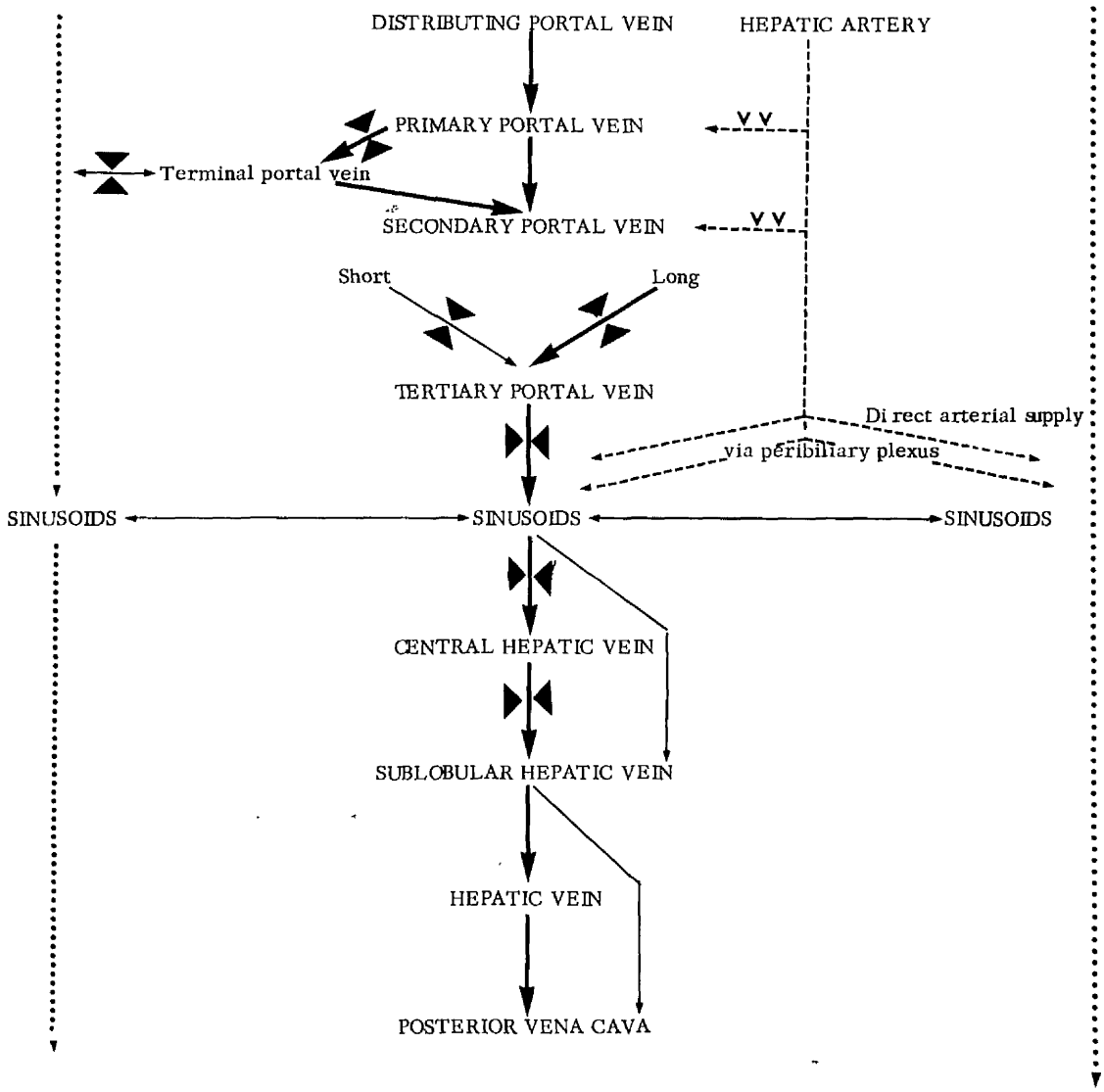

DISTRIBUTING PORTAL VEIN
HEPATIC ARTERY
PRIMARY PORTAL VEIN
Terminal portal vein
SECONDARY PORTAL VEIN
Short
Long
TERTIARY PORTAL VEIN
Di rect arterial supply
via peribiliary plexus
SINUSOIDS
SINUSOIDS
SINUSOIDS
CENTRAL HEPATIC VEIN
SUBLOBULAR HEPATIC VEIN
HEPATIC VEIN
POSTERIOR VENA CAVA

Fig.55. Effects of F. hepatica infection on ovine intrahepatic blood flow routes: occlusion of major ========►, minor - - - - - - - - ► and anastomotic ◄- - - - - -► routes, development of anastomoses ◄═══► and arterial hyperplasia ═══► and possibly a reduction in portal vein vasa vasorum supply - - V V - - ► . Patent sinusoidal connections to sublobular veins may provide drainage routes (—?—►). Compare with Fig. 2 Section 1 and Fig. 54 this Section.

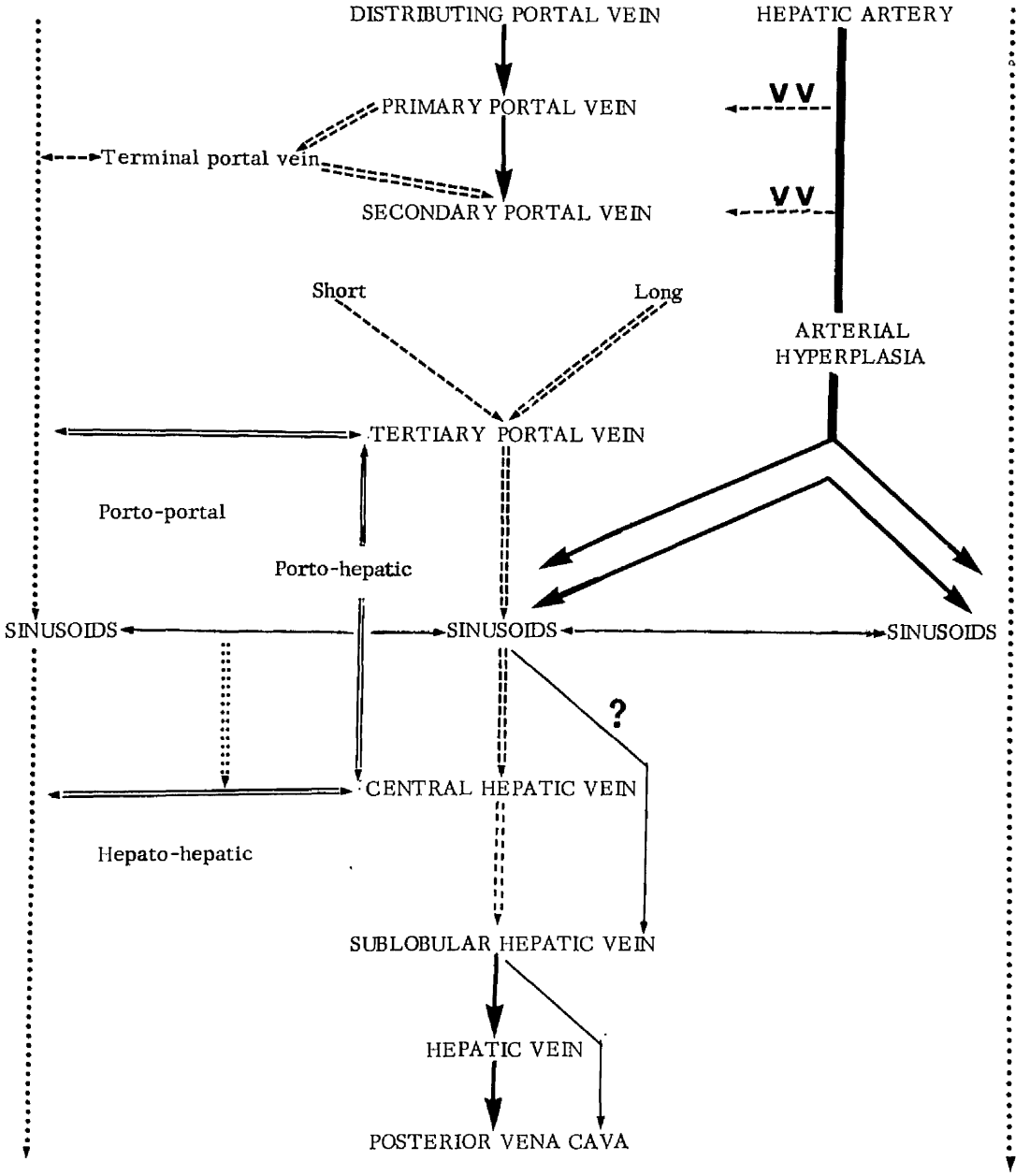

DISTRIBUTING PORTAL VEIN
HEPATIC ARTERY
PRIMARY PORTAL VEIN
Terminal portal vein
SECONDARY PORTAL VEIN
Short
Long
ARTERIAL HYPERPLASIA
TERTIARY PORTAL VEIN
Porto-portal
Porto-hepatic
SINUSOIDS
SINUSOIDS
SINUSOIDS
?
CENTRAL HEPATIC VEIN
Hepato-hepatic
SUBLOBULAR HEPATIC VEIN
HEPATIC VEIN
POSTERIOR VENA CAVA

Fig. 56. Cast of the portal (blue), arterial (yellow) and biliary (red) systems. The ventral lobe primary portal veins are shorter than normal and giving rise to only a few secondary and terminal portal veins. Note the tortuosity of the primary artery (arrow) and the profusion of yellow Tensol-filled sinusoids in the central lobe. Compare with Fig. 3, Section I.

Fig. 57. Cast of the portal system showing a primary portal vein (1P) giving rise to a few long secondary portal veins. A small vein, possibly a tertiary portal vein, is arising from a secondary portal vein and connecting with an acinar-shaped group of sinusoids. Note the absence of short secondary portal veins and compare with Fig. 6, Section I.


Y100

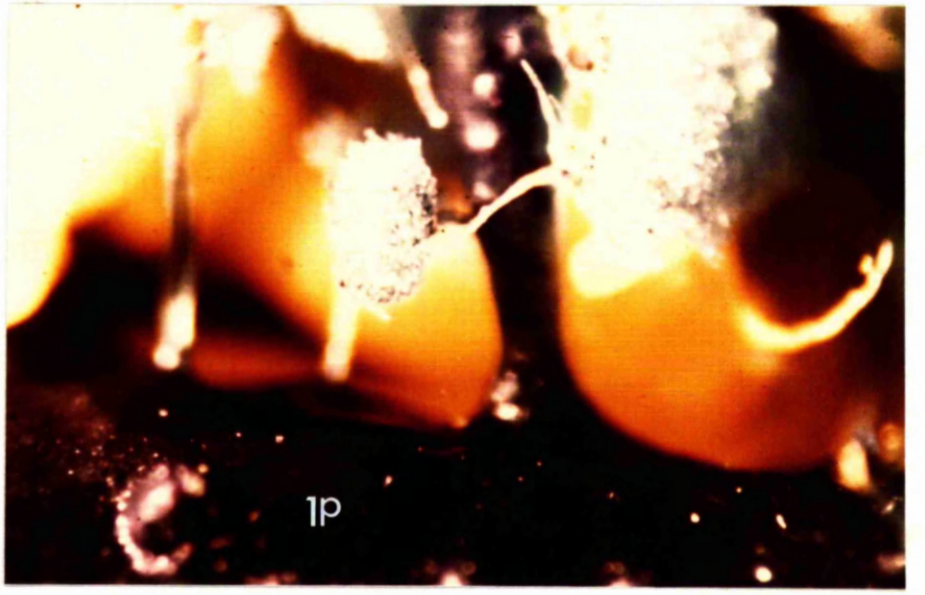
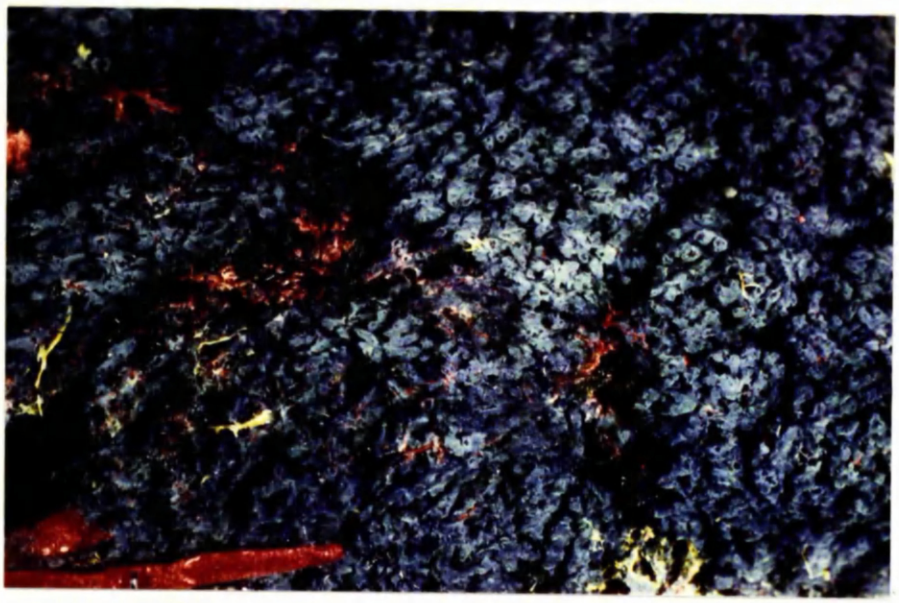

1P

Fig. 58. Cast of the portal system showing focal areas in the central lobe of poor secondary and tertiary vein perfusion surrounded by areas of normal perfusion.

Fig. 59. Cast of the portal system showing multiple, irregularly arranged interconnecting porto-portal anastomoses.



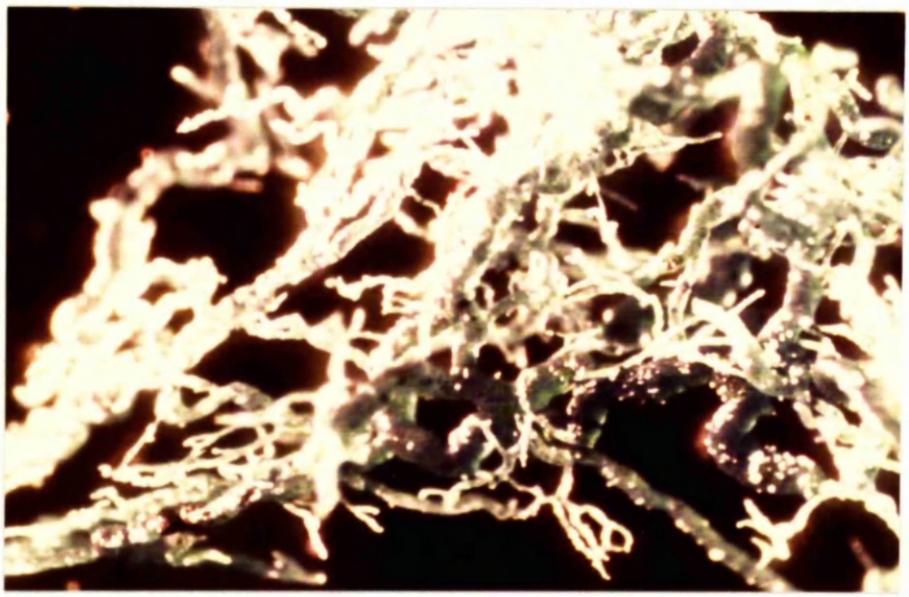
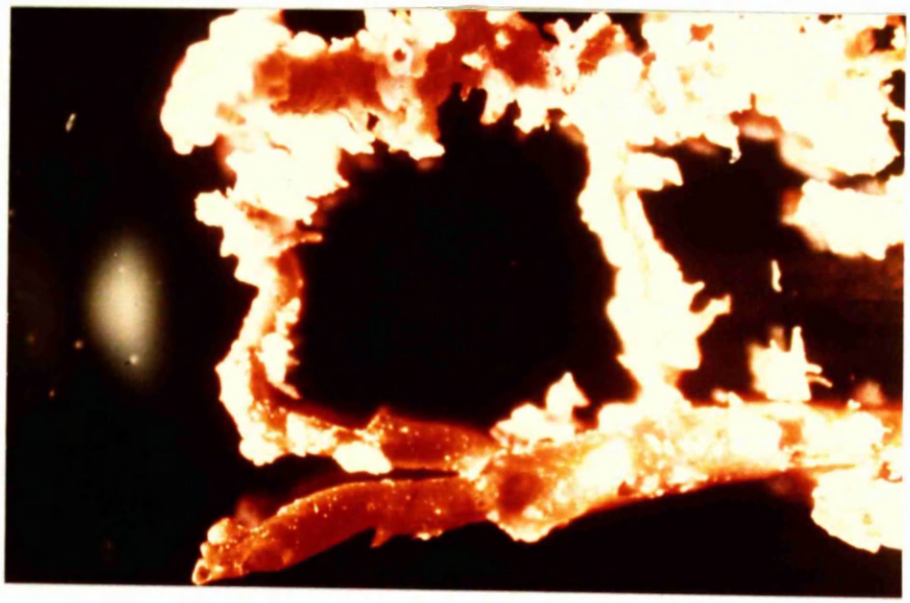


Fig. 60. Cast of the hepatic system showing hepato-hepatic anastomoses with sinusoidal connections.

Fig. 61. Cast of the portal (blue) and hepatic (red) systems interconnected by a porto-hepatic anastomoses.



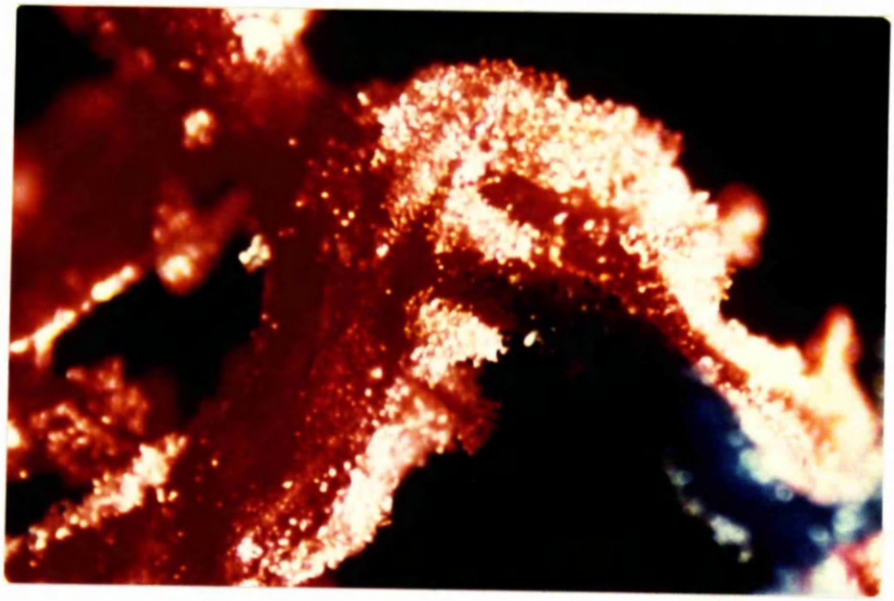
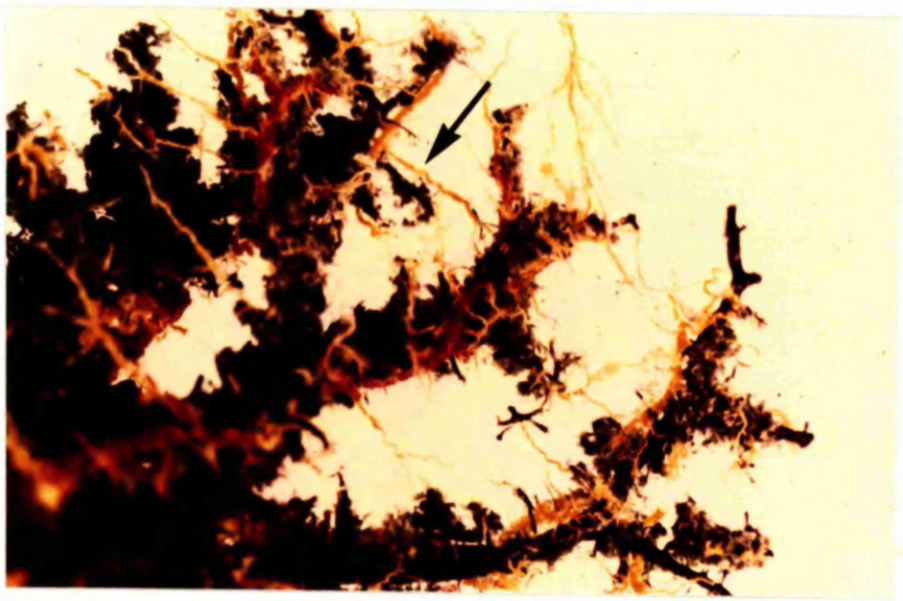


Fig. 62. Cast of the arterial (yellow), portal (blue) and biliary (red) systems. A thickened tortuous artery can be seen paralleling a narrow primary portal vein which has only a few stunted secondary veins arising from it. Compare with Fig. 15, Section I.

Fig. 63. Cast of the arterial (yellow), portal (blue) and biliary (red) systems. Tortuous arteries can be seen together with numerous interconnecting arteries (arrow).



flukes in Groups 1 and 2 migrated into areas minimally or unaffected by the primary infection producing eventually increased and more widespread hepatic damage and fibrosis.

In Section V serum samples collected from the primary infection sheep in Section II and the reinfection sheep in Section IV were screened with standardised liver antigens in AGP, CF and PH tests for auto-antibodies. Normal sheep serum was shown to contain low titres of CF and PH but not precipitating auto-antibodies. The CF and PH titres increased between weeks 3 and 12 of a primary infection of sheep with F. hepatica with a peak being reached at week 6. Upon reinfection the titres increased within the first week and maintained increased but irregular levels for up to 16 weeks, then declined. Liver cell components released during the migrations of the flukes by possibly acting as "antigens" were suggested as stimulating auto-antibody production to aid removal of the cell debris. AGP testing for circulating tissue "antigens" was not successful.

To account for the apparent delay between the first evidence of liver damage and the increases in primary CF and PH titres and titre fluctuations following reinfection, three possibilities were proposed: the destruction of a heat-labile but not stable auto-antibody; the type of auto-antibody produced; the complexing of auto-antibody and "antigen".

STUDIES ON THE PATHOLOGY

OF EXPERIMENTAL OVINE FASCIOLIASIS

By

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A summary of a thesis submitted for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, University of Glasgow Veterinary School, December 1974.

This thesis is divided into 5 sections.

In Section I Tensol casts of the portal, hepatic and arterial systems and histological sections were used in a study of normal ovine hepatic morphology. The morphology of the ovine liver was not markedly different from that of other mammalian species; differences were encountered in the portal and hepatic vasculature and perisinusoidal reticular fibre structure. Based upon the location, sequence of branching and histological characteristics a nomenclature for the division of the portal vascular system was proposed. The names of distributing, primary, secondary (long and short), tertiary and terminal veins were adopted to identify all the portal vein branches. These names can also be applied to branches of the arterial and biliary systems. The same criteria allowed the identification of three hepatic vein branches, central, sublobular and hepatic; the central hepatic veins and to a much lesser extent the sublobular hepatic veins drained the sinusoids. Casts of the arterial system demonstrated an arterial blood supply to the biliary system and to the sinusoids and to the portal veins as vasa vasorum.

The ovine hepatic parenchyma was composed of anastomosing sheets of hepatocytes one cell thick, a fine reticular fibre network and a continuous limiting plate of hepatocytes surrounding portal canals, hepatic cells and lining the capsule of Glisson. Stellate cells were identified.

Section II traced the course of a primary experimental F. hepatica infection in sheep from one week after infection to week

30. The flukes produced a system of necrotic tracts in the parenchyma during their migratory and feeding activities that expanded between weeks 1 and 12 from a few localised areas in the ventral lobe to include most of the ventral lobe and focal areas of the other lobes. In addition, trauma, stress, waste products, ischaema and possibly immunological mechanisms may have contributed towards liver damage in and around these tracts especially during weeks 10 to 12 after infection. Accompanying the formation of these tracts was the development of bile ductules apparently from hepatocytes, portal and hepatic vein occlusion, and capsular thickening and eventually fibrosis.

Organisation of the fluke tracts and accompanying lesions produced post-necrotic, pericellular, monolobular, portal canal and portal and hepatic vein fibrosis. The identifiable stimuli for these fibrotic reactions were dead and dying hepatocytes, iron-laden macrophages, portal canal reactions and possibly in the case of monolobular fibrosis parenchymal expansion due to regeneration.

Following colonisation of the bile ducts a chronic hyperplastic cholangitis ensued. Fluke eggs discharged into the bile ducts may have stimulated cell-mediated egg granuloma which resulted in the destruction of secondary bile ducts.

Section III investigated the effects of a primary F. hepatica infection on the intrahepatic vascular system. F. hepatica during its migratory phase in the ovine liver produced extensive changes in the intrahepatic vasculature. Portal and hepatic veins and hepatic arteries directly involved with fluke tracts were necrosed and in the case of hepatic veins thrombosed. Sinusoids were extensively destroyed as a result of fluke migration and many became thrombosed.

Portal veins in close proximity to fluke tracts were infiltrated first by lymphoid cells then by eosinophils and oedema and finally collagen was deposited in the vein wall producing venous occlusion. Terminal, secondary and tertiary portal veins were the most severely affected particularly in the ventral lobe. Lymphoid cells also infiltrated into hepatic veins near acute tracts but were followed by a mixed cell population consisting of lymphocytes, eosinophils, plasma cells and polymorphonuclear leukocytes. Venous occlusion

developed as a result of organisation of this lesion and thrombi and due to the involvement of hepatic veins with healing tracts. Where central veins were involved sinusoidal connections were severed.

The effects of portal and hepatic vein occlusion was to reduce primarily the major routes of sinusoidal blood supply and drainage to the ventral lobes.

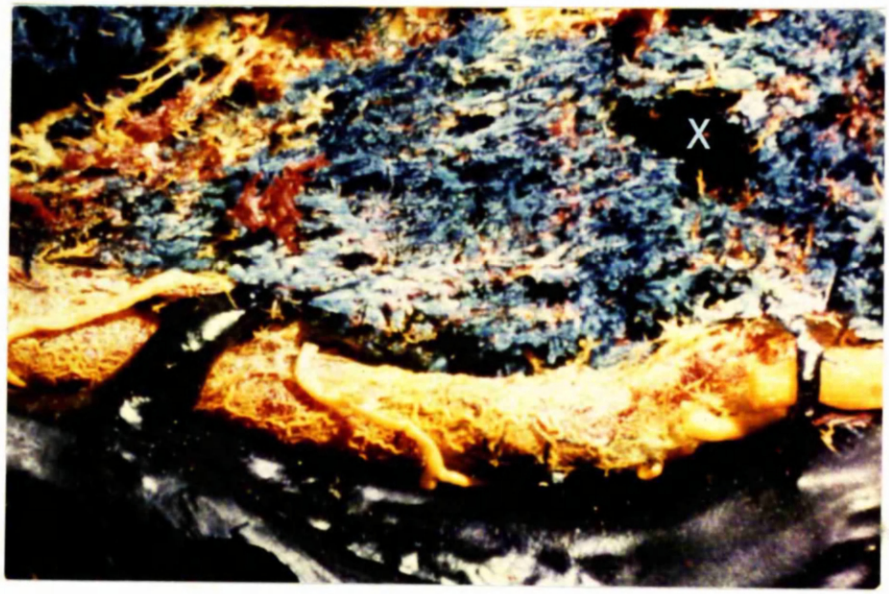
Paralleling the development of the above lesions the hepatic artery became hyperplastic, tortuous and intimal fibrosis developed. These morphological changes were indicative of increased arterial flow which may have been compensating for the reduced portal supply. A restructuring of the sinusoids also occurred producing intrahepatic anastomoses which may have been acting as by-pass routes around occlusive lesions. The vascular occlusive lesions, the anastomoses and the arterial changes were suggestive of a degree of portal hypertension developing.

In Section IV the parasitological, biochemical and pathological consequences of reinfection with F. hepatica in sheep were investigated in 2 Groups of sheep (Groups 1 and 2); Group 1 was treated with anthelmintic to terminate a 10 week primary infection 1 week prior to reinfection and in Group 2 the primary infection was allowed to persist. A third Group, Group 3, was given a primary infection at the time of reinfection to act as challenge controls.

There was not any evidence of acquired resistance to reinfection. Compared to Group 3, the growth rate of the reinfection flukes in Groups 1 and 2 was reduced up to week 12 after reinfection. A delay in the onset of sexual maturity was also observed at week 12 in one sheep in Group 1.

The biochemical results indicated that in both reinfection Groups the flukes in the majority of sheep had a shorter than normal phase of parenchymal migration while a minority had apparently normal phases. In one sheep in Group 1 the flukes had a prolonged and destructive phase of migration that was reflected biochemically in higher and more sustained SGOT levels than in the remaining sheep and on necropsy in the number of acute tracts present. The reinfection

Fig. 64. Cast showing the expanded arterial plexus (yellow) around the distributing bile duct (red) containing numerous small tortuous arteries. Note the perfusion of secondary portal veins and an area where no secondary veins have been perfused (X).


X

SECTION IV

REINFECTION OVINE FASCIOLIASIS

INTRODUCTION

Although under natural grazing conditions sheep are continually exposed to infection with the liver fluke Fasciola hepatica, experimental studies have been confined almost entirely to the disease produced by single infections. Any experimental investigations that have taken place on the reinfection aspects of the disease have concentrated on parasitological and haematological effects with only brief references to pathological results (Sinclair, 1971, 1973).

In addition, preliminary investigations in Glasgow have indicated that the hepatic damage produced as a result of reinfection of sheep with F. hepatica was greater than would be expected from the superimposed effects of one fluke population upon another and suggested that a variety of mechanisms were operative in the pathogenesis of reinfection in cases of fascioliasis.

Thus, this study was designed to further investigate the pathological, biochemical and parasitological consequences of reinfection of sheep with F. hepatica.

MATERIALS AND METHODS

Experimental design

Sixteen sheep were randomised into two groups (Groups 1 and 2) and inoculated orally with 400 F. hepatica metacercariae. Two sheep (R24 and R27, see Appendix 1, Table 3) died two weeks after inoculation from uroliathiasis. Ten weeks after inoculation the seven remaining sheep in Group 1 were treated with rafoxanide at 7.5 mg per kg live body weight. One week later, one sheep from Group 1 was slaughtered to assess the effectiveness of the anthelmintic treatment (anthelmintic control) and one sheep was slaughtered from Group 2 to provide an estimate of the level of the fluke burdens established by the 400 metacercariae (infectivity control). At the same time the remaining sheep in both groups were reinoculated orally with 400 metacercariae. One sheep from each group was slaughtered at weeks 1, 2, 6, 12, 20 and 30 after challenge. Since the same batch of metacercariae were used to establish the primary infection in Section II and the reinfection of this Section, the parasitological results of the

sheep slaughtered at weeks 1, 2, 6, 12, 20 and 30 in Section II are included in this Section to act as challenge controls (Group 3).

In the results and discussion, all times of slaughter are given in weeks after reinoculation. To convert to the primary inoculation time scale add 11 weeks.

Parasitological techniques

Recovery of F. hepatica burdens

F. hepatica burdens were recovered and the mean length, SE, range and percentage of immatures determined for each sheep as described in the general materials and methods.

Faecal egg counts

Faecal samples were collected at regular intervals from the sheep in Groups 1 and 2 after primary and secondary inoculations. The samples were first screened for fluke eggs using the McMaster technique (Gordon and Whitlock, 1939). The number of eggs found was multiplied by 50 and expressed as eggs of faeces. In those samples where eggs were absent the more sensitive sedementation technique of Happich and Boray (1969) was used. Eggs detected by this method were counted and enumerated up to 25 and samples in excess of this number were written as having more than 25 but less than 50.

Pathological techniques

These were as described in the general materials and methods.

Biochemical estimations

Sheep were bled at regular intervals after the primary inoculation and reinoculation and their SGOT levels estimated as described in the general materials and methods.

RESULTS

Pathology

Initially, in both reinfection groups the gross and histological lesions produced by the primary infection and by the reinfection could be distinguished. By week 12, however, this was not possible. The histological findings are, therefore, described as

those attributable to the primary infection, or to reinfection or to those of indistinguishable origin. The gross findings are described as a whole.

In Section II (Group 3) a description was given of a primary F. hepatica infection in sheep. Therefore, in this Section only differences evident between that infection (Group 3) and the reinfections (Groups 1 and 2) induced in this Section will be described. In addition, any divergencies that developed between the two reinfection groups will be noted.

Gross findings

Reinfection flukes departed from the migratory pattern seen in Group 3 and produced liver damage in previously minimally affected areas. In addition, there were notable differences between the two reinfection groups at all stages of reinfection. Initially, this was due to the absence of the primary infection in Group 1, then to divergencies in reinfection fluke migration and, finally, to organisation of primary and secondary infection damage.

In Group 1 in the anthelmintic control sheep and the sheep slaughtered at weeks 1, 2, 6 and 12, the bile ducts were not distended nor fibrosed. The ventral lobes of these sheep while being fibrosed were not severely so nor were they particularly reduced in size. In the equivalent sheep in Group 2 the changes present were characterised by distended, fibrosed bile ducts containing adult flukes and small fibrosed ventral lobes with deep irregularly fissured lobar surfaces. Exceptions to these patterns were seen in both Groups; the livers of the sheep slaughtered at week 2 in Group 1 and the control sheep in Group 2 (as the low F. hepatica burden would indicate - see Appendix 1, Table 1) were less severely affected than the livers of the other sheep in their Groups.

In addition to the above changes in Groups 1 and 2, acute and healing tracts were identified. These were considered in the control and week 1 and 2 sheep to be due to the primary infection and in the week 6 and 12 sheep the reinfection (Figs. 65, 66 and 67). The primary infection tracts were similar in morphology and distribution to the primary infection of Group 3. By contrast, the reinfection tracts in both Groups, although mainly confined to the ventral lobes at week 6, were found at week 12 extending in to all lobes. Moreover, in Group 1 at week 12 many of these reinfection tracts were

large and haemorragic (Figs. 66 and 67) while only a few were so in Group 2 (Fig. 65).

The combined effects of the first and second infections in Group 2 at week 17 (Fig. 68 and see Appendix 1, Table 1) and week 30 produced fibrosis of all lobes with the ventral lobes being more severely affected than in Group 3. All lobe surfaces were deeply and irregularly fissured and nodules of regeneration were identified in the central and dorsal lobes. Fibrosis of the ventral lobes had produced small contracted lobes and the presence of nodules of regeneration slightly enlarged central and dorsal lobes. The bile ducts were markedly distended, varicosed and heavily fibrosed. In Group 1 at weeks 20 (Fig. 69) and 30 reinfection flukes, while being located in the bile ducts, had not produced marked biliary distension nor fibrosis. However, all liver lobes, particularly the ventral lobes, were more diffusely fibrosed than in Group 3. The ventral lobes were only marginally reduced in size and the other lobes were of normal size.

Histological findings

Lesions attributable to the primary infection

The range and distribution of lesions produced by the primary infection in Group 2 between weeks 0 and 6 after reinfection were equivalent to the lesions seen during weeks 10 to 16 of the primary infection in Group 3. The low F. hepatica burden (see Appendix 1, Table 1) of the infectivity control sheep produced only mild liver damage and fibrosis.

One prominent and unusual feature at week 1 was a large irregularly shaped area of infarction. This area contained sheets of necrotic hepatocytes surrounding portal canals enclosing necrosed bile ducts and thrombosed portal veins and hepatic arteries. Immediately surrounding the infarcted area was a zone of macrophages and fibroblasts intermingled with lymphocytes and plasma cells.

During the first 6 weeks after reinoculation in Group 1, while there was an equivalent range of primary infection lesions with similar distributions to Groups 2 and 3, there were differences in severity. Compared to Groups 2 and 3, organisation of the primary infection tracts in Group 1 was proceeding more rapidly with less post-necrotic collagen being deposited. The fibrous septa interlinking these tracts to adjacent portal canals, hepatic veins and capsule were

thinner and less numerous. In addition, portal canal, portal and hepatic vein and capsular fibrosis in Group 1 were less severe. Although live primary infection flukes were not seen in the bile ducts, that they had been there was evident from the slight hyperplasia and erosion of the biliary epithelium of the distributing and primary bile ducts. These ducts were not fibrosed but within the lamina propria were many lymphocytes, plasma cells, eosinophils and occasional small lympho-reticular nodules.

At week 6 in Group 1, located in the primary and terminal bile ducts (Fig. 70) and lying free within the parenchyma (Fig. 71), were irregularly-shaped pale pink structures identifiable as dead primary infection flukes by their large size and ghost-outline remnants of reproductive and digestive organs; thus, confirming that the primary infection became established but was killed by anthelmintic treatment. Surrounding the dead flukes was a narrow inner layer of active macrophages and giant cells, a wider intermediate layer composed of variable numbers of eosinophils, lymphocytes and plasma cells and an outer mononuclear cell-rich layer containing lympho-reticular nodules and strands of collagen. Where the dead flukes were located in the parenchyma the peripheral cellular reaction had replaced large areas of hepatocytes (Fig. 71). In the case of dead intra-biliary flukes, whole or part of the bile duct wall had been destroyed (Fig. 70) and the accumulating cells had surrounded the flukes and spread out into the surrounding portal canal and parenchyma. Globule leukocytes packed the intact areas of biliary epithelium around the dead flukes. One encapsulated inspissated fluke remnant was identified lying free in a primary bile duct at week 12. Thereafter dead flukes were not positively identified.

Lesions attributable to the reinfection

In both reinfection Groups, acute and healing reinfection tracts were identified at weeks 2 (not Group 2 - see Appendix 1, Table 1), 6 and 12. The morphology of these tracts and their associated portal canal, portal and hepatic vein and parenchymal reactions up to week 6 in Group 1 and week 12 in Group 2 were similar to the primary infection of Group 3. The distribution of the tracts was as stated in the gross findings. Where acute reinfection tracts had passed close to portal veins fibrosed as a result of the primary infection, any venous-occlusion present was exacerbated by the reinfiltration of eosinophils and oedema. Compared to the primary infection in Group 3

there were many more lympho-reticular nodules within the portal canals and adjacent to healing tracts at week 12 in both reinfection Groups.

At week 12 in Group 1, the numerous acute tracts identified grossly were seen histologically to be disproportionately larger than the flukes. Once vacated by the flukes the tracts became packed with red blood corpuscles and eosinophils. Portal canals and portal veins adjacent to these tracts were more oedematous and heavily infiltrated with eosinophils than at the same time in Group 3. As a result, portal vein occlusion was invariably severe especially in veins already fibrosed. The walls of many secondary arteries contained within these canals were also oedematous and very occasionally eosinophils were seen subendothelially. Primary arteries, mainly in the ventral lobe were hypertrophied and tortuous. Lympho-reticular nodules were commonly seen next to healed tracts and in the portal canals. Occasional small live flukes were sectioned in slightly distended but not fibrosed bile ducts surrounded by hyperplastic epithelium, cell debris and mucin but not fluke eggs.

Lesions produced by the first and second infections

In both Groups the combined effects of the first and second infections were evident at weeks 12, 20 (17 in the case of Group 2 - see Appendix 1, Table 1) and 30. At these times there was an equivalent range of lesions to the primary infection of Group 3 but there were differences in severity and distribution. Moreover, there were divergencies between the two reinfection Groups in the severity of the parenchymal, portal canal and peribiliary fibrosis. Reinfecting without treating increased considerably the amount and distribution of all the types of fibrosis identified in Section II. Treating before reinfecting resulted in only a marginal increase in amount but a significant increase in distribution of hepatic fibrosis due mainly to post-necrotic fibrosis in all liver lobes.

Healing reinfection and healed tracts of unknown origin were located in all the lobes of the two reinfection Groups with the ventral lobes containing most. Interlinking these tracts were irregularly-orientated septa that in Group 2 were similar in morphology to the primary infection of Group 3 but in Group 1 they were much thinner and relatively acellular. Monolobular fibrosis (Section II) was an infrequent finding in both reinfection Groups. By contrast with the primary infection in Group 3 the portal canals, hepatic veins

and areas of the capsule of Glisson to which the septa interlinked were more severely fibrosed in Group 2 and slightly less severely fibrosed in Group 1. The difference in portal canal fibrosis was due mainly to the presence in Group 2 of enlarged heavily fibrosed bile ducts whereas in Group 1 while the ducts were enlarged they were minimally fibrosed. In the portal canals of both Groups were many lympho-reticular nodules, fibrosed portal veins and thickened hyperplastic arteries. Lympho-reticular nodules were plentiful next to healing and healed tracts in both reinfection Groups. At week 30 in Group 1, an encapsulated irregular-shaped eosinophilic structure larger than healing and healed fluke tracts was seen which could possibly have been the remains of a dead fluke.

Parasitological findings

F. hepatica burdens

From each sheep the total number of flukes recovered, the percentage recovery together with their mean lengths, standard errors, range in size and percentage are given in Appendix 1, Table 1. These results show that anthelmintic treatment of the sheep in Group 1 with rafoxanide was completely effective in removing all the flukes established from the primary infection. However, the presence of pathological lesions characteristic of a primary fluke infection (see pathology results) demonstrated that these sheep had been infected. In Group 1 it was not technically possible to recover flukes from the sheep slaughtered at weeks 1 and 2 after the second infection. However, early necrotising tracking lesions in these sheep indicated the presence of migrating parasites (see pathology results).

The mean number of flukes established as a result of reinfection of the sheep in Group 1 and killed at weeks 6, 12, 20 and 30 was 81$\pm$19.2mm (Appendix 1, Table 1). This was lower but not significantly so than that, 138 $\pm$ 22.8,obtained from the sheep in Group 3 which received the same number and batch of metacercariae as a primary infection. This difference was marked at week 30 when the number of flukes recovered from the sheep in Group 1 was 30 and in Group 3, 197. There was a significant difference ($p < 0.001$) in the mean length of the flukes established at week 12 between Group 1, 8 mm, and Group 3, 18.4mm (Appendix 1, Table 1).

In Group 2, the sheep sacrificed to act as an infectivity control had a relatively low burden of 30 flukes. However, the fluke

burdens present at weeks 1 and 6 were 72 and 150 respectively and were identified on their mean lengths as originating from the primary infection. Thereafter, the presence of flukes resulting from the reinfection became apparent in that increased numbers were recovered (171, 241 and 230 compared to 30, 72 and 150). Moreover, a decrease in mean length of the total fluke population occurred at week 12 due to the influence of smaller flukes developing from the reinfection (Appendix 1, Table 1).

The differences in length between the fluke populations of the 3 Groups at weeks 6, 12 and 20 are highlighted when their lengths are plotted in histogram form (Figs. 72, 73 and 74). Thus, in Groups 1 and 2 at week 12 the reinfection flukes were considerably shorter than the equivalent 12 week primary infection in Group 3 (Appendix 1, Table 1 and Fig. 73). Thereafter, the lengths of the flukes in the 3 Groups became similar (Appendix 1, Table 1 and Fig. 74).

Faecal egg counts

The mean egg counts for the 3 Groups are given in Appendix 1, Table 2. The presence of eggs at the time of challenge in Group 2 and their absence at this time in Group 1 confirmed the efficacy of treatment in terminating the primary infection. While eggs appeared in the faeces at approximately the same time in Groups 1 and 3, it was significant that one sheep in Group 1, R21 (see Appendix 1, Table 2), remained negative up to its time of slaughter at week 12. Such a finding was consistent with 98% of the fluke population being immature in this sheep (Appendix 1, Table 1) and the absence, histologically, of eggs in the bile ducts (see pathology results).

Biochemical results

The individual and mean SGOT results for all Groups are shown in Table 2, Section II and Appendix 1, Tables 3 and 4. Following the primary inoculation of Groups 1, 2 and 3 the SGOT levels increased from less than 100 S-F units to a maximum at week 9 of 397 $\pm$ 33 in Groups 1 and 2 and 191 $\pm$ 34 in Group 3 (Table 2, Section II, Appendix 1, Table 3 and Figs. 75 and 76). In Group 3 the week 9 peak was followed by a decline to approximately 100 S-F units at week 12. In Group 1, terminating the primary infection at week 10 followed by challenging at week 11 resulted in a comparable decline in SGOT levels which then gradually but slightly irregularly increased to a

maximum of 233 ± 90 at week 9 post-challenge (Fig. 76).

Inspection of the results of Group 1 (Appendix 1, Table 4) show considerable variation with peak values occurring in sheep at weeks 4, 6, 9 and 10 after reinfection. The week 9 peak and the SGOT levels that followed it were notable for their extremely high values.

Following challenge in Group 2 there was a slight fall in mean SGOT values followed by a rise to maximum levels of 195 ± 38 and 191 ± 21 S-F units at weeks 3 and 4 (Appendix 1, Table 4 and Fig. 76). Thereafter, a gradual reduction in mean levels was seen to less than 100 S-F units at week 12 post-challenge. All the sheep in this Group had relatively high levels at weeks 3 and/or 4 post-challenge. Except for one sheep the individual SGOT levels decreased after week 4. In this one sheep the SGOT level rose to a maximum at week 10 which was only slightly in excess of previous levels.

The differences between the mean peak SGOT levels of the reinfection Groups at weeks 4 and 9 and between the means of Groups 2 and 3 at week 9 were not statistically significant ($p > 0.05$).

DISCUSSION

Based on the F. hepatica burdens recovered from both reinfection regimes compared to the numbers recovered from Group 3 there was not any evidence of an acquired resistance to reinfection. However, in Group 1 the number of reinfection flukes established was lower but not significantly so than in Group 3. Also, by week 30 after reinfection in Group 1 there appeared to have been some loss of fluke burden compared to the control sheep in Group 3. Since these observations were based upon single animals no conclusions can be drawn. Sinclair (1973) also found a reduced take although not apparently significant in sheep treated and reinfected with F. hepatica. The absence of a demonstrable resistance to reinfection was compatible with the results recorded by Sinclair (1971, 1973) and provided further support for the view that sheep are highly susceptible to reinfection with F. hepatica (Ross, 1967).

When the rate of growth and maturation of the reinfection flukes was considered at week 12, the flukes in Groups 1 and probably 2 were found to be retarded in length and maturity. The retardation in maturity in Group 1 was conclusively demonstrated when 98% immature reinfection flukes were recovered and F. hepatica eggs were not

detected either in the faeces or histologically in the bile ducts. An immune reaction (see later) directed against but unable to kill the parasites may have been responsible for the delay in growth rate and onset of maturity. Retardation of growth rate to a degree comparable to that seen in this study between 6 and 12 weeks after reinfection has been observed previously in reinfected and treated and reinfected sheep (Boray, 1967; Sinclair, 1971, 1973). This retardation was attributed to hypersensitivity reactions established as a result of reinfection. In addition, following heavy primary infections in sheep (Ross, 1967 ;Boray, 1967; Roberts, 1968) retardation of growth rate was observed before week 6 and continuing after week 12. Competition between the parasites for nutrients was considered to be the cause in these sheep.

SGOT levels have been shown to be an accurate index of liver damage (Thorpe, 1965b) and their increase, therefore, in F. hepatica infections reflects the parenchymal migratory activities of the flukes. In this study, because of the small number of sheep used and the variability in individual maximum SGOT levels in Groups 1 and 2 it was not possible to come to any hard and fast conclusions on reinfection fluke migratory activity. However, if one considers individual results correlated with pathology, certain trends become apparent. In Group 1 the sheep slaughtered at week 12 had high SGOT levels at weeks 9, 10 and 12 which were consistent with the finding at necropsy of large numbers of acute haemorrhagic tracts and numerous flukes still in the parenchyma. Since at these weeks in a primary infection the SGOT levels while elevated were considerably lower and few flukes are located in the parenchyma (Section II), the reinfection flukes in the above sheep appear to have had a prolonged period of migration. By contrast, as most of the sheep in Groups 1 and 2 had individual maximum SGOT levels earlier (3-7 weeks) than in a primary infection (week 9), the parasites in these sheep had perhaps shorter than normal periods of migration. Departures from the normal length of migration by reinfection flukes have been recorded previously in sheep (Sinclair, 1970, 1973). Based upon later and earlier than normal rises in serum enzyme levels and onset of anaemia and clinical symptoms, longer (Sinclair, 1970) and shorter (Sinclair, 1973) periods of migration have been deduced as occurring. Likewise, contracted and protracted migratory periods have been observed in reinfected mice (Lang, 1967) and cattle (Ross, 1967), respectively.

In addition to having migratory periods of varying lengths,

the reinfection flukes in both Groups were seen by week 12 to have migrated into areas of the liver minimally affected by primary infections; namely, the central, dorsal and caudate lobes (Section II). Moreover, the acute reinfection tracts in Group 1 were unusual in that they were larger and more haemorrhagic than could be accounted for by direct trauma from the reinfection flukes as they were about half their normal size. Histologically, these reinfection tracts were associated with intense eosinophil cell and diffuse oedema reactions in adjacent portal canals. The reasons for these gross and histological changes were not immediately apparent but haemorrhage has been reported as a feature of the disease process in primary infections after week 6 (Dow et al., 1968; Section II) and in infected and immunosuppressed sheep (Sinclair, 1968). Under these conditions the haemorrhage was attributed largely to the effects of avid feeding and rapid growth of the flukes (Section II). Because the flukes were stunted at week 12 in this study factors other than growth rate must have been responsible for the haemorrhage. One possibility was that liver damage around the fluke tracts was enhanced by the formation and deposition of immune complexes containing fluke antigen. This type of liver damage has been suggested (Sinclair and Poyner, 1974) as occurring to explain the rise in SGOT levels that followed the injection of fluke antigen into the mesenteric veins of fluke-infected rabbits. The presence of immune-complexes could also have accounted for the intense eosinophil cell reaction (Litt, 1964).

The outcome of reinfection in both Groups was to increase the severity and distribution of hepatic fibrosis. In Group 2, this was due to an increase in all the types of fibrosis identified in Section II while in Group 1 post-necrotic fibrosis was mainly responsible. This difference in fibrosis between the two Groups was the result of anthelmintic treatment eliminating the primary infection in Group 1, thereby allowing resolution of some primary infection damage. Nevertheless, hepatic fibrosis was increased following reinfection in this Group. Therefore, in the field, with continuing infections even if eliminated by repeated anthelmintic treatments hepatic fibrosis will, in all probability, accumulate producing progressively more severe liver fibrosis. This is what appears to have happened in the naturally and repeatedly infected and treated sheep studied by Irfan and Lee (1968) which had severe liver fibrosis.

Fibrosis of the ventral lobes as a result of the primary infection was possibly responsible for deviating the flukes to the

other lobes. In cattle (Dow et al., 1967) and in pigs (Ross et al., 1967b)it has been suggested that fibrous tissue has been shown to restrict fluke migration (see Introduction to this thesis, pages 5 and 6). Similarly, capsular fibrosis developing from the primary infection may have caused the flukes to seek less fibrous areas over the central, dorsal and caudate lobes before penetrating. Once established in the parenchyma, cholangitis and peribiliary fibrosis (Boray, 1969) have been suggested as hindering the entry of flukes to the bile ducts thus prolonging their migrations. Any peribiliary fibrosis and cholangitis developing from the primary infection may, therefore, have been the cause of the prolonged migrations in R21 (see Appendix 1, Table 1). However, this seems unlikely because most of the reinfection flukes appeared to have vacated the parenchyma faster than normally in most of the remaining sheep in this Group. Even in Group 2, where the bile ducts were more severely inflamed and heavily fibrosed than in Group 1, a more rapid period of migration appeared to have occurred.

There is now strong evidence from cell and serum transfer studies that both humoral and cell-mediated mechanisms are involved in the acquisition of resistance to F. hepatica (Corba, Armour, Roberts and Urquhart, 1967; Armour and Dargie, 1974). Although there was not any evidence of an acquired resistance in sheep to reinfection the increase in the numbers of lympho-reticular nodules following reinfection and the reactions provoked by the flukes killed by anthelmintic treatment indicated that immunological mechanisms were operative. In infections with the lung worm, Dictyocaulus viviparous, lympho-reticular nodules and dead worm reactions have been used as morphological markers of a hosts immunological status (Jarrett and Sharp, 1963; Pirie, Doyle, MacIntyre and Armour, 1970). Since following vaccination and repeated challenge the calves developed a good resistance and pulmonary lympho-reticular nodules considerably increased in numbers and were often associated with dead lung worms, Pirie et al. (1970) concluded that resistance to D. viviparous was likely to be related to the number of nodules that developed. In this study the increased number of nodules was highly suggestive of an enhanced immunological response which was possibly enhanced further by the dead fluke reactions. The deviations in migration and the retardation of fluke growth rate may, therefore, have been manifestations of this enhanced immunological response. This conclusion was compatible with Sinclair's (1973) and Lang's (1967) suggestion of a state of hyper-

sensitivity being induced upon reinfection in sheep and mice respectively. Further evidence demonstrating the controlling influence the hosts immunological system has upon fluke development was provided by Sinclair (1968, 1970) when he immunosuppressed sheep; the flukes in these sheep had faster than normal rates of growth.

Finally, this study provided evidence for an elevated immunological response which may have been responsible for the abnormal growth and migratory patterns of reinfection flukes. In individual treated sheep acute liver damage was produced that was more severe than expected from the superimposition of one fluke population upon another. In view of the common use of anthelmintics in the field similar deviations in migration and liver damage could result with possible important consequences. Such a situation may have been present in sheep kept on fluke-infested pastures that were repeatedly treated but still died and at post-mortem were found to have haemorrhagic livers harbouring relatively small numbers of parasites (Reid, Armour and Jennings, 1970).

SUMMARY

The parasitological, biochemical and pathological consequences of reinfection with F. hepatica in sheep were investigated in 2 Groups of sheep (Groups 1 and 2); Group 1 was treated with anthelmintic to terminate a 10 week primary infection 1 week prior to reinfection and in Group 2 the primary infection was allowed to persist. A third Group, Group 3, was given a primary infection at the time of reinfection to act as challenge controls.

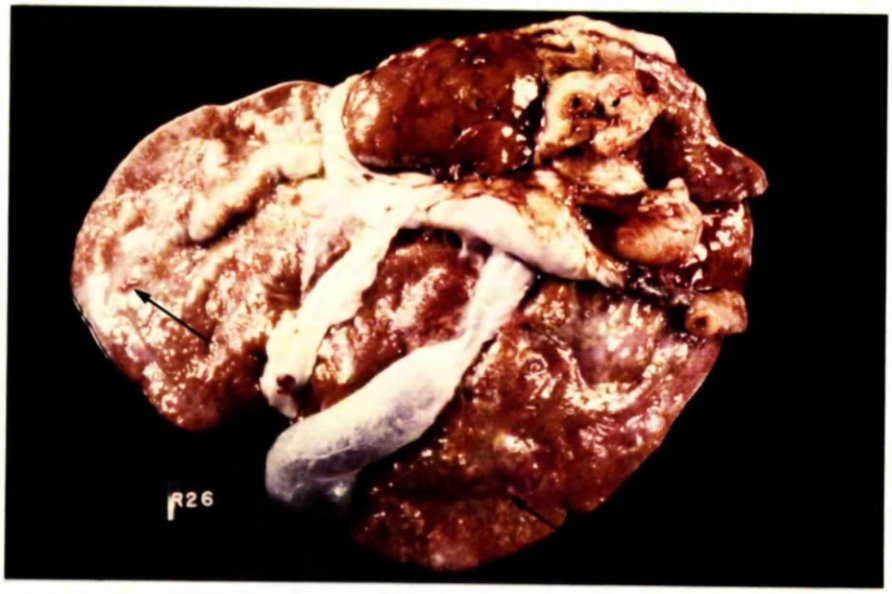
There was not any evidence of acquired resistance to reinfection. Compared to Group 3, the growth rate of the reinfection flukes in Groups 1 and 2 was reduced up to week 12 after reinfection. A delay in the onset of sexual maturity was also observed at week 12 in one sheep in Group 1.

The biochemical results indicated that in both reinfection Groups the flukes in the majority of sheep had a shorter than normal phase of parenchymal migration while a minority had apparently normal phases. In one sheep in Group 1 the flukes had a prolonged and destructive phase of migration that was reflected biochemically in higher and more sustained SGOT levels than in the remaining sheep and on necropsy in the number of acute tracts present. The reinfection

flukes in Groups 1 and 2 migrated into areas minimally or unaffected by the primary infection producing eventually increased and more widespread hepatic damage and fibrosis.

Fig. 65. Group 2, Week 12: In all the lobes are many healing and only an occasional acute haemorrhagic tract (arrows).

Fig. 66. Group 1, Week 12: Numerous acute haemorrhagic tracts (arrows) of varying size can be seen in all the lobes. Compare with Fig. 25, Section II and Fig. 65 of this Section.


R26

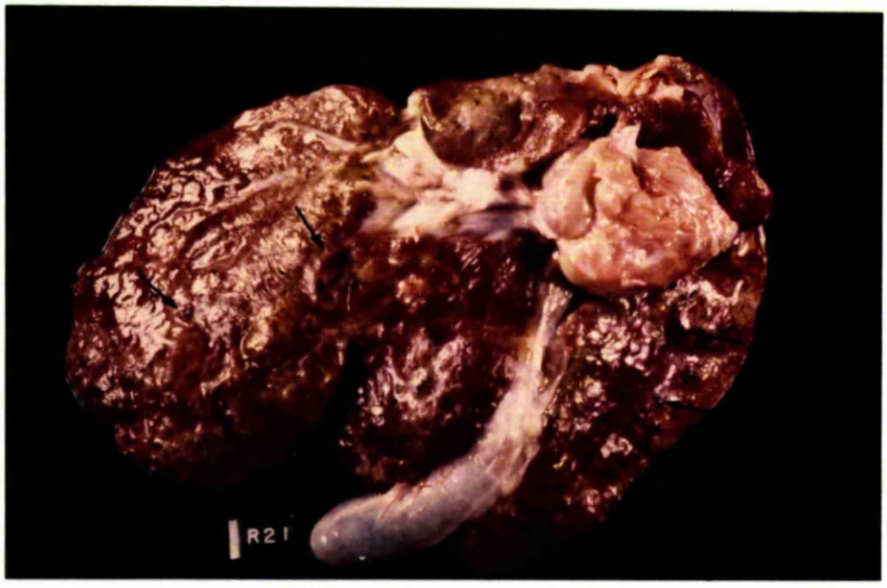

R21

Fig. 67. Group 1, Week 12: Closer view of a large acute haemorrhagic tract in the ventral lobe.

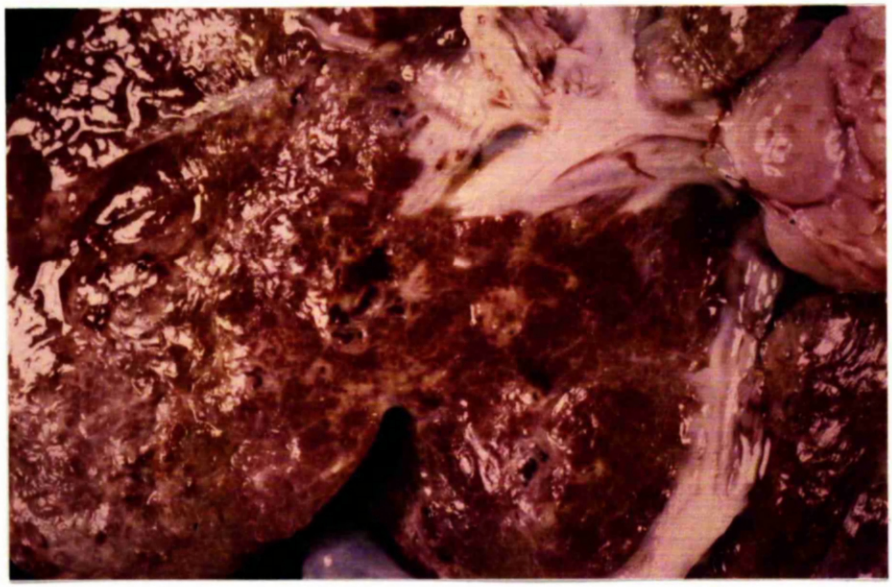
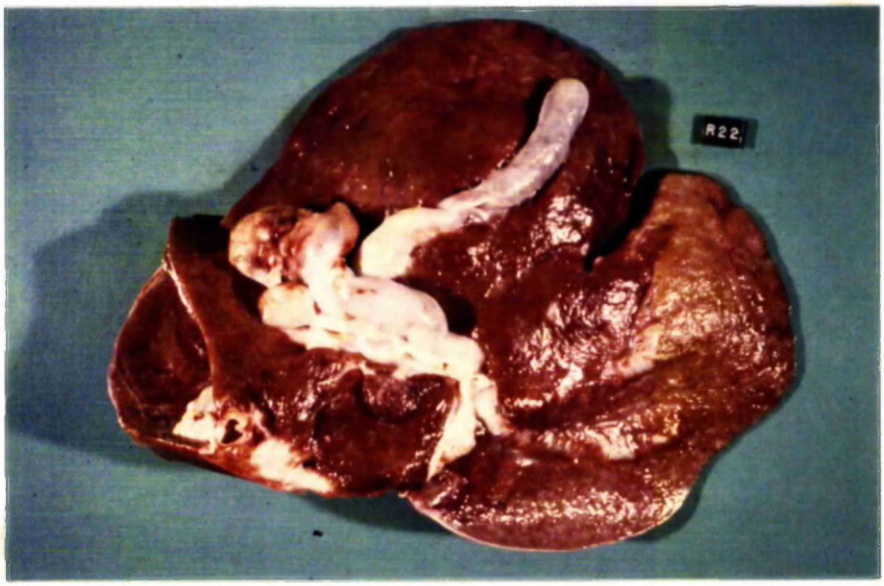


Fig. 68. Group 2, Week 17: A diffusely pale liver (indicative of copper poisoning) with a small contracted ventral lobe, distended bile ducts and nodules of regeneration in the central and dorsal lobes. Compare with Fig. 41, Section II.

Fig. 69. Group 1, Week 20: This liver has diffuse but not deep surface fissuring of all lobar surfaces, slight distension of its distributing and ventral lobe primary bile ducts and a marginal reduction in ventral lobe size. Compare with Fig. 41, Section II.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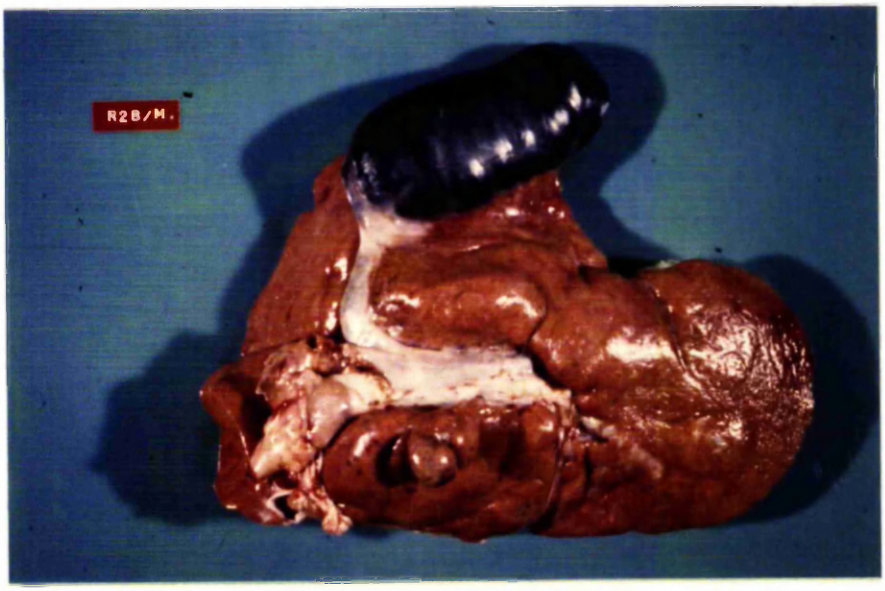
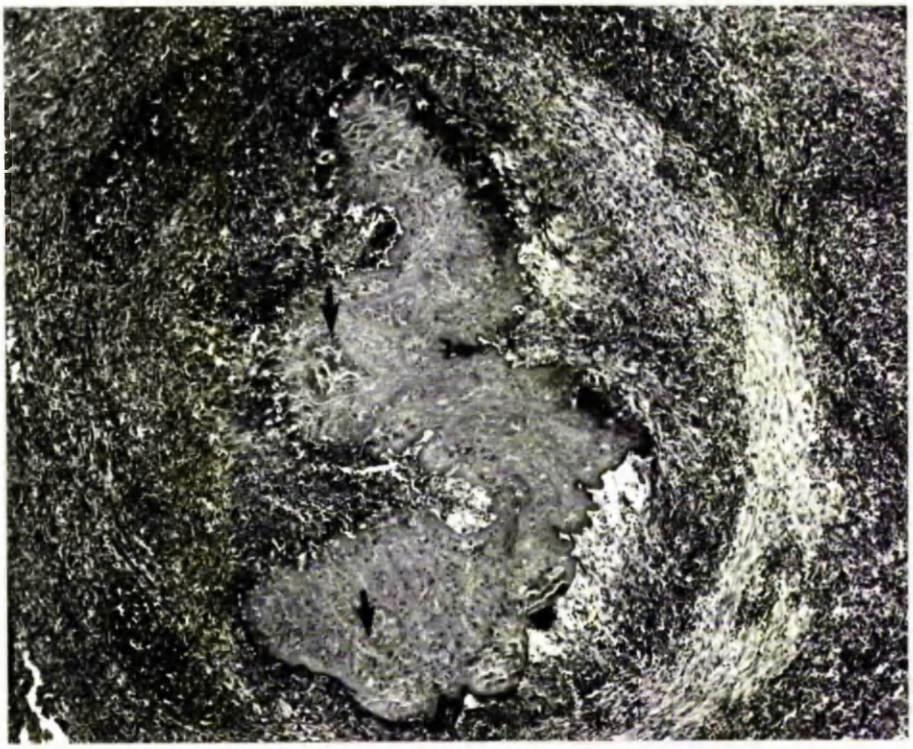

R28/M.

Fig. 70. A dead fluke identified by remnants of reproductive and digestive organs located partially in a bile duct (remains of the bile duct wall can be seen ➔) and surrounded by a diffuse cellular reaction.

HE x 12.

Fig. 71. A dead fluke identified by remnants of reproductive and digestive organs (arrows) located in the parenchyma surrounded by a diffuse cellular reaction, a few lympho-reticular nodules (stars) and strands of collagen.

HE x 12.



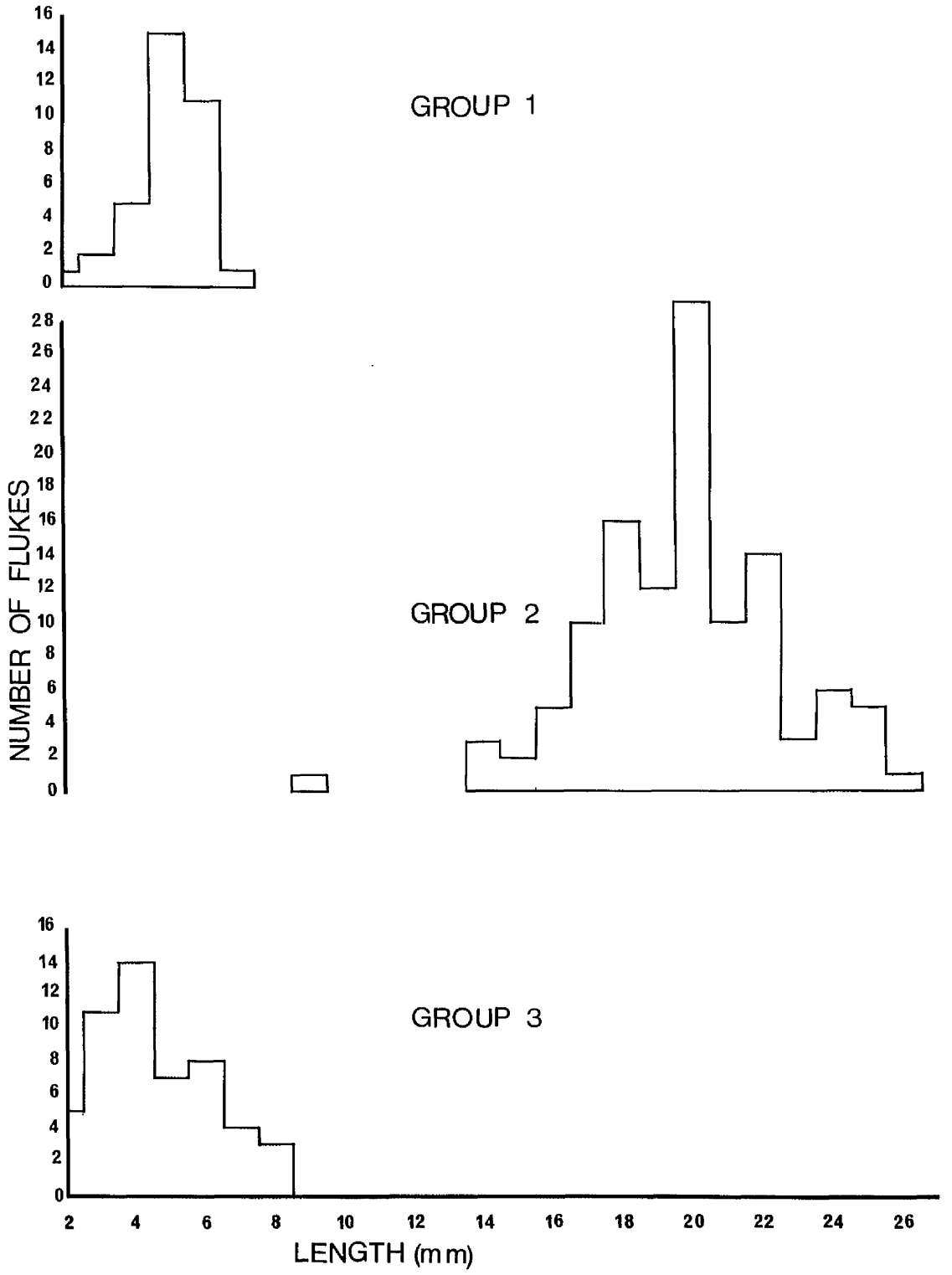


Fig. 72. Length distribution of two F. hepatica populations. Groups 1 and 2 were given 400 metacercariae then challenged 11 weeks later with a further 400 metacercariae. Group 1 was treated with rafoxanide 1 week prior to challenge. Group 3 was given the same number and batch of metacercariae as used in the challenge infection. The sheep were slaughtered 6 weeks after challenge.

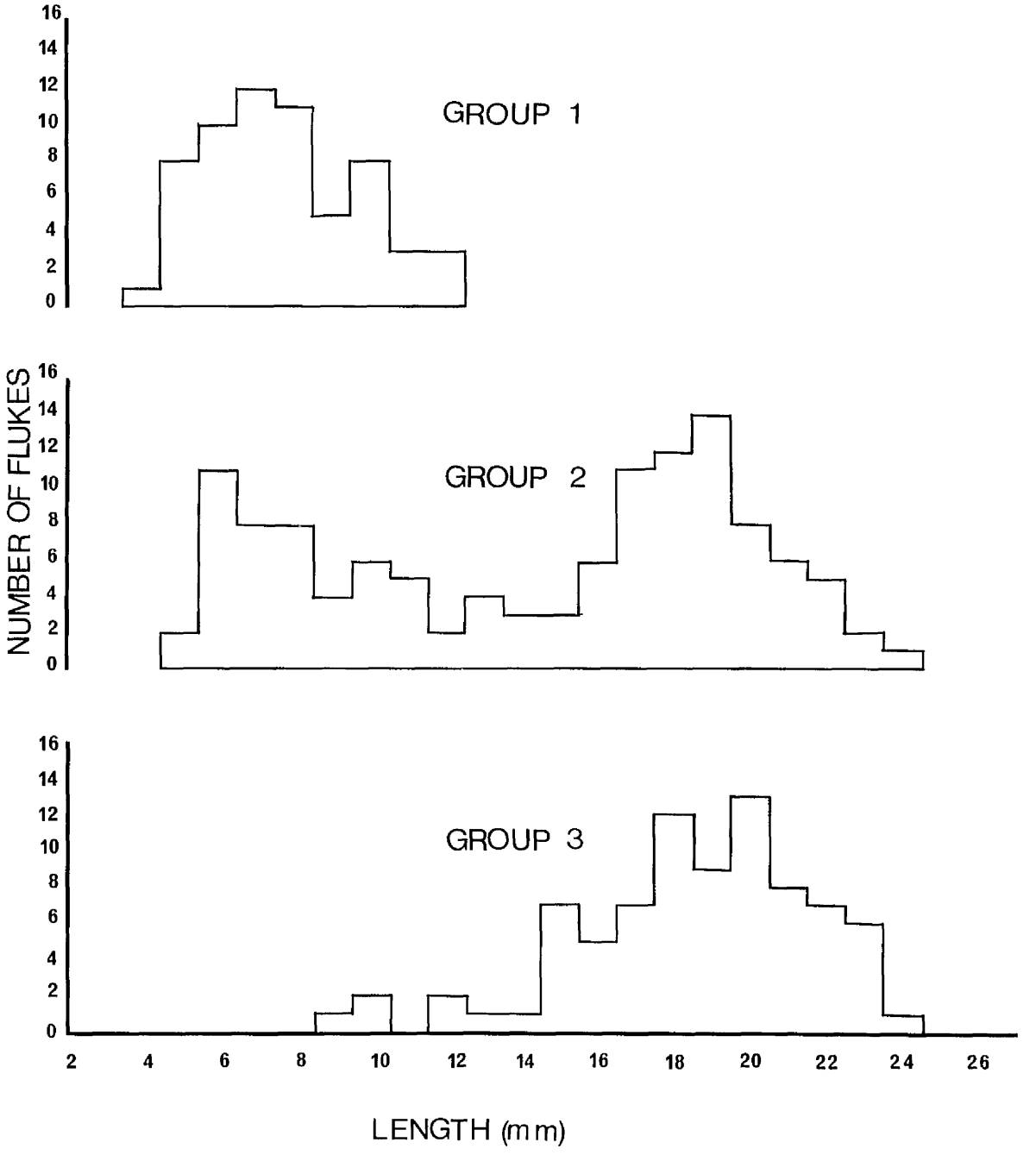


Fig. 73. Length distribution of two F. hepatica populations. Groups 1 and 2 were given 400 metacercariae then challenged 11 weeks later with a further 400 metacercariae. Group 1 was treated with rafoxanide 1 week prior to challenge. Group 3 was given the same number and batch of metacercariae as used in the challenge infection. The sheep were slaughtered 12 weeks after challenge.

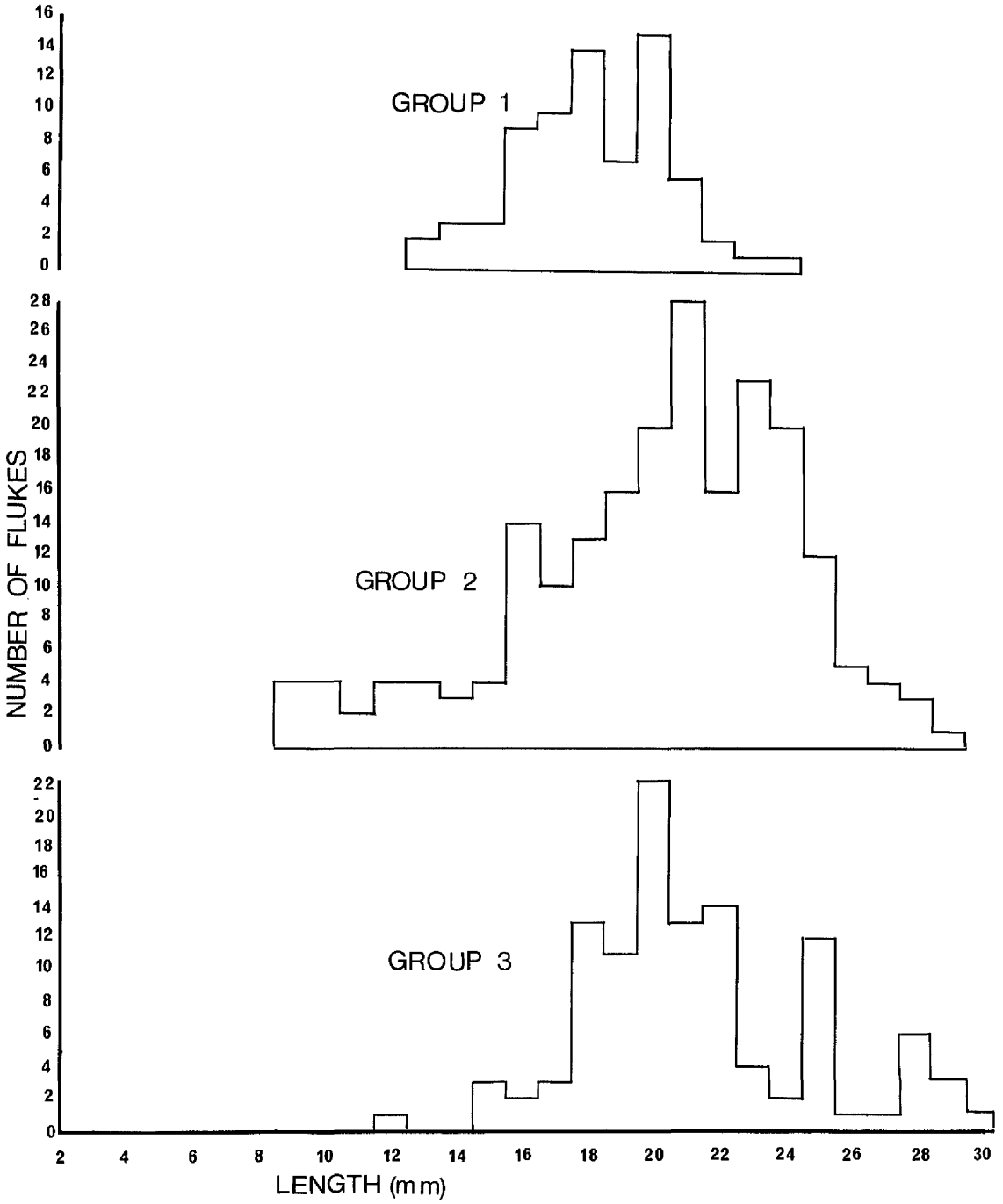


Fig. 74. Length distribution of two F. hepatica populations. Groups 1 and 2 were given 400 metacercariae then challenged 11 weeks later with a further 400 metacercariae. Group 1 was treated with rafoxanide 1 week prior to challenge. Group 3 was given the same number and batch of metacercariae as used in the challenge infection. The sheep were slaughtered 20 weeks after challenge.

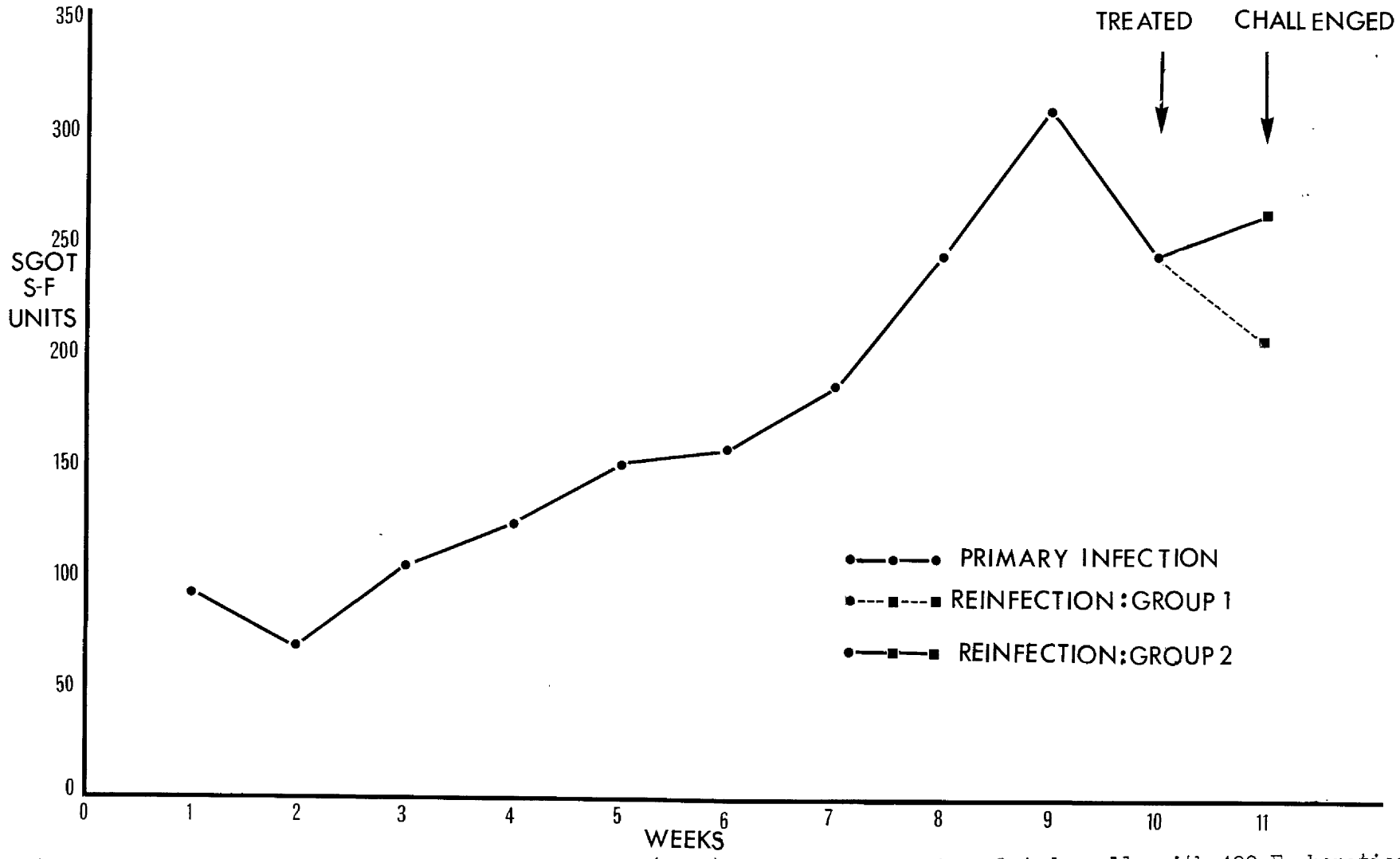


Fig. 75. Serum glutamic oxaloacetic transaminase (SGOT) levels of sheep inoculated orally with 400 F. hepatica metacercariae then challenged with a further 400 metacercariae, half the sheep (Group 1) being treated with rafoxanide 1 week prior to challenge.

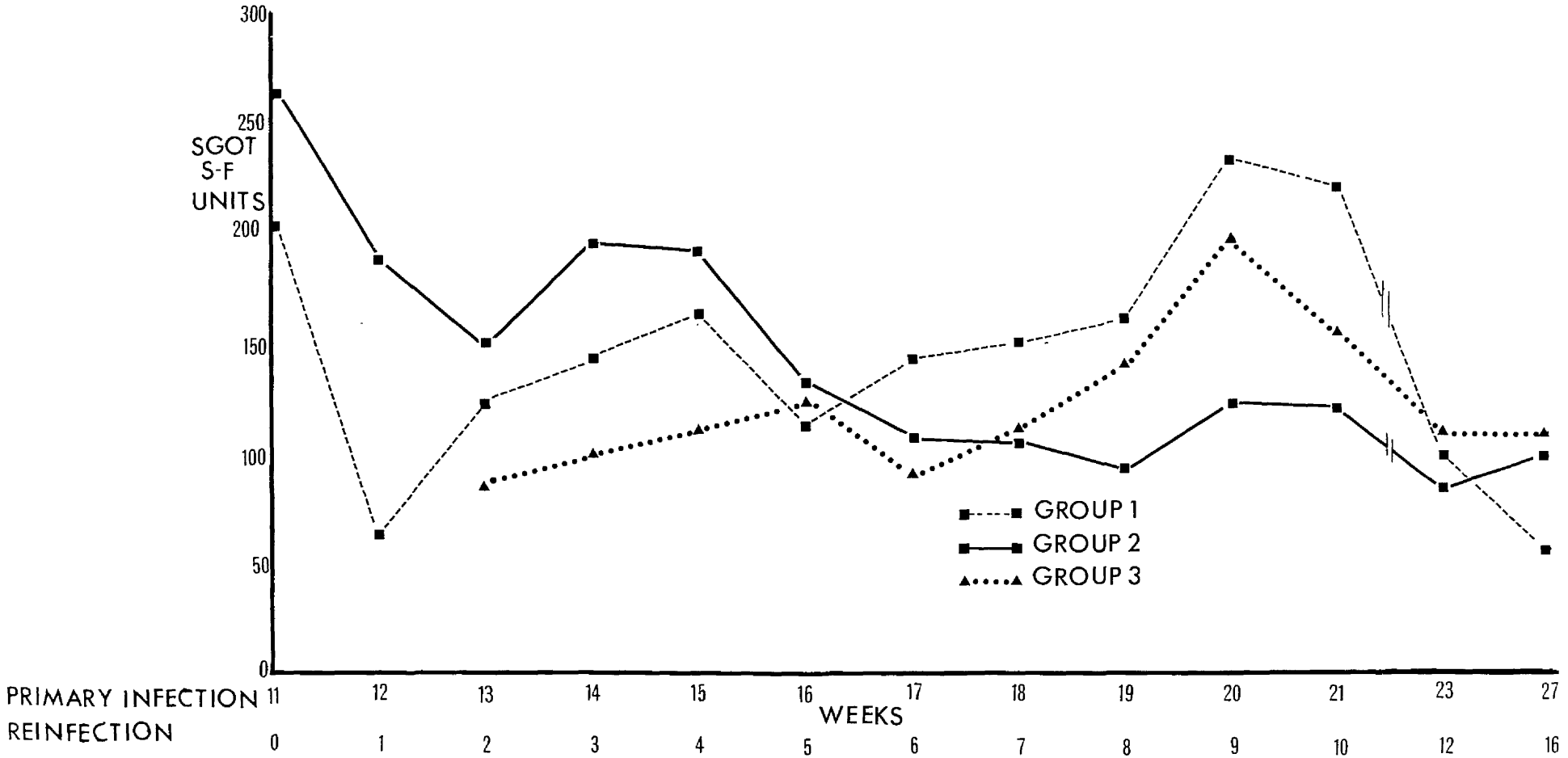


Fig. 76. Serum glutamic oxaloacetic transaminase (SGOT) levels of sheep (Group 3) inoculated orally with 400 F. hepatica metacercariae and two groups of sheep (Groups 1 and 2) inoculated then reinoculated 11 weeks later with 400 metacercariae. Group 1 was treated with rafoxanide 1 week prior to reinoculation.

SECTION V

AUTO-ANTIBODIES IN OVINE FASCIOLIASIS

INTRODUCTION

Auto-antibodies have been detected in a wide range of tissue destructive (reviewed by Weir, 1973) and parasitic diseases (Asherson and Rose, 1963; Kurata and Noda, 1965; Shamina et al., 1965; Mansfield and Kreirer, 1972; Mackenzie and Boreham, 1974). In view of the fact that F. hepatica produces extensive hepatic damage there was the likelihood that auto-antibodies would be produced. This section investigates this possibility.

Agar gell precipitation (AGP), passive haemagglutination (PH) and complement fixation (CF) tests incorporating either homologous whole tissue homogenates or subcellular fractions as antigens are the serological tests most frequently employed to detect auto-antibodies in non-autoimmune diseases (reviewed by Weir, 1973). Of the three tests, the CF test has been used most frequently for detecting auto-antibodies because this test, unlike the PH test, does not depend upon the use of defined antigens for its successful completion (Herbert, 1970). This test is, therefore, particularly useful for screening sera for auto-antibodies with an antigen that may contain a number of antigens, e.g., a tissue homogenate.

The most common method of preparation of tissues for use as antigens involves the homogenisation of a tissue in an isotonic solution followed by fractionation of the homogenate in an ultracentrifuge into nuclear, mitochondrial microsomal and cell sap components (Pinckard and Weir, 1966; Davies, 1973). However, the serological responses produced by these fractions appear to depend upon the serological test used, the species under investigation and the fraction incorporated to act as antigen. Pinckard and Weir (1966) have demonstrated that when rat liver homogenate and subcellular fractions were used in CF tests with positive anti-rat liver anti-serum, the titres produced varied considerably from fraction to fraction with the mitochondrial fraction producing the highest titre. On the other hand Emetaron, Nelkon and Boss (1967), working with the rat liver and AGP test, identified the microsomal fraction as producing the maximum precipitation response of all the tissue fractions. Büschenfelde and Miescher (1972) have shown that in the human liver soluble antigens reside in the hepatocyte cell wall. Contrary to this result, Pinckard and Weir (1966) found that the fraction of the rat liver containing soluble constituents did not

produce CF titres with anti-sera indicating the absence of soluble antigens in the rat hepatocyte.

Sargent, Myers, Rose and Richter (1966) recorded extremely high PH titres between red blood corpuscles sensitised with a whole rat liver homogenate and a rabbit anti-rat liver anti-serum but they did not test individual fractions. Much lower PH titres were detected by Weir, Pinckard, Elson and Suckling (1966) with a whole rat liver homogenate antigen and serum from rats with hepatocellular damage but they, too, did not identify the antigenically active fractions of the liver.

The one reaction common to all the above tests is the union of antibody and antigen. For this to occur to the maximum degree antibody and antigen must be specified for each other and be present in optimal concentrations, i.e., at equivalence, as excess of either can alter the results obtained, e.g., precipitation is reduced under conditions of antigen excess (Herbert, 1970). To ensure, therefore, that optimal results are obtained with a particular serological test an antigen suitable for the test and its optimal concentration have to be standardised. Such procedures involve reacting doubling dilutions of antigen and anti-serum in chequer-board titrations. The antigen dilution to produce the maximum effect, i.e., the point where antigen and antibody are at equivalence, is then used in subsequent tests involving sera with unknown antibody titres (Herbert, 1970).

Before testing sera from sheep infected with F. hepatica for auto-antibodies to liver antigens could be undertaken, the preliminary measures considered necessary consisted of homogenisation and fractionation of sheep liver, preparation of anti-liver anti-serum and recognition and standardisation of an antigenic fraction appropriate for each test employed.

MATERIALS AND METHODS

Preparation of liver fractions

Normal sheep liver was collected as soon after necropsy as possible, cooled to 4°C, washed with ice-cold 0.25 mol/l sucrose and approximately 1 part of the tissue was homogenised with 9 parts of ice-cold 0.25 mol/l sucrose for 5 minutes in a Silverson homogeniser (Silverson Machines Ltd., Chesham, Bucks.). The resultant 1/10 whole liver homogenate (1/10 WLH) was spun at 1000 g/min to remove tissue

debris which was discarded, divided into two, one half being aliquoted and stored at -70°C and the other fractionated by differential ultra-centrifugation using a slight modification of the method of Pinckard and Weir (1966). The gravitational forces and times used and the fractions obtained were as follows:

Fraction	Gravity	Time
Nuclear	600g	10 mins
Mitochondrial	26,000g	10 mins
Microsomal	100,000g	60 mins
Cell sap	supernatent to above	

The first three fractions were resuspended in ice-cold 0.25 mol/l sucrose and recentrifuged twice. The final sediments and cell sap volume were made up to a 1/10 dilution, aliquoted and stored at -70°C.

Preparation of rabbit anti-whole sheep liver anti-serum

Equal volumes of 1/10 WLH and complete Freunds adjuvant (Difco, Detroit, Michigan) were homogenised. Three rabbits each had 1 cc of the 1/10 WLH-adjuvant homogenate injected subcutaneously and 1 cc intramuscularly. One month later 1 cc of 1/10 WLH was injected intra-peritoneally and then the rabbits were bled 1 week later and the serum collected and decomplemented by heating at 56°C for 30 minutes. The rabbits were bled at regular intervals with an intra-peritoneal injection being given 1 week before each bleeding. The serum of one rabbit based upon its more potent anti-liver response in AGP tests was selected as the positive serum (PRS) to be used to standardise the liver antigens.

Specificity of rabbit anti-whole sheep liver anti-serum (PRS)

Since the 1/10 WLH used to immunise the rabbits probably contained blood components capable of stimulating the production of antibodies that may have interfered with future tests, the PRS was tested in agar gell plates against normal sheep serum for any anti-serum activity. This was carried out by placing undiluted PRS in the centre well of an agar gell plate and doubling dilutions ($\frac{1}{2}$ to 1/128) of normal sheep serum in the outer wells. Lines of precipitation did not develop between any of the wells indicating the absence of anti-serum antibodies.

Standardisation of antigens

Agar gell precipitation test (AGP)

Ionagar (Oxoid No. 2) (Oxoid Ltd., London) was made up to a 3.0% solution with citrate buffer pH 7.2 which was prepared as follows:

Solution A 35.6 g $Na_2 HPO_4 . 2H_2O$ dissolved in 1 litre of distilled water.

Solution B 21.01 g $C_6 H_8 O_7 . H_2O$ dissolved in 1 litre of distilled water.

82 ml of solution A was added to 18 ml of solution B to make up 100 ml of buffer. All serum and antigen dilutions incorporated into the tests were diluted with half strength citrate buffer.

Initial testing of the PRS and WLH showed that when anti-serum and antigen were placed in their respective wells at the same time, lines of precipitation developed very close to the well containing the anti-serum. To obtain lines spaced well apart, PRS and all other sera used were placed in their wells 12 hours before adding antigen. All plates were kept moist and incubated at 37°C for 3 days before reading.

Undiluted PRS was tested against 1/10 dilutions of WLH and the four liver fractions. The 1/10 WLH produced the best precipitating response of all the fractions (see results). This dilution was, therefore, tested further to identify the dilution to produce the optimal precipitating response by preparing doubling dilutions of 1/10 WLH (1/10 to 1/1024) against doubling dilutions of PRS (1/2 to 1/64). One dilution of the PRS was placed in the central well of an agar gell plate surrounded by 8 wells containing the WLH dilutions. One additional plate contained the WLH dilutions and normal rabbit serum in place of PRS to act as a serum control.

The WLH at a 1/10 dilution was judged on its precipitation response as the dilution most suitable for testing sheep sera for precipitating anti-liver antibodies. However, since the precipitation response produced by the dilutions of WLH was found to vary for each dilution and fraction tested (see results), all sheep samples found to contain precipitating antibodies were to be screened further with the dilutions of WLH used above.

Complement fixation test (CF)

All CF tests were carried out using the Microtitre Kit (Cooke Engineering, Alexandria, Virginia) and Bio-cult (Bio-cult Laboratories Ltd., Paisley, Scotland) disposable microtitre plates. Care was always taken to ensure regular and careful washing of the microdiluters and pipettes at all times, and that other equipment used was scrupulously clean and sterilised. In the CF tests every effort was made to keep the temperature of all reagents and plates below 4°C by storing in the fridge and by using ice baths.

Standardisation of complement

Commercial freeze-dried complement (C′) (Wellcome Reagents Ltd., Beckenham) was used in all the tests and was standardised as follows:

(i) 2 ml of freeze-dried complement was made up to a 1/10 solution by adding 14 ml of distilled water, aliquoted into 1 ml volumes and stored at - 20°C. One aliquote was thawed then standardised.

(ii) 0.5 ml of the thawed 1/10 solution was added to 2.5 ml Veronal buffer (VB) (Oxoid Ltd., London) to produce a 1/60 dilution.

(iii) 10 tubes were set up containing 0.5 ml VB.

(iv) 2 ml of the 1/60 C′ was added to the first tube, mixed, 2 ml removed and added to the next .... etc. This gave a dilution series of 1 in 60, 75, 94, 128, 148, 184, 230, 288, 360, 450 and 463.

(v) 0.025 ml VB was pipetted in to all wells of a microtitre plate. Into the first 6 verticle wells 0.025 ml of 1:60 C′ was pipetted, into the next 1:75 ..... etc., the last set of wells had only 0.025 ml of VB added to act as haemolytic controls. 0.025 ml of VB was added to each well in place of the antigen. The plates were covered with a plastic top and kept overnight at 4°C.

(vi) The haemolytic system was prepared by incubating a 2% solution of sheep red blood cells (RBC's) (Wellcome Reagents Ltd., Beckenham) washed with VB and incubated with an equal volume of one dilution from a doubling dilution (1/100 to 1/1,600) of rabbit anti-sheep red blood cell anti-serum (Wellcome Reagents Ltd., Beckenham) for 30 minutes at 37°C. 0.025 ml of sensitised cells were pipetted into the wells horizontally starting with the 1:100 dilution in the top row and working down with each dilution. The sixth and final row had non-sensitised RBC's added to act as C′ controls.

(vii) The plates were incubated at 37°C for 45 minutes.

During this time the RBC's were kept suspended by shaking by hand. The reaction was stopped by placing in a fridge and when the cells had settled the test scored as shown below to obtain the haemolytic dose 50 (HD_{50}) dilution of C′.

Scoring system

The percentage of red cells remaining in each well was assessed visually and scored as follows:

Percentage of RBC's remaining	Score
0	0
10%	Trace (T)
25%	1
50%	2
75%	3
100%	4

The HD_{50} dilution for the batch of C′ under test was determined by identifying the C′ dilution to produce the best score of 2 with a preceding score of 1 and a succeeding score of 3. In the following tests 4 units of the HD_{50} C′ dilution were used together with a haemolytic system prepared from RBC's sensitised with anti-serum at the dilution to produce the HD_{50} reading.

Standardisation of antigen for CF test

Doubling dilutions (1/100 to 1/128,000) of WLH and the four liver fractions were tested against doubling dilutions (1/2 to 1/32,768) of PRS. The PRS dilutions were made in microtitre plates using the microdiluters and the tissue dilutions were pipetted in horizontally with one row accommodating 1 tissue dilution. Duplicate plates were prepared which contained only VB and either the PRS dilutions or tissue dilutions to measure any anti-complementary activity in the anti-serum and tissues and thus act as serum and antigen controls. The above procedures were repeated with normal rabbit serum being substituted for PRS to act as negative serum controls.

To all the plates was added 0.025 ml of 4 HD_{50} units of C′ and, at the same time, C′ controls (C′ at 4, 2, 1, $\frac{1}{2}$ and 0 HD_{50} units with only 0.05 ml of VB) were included. The plates were then handled and scored as described previously. The end point was taken at the HD_{50} reading for each test.

The tissue dilution to produce the maximum titre (a 1/64,000 dilution of WLH) was selected as the antigen for use in the following tests. Two units (a 1/3,200 dilution) of antigen were used

in these tests.

Passive haemagglutination test (PH)

Sheep red blood cells (Wellcome Reagents Ltd., Beckenham) were washed twice with phosphate buffered saline (PBS) (Oxoid Ltd., London) pH 7.2. A 2% solution of cells was shaken vigorously by hand with a 1/40,000 solution of tannic acid. The tanned cells were then washed twice with PBS. Doubling dilutions (1/100 to 1/6,400) of the WLH and the four liver fractions were prepared with PBS. Each tissue dilution was incubated for 30 minutes at 37°C with an equal volume of tanned RBC's then washed three times with PBS to prepare sensitised RBC's.

PRS, prior to use, was absorbed with washed sheep RBC's for 30 minutes at room temperature to remove heterophil agglutinins. Following this, doubling dilutions (1/2 to 1/2,096) of absorbed PRS were tested against the RBC's sensitised with the doubling dilutions of the WLH and the four liver fractions in microtitre plates. The PRS dilutions were prepared in the plates using microdiluters and the sensitised cells were pipetted in horizontally. To check for any spontaneous agglutination of the sensitised and non-sensitised RBC's, the sensitised cells at all the dilutions used were pipetted into plates containing only PBS and non-sensitised cells were added to PBS in one well of a plate. The presence of any remaining heterophil agglutinins in the PRS was also checked by adding non-sensitised tanned cells to a doubling dilution series of PRS.

The above procedures were repeated using normal rabbit serum in place of PRS to act as negative serum controls.

Once all reagents were added, the plates were allowed to stand covered with plastic caps in a vibration-free place on the bench. The plates were read when all the cells had settled and the tissue dilution to produce the maximum agglutination titre (a 1/400 dilution of the mitochondrial fraction) was selected to sensitised tanned RBC's in future tests.

Protein estimations

The total protein contents of a 1/400 WLH dilution and 1/100 dilutions of the liver fractions (the tissues being adjusted to these dilutions to suit the method used) were determined by the method of Lowry and Farr (1966). This procedure was carried out to enable

new batches of tissue antigens to be made up to the immunologically standardised dilutions by measuring the total protein contents of the new batches and then diluting to the required protein contents with 0.25 mol/l sucrose without having to repeat the immunological tests. In the event, new batches were not necessary as sufficient antigens were prepared and standardised in the initial immunological tests to cover all the investigations carried out.

Testing of sheep serum

Serum samples collected from the sheep as described in Sections II and IV of this thesis were tested with the standardised liver antigens in AGP, PH and CF tests. All CF and PH titres were recorded as $\log_2$ units. For the purposes of this section the sera from the reinfection Groups in Section IV were treated as one Group. To facilitate description the primary infection results of Section II were called Group A, the primary infection of Section IV, Group B, and combined reinfection results of Section IV, Group C.

During the collection of the serum samples care was taken to prevent excessive haemolysis by avoiding rapid temperature changes and by careful "ringing" of the blood clots since serum containing haemolysed RBC's was found to produce high anti-complementary readings in the CF tests.

At all stages of testing, controls were incorporated into the PH tests to measure any spontaneous agglutination of the sensitised RBC's (sensitised cell control) and non-sensitised RBC's (non-sensitised cell control) and to determine the presence of any heterophil agglutinins in the sheep serum (serum control). The sensitised and non-sensitised cell controls consisted of sensitised and non-sensitised tanned RBC's in PBS and the serum control was prepared by adding non-sensitised tanned cells to duplicate dilutions of serum tested. The controls incorporated in the CF test were serum (no antigen just C' in duplicate serum dilutions to measure anti-complementary effects), antigen (two units of standardised antigen and no serum to detect any anti-complementary effects of the antigen) and complement. Complement controls consisted of C' at 4, 2, 1, $\frac{1}{2}$ and 0 HD_{50} levels to check that the deep frozen batches of complement were continuing to produce 50% haemolysis at their HD_{50} standardised dilutions. The well with no complement contained only sensitised RBC's and this served as a haemolytic serum control to register any

spontaneous haemolysis of the sensitised cells.

Serum samples from the sheep at times of peak liver damage (week 9 in the primary infections and weeks 4 and 9 in the reinfections as indicated by the SGOT levels - see Table 2 and Appendix 1, Tables 3 and 4) were tested in agar gell plates against undiluted PRS for the presence of circulating liver antigens.

Biochemical investigations

The SGOT results recorded in Sections II and IV (see Table 2 and Appendix 1, Tables 3 and 4) were used in this section to provide an estimate of the severity of liver cell damage for comparison with the CF and PH titres. The results from the two reinfection Groups in Section IV were combined to form one Group.

RESULTS

Standardisation of antigens

Agar gell precipitation test (AGP)

Undiluted PRS produced multiple strong precipitation lines with the 1/10 WLH, a lesser number of lines but of equivalent strength with the mitochondrial, microsomal and cell sap fractions and a few weak lines with the nuclear fraction (Fig. 77). Reactions of identity, partial identity and non-identity were seen between some of these precipitation lines (Fig. 79). The precipitating response between the WLH and undiluted PRS apart from a few lines (see below) was not enhanced by diluting the 1/10 WLH (Fig. 78 and Table 5). Precipitation lines did not develop between the WLH dilutions and the normal rabbit serum (Table 5). Because of the maximum precipitating capacity of the WLH at a 1/10 dilution this dilution was selected as the dilution to act as antigen in the AGP tests used to screen the sheep serum for precipitating antibodies.

Although diluting the 1/10 WLH resulted eventually in the total loss precipitation, some of the precipitation lines that developed at the higher WLH dilutions became more distinct and stronger (Table 5). This increase in precipitation with dilution demonstrated that not all the liver antigens had the same equivalence points with the PRS. Therefore, for this reason, any sheep serum samples found to contain precipitating antibodies with the 1/10 WLH dilution were to have been screened further with the higher dilutions of the WLH.

TABLE 5.

AGAR GELL PRECIPITATION STANDARDISATION TEST:
PRECIPITATING RESPONSES BETWEEN DOUBLING DILUTIONS
OF WHOLE SHEEP LIVER HOMOGENATE (WLH) AND RABBIT
ANTI-SHEEP LIVER ANTISERUM (PRS) AND NORMAL RABBIT SERUM

WLH dilutions	PRS dilutions (centre well) Neat	1/2	1/4	1/8	1/16	1/32	1/64	Normal Rabbit Serum.
1/10	+++++	+++++	+++++	++	++	-	-	-
1/20	+++++	+++++	+++++	++	++	+	+	-
1/40	+++++	+++++	+++++	+	++	+	++	-
1/80	++++	++++	++++	++	+	+	+	-
1/160	++++	++++	++++	+	++	+	±	-
1/320	+++	+++	++	+	++	+	+	-
1/640	++	++	++	+	+	+	+	-
1/1280	++	++	-	-	+	-	-	-
1/2650	+	NT	NT	NT	NT	NT	NT	NT
1/5120	-	NT	NT	NT	NT	NT	NT	NT

NT Not tested

++++++ → ± Multiple strong precipitation lines to one weak line.

\- No precipitation visible.

——— Screening dilution.

TABLE 6.

COMPLEMENT FIXATION STANDARDISATION TEST:
MAXIMUM TITRES DEVELOPING BETWEEN DOUBLING DILUTIONS OF
RABBIT ANTI-SHEEP LIVER ANTISERUM (PRS) AND NORMAL RABBIT
SERUM AND A WHOLE SHEEP LIVER HOMOGENATE
AND FOUR SUBCELLULAR LIVER FRACTIONS.

1. Rabbit Anti-Sheep Liver Antiserum (PRS)

Dilution	WLH	Nuclear Fraction	Mitochondrial Fraction	Microsomal Fraction	Cell Sap Fraction
1/100	A C	A C	A C	A C	1/512
1/200	A C	A C	1/1024	1/512	1/1024
1/400	1/1024	1/1024	1/1024	1/512	1/1024
1/800	1/2048	1/1024	1/2048	1/1024	1/2048
1/1600	1/2048	1/512	1/4096	1/1024	1/4096
1/3200	1/4096	1/512	1/512	1/2048	1/2048
1/6400	[1/8192]	1/256	1/1024	1/2048	1/2048
1/12800	1/2048	1/256	1/512	1/1024	1/2048

2. Normal Rabbit Serum.

All dilutions produced a titre of 1/256 or less.

3. Controls.

Serum control (anti-complementary titre)

PRS 1/64

Normal Rabbit Serum 1/32

Complement at 4, 2, 1, 1/2, 0 HD_{50} units

% haemolysis 100, 50, 10, 10, 0

A C Tissue dilution was anti-complementary.

——— Screening antigen and dilution, 2 units (1/3,200 dilution) being used.

[ ] Maximum titre.

TABLE 7.

PASSIVE HAEMAGGLUTINATION STANDARDISATION TEST:
MAXIMUM TITRES DEVELOPING BETWEEN DOUBLING DILUTIONS OF
RABBIT ANTI-SHEEP LIVER ANTISERUM (PRS) AND NORMAL RABBIT
SERUM AND A WHOLE SHEEP LIVER HOMOGENATE (WLH)
AND FOUR SUBCELLULAR LIVER FRACTIONS.

1. Rabbit Anti-Sheep Liver Antiserum (PRS)

Dilution	WLH	Nuclear Fraction	Mitochondrial Fraction	Microsomal Fraction	Cell Sap Fraction
1/100	1/128	1/256	1/512	1/512	1/256
1/200	1/128	1/128	1/512	1/512	1/512
1/400	1/256	1/128	1/512	1/256	1/256
1/800	1/256	1/64	1/512	1/256	1/256
1/1600	1/256	1/64	1/512	1/256	1/256
1/3200	1/256	1/32	1/256	1/256	1/128
1/6400	1/256	1/32	1/256	1/256	1/256

2. Normal Rabbit Serum.

All dilutions produced a titre of 1/4 or less.

3. Controls.

Serum control

PRS 1/4

Normal Rabbit Serum 1/4

Non-sensitised RBC's - no agglutination.

Sensitised RBC's - no agglutination.

_____ Screening antigen and dilution.

[_____] Optimal titre.

TABLE 8.

TOTAL PROTEIN CONTENTS OF WHOLE SHEEP LIVER HOMOGENATE (WLH) AND FOUR SUBCELLULAR LIVER FRACTIONS.

Test Tissue	Test Dilution	Colourimeter Reading (M)	Total Protein Content of 0.2 ml of Test Dilution	Total Protein Content of the Standardised Dilutions
WLH	1/400	0.290	0.603 mg/ml	AGP test 1/10 dilution 24.2 mg/ml
Nuclear fraction	1/100	0.060	0.012 mg/ml	CF test 1/6400 dilution 0.039 mg/ml
Mitochondrial fraction	1/100	0.135	0.28 mg/ml	PH test 1/400 dilution 0.07 mg/ml
Microsomal fraction	1/100	0.056	0.012 mg/ml	
Cell sap fraction	1/100	0.260	0.540 mg/ml	
Test standard		0.150		

Formula for calculating total protein content (mg/ml) of test dilution = $\frac{62.5}{\text{Test standard}} \times M \times \frac{1}{1000} \times \text{dilution}$.

Complement fixation test (CF)

The maximum CF titres obtained with the doubling dilutions of the WLH and the four liver fractions are contained in Table 6. Apart from the 1/100 dilutions of the WLH, nuclear, mitochondrial and microsomal fractions and the 1/200 dilutions of WLH and the nuclear fraction being anti-complementary, all other tissue dilutions produced titres with the PRS in excess of the maximum titre (1/256) obtained with the normal rabbit serum and the anti-complementary titre (1/64) of the PRS. The titres with the PRS ranged from a minimum of 1/512 with a few dilutions of all the liver fractions to a maximum of 1/8,192 at the 1/6,400 dilution of the WLH. Two units (a 1/3,200 dilution) of the 1/6,400 WLH dilution were prepared and used as antigen in the CF tests incorporating sheep serum.

Passive haemagglutination test (PH)

The PH titres obtained with the doubling dilutions of the WLH and the four liver fractions are detailed in Table 7 together with the results of the control tests incorporated. The maximum titre (1/512) that developed was obtained with 5 dilutions (1/100 to 1/1,600) of the mitochondrial fraction, 2 dilutions (1/100 to 1/200) of the microsomal fraction and one dilution (1/200) of the cell sap fraction. All titres that developed between the normal rabbit serum and tissue dilutions and the titres in the serum controls were less than 1/4. Spontaneous agglutination did not occur between the sensitised and non-sensitised cells.

The central dilution (1/400) of the plateau of titres for the mitochondrial fraction was selected to sensitise sheep RBC's in future tests.

Protein estimations

The total protein contents of the WLH and the four liver fractions at their tested dilutions and the standardised dilutions for the AGP (1/10 WLH), CF (1/6,400 WLH) and PH (1/400) tests are given in Table 8.

Sheep sera

Agar gell precipitation test (AGP)

Precipitating anti-liver antibodies were not detected at any stage in Groups A, B and C using a 1/10 WLH as antigen. Circulating

liver antigen components were not identified with the PRS at the times of maximum liver damage.

Complement fixation test (CF)

Individual CF titres, weekly mean CF titres together with SE's and individual serum anti-complementary titres for Group A are given in Appendix 1, Table 5, for Group B Appendix 1, Table 6 and for Group C Appendix 1, Table 7. In Groups A and B mean CF titres of less than 6 were present up to week 2 after infection (Figs. 79 and 80). After week 2 a rise in titre was evident in both Groups that reached a maximum of approximately 8 at week 6 then declined to 6 and less between weeks 8 and 9 (Figs. 79 and 80). The titre increase between weeks 2 and 6 in Group A was significant ($P < 0.001$) but not in Group B ($P > 0.05$). A slight increase in titre to 6 and over occurred in both Groups between weeks 9 and 12 (Figs. 79 and 80). Thereafter, in Group A the mean titre remained reasonably constant around 6.

Reinfection at week 11 in Group C produced a rapid rise in mean titre to a maximum level of 8.2 ± 0.5 at week 3 after reinfection (Appendix 1, Table 7, Fig. 81). The titre fluctuated from 6.5 to 7.8 between weeks 4 and 9 and then declined to less than 6 at week 16 after reinfection (Fig. 81).

Antigen controls incorporated at all stages were not anti-complementary and the C' controls showed that the batches of C' were producing 50% haemolysis at their HD_{50} dilutions. Haemolysis was not seen in the haemolytic controls.

Passive haemagglutination test (PH)

Individual PH titres and mean titres with SE's are given in Appendix 1, Table 8 for Group A, Appendix 1, Table 9 for Group B and Appendix 1, Table 10 for Group C.

The mean PH titre in Groups A and B up to week 2 after infection was less than 1 (Figs. 79 and 80). A significant rise ($P < 0.01$) in titre to about 2 took place for both Groups between weeks 3 and 5 followed by a decline to less than 1 at week 10 (Figs. 79 and 80). The mean titre in Group A increased rapidly to 5.0 ± 0.3 at week 12 then fluctuated but fell to less than 1 at week 24 (Fig. 79). In Group B a rise in titre occurred at the same time but only reached a mean level of 1.3 ± 0.4 at week 12 (Fig. 79). Reinfection

produced a fluctuating titre (Fig. 81) with a maximum titre of 2.6 ± 0.2 at week 6.

All serum controls did not register any titres and spontaneous agglutination did not occur in the sensitised and non-sensitised cell controls.

Biochemical results

The SGOT results for Groups A, B and C are presented in Sections II (Table 2) and IV (Appendix 1, Tables 3 and 4). The combined mean SGOT levels of the 2 reinfections in Group C are given in Appendix 1, Table 11.

Following primary infection in Groups A and B, the SGOT levels rose from less than 100 S-F units to maximums of 237 and 307 respectively at week 9 (Figs. 79 and 80). In Group A a gradual decline then occurred to less than 100 units at week 16. Challenging Group C produced a gradual but slightly irregular decrease in the mean SGOT level from a peak of 234 units at the time of challenge to 76 units 16 weeks after challenge (Appendix 1, Table 11and Fig. 82).

DISCUSSION

During the standardisation of the AGP test multiple precipitation lines developed that indicated the presence of numerous liver antigens. Some of these antigens appeared to be common to all the fractions, others were not. That these precipitation responses were produced by the interaction of liver antigens and anti-liver antibodies and not by trapped serum components in the tissue fractions and anti-serum component antibodies in the PRS was shown by the absence of any reactivity between PRS and normal sheep serum. Contamination of the fractions by other fraction antigens may have occurred during the fractionation process accounting possibly for the presence of the antigens common to each fraction. However, the divergencies in precipitation response as well as the variations in the CF and PH standardisation titres demonstrated that the liver fractions differed antigenically. Moreover, these variations in serological response demonstrated the necessity of conducting standardisation tests as only after carrying out these procedures was an antigen suitable for each test employed identified.

Screening the sera from Groups A and B with the standardised antigens detected low CF and PH titres in normal sheep serum that increased 2 to 3 weeks after a primary infection of F. hepatica and within the first week of a reinfection in Group C. Observations similar to these results have been made in rabbits with liver-induced parasitic damage (Asherson and Rose, 1963; Kurata, 1966), in rats with hepatocellular injury (Weir, 1963) and in a wide variety of tissue-destructive diseases in man and animals (see Mackay and Gajdusck, 1958; Gajdusck, 1958; Weir, 1973). Asherson and Rose (1963) using the CF test and Kurata (1966) the PH test recorded increases in low normal levels of auto-antibody following infection of rabbits with S. japonca and E. stediae respectively. In common with F. hepatica both these parasites damage the liver, S. japonca through the release of eggs and formation of egg granulomata and E. stediae by producing a chronic cholangitis (Smith and Jones, 1966). The PH titres produced with E. stediae (Kurata, 1966) differed from those detected in this study in that they reached considerably higher levels (up to 1/512) and one peak was produced 8 weeks after infection followed by a decline in titres. Asherson and Rose (1963) measured CF titres only over the first 7 weeks of infection and reported titres comparable to those detected in this study.

Successive phases of liver damage have been produced by Weir (1963) administering CCl_4 to rats and the auto-antibody response monitered with the CF test. Following the first administration of CCl_4, CF auto-antibody titres rose rapidly to a peak within 2 days, persisted for about 1 week then declined (Weir, 1963). Repeated administration produced equally rapid but slightly lower responses (Weir, 1963). Since circulating liver antigens were also detected following the administration of CCl_4 (Pinckard and Weir, 1966), Weir (1963; 1967) concluded that the rapid titre increases after the first and subsequent administrations of CCl_4 were suggestive of a secondary antibody response to the products of tissue breakdown. Weir (1964) provided support for the antibody nature of this response by identifying raised serum IgM levels (Weir, 1964), by preventing the antibody increase with X-irradiation and splenectomy (as stated by Weir and Suckling, 1968) and by demonstrating increased immunocyto-adherence of rat spleen cells from CCl_4 -injected rats for mitochondrial-coated RBC's (Weir and Suckling, 1968).

In this study, liver cell death due to fluke migration was seen histologically one week after infection (Section II) and was

reflected in raised SGOT levels 2 to 3 weeks after infection (Sections II and IV). In spite of the fact that circulating liver antigens were not detected, the increased auto-antibody titres were most probably stimulated by the release of liver components. However, the delay of up to 2 weeks between the first evidence of liver cell death and the rise in CF and PH titres was indicative of a primary antibody response and not a secondary response as would have been expected because of the low normal titres (Weir, 1964; Herbert, 1970). The more rapid increase in titres following reinfection was consistent with a secondary response but not the low fluctuating CF and PH titres that developed. These results suggest that factors other than liver damage were influencing the auto-antibody titres. The delayed rise in primary infection titres and the low fluctuating reinfection titres could be due to a number of possibilities acting either individually or together.

First, decomplementing serum by heating to 56°C for 30 minutes reduces but does not eliminate the CF and PH activity of normal rat serum (Pinckard and Weir, 1966) indicating the presence of heat-labile and stable auto-antibodies. A similar reduction in activity is not observed in the rabbit until a temperature of 65°C is reached and maintained for one hour (Asherson and Rose, 1963). Decomplementing the sera in this study may, therefore, have destroyed a heat-labile but not a heat-stable auto-antibody; thus, permitting detection of auto-antibody but possibly delaying the rise in the primary infection titres and reducing the CF and PH and reinfection titres.

Second, the rate of increase, level reached and time maintained by an antibody titre depends upon the type of antibody produced. The auto-antibodies in the rat were identified by Weir (1964) as probably belonging to the IgM class of antibody. On the other hand, Asherson and Rose (1963) detected IgG auto-antibodies in the rabbit. Characteristically, IgM antibodies are produced first in a primary immune response in low titres which are not markedly elevated upon secondary stimulation and neither primary nor secondary titres are maintained for long periods of time (Roitt, 1970). By contrast, IgG is produced later than IgM in a primary response but in higher titres which are considerably increased and maintained upon secondary stimulation (Roitt, 1970). In this study, the CF and PH primary and reinfection titres were suggestive of IgM auto-antibody production. If IgG auto-antibodies had been produced they may have missed detection

because of their relatively poor CF and PH ability compared to IgM antibodies (Herbert, 1970).

Third, the formation of immune complexes between circulating liver tissues and auto-antibodies (Weir, 1963) could determine the amount of free auto-antibody available for detection. By comparing the primary infection CF and PH titres and the SGOT graphs (see Figs. 79 and 80) two phases of immune complex formation can be postulated; one under conditions of antibody excess and the other under conditions of "antigen" excess. These phases are shown diagramatically in Fig. 82. Between weeks 3 and 6 of the primary infections, CF and PH titres and circulating "antigens" (as indicated by the SGOT graph) were all increasing but because of the rising titres auto-antibodies were apparently in excess of "antigen". From week 6 to 9 the "antigen" level continued to increase while the titres declined. During this period complexing of "antigen" and auto-antibody might under conditions of "antigen" excess have prevented further increases in auto-antibody titre, decreased the titres already present but still permitted an increase in "antigen" level. Following the decrease in "antigen" level between weeks 9 and 12 a rise in CF and PH titres occurred briefly which indicated a slight excess of antibody over antigen. The fluctuations in the CF and PH reinfection titres may have been due to immune complex formation removing free auto-antibody and hence depressing the titres.

Although ovine fascioliasis cannot be classified as an auto-immune disease (criteria for classification are detailed on pages 9 and 10), auto-antibodies were produced during the course of the disease. Recent evidence is available which points to the function of the auto-antibodies produced in such tissue destructive diseases as fascioliasis as being essentially physiological. This evidence implicates the auto-antibodies in aiding removal of tissue breakdown products by binding to the products together with complement and attracting polymorpho-nuclear leukocytes and possibly macrophages (Elson and Weir, 1967). By contrast, auto-antibodies in auto-immune diseases are of particular importance because of their pathogenic roles in such diseases as auto-immune haemolytic anaemia and possibly Hashimotos thyroiditis and their value as diagnostic markers in the auto-immune liver disorders (Roitt, 1970).

Finally, the pathway whereby auto-antibodies are produced in non-auto-immune diseases has been elucidated because it may provide,

under suitable circumstances, a route for cytotoxic auto-antibody production. This does appear to be so. In non-auto-immune diseases the pathway involves the induction of a state of tolerance in thymus-derived lymphocytes (T-cells) by soluble tissue antigens in low concentrations, e.g., cell sap (Dresser, 1962; Allison, 1971; Iverson and Lindenman, 1971). At the same time, particulate tissue antigens, e.g., mitochondria, are able to stimulate directly bone-marrow derived lymphocytes (B-cells) to produce IgM auto-antibodies (Torrigiani and Roitt, 1965) without the normal intervening T- and B-cell co-operative stage preceding B-cell antibody production (Allison, 1971; Feldman, 1972; Iverson and Lindenman, 197). The production of auto-antibodies in auto-immune diseases while of unknown cause can be achieved experimentally by the use of drugs and infectious agents acting possibly by altering a "self-antigen" to stimulate T-cells to co-operate with B-cells to produce auto-antibodies capable of cross-reacting with the original "self-antigen" (Roitt, 1970). In auto-immune diseases a basic genetic abnormality is also considered to play a part in their causation (reviewed by Burnet, 1963; Nossal, 1973). In addition to the use of drugs and infectious agents transition from a physiological state of auto-antibody production to a pathological state can be induced by immunising animals with non-antigenic T-cell stimulators (adjuvants) (Allison, Denman and Barnes, 1971; Nossal, 1973) and tissue homogenates to produce cytotoxic auto-antibodies. In view of this, the use of an adjuvant in combination with an ovine liver homogenate may, therefore, induce liver changes in normal ovine livers and enhance possibly the damage produced by F. hepatica perhaps to the extent seen in R21 in Section IV.

SUMMARY

Using standardised ovine liver antigens in AGP, PH and CF tests normal sheep serum was shown to contain low titres of CF and PH but not precipitating auto-antibodies. The CF and PH titres increased between weeks 3 and 12 of a primary infection of sheep with F. hepatica with a peak being reached at week 6. Upon reinfection the titres increased within the first week and maintained increased but irregular levels for up to 16 weeks, then declined. Liver cell components released during the migrations of the flukes by possibly acting as "antigens" were suggested as stimulating auto-antibody production to aid removal of the cell debris. AGP testing for

circulating tissue "antigens" was not successful.

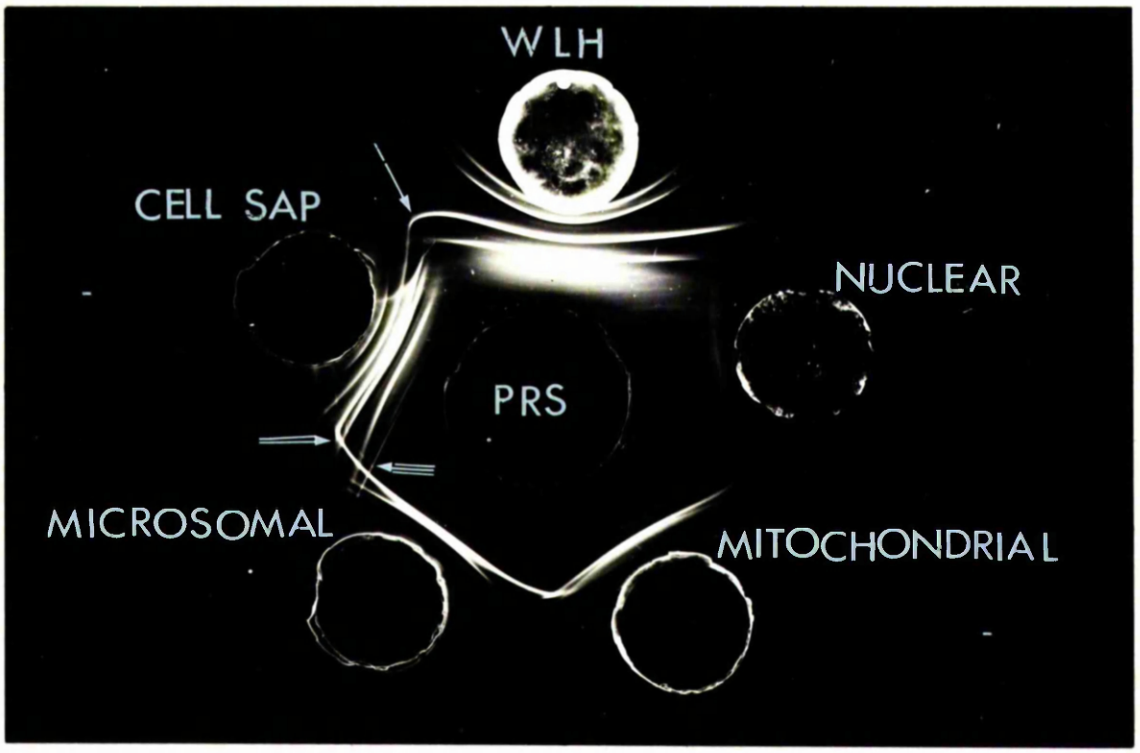
To account for the apparent delay between the first evidence of liver damage and the increases in primary CF and PH titres and titre fluctuations following reinfection, three possibilities were proposed: the destruction of a heat-labile but not stable auto-antibody; the type of auto-antibody produced; the complexing of auto-antibody and "antigen".

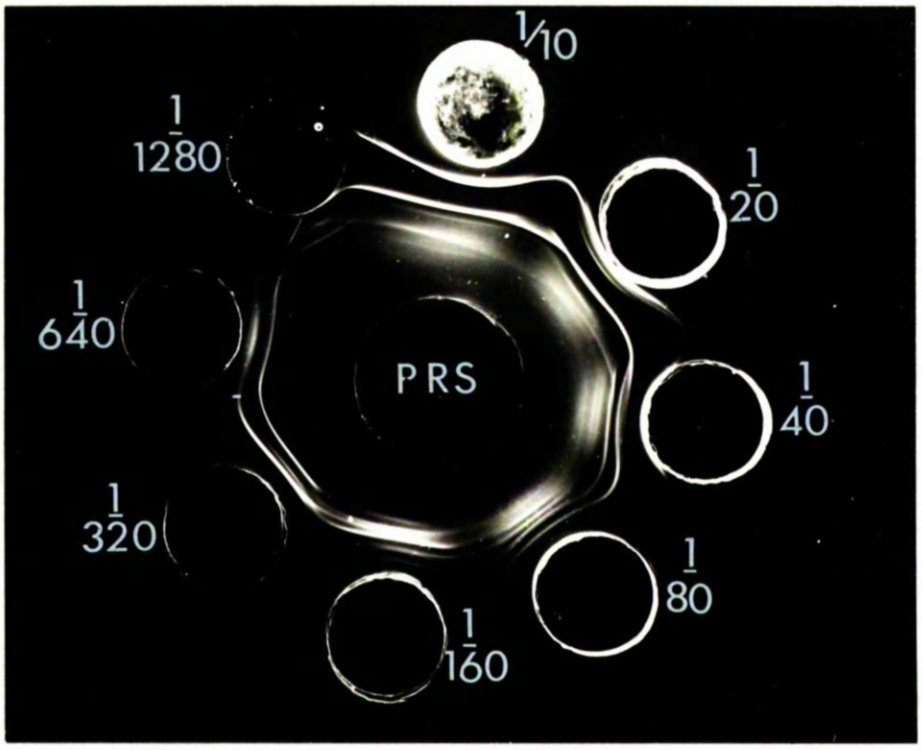
Fig. 77. Agar gell standardisation test:

1/10 dilutions of whole liver homogenate (WLH) and the four liver fractions, nuclear, mitochondrial, microsomal and cell sap against undiluted positive rabbit serum (PRS) in the centre well. Lines of identity (——►), partial identity (═══►) and non-identity (≡≡≡►) can be seen.

Fig. 78. Agar gell standardisation test:

Doubling dilutions of whole liver homogenate (WLH) in the outer wells against undiluted positive rabbit serum (PRS) in the centre well.


WLH
CELL SAP
NUCLEAR
PRS
MICROSOMAL
MITOCHONDRIAL


1/10
1/1280
1/20
1/640
PRS
1/40
1/320
1/80
1/160

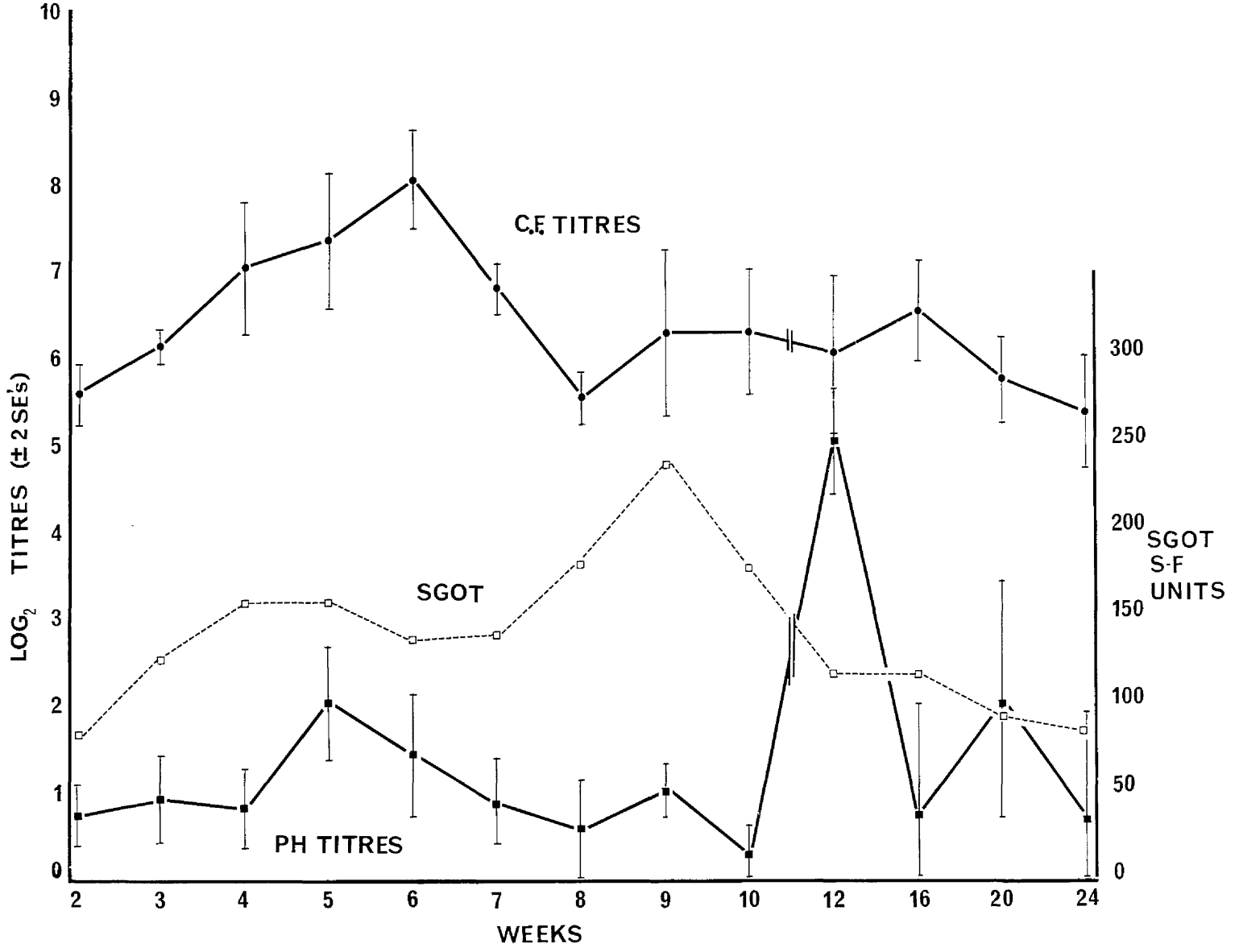


Fig. 79. Complement fixation (CF) and passive haemagglutination (PH) auto-antibody titres (± 2 SE's) and serum glutamic oxaloacetic transaminase (SGOT) levels from sheep (Group A) given a primary F. hepatica infection.

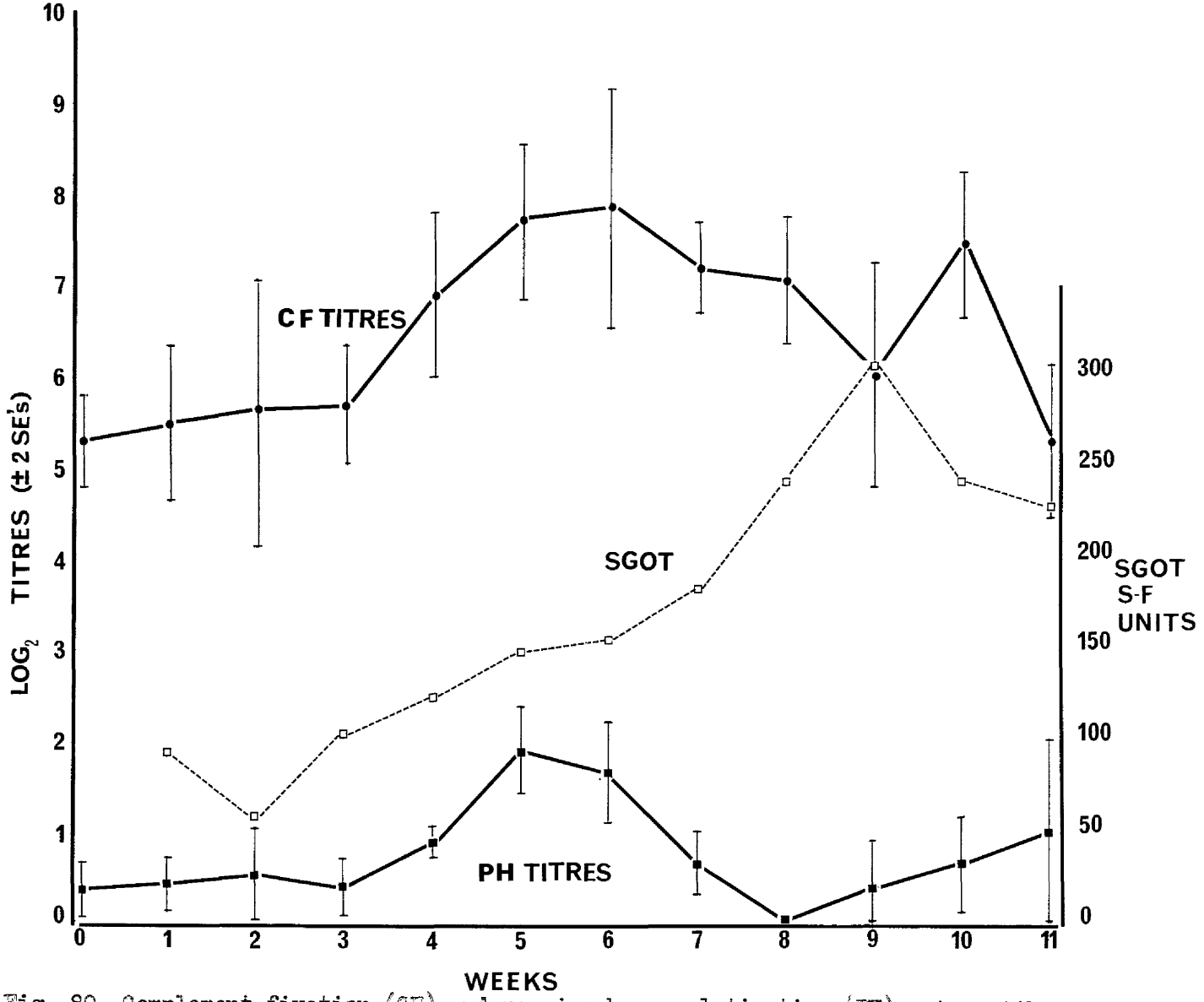


Fig. 80. Complement fixation (CF) and passive haemagglutinating (PH) auto-antibody titres (± 2 SE's) and serum glutamic oxaloacetic transaminase (SGOT) levels from sheep (Group B) given a primary F. hepatica infection.

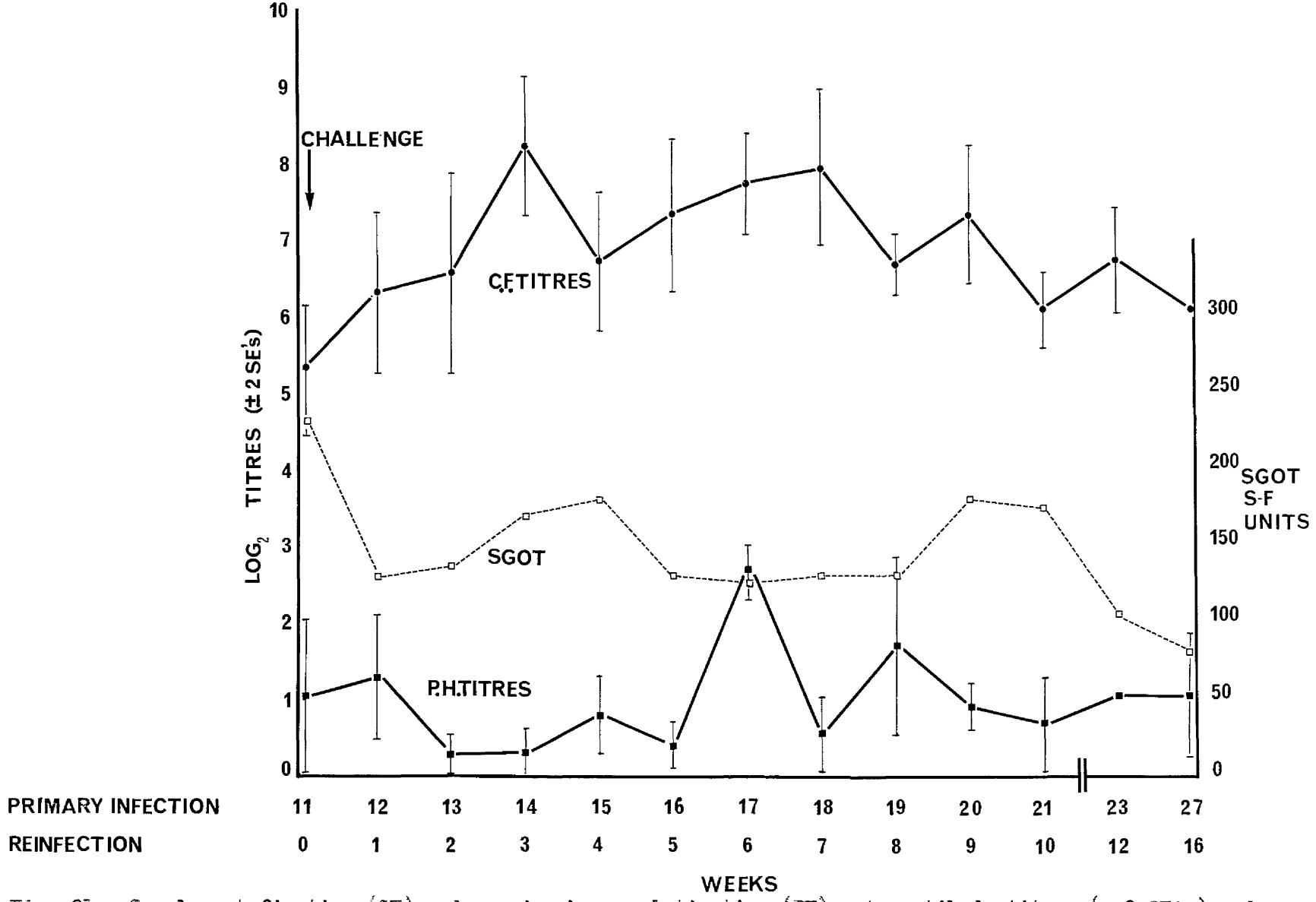


Fig. 81. Complement fixation (CF) and passive haemagglutination (PH) auto-antibody titres (± 2 SE's) and serum glutamic oxaloacetic transaminase (SGOT) levels from sheep (Group C) given primary and secondary F. hepatica infections.

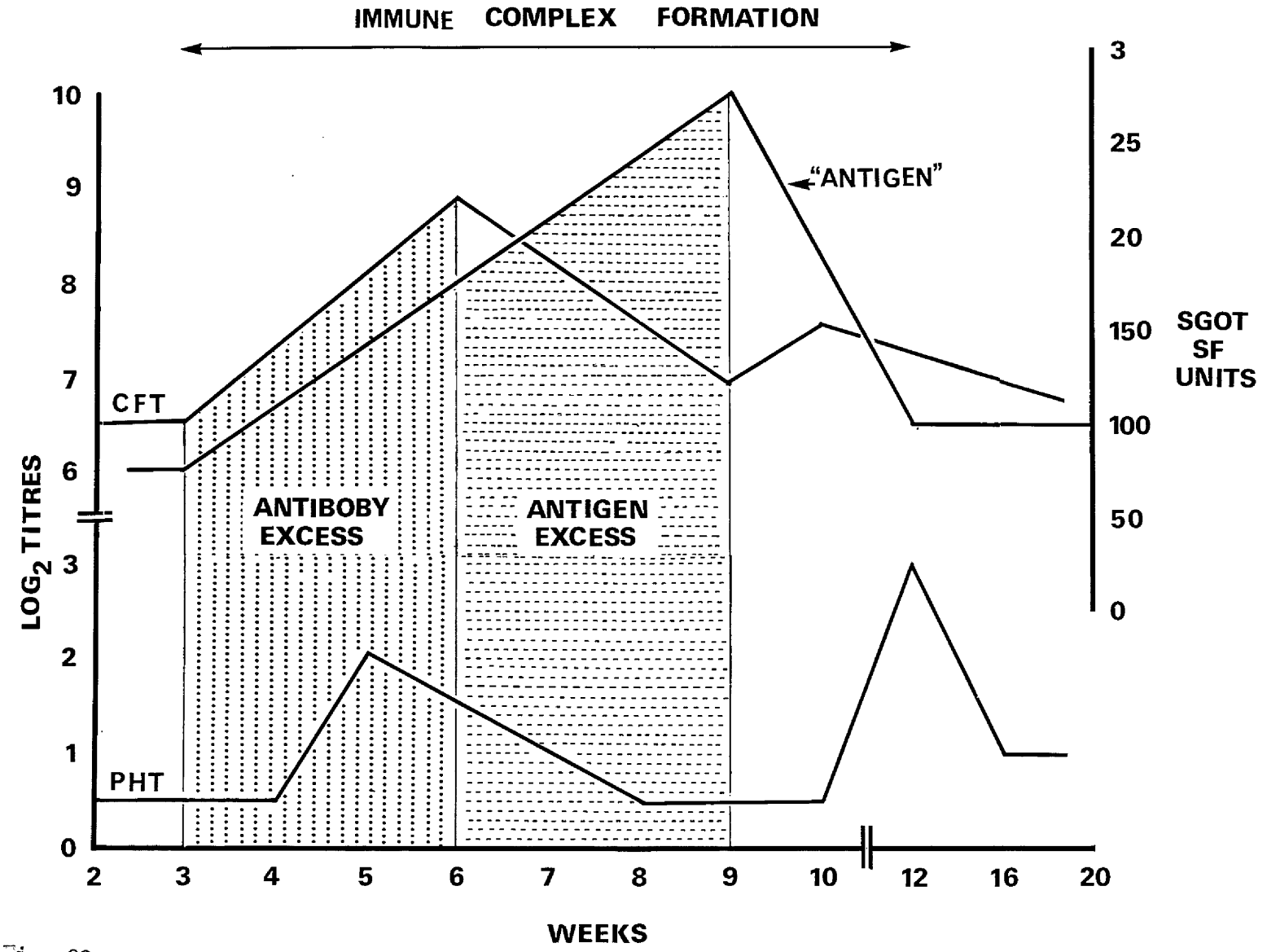


Fig. 82. Diagram of two phases of immune complex formation in a primary F. hepatica infection in sheep as judged from the complement fixing (CF) and passive haemagglutinating (PH) titres and the serum glutamic oxaloacetic transaminase (SGOT) levels representing the amount of circulating liver "antigen".

APPENDIX 1

APPENDIX 1. TABLE 1.

F. hepatica Burdens of Sheep Subject to Primary and Secondary Inoculations of 400 Metacercariae.

	Time after Inoculation in Weeks: Primary	Secondary	Total Number of Flukes Recovered	Percentage Recovery	Mean Length in mm.	SE	Range in mm.	% Immature (> 12 mm. length)
	GROUP 1							
R31	11	Control	0	-	-	-	-	-
R32	12	1	0	-	-	-	-	-
R33	13	2	0	-	-	-	-	-
R25	17	6	120	30	5.0	0.2	2-7	100%
R21	23	12	79	20	8.0[1]	0.3	4-15	98%
R22	31	20	98	24.5	18.3	0.3	13-22	0
R23	41	30	30	7.5	15.0	0.6	11-20	16%
			81[2]			19.2		
	GROUP 2							
R34	11	Control	30	7.5	15.1	0.5	8-20	16%
R35	12	1	72	18	15.5	0.6	9-23	24%
R36	13	2[3]	-	-	-	-	-	-
R30	17	6	150	37.5	19.7	0.2	9-25	1%
R26	23	12	171	-	15.2	0.9	6-23	32%
R28	28	17[4]	241	-	19.3	0.3	8-27	3%
R29	41	30	230	-	19.0	0.2	13-26	0
	GROUP 3							
G88	1		0	-	-	-	-	-
G99	2		0	-	-	-	-	-
G96	6		86	21.5	4.5	0.2	2-8	100%
G90	12		133	33	18.4[1]	0.3	10-23	2%
G89	20		139	35	21.3	0.3	12-30	1%
G100	30		197	49	17.7	0.2	10-25	2%
			138[2]			22.8		

1 Difference highly significant ($P < 0.001$)
2 Difference not significant ($P > 0.05$)
3 Primary infection did not become established
4 Died at Week 17 from copper poisoning

APPENDIX 1. TABLE 2.

Faecal Egg Output in Sheep subjected to Primary and Secondary Inoculations of F. hepatica, with Group 1 being Treated with Rafoxonide prior to the Secondary Inoculation.

Time after Inoculation in weeks:																	
Primary:	8	9	10(T)	11(C)	12	13	14	15	16	17	18	19	20	21	23	27	31
Secondary:				0	1	2	3	4	5	6	7	8	9	10	12	16	20
GROUP 1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	-
	N	N	N	N	N	N	N	N	N	N	N	N	N	2	50	500	350
	N	N	2	N	N	N	N	N	N	N	N	N	N	2	50	50	150
	N	N	1	N	N	N	N	N	N	N	-	-	-	-	-	-	-
GROUP 2	N	N	2	50	50	100	150	300	150	50	350	300	50	50	50	-	-
	N	2	12	50	150	350	850	300	200	250	700	150	50	50	50	200	200
	N	N	N	N S	N S	N	100	N	N	N	N	50	50	50	50	150	100
	N	N	8	50	100	100	950	600	200	200	-	-	-	-	-	-	-

(T) : Time of Treatment
(C) : Time of Challenge
N S : No Sample
N : No Eggs

APPENDIX 1. TABLE 3.

Serum Glutamic Oxaloacetic Transaminase (SGOT) Levels of Sheep Inoculated Orally with 400 *F. hepatica* Metacercariae.

	Weeks After Inoculation									
	1	2	3	4	5	6	7	8	9	10
R21	148	65	112	101	98	136	158	190	256	120
R22	63	60	77	75	117	99	104	224	238	261
R23	73	79	109	185	226	255	238	273	336	295
R24	92	DIED								
R25	91	62	92	168	208	201	288	386	470	294
R26	119	58	80	98	122	129	184	206	332	295
R27	56	DIED								
R28	83	49	86	87	125	142	147	201	272	162
R29	N S	99	96	123	134	113	123	170	170	293
R30	N S	74	172	146	156	167	233	292	390	226
Mean	91	68	103	123	148	155	184	242	307	243
SE	10	5	10	14	16	18	22	25	33	24

N S : No Sample

APPENDIX 1. TABLE 4.

Serum Glutamic Oxaloacetic Transaminase (SGOT) Levels of Sheep Inoculated and Reinoculated Orally with 400 *F. hepatica* Metacercariae (Groups 1 and 2), Group 1 being Treated with Rafoxanide prior to Reinoculation. SGOT Results from Sheep Receiving the same Number and Batch of Metacercariae as the Reinoculation as a Primary Inoculation are Included to Act as Challenge Controls (Group 3)

Time after Inoculation in Weeks:														
Primary:		11(C)	12	13	14	15	16	17	18	19	20	21	23	27
Secondary:		0	1	2	3	4	5	6	7	8	9	10	12	16
GROUP 1	R21	169	56	67	57	90	85	136	122	230	414	364	214	-
	R22	85	66	126	97	152	118	145	150	128	151	175	70	87
	R23	291	64	154	191	157	138	195	177	122	134	123	80	23
	R25	274	69	140	227	250	145	89	-	-	-	-	-	-
	Mean	204	64	121	143	162[1]	121	141	150	160	233[2]	220	121	55
	SE.	48.	2.	19.	39.	33.	13.	21.	15.	35.	90.	73.	46.	23.
GROUP 2	R26	257	243	180	300	250	117	88	86	87	131	118	80	-
	R28	295	222	193	204	157	111	101	84	68	70	78	38	109
	R29	207	107	90	146	160	150	135	44	125	162	165	132	87
	R30	293	185	185	130	197	152	103	-	-	-	-	-	-
	Mean	203	189	150	195	191[1]	132	106	104	93	121[2,3]	120	83	98
	SE.	20.	29.	24.	38.	21.	10.	10.	9.	19.	27.	25.	25.	27.
GROUP 3	G96	N S	N S	76	109	114	128	111	-	-	-	-	-	-
	G90	N S	N S	78	95	116	119	82	84	109	133	101	58	-
	G89	N S	N S	66	80	87	94	87	95	156	240	241	148	88
	G100	N S	N S	60	113	137	148	144	167	154	202	131	107	137
	Mean	-	-	70	99	113	122	81	115	139	191[3]	157	101	112
	SE.	-	-	4.	7.	10.	10.	27.	26.	15.	31.	42.	26.	24.

N S : No Sample
(C) : Time of Challenge

1. Difference not significant ($P > 0.05$)
2. Difference not significant ($P > 0.05$)
3. Difference not significant ($P > 0.05$)

APPENDIX 1. TABLE 5.

Log2 Complement Fixing (CF) and Serum Anti-complementary Titres of Sheep (Group A) Inoculated Orally with 400 F. hepatica Metacercariae (Anti-complementary Titre in First Column, Test Titre in Second).

	Time after Inoculation in Weeks												
	2	3	4	5	6	7	8	9	10	12	16	20	24
G89	3 5	4 6	4 6	4 6	4 8	2 7	2 5	3 6	3 6	1 5	5 7	4 6	-
G90	3 6	2 6	2 7	3 7	4 8	5 7	5 6	5 7	5 7	NS	-	-	-
G91	3 5	3 6	3 7	3 7	2 9	4 7	3 5	5 AC	DIED	-	-	-	-
G92	3 6	3 7	3 7	2 9	4 9	4 7	4 6	-	-	-	-	-	-
G93	4 6	3 6	2 9	3 9	4 9	4 8	4 6	5 AC	5 8	5 7	5 7	4 5	4 6
G94	3 7	3 6	6 3	7 2	5 8	4 7	3 5	6 AC	3 6	5 7	-	-	-
G95	3 5	3 6	3 9	3 8	3 8	3 5	4 5	4 5	3 5	5 6	4 6	3 6	3 5
G96	3 6	3 6	2 6	2 7	3 8	-	-	-	-	-	-	-	-
G97	3 5	3 6	3 7	1 6	4 6	4 7	3 6	4 7	4 6	-	-	-	-
G98	3 6	3 6	2 5	-	-	-	-	-	-	-	-	-	-
G99	4 5	-	-	-	-	-	-	-	-	-	-	-	-
G100	3 5	4 5	4 5	3 6	2 6	3 6	3 6	3 5	3 6	4 5	4 6	3 6	3 5
Mean Log2 Titre	5.5[1]	6.0	7.0	7.3	8.0[1]	6.8	5.5	6.0	6.3	6.0	6.5	5.8	5.4
SE	0.2	0.1	0.4	0.4	0.3	0.3	0.2	0.4	0.3	0.4	0.3	0.3	0.3

1 Significant difference $P < 0.001$
AC Serum Anti-complementary
NS No Sample

APPENDIX 1. TABLE 6.

Log2 Complement Fixing (CF) and Serum Anti-complementary Titres of Sheep (Group B) Inoculated Orally with 400 *F. hepatica* Metacercariae (Anti-complementary Titre in the First Column, Test Titre in the Second).

Time after Inoculation in Weeks

	0	1	2	3	4	5	6	7	8	9	10
R21	4 5	4 6	4 5	2 6	5 6	8 AC	4 AC	5 7	5 7	7 AC	8 AC
R22	4 AC	2 5	2 3	1 5	3 5	4 6	4 7	4 6	4 6	4 5	5 6
R23	4 AC	4 6	5 AC	5 7	6 7	5 8	7 8	6 7	7 8	4 AC	7 AC
R24	4 AC	4 7	DIED	-	-	-	-	-	-	-	-
R25	4 5	AC 6	6 AC	3 5	8 AC	5 8	7 AC	6 7	4 AC	7 AC	7 8
R26	4 5	2 4	5 6	3 6	6 8	10 AC	7 8	6 8	4 AC	5 7	6 8
R27	4 AC	2 4	DIED	-	-	-	-	-	-	-	-
R28	3 6	3 6	4 7	3 4	6 AC	5 7	5 AC	5 7	5 6	5 6	6 8
R29	NT	NT	5 7	3 6	6 7	5 9	5 6	8 AC	6 8	7 AC	6 7
R30	NT	NT	5 AC	3 6	6 8	4 8	6 10	6 8	6 8	7 AC	8 AC
Mean Log2 Titre	5.3	5.5	5.6	5.6[1]	6.8	7.7	7.8[1]	7.2	7.0	6.0	7.4
SE	0.2	0.4	0.7	0.3	0.5	0.4	0.7	0.2	0.4	0.6	0.4

1 Difference not significant $P > 0.05$
AC Serum Anti-complementary
NT Not Tested

APPENDIX 1. TABLE 7.

Log_2 Complement Fixing (CF) and Serum Anti-complementary Titres of Sheep (Group C) Inoculated then Reinoculated Orally with 400 F. hepatica Metacercariae. (Anti-complementary Titre in the First Column, Test Titre in the Second).

	Time after Inoculation in Weeks												
Primary	11	12	13	14	15	16	17	18	19	20	21	23	27
Secondary	0	1	2	3	4	5	6	7	8	9	10	12	16
R21	2 6	6 AC	5 AC	7 9	4 7	6 7	5 8	5 7	5 7	6 7	4 6	5 6	-
R22	1 3	2 4	1 5	5 6	1 5	3 5	4 7	3 6	4 6	4 6	4 6	5 AC	5 6
R23	2 6	6 8	4 7	7 9	4 9	5 7	6 8	6 9	5 7	6 8	5 7	5 7	7 AC
R25	2 5	4 6	5 AC	7 AC	5 9	4 6	6 8	-	-	-	-	-	-
R26	5 AC	6 8	6 8	7 9	4 7	5 10	6 9	6 9	5 7	7 8	5 6	4 7	-
R28	3 6	5 6	4 6	6 8	2 6	4 7	5 8	5 8	2 7	6 AC	4 6	4 AC	7 AC
R29	3 5	5 6	5 AC	6 8	1 5	5 8	3 7	5 8	4 6	5 AC	2 5	6 AC	5 6
R30	3 6	2 6	5 AC	7 AC	2 7	4 8	4 6	-	-	-	-	-	-
Mean Log_2 Titre	6.3	6.3	6.5	8.2	6.6	7.3	7.7	7.8	6.7	7.3	6.0	6.7	6.0
SE	0.4	0.5	0.6	0.5	0.5	0.5	0.3	0.5	0.2	0.5	0.2	0.3	0

AC Serum Anti-complementary

APPENDIX 1. TABLE 8.

Log_2 Passive Haemagglutinating (PH) Titres of Sheep (Group A) Inoculated Orally with 400 F. hepatica Metacercariae.

	Time after Inoculation in Weeks												
	2	3	4	5	6	7	8	9	10	12	16	20	24
G89	1	1	1	1	0	1	2	1	1	2	0	1	-
G90	1	2	2	3	3	1	2	1	0	N S	-	-	-
G91	0	1	1	1	1	1	0	0	DIED	-	-	-	-
G92	0	0	0	1	1	0	0	-	-	-	-	-	-
G93	0	0	0	4	2	1	1	1	0	2	0	2	1
G94	1	1	0	2	0	0	0	1	0	2	-	-	-
G95	0	0	0	3	0	0	0	1	0	1	0	1	0
G96	1	2	1	2	3	-	-	-	-	-	-	-	-
G97	1	1	1	2	2	2	0	2	0	-	-	-	-
G98	1	0	2	-	-	-	-	-	-	-	-	-	-
G99	0	-	-	-	-	-	-	-	-	-	-	-	-
G100	2	2	1	1	2	2	0	1	1	3	3	4	1
Mean Log_2 Titre	0.7[1]	0.9	0.8	2.0[1]	1.4	0.9	0.5	1.0	0.3	5.0	0.8	2.0	0.7
SE	0.2	0.2	0.2	0.3	0.4	0.3	0.3	0.2	0.2	0.3	0.8	0.7	0.3

1 Significant difference $P < 0.01$.
N S No Sample.

APPENDIX 1. TABLE 9.

Log_2 Passive Haemagglutinating Titres of Sheep (Group B) Inoculated Orally with 400 F. hepatica Metacercariae.

	Weeks after Inoculation										
	0	1	2	3	4	5	6	7	8	9	10
R21	1	0	1	0	1	1	1	1	0	0	1
R22	0	0	2	1	1	2	2	0	0	0	0
R23	0	1	0	1	1	1	1	1	0	2	1
R24	0	1	DIED								
R25	0	0	0	0	1	2	1	1	0	0	2
R26	1	0	0	0	1	3	2	1	0	1	1
R27	0	0	DIED								
R28	0	1	0	1	1	2	1	0	0	0	0
R29	NT	NT	1	0	0	2	2	0	0	0	0
R30	NT	NT	0	0	1	2	3	1	0	0	0
Mean Log_2 Titre	0.3	0.4	0.5[1]	0.4	0.9	1.9	1.6[1]	0.6	0	0.4	0.6
SE	0.2	0.2	0.3	0.2	0.1	0.2	0.2	0.2	0	0.2	0.2

1 Significant difference $P < 0.01$.
NT Not Tested.

APPENDIX 1. TABLE 10.

Log_2 Passive Haemagglutinating (PH) Titres of Sheep (Group C) Inoculated then Reinoculated Orally with 400 F. hepatica Metacercariae.

Weeks after Inoculation

Primary	11	12	13	14	15	16	17	18	19	20	21	23	27
Secondary	0	1	2	3	4	5	6	7	8	9	10	12	16
R21	0	0	0	0	0	0	3	1	2	1	2	1	-
R22	0	0	0	0	0	0	2	0	0	0	0	1	1
R23	0	2	0	0	0	1	3	1	4	1	1	1	2
R25	1	3	0	0	2	1	3	-	-	-	-	-	-
R26	2	2	1	1	1	1	3	1	1	1	1	1	-
R28	0	0	0	0	1	0	2	0	2	1	0	1	1
R29	4	2	1	1	1	0	3	0	1	1	0	1	0
R30	1	1	0	0	1	0	2	-	-	-	-	-	-
Mean Log_2 Titre	1.0	1.3	0.3	0.3	0.8	0.8	2.6	0.5	1.7	0.8	0.7	1.0	1.0
SE	0.5	0.4	0.1	0.1	0.2	0.2	0.2	0.2	0.6	0.1	0.3	0	0.4

APPENDIX 1. TABLE 11.

Mean Serum Glutamic Oxaloacetic Transaminase (SGOT) Levels of Sheep Inoculated then Reinoculated Orally with 400 F. hepatica Metacercariae.

	Weeks after Reinoculation												
	0	1	2	3	4	5	6	7	8	9	10	12	16
Mean	234	127	136	169	177	127	124	127	127	177	170	102	76

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