EXPERIMENTAL FASCIOLIASIS

IN THE RABBIT

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE FACULTY OF MEDICINE, THE UNIVERSITY OF GLASGOW

by

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GENERAL INTRODUCTION.

INTRODUCTION.

Between 1881 and 1883 Leuckart in Germany and Thomas in England independently elucidated the first trematode life-cycle, that of Fasciola hepatica. Since then a large mass of information has been obtained about this parasite, but it is still encountered today as a major cause of mortality and morbidity in sheep and cattle all over the world.

In Britain in 1942, Peters and Clapham estimated, on an analysis of slaughterhouse returns covering over 73,000 cattle, that the economic loss to the meat industry was probably about 1,500 tons of liver per annum, representing a retail price of about one-quarter of a million pounds; this estimate did not include reduced meat and milk production in infected animals. Also in this country during the winters of 1946-47 and 1947-48 severe epidemics of fascioliasis occurred among sheep and mortality was extremely high (Taylor 1949). The incidence of the disease in other countries is reviewed by Hutyra, Marek and Manninger (1946).

The adult parasite (Fig. 1) is found in the bile-ducts of cattle and sheep, and has also been observed on numerous occasions in the livers of rabbits, horses, pigs and man. It is leaf-shaped, broader anteriorly than posteriorly and is about 30 mm. long and 13 mm. wide. At the anterior end there is a conical projection terminating in the oral sucker; the ventral sucker is situated at the level of the shoulders. The cuticle is covered with sharp spines. The digestive tract possesses branched caeca which extend to the posterior end of the worm.

The/

The parasites are hermaphrodite and lay large yellowish ovoidal operculate eggs (Fig. 2) about 140μ x 70μ. The eggs enter the intestine with the bile and pass out in the faeces. After about 9 - 15 days at an optimal temperature of 22° - 26°C. the eggs hatch and the pyriform ciliated miracidia emerge (Fig. 3). The anterior end of each miracidium is blunt and possesses a small papilliform protrusion. These miracidia move actively in water and may survive for about 24 hours. If during this time a suitable snail host (in Britain, principally Lymnaea truncatula (Fig. 4)) is encountered, the miracidium actively penetrates the soft tissues of the snail, using the papilliform protrusion as a boring apparatus. Once in the snail, the miracidum loses its ciliated covering and is known as a sporocyst; this structure increases in size, and by growth and multiplication of its germinal cells produces numerous larvae known as rediae; these grow, rupture the sporocyst, and are liberated into the snail tissues. The redia (Figs. 5 and 6) is cylindrical and possesses anteriorly a collar-like structure and posteriorly a pair of marginal processes. During movement (Wright, 1928) the collar disappears and the anterior part of the body is thrust forward; the collar then reappears and acts as an anchor while the marginal processes are withdrawn and the posterior part of the body is drawn forward. The germinal cells of the redia produce the next stage of the parasite, the cercariae. These are shed in considerable numbers through a birth pore behind the collar of the redia, emerge from the snail and may be seen by the naked eye as minute tadpole-like structures which swim freely in water.

Each/

Each cercaria possesses oral and ventral suckers, a bifurcating intestine and dark granular cystogenous glands. After about one hour the cercaria attaches itself to a grass blade or other suitable object, surrounds itself with secretion from the cystogenous glands and discards its tail (Fig. 7). The time which elapses between the infection of the snail and the shedding of cercariae occupies about 6 - 7 weeks under optimal conditions.

These encysted cercariae may remain infective for several months. If, during this time, they are ingested by a suitable host they excyst in the intestine, penetrate the wall of the gut and migrate across the peritoneum to the liver which they enter by boring through the capsule (Schumacher, 1939); the young flukes may be found in the liver as soon as 48 hours after infection; they grow and wander apparently at random in the liver parenchyma for several weeks and eventually enter the bile-ducts in which they become mature about 8 or 9 weeks after infection.

Death of the host may occur during the acute or migratory stage of the infection due to extensive destruction of liver tissue or to haemorrhage from this organ. The chronic form of the disease associated with the presence of adult parasites in the bile-ducts is more frequently encountered and is usually characterised by emaciation, anaemia and oedema; the oedema is usually present as ascites and oedema of the intermandibular space (the latter is the classical sign of "bottle-jaw"). At autopsy the liver is cirrhotic and the large bile ducts containing the parasites are thickened and fibrous.

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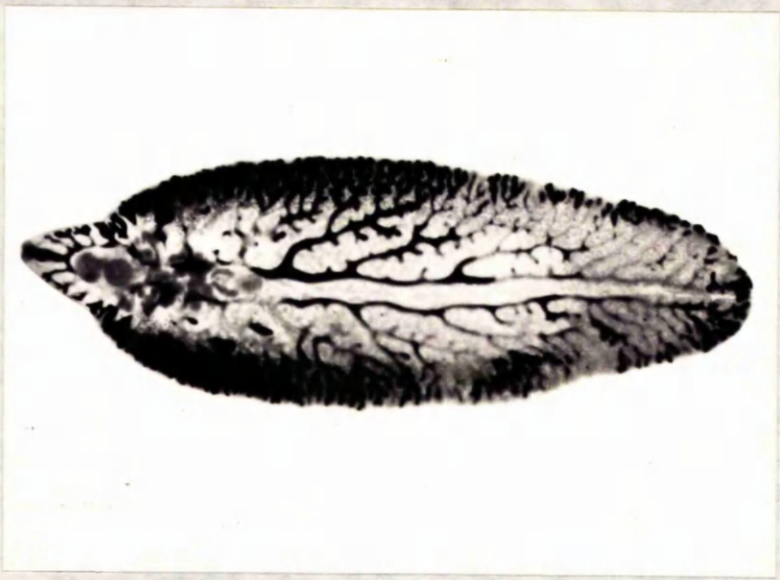


Fig. 1. Adult Fasciola hepatica. The branching caeca are filled with a dark brown fluid; the oral and ventral suckers and the genitalia may be seen. x 5.

Fig. 2. Egg of F. hepatica recovered from gall bladder bile. x 550.

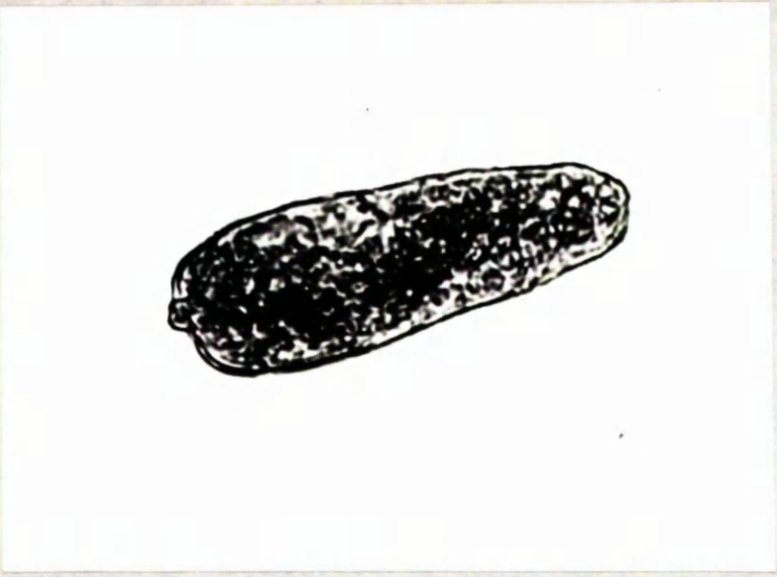


Fig. 3. Miracidium of F. hepatica showing conical boring apparatus at the blunt anterior end. x 450.

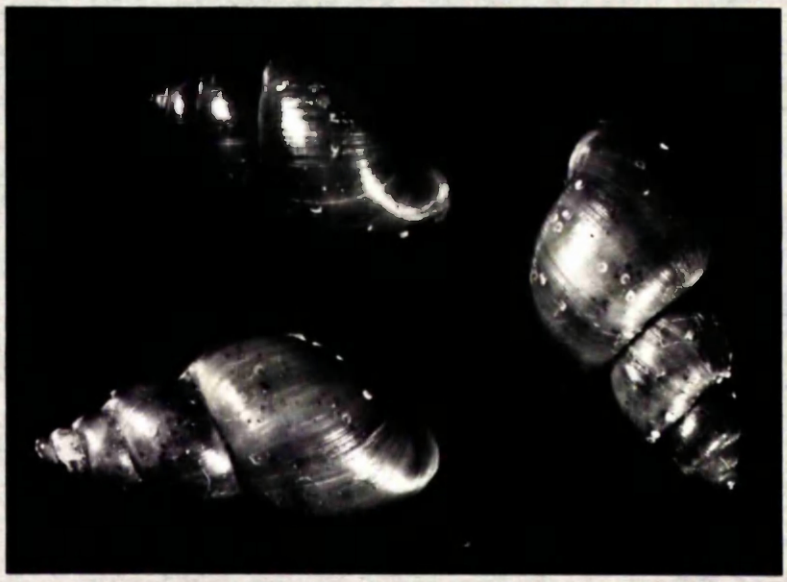


Fig. 4. The snail host, Lymnaea truncatula. A few cercariae have encysted on the shells. x 10.

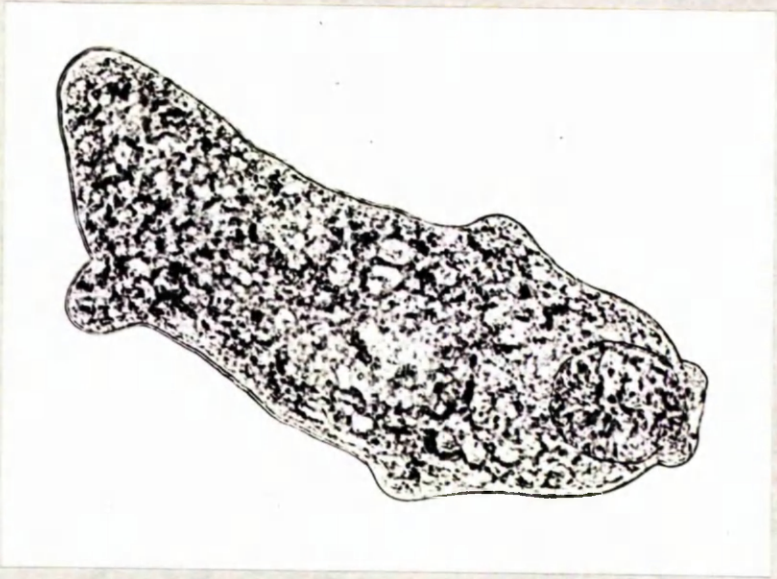


Fig. 5. Young redia. The pharynx, the collar, and the lateral processes are visible. x 150.

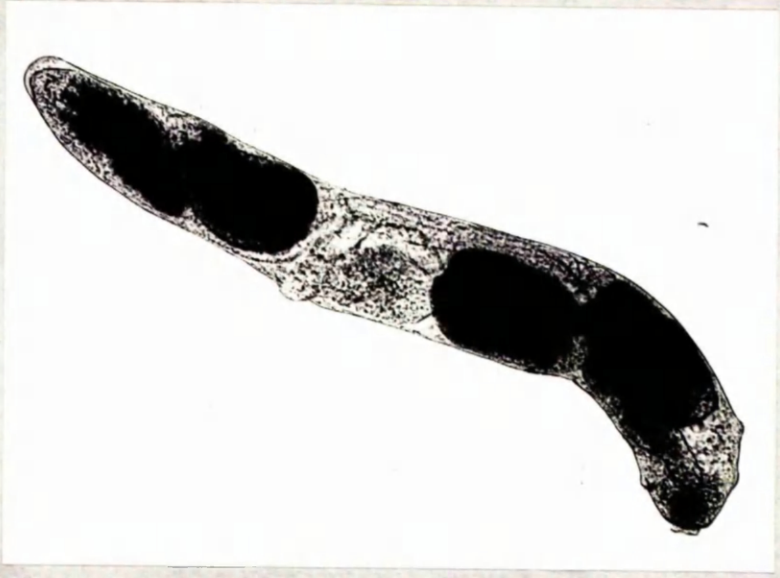


Fig. 6. Mature redia. There are four cercariae within the body. x 70.

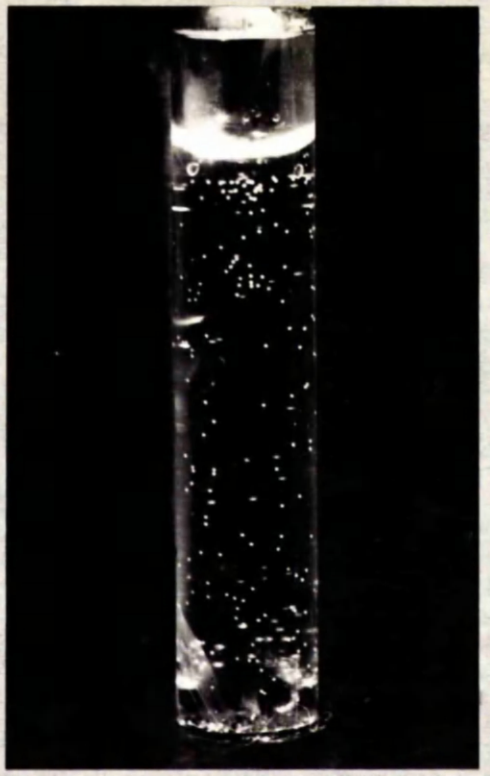


Fig. 7. Cercariae encysted on Cellophane lining of glass tube. x 2.

In this investigation an attempt has been made to study three aspects of the disease as it affects the final host; they are the anaemia, the liver lesions, and the response to artificial immunisation with an antigen prepared from the parasites. The literature on each of these subjects is reviewed in the relevant sections of the thesis.

Because of lack of accommodation when these experiments were started, it was not possible to use sheep or calves, and the rabbit was selected as a suitable laboratory host. The first section of the thesis is, therefore, concerned with a study of the rabbit as host in experimental fascioliasis.

SECTION I

THE RABBIT AS HOST IN EXPERIMENTAL FASCIOLIASIS.

As a preliminary to the rest of the work reported in this thesis, and with particular regard to the proposed studies on acquired immunity, it was found necessary to determine some of the factors influencing the number of adult flukes which develop from a given number of cercariae administered to an unimmunized rabbit or to a previously uninfected rabbit.

Such factors might include the breed, age, weight, sex and individual natural resistance of the rabbit, the age of the cercariae, a variation in virulence of cercariae from different snail hosts, the age of the snail, the technique of infection of the rabbit and the size of the infecting dose.

The determination of the precise importance of each of these factors would require a large number of experiments, but sufficient information was obtained to be of value for practical purposes.

There is little published information available on the rabbit as an experimental host for F. hepatica. Montgomerie (1931) recovered from three rabbits infected with 6, 15, and 40 cercariae, 4, 7, and 11 flukes after 13, 31 and 37 months, respectively, while Kerr and Petkovitch (1935) found that in three rabbits each infected with 13 cercariae and killed 111, 116, and 124 days after infection, there were 5, 9, and 11 flukes, respectively, in the liver.

METHODS AND MATERIALS.

The snails, Lymnaea truncatula, and suitable mud were obtained from the field (see Appendix A); the former were cultured by the method/

method of Taylor and Mozley (1948) and were infected when about 6 weeks old with miracidia obtained by culturing in water, eggs recovered from sheep bile.

The cercariae were stored in a cool, dark, water-saturated atmosphere for 7 days. At the end of this period they were administered in doses of 50 cercariae to each rabbit. The method of infecting the rabbits was varied and the techniques are described in detail below.

The rabbits, all obtained from the same source, were from 6 months to 1 year old and were either Dutch or Old English-Chinchilla crosses (O.E. x C.), both females and castrated males being used.

RESULTS

Experiment 1: The Development of a Simple, Rapid and Efficient Technique for the Infection of Rabbits with Cercariae of *F. hepatica*.

In experimental infection of rabbits with cercariae of *Fasciola hepatica*, particularly where large numbers of cysts are being fed, it is desirable to use a method which is satisfactory in the following respects.

(a) The preparation of specific numbers of viable cercariae should be simple and quick.

(b) The cercariae should be handled as little as possible in order to minimize damage to them and danger to the investigator.

(c) The method of administration of the cercariae to the animal should be simple and reliable.

The/

The advantages and disadvantages of a number of techniques are described below and in conclusion details are given of one which has proved superior in the above respects.

The method originally used to collect cercariae from the host snail, Lymnaea truncatula, was as follows. Each snail was placed in a glass test tube (5 x 1 cm) three-quarters full of water. The tube was then corked. When the cercariae emerged, they encysted on the glass, the majority often being found at the water line.

The technique of infection was to lift the cercariae off the glass with a thin glass rod and place them in a watchglass or small test tube. When the requisite number had been collected, a few drops of water were added to the watchglass, and the cercariae drawn up into a capillary pipette to be administered orally to the rabbit. Alternatively, water was added to the small test tube, the latter inverted several times and the contents forcibly administered to the rabbit.

The disadvantages of this technique were :

(a) The task of transferring large numbers of cercariae from one container to another by means of a glass rod was extremely tedious.

(b) During transference, the outer cysts of the cercariae were liable to be ruptured or to be lost entirely, thus introducing an unnatural element during infection.

(c) The cercariae adhered to the wall of the dosing tube or to the sides of the pipette. The use of liquid paraffin, silicone, wax or wetting agents to coat the glass was of little value.

(d)/

(d) During administration the rabbits tended to cough and so to lose some of the fluid.

When this method was used the following numbers of flukes were obtained when each of 12 rabbits was infected with 50 7-day old cercariae and killed 63 days after infection :

1, 2, 2, 3, 6, 6, 8, 12, 13, 14, 17 and 20.

The average number of flukes in each rabbit was 8.7 ± 6.4.

Various other techniques of infection were tried, including gelatine capsules and the stomach tube. The gelatine capsules tended to dissolve in contact with drops of water; it was impossible to count the cercariae once they were in the capsule; and dosing was extremely difficult because of the long palate of the rabbit. The stomach tube had the disadvantage that the cercariae adhered to the sides of the syringe and tube.

The following method was finally adopted :

The "shedding" tube was lined with a Cellophane cylinder made by folding a rectangle of Cellophane around a glass rod. It was then filled three-quarters full with water, the snail inserted and the tube corked. The cercariae encysted on the Cellophane, usually at water level. The snail was removed, the water drained off, and the tube was stored in a cool dark cupboard in a saturated atmosphere.

When the cercariae were required, the Cellophane was removed from the tube with forceps and spread out flat on a watchglass. A piece of Cellophane containing the requisite number of cercariae was cut out with a scalpel blade. It was usually convenient to take a

piece/

piece of Cellophane which was at water level and which contained a large number of cercariae in a small area. This piece of Cellophane was placed on a slide and examined microscopically to ensure that none of the cercariae was damaged. The Cellophane was then inserted, unfolded if possible, in a small piece of cabbage stalk and fed to the rabbit by hand. Ordinarily the rabbit ate the cabbage and Cellophane without any difficulty, especially if fed at the usual feeding time.

This method when used to infect each of 32 rabbits with 50 7-day old cercariae gave the following number of flukes when the rabbits were killed after 63 days :

4, 6, 6, 6, 6, 6, 7, 11, 12, 13, 15, 16, 18, 18, 18, 19, 20, 20, 22, 22, 25, 25, 25, 25, 25, 25, 27, 27, 27, 30, 34, and 35.

The average number of flukes in each rabbit was 18.6 ± 8.8.

This method would probably also be satisfactory for infecting sheep or cattle with large or small numbers of cercariae.

TABLE I/

TABLE I

Number of Adult Flukes Obtained from 4 Groups of Rabbits Infected by Feeding Cercariae Encysted on Cellophane. Each Group Received Cercariae from a Different Snail and Each Rabbit Received 50 Cercariae

	Breed (Sex)*	No. of flukes	Av. No. of flukes in each group
Snail D	Dutch (F)	13	16.7
	Dutch (F)	15	
	Dutch (F)	22	
Snail E	Dutch (F)	12	16.3
	Dutch (F)	15	
	Dutch (F)	22	
Snail F	O.E. x C. (F)	19	24.3
	Dutch (F)	27	
	O. E. x C. (M)	27	
Snail G	Dutch (F)	11	20.5
	Dutch (F)	30	

Average Number of flukes per rabbit, 19.4 ± 6.6.

* (F) refers to female; (M) refers to male.

Experiment 2: To Study the Effect of the Uses of Cercariae from Different Snails on the Infectivity of Cercariae of F. hepatica

In this experiment 4 groups of rabbits were each infected with cercariae from different snails, each rabbit receiving 50 7-day old cercariae. The rabbits were killed 63 days after infection and the results are recorded in Table I.

It is evident that the use of cercariae from different snails does/

does not markedly influence the number of adult flukes in an infected rabbit. However, in this experiment the rabbits with one exception were all female. In order to determine if the infectivity of cercariae is influenced by the sex of the rabbit a further experiment was carried out.

Experiment 3: To Study the Effect of the Sex of the Rabbit on the Infectivity of Cercariae of F. hepatica

Ten castrated male rabbits and 10 female rabbits were each infected with 50 7-day old cercariae encysted on Cellophane, and killed 63 days later. The results are recorded in Table II.

TABLE II

The Average Number of Adult Flukes in Male and Female Rabbits 63 Days after Each was Infected with 50 Cercariae Encysted on Cellophane

Males		Females	
Breed	No. of flukes	Breed	No. of flukes
Dutch	4	?	6
?	6	Dutch	11
?	7	Dutch	12
O.E. x C.	16	Dutch	13
Dutch	18	Dutch	15
O.E. x C.	20	Dutch	22
Dutch	25	Dutch	22
Dutch	25	O.E. x C.	27
O.E. x C.	27	Dutch	27
?	35	Dutch	30
Average number of flukes 18.3 ± 10.2		Average number of flukes 18.5 ± 8.2	

It appears therefore that the sex of a rabbit does not influence its susceptibility to infection.

DISCUSSION/

DISCUSSION

In these experiments an attempt has been made to determine the average number of Adult Fasciola hepatica which developed from a given number of cercariae fed to a rabbit. The variations around this average, and some of the factors influencing these, have also been studied.

It would appear that, when cercariae of the same age are used, two of the main factors influencing the "take" are the method of infection and the natural resistance of the individual rabbit. Variation due to the first factor may be almost eliminated by the use of a technique in which it is certain that the rabbit has swallowed the requisite number of viable cercariae. The advantages of the Cellophane infection technique have been described previously and, assuming that the rabbit has eaten all the cabbage containing the Cellophane, the only variable with this method is the possibility of damage to the cercariae by the teeth of the rabbit. The term "natural resistance" is used here in a broad sense and includes, as well as resistance of a genetic origin, such factors likely to influence natural resistance as the amount of food in the alimentary tract at the time of infection or the possibility of damage to the cercariae by the teeth of the rabbit.

In these experiments, the natural resistance of the rabbits to infection with cercariae of F. hepatica appeared to vary within quite wide limits, and it is interesting to compare the results recorded/

recorded here with those obtained by Montgomerie (1928) in the artificial infection of sheep with F. hepatica. Thirty-two sheep were infected with a total of 2,550 cercariae; 37.4% of these cercariae developed and were recovered at autopsy 14 weeks after infection; the smallest infection was 8% and the heaviest 76%. The results recorded here show that the average number of flukes in 32 rabbits infected with a total of 1,600 cercariae was 37.2%; the smallest infection was 8% and the heaviest 70%.

The age range of the cercariae used in Montgomerie's experiment is not stated, but it appears that the natural resistance of the sheep to infection with F. hepatica is about the same level and within the same limits as that of the rabbit.

Evans and Stirewalt (1951), discussing the infectivity of Schistosoma mansoni in mice, concluded that statistically significant variations occurred in the infectivity of the cercariae obtained throughout the life of the host snail and they suggest that these variations are directly related to the fluctuating physiological condition of the host snail. In our experiments the snails invariably died within 3 or 4 days after first shedding cercariae and this factor was not studied.

The use of cercariae from different snails and the sex of the rabbits, at least as far as females and castrated males are concerned, do not appear to influence markedly the degree of infection.

The two breeds of rabbits used were not ideally distributed to determine their precise importance but, on the available evidence/

evidence, the breed seems to have no effect on the degree of infection.

SUMMARY

1. The possible factors which influence the number of adult flukes developing in a rabbit from a given number of cercariae are described.

2. Experiments were carried out to determine the importance of some of these factors.

3. It is concluded that two of the most important factors determining the number of flukes developing from a given number of 7-day old cercariae are the technique of infection and the natural resistance of the rabbit.

4. Several methods of infecting rabbits with cercariae of F. hepatica are described and discussed, and details are given of a simple, rapid and efficient technique.

5. The natural resistance of the sheep to infection with F. hepatica appears to be about the same level and within the same limits as that of the rabbit.

6. When a technique of infection was used in which the cercariae were encysted on Cellophane and, when 7 days old, fed in cabbage in doses of 50 cercariae, the average number of flukes in 32 rabbits killed 63 days after infection was 18.6 ± 8.8.

7. The breed and sex of the rabbits used or the use of cercariae from different snails played no significant part in determining the degree of infection.

NOTE:/

NOTE: Recently, a slightly different method of infecting rabbits with cercariae has been employed. The piece of Cellophane containing the requisite number of cercariae is inserted into a small gelatine capsule; the latter is placed in a "balling gun" constructed of two short lengths of polythene tubing, one inside the other, and the capsule is forcibly fed to the animal.

With this method, the rabbit cannot refuse to swallow the Cellophane and the possibility of damage to the cercariae by its teeth is eliminated. Only a small number of rabbits has been infected by this method and the results are similar to those described above.

SECTION 2

A STUDY OF THE ANAEMIA ASSOCIATED WITH *FASCIOLA HEPATICA* INFECTION OF RABBITS

INTRODUCTION

A degree of anaemia is recognised as a characteristic feature of chronic infection with Fasciola hepatica in sheep and cattle and each of three standard textbooks on veterinary helminthology makes reference to this fact. Cameron (1951) states that, "the flukes feed on blood and bile but any loss from this source is generally easily replaced by the host ..... the most profound general effect, however, appears to be due to a specific toxin secreted by the fluke, causing a more or less profound anaemia." Clunies, Ross and Gordon (1936) record that "the production of a haemolytic toxin by the fluke leads to the destruction of the red blood corpuscles." Finally, Monnig, (1950) states that the flukes feed "on liver tissue and blood", and that the parasites "produce a haemolytic toxin."

In each of these books, reference is made to a toxin, in two of them to a haemolytic toxin, as being responsible for the anaemia associated with fascioliasis. Although none of the authors cites any reference in support of this statement, the latter is probably extracted from the conclusions of Marconé and of Balian. Marconé (1940) claimed that the sera of fluke-infected sheep had a haemolytic effect on normal red cells. Balian (1940) in a study of the anaemia in naturally infected sheep observed macrocytosis and concluded, apparently by analogy with some human haemolytic anaemias, that a haemolytic factor was present; he also postulated, in order to explain the severity of the anaemia, that the flukes had/

had a specific depressant action on the haemopoietic tissue.

Apart from the papers of these two authors, there are few references to the etiology of this anaemia in scientific journals. In 1908 Guerini demonstrated two types of secretory cells in the cuticulum of F. hepatica and suggested that the secretions of these cells may be absorbed and destroy the red cells; more recently, Holman (1945) has suggested that an aplastic anaemia might occur in fluke-infected sheep, due to extensive liver destruction.

The nature of the food of F. hepatica, a problem which has been the subject of prolonged controversy, is of possible interest with regard to the etiology of the anaemia. Muller (1923) examined the gut contents of flukes microscopically and found neither haemoglobin nor red blood cells. Weinland and Brand (1926) examined microscopically 12,000 F. hepatica from 148 sheep; they concluded that the flukes feed on tissue elements and inflammatory exudates but not on blood. They also claimed that the parasites inhabit the larger bile ducts and gall bladder and migrate into the smaller bile ducts to feed. Recently, however, Stephenson (1947) on the basis of histological, histochemical and spectroscopic examination of the gut contents of F. hepatica concluded that the adults feed mainly, if not exclusively, on blood.

Since it is known that the rabbit is a suitable host of F. hepatica (Montgomery 1931; Taylor, 1949; Urquhart, this thesis) a series of experiments on rabbits was planned in

an/

an attempt to obtain details of the anaemia. Later, it was found desirable to compare these details with the blood changes produced in rabbits by repeated haemorrhage and by ligation of the common bile duct. From the results obtained it was possible to discuss some factors concerned in the etiology of the anaemia.

MATERIALS AND METHODS

The snail host of F. hepatica, Lymnaea truncatula was cultured according to the method described by Taylor and Mozley (1948). The snails were infected when about 6 weeks' old with miracidia obtained by incubating bile from infected sheep livers at 26°C. The cercariae were collected and the rabbits infected by the methods described in the first section of this thesis.

Haematocrit estimations were carried out using Wintrobe haematocrit tubes which were spun at 3,000 r.p.m. for 1 hour in an M.S.E. Minor Centrifuge and haemoglobin estimations with the M.R.C. Grey Wedge Photometer (King et al. 1948). Plasma bilirubin estimations were also carried out with this instrument or with the E.E.L. Colorimeter, following the procedure described by King (1951). The spectral absorption curve of the plasma was examined for evidence of the presence of biliverdin using a Unicam S.P. 500 Photoelectric Spectrophometer (see Appendix B). The method used for the estimation of alkaline phosphatase was essentially that described by King (1951) except that the reaction was carried out at pH 9 using a barbitone buffer. Blood films and marrow smears were stained by Leishman, and

if/

if a reticulocyte count was desired, a 1% solution of brilliant cresyl blue in normal saline was used. The marrow sections were stained with Giemsa, Maximow, haematoxylin and eosin, and Prussian blue. The red cell fragility test was carried out by the method described by Whitby and Britton (1950) which is based on Creed's technique; the points recorded were those at which haemolysis commenced and those at which it was complete. The coagulation time of the blood was determined by the capillary tube method of Wright and Colebrook (1921). Plasma proteins were estimated either by the Biuret method or by the copper sulphate specific gravity method described by Phillips et al. (1947). The benzidine and haemin tests were carried out as described by Hawke, Oser and Summerson (1947); all solutions for testing were boiled before the benzidine test was performed.

The diameters of the red cells were measured on heat-fixed fresh smears, stained by Leishman. The technique of measurement was essentially that described by Goldberg, Hammarsten, Hellstrom, Lindgren and Plum (1950); 200 cells in each smear were measured. Red cell diameter distribution curves were constructed from these measurements; these curves were smoothed by the method described by Mogensen (1938).

Ten rabbits were infected with 100 cercariae and 25 rabbits with 50 cercariae. The blood of the first group was examined regularly over a period which in some of the rabbits extended over 18 months; the blood of the second group was examined at infrequent/

infrequent intervals. Each blood sample was obtained from the ear vein by the following method: The ear was lightly swabbed with xylol except around the marginal vein. This area, previously shaved, was swabbed with alcohol and smeared with Vaseline. The vein was raised by digital compression and then cut. When the blood flowed freely 2.5 ml.were collected in a bijou bottle which contained Wintrobe's anticoagulant crystals. After the sample was collected, bleeding was stopped immediately and the ear swabbed with alcohol and dried. Bone marrow biopsies were carried out, under pentothal anaesthesia in the following manner. A hole was drilled in the proximal end of the tibia; marrow fragments were aspirated from this hole with a 1 cc. syringe fitted with a blunt-pointed hypodermic needle. The aspirated material was expelled into a watchglass and smears were made from the marrow fragments. When each rabbit died or was killed, it was autopsied and the number of F. hepatica in the liver was determined; blocks of liver, spleen and bone marrow were removed for histological examination.

Approximately 25 normal rabbits were killed at various times in order to obtain sections of normal tissues. In the two rabbits in which an artificial post-haemorrhagic anaemia was produced, blood was removed in the manner described above. The quantity and frequency of removal are stated in the relevant section.

The three rabbits which were subjected to ligation of the common bile duct were operated on under pentothal anaesthesia. A longitudinal/

longitudinal incision was made just to the right of mid-line, and ¼" behind the last rib. The common bile duct was exposed and this was ligated with silk thread at two points just above the duodenum.

RESULTS

In each of the infected rabbits a degree of anaemia was produced which in every animal appeared about 6 weeks after infection. It is interesting that it is about this time that the young flukes settle down in the bile-ducts and commence their adult activities.

Eighteen of the 25 rabbits which received 50 cercariae were killed 63 days after infection. Fig. 8 shows the number of flukes found in the bile ducts of each of these rabbits compared to the blood haemoglobin of each individual immediately before death. It is apparent that the degree of anaemia is approximately proportional to the number of flukes found in the bile-ducts at autopsy.

The bloods of the 10 rabbits each of which received 100 cercariae were studied in detail; the results are described below under the appropriate headings and in Table 5. Figure 9 consists of graphs constructed from this table to facilitate comparison of the red cell counts, mean corpuscular volumes, mean corpuscular haemoglobin concentrations, and reticulocyte counts of the individual rabbits.

Red cell count, haemoglobin and haematocrit.

The average red cell count of the 10 rabbits prior to infection was 6.04 ± 0.16 million per c.mm. This remained unaltered until about 42 days after infection when the counts of each of the rabbits, except that of the lightly infected N217, started to fall and by 60-70 days/

days were below 4 million in each case.

About 80 days after infection the sharp drop in the red cell count ceased and thereafter fell fairly slowly or continued to fluctuate around a low level. One of the rabbits, 0157, died 76 days after infection; on this date the red cell count was 2.7 million and the number of flukes recovered from the bile ducts was 31. The lowest red cell count recorded was 1.8 million which was that of 0137, 84 days after infection; at autopsy this rabbit proved to be the most heavily infected of the ten and had 35 flukes in its bile ducts. At no time during the course of the experiments did the red cell count of any of the parasitised rabbits return to the normal level.

Prior to infection the average value obtained for haemoglobin in the ten rabbits was 12.28 ± 0.78 gm. per 100 ml. and for the haematocrit 41.16 ± 1.97. The reductions in the haemoglobin and haematocrit level after infection were approximately proportioned to those of the red cell counts and the significance of these changes is discussed under the headings of M.C.V. and M.C.H.C.

Mean corpuscular volume.

Before infection the M.C.V. of the red cells of the 10 rabbits was 67.3 ± 2.7 c.μ. As anaemia developed in each of these rabbits the M.C.V. rose to between 70 and 90 c.μ. and almost without exception remained elevated. The two highest mean corpuscular volumes which were recorded were 108 c.μ. and 96 c.μ. The former occurred in H251 128 days after infection when the red cell count was 3.3 million and

the latter in 0703, and 0469, 120 and 124 days after infection respectively; the red cell count of each at that time was 3.4 million.

Appearance of red cells.

If a blood film from any of the anaemic rabbits was stained with Leishman, and the red cells compared to those of a similarly stained film of normal rabbit blood, it was usually obvious that the red cells of the fluke-infected rabbits were larger (Figs. 10 and 11).

Polychromasia was also a characteristic feature of the red cells from the anaemic rabbits. In severe cases anisocytosis was considerable, the red cells were pale and showed marked ring staining and normoblasts were occasionally noted. Basophilic stippling or spherocytosis was not observed.

To confirm the presence of macrocytosis, the diameters of 200 red cells from each of four anaemic rabbits were measured, and the mean diameter of the red cells of each rabbit compared to the mean diameter of each before infection. The results in Table 3 show that macrocytosis is a characteristic feature of the anaemia. This aspect of the anaemia is dealt with at some length in the discussion.

Table 3/

Table 3: The increase in the mean diameter of the red cells of 4 rabbits suffering from anaemia due to F. hepatica infection.

Rabbit	Mean Diameter before Infection. (μ)	Mean Diameter after Infection. (μ)
0137	6.95	7.4
0460	7.05	7.35
0469	6.45	7.45
0703	6.2	7.2

Mean corpuscular haemoglobin concentration.

The M.C.H.C. of the ten rabbits before infection averaged 30.47 ± 1.07. After infection, and as anaemia developed in each of the rabbits, the M.C.H.C. dropped to about 25%; on those occasions when the erythrocyte count fell below 3 million the M.C.H.C. dropped still further. The lowest M.C.H.C. recorded was 15% which was that of 0137, 84 days after infection; the red cell count on this date was 1.8 million.

Reticulocyte count.

In the rabbits before infection the reticulocyte count averaged 11 per 1,000 red cells. In the infected rabbits the count started to rise about 60 days after infection and remained high throughout the course of infection, usually there were between 75 and 150 reticulocytes/

reticulocytes per 1,000 red cells (Fig. 12), although on one occasion (0469 - 178 days past infection) the count was raised to 328 reticulocytes per 1,000. Blood smears which had been stained supravitally with brilliant cresyl blue and then with Leishman showed that the reticulocytes were often larger than the mature corpuscles and were slightly basophilic.

Marrow examination.

In the normal rabbit, the shaft of the tibia is filled with yellow marrow. In these anaemic rabbits this was almost completely replaced by a red cellular marrow. Microscopically, the marrow picture in each anaemic rabbit was that of normoblastic hyperplasia. Fig. 13 illustrates a typical section of normal rabbit marrow from the proximal region of the tibia; the marrow consists largely of fat and the haemopoietic tissue occupies only a small proportion of the available space. In contrast Fig. 14 shows a marrow section from the proximal end of the tibia of an anaemic rabbit (Rabbit 0137); The haemopoietic tissue has undergone hyperplasia and the marrow fat has diminished proportionately. The cells which are responsible for the hyperplasia are those of the erythrocytic series and this is illustrated by Fig. 15 which shows a marrow smear from another severely anaemic rabbit (Rabbit 0469); there is a marked increase in the proportion of normoblasts and this was typical of the marrow of these infected rabbits throughout the course of the anaemia. Similar changes were described by Barbieri (1935) who examined the marrow of a number of naturally infected sheep. The large

partially/

partially haemoglobinated cells with reticulated nuclei which are characteristic of megaloblastic erythropoiesis, and indicative of a deficiency of liver principle, were not observed on any occasion.

The amount of stainable iron in normal rabbit marrow is variable. In some marrow sections iron-containing macrophages may be found in every high-power field while sections from other rabbits will show only an occasional iron-containing macrophage. In the anaemic rabbits iron-bearing cells were always few in number, being present in small numbers in some sections and almost completely absent in others.

Plasma and bile pigments.

Normal rabbit plasma is lightly straw-coloured and is almost completely clear. According to Martin, Harrero and Lichtenstein (1949) biliverdin alone is produced by the normal rabbit, although if hepatic disease is present bilirubin may also be produced; it was necessary, therefore, to examine the plasma of the infected rabbits for the presence of both of these pigments.

In each of the 35 infected rabbits, the plasma colour was normal at all times except at the points marked on the haematological tables of O137, H318, H475 and N217 (Table 5). On two of these occasions (rabbit O137) the plasma was whitish; this was due to lipaemia and may be reasonably attributed to liver dysfunction as the liver of this rabbit was found to be very severely damaged at autopsy. On the other occasions, the plasma was slightly greenish in colour and was positive to Van den Bergh's reaction, a brick-red ring being obtained for the direct test and a very faint pink for the indirect. On no other occasion was a positive Van den Bergh reaction/

reaction obtained although the test was carried out repeatedly on the plasma of all 10 infected rabbits.

When these observations were made it was not possible to examine the plasma spectrophotometrically for the presence of biliverdin. Since then several rabbits infected for purposes outwith the scope of this paper have been encountered whose plasma was also greenish in colour some weeks after infection. When the plasma from these rabbits was subjected to spectrophotometric examination (see Appendix B) there was no dip in light transmission between 600 and 700 mμ which is indicative of the characteristic absorption band of biliverdin, but in each case there was a dip at 430 mμ which is evidence of the presence of bilirubin. Samples of clear sera from 0150, 0703 and 0469, all 125 days after infection, were also examined. Each sample was negative for the presence of bilirubin or biliverdin.

It appears, therefore, that bilirubinaemia or biliverdinaemia is not a characteristic feature of the anaemia which is associated with fascioliasis. The temporary presence of bilirubin in the plasma of a proportion of rabbits some weeks after infection was probably due to extensive liver damage.

The plasma protein values for 6 of the rabbits before infection averaged 7.5 ±0.68 gm./100 c.c. There was no significant alteration in this during the course of the infection.

The Coombs' Test.

In many cases of acquired haemolytic anaemia in humans it has been shown that the haemolysis is associated with the presence of an abnormal/

abnormal globulin which is adsorbed on to the red cells, and which may render them susceptible to agglutination in vitro. This globulin may be demonstrated by the Coombs' test (Coombs' et al. 1945). A thrice washed suspension of the cells under test is mixed with anti-human globulin serum obtained from a suitably immunised rabbit. If the abnormal human antibody is present on the red cells it will combine with the rabbit antiserum with consequent agglutination of the red cells. Haemolytic anaemias, which depend on the presence of an abnormal antibody attached to the red cells have also been demonstrated in experimental animals. (Dameshek, Schwartz and Gross, 1938; Tigertt, Duncan and Hight, 1940).

Samples of red cells from O137, 57, 72 and 102 days after infection, when the red cell count was 5.2, 3.0 and 2 million respectively, were examined by the Coombs' test for the presence of adsorbed antibodies (see Appendix C). The results were negative on each occasion.

Spleen.

A degree of erythrophagocytosis is one of the normal functions of the macrophages of the red pulp of the spleen (Maximow and Bloom, 1950). In haemolytic anaemia this function may be greatly increased due to the presence of excessive numbers of erythrocyte particles or of sensitised red cells in the plasma (Whitby and Britton, 1950; Wintrobe, 1946).

The spleens of the infected rabbits were normal in size and consistency and showed no evidence of increased erythrophagocytosis when compared to those of normal rabbits.

The/

The Prussian blue reaction showed that the stainable iron, normally present in abundance, was almost entirely absent.

Fragility of the red corpuscles.

If red corpuscles are placed in hypotonic salt solution they become swollen and finally rupture, the corpuscular contents going into solution; the highest concentrations of salt solutions in which haemolysis first occurs and in which it is complete are fairly constant for any given species.

In certain disease conditions in man, the red cells show an increased resistance to lysis and this is termed decreased fragility; in other conditions the red cells are more susceptible to lysis, i.e. increased fragility. In general, variations in red cell fragility are dependent on corpuscular shape, and it has also been reported that young cells and reticulocytes are more resistant to haemolysis (Daland, G., and Worthley, K. 1935; Davis et al. 1954). On the other hand, Cruz et al. (1941) reported that young cells are less resistant to haemolysis.

An extensive study of red cell fragility was not made in this experiment, the blood of only 4 infected and 6 normal rabbits being tested. The results are shown in Table 4.

Table 4:/

Table 4: Results of red cell fragility test on 4 infected and 6 normal rabbits.

No. of Rabbit	Infected A1	A2	A11	A14	Normal 1	2	3	4	5	6
Point* at which haemolysis first commenced	.52	.48	.48	.56	.56	.56	.56	.56	.6	.56
Point at which haemolysis was complete	.36	.28	.32	.36	.40	.40	.40	.40	.36	.32

* Points represent concentrations of NaCl in gm. per 100 ml. water.

Alkaline phosphatase estimations

The value for alkaline phosphatase in the serum of a number of normal rabbits was 2.0 - 3.0 King-Armstrong units. There was no alteration in this in the infected rabbits.

Sedimentation rate

The normal sedimentation rate in Wintrobe tubes after 2 hours varied between 0 and 2%. There was no alteration in this rate in the anaemic rabbits.

Coagulation time

In normal rabbits, the average coagulation time was 3.7 minutes. There was no alteration in this time in the infected rabbits, except in one rabbit, H318, 178 and 180 days after infection, when the coagulation time was increased to 8 and 11 minutes respectively.

Examination of bile

Immediately each control or infected rabbit was killed the abdomen was opened and the gall-bladder and common bile duct was exposed./

exposed. In the infected rabbits the gall-bladder was always enlarged and the common bile grossly distended; the normal diameter of the common bile duct is about 1 mm. and in those infected rabbits this was often increased to 12 mm. It was usual to find the majority of the parasites in this distended bile duct.

The surface of the gall-bladder of each rabbit was seared with a flamed spatula to eliminate haemorrhage and the bile was withdrawn into a capillary pipette. In a similar manner samples of bile were obtained from the common bile duct of the infected rabbits; it was not found possible to obtain any bile from the common bile duct of normal rabbits with this technique because of the narrow lumen of the duct. Bile samples from the gall-bladder of normal rabbits were always clear and green; those from the gall-bladders of infected rabbits were invariably brown, while those from the common bile duct were dark brown, black or red. The benzidine test examination for haemin and spectroscopic examination of the bile of 15 normal rabbits were negative for the presence of occult blood. The gall-bladder bile of infected rabbits was always positive to the benzidine test, and almost always contained haemin. Bile from the common bile duct of these rabbits was always positive to the benzidine test and for the presence of haemin. In addition bile from this source when suitably diluted and examined spectroscopically frequently showed the typical absorption bands of oxyhaemoglobin. In addition to the 25 rabbits under experiment these tests yielded similar results when carried out on another 20 infected rabbits.

White/

White cell count.

There was a considerable variation in the white cell counts of the ten rabbits before infection but the average count per cu./mm. was 8.55 ± 1.87. About 35 days after infection the total white cell count in all the infected rabbits, excepting 0460 and H318, started to increase, reaching a peak about the fiftieth day, at which time it was usually approximately double the normal value. By about the eightieth day the white cell count had usually returned to normal and thereafter remained fairly steady.

Examination of the caeca of F. hepatica for the presence of blood or its products.

When the parasites were removed from the bile duct they were rinsed in saline and then crushed or squeezed to eject the intestinal contents. The brownish fluid which was obtained was positive to the benzidine and haemin tests in every case.

Numerous adult parasites were fixed in corrosive formal, embedded, sectioned and stained with haemotoxylin and eosin. In many cases only a brownish mass could be distinguished in the caeca of the flukes, but in numerous cases the caeca were filled with intact red cells and leucocytes in the same proportion as they occur in the blood. (Fig. 16).

Table 5/

TABLE 5.

Rabbit 0137. 35 F. hepatica at autopsy.

	Days after Infection.																			
	-20	-10	0	14	24	42	50*	57*	72*	84x	92x	102	112*	120*	129*	142	163	186	224	226
Red Cell Count (million per c.mm.)	5.9	6.1	6.0	6.0	6.3	5.9	5.7	5.2	3.0	1.8	2.2	2.0	2.3	2.1	2.4	2.2	2.2	2.9	3.4	2.7
Haemoglobin (gm.per 100 ml.)	12.0	13.4	13.2	14.0	12.7	11.2	10.6	9.6	5.6	2.0	4.6	3.0	4.0	4.4	5.6	4.3	4.3	5.3	7.0	6.0
Haematocrit	40.0	42.0	41.5	41.5	43.0	37.0	39.0	32.5	22.0	13.0	-	18.0	20.0	18.5	20.5	20.5	20.5	23.0	23.0	22.5
M.C.V. (c.μ)	68	70	70	70	69	63	68	63	74	72	-	90	87	89	85	94	94	80	83	84
M.C.H.C.	30.0	32.0	33.0	34.0	29.5	30.3	27.3	29.8	25.5	15.0	-	17.0	20.0	23.7	27.0	21.0	21.0	22.6	25.0	27.0
Sedimentation Rate	1.0	0	-	0	-	0	0	0.5	0.5	3.0	2.0	1.0	0	-	-	-	2.0	1.0	0.5	0.5
Reticulocytes per 1000 red cells	5	1	10	16	20	-	-	25	166	160	264	184	125	201	125	260	236	178	160	-
Coagulation Time (in mins.)	4.0	3.5	3.5	4.5	3.0	3.5	4.0	4.0	3.5	3.0	3.5	4.0	3.5	3.0	4.0	3.5	2.5	3.0	3.5	3.0
White Cell Count (thousands per c.mm)	8.0	-	12.2	7.1	11.8	16.5	21.4	18.9	12.9	7.2	8.3	9.6	7.9	8.5	7.4	8.2	11.2	14.4	13.9	7.6

* plasma greenish
x plasma white

TABLE 5.(Contd.)

Rabbit 0460. 13 F hepatica at autopsy.

	Days after Infection.																	
	-28	-18	-7	1	3	5	8	15	32	46	60	70	80	87	101	126	154*	156
Red Cell Count (million per c.mm.)	6.2	6.2	6.0	-	-	-	6.2	6.2	6.3	6.0	5.3	4.6	4.8	4.5	4.8	4.5	3.9	4.2
Haemoglobin (gm.per 100 ml.)	12.6	12.5	11.6	-	-	-	12.0	13.0	12.3	10.6	9.5	7.7	10.2	8.9	9.5	9.1	6.6	5.1
Haematocrit	40.0	40.5	39.5	-	-	-	38.5	42.0	41.0	38.5	35.0	33.0	36.0	35.0	35.0	31.5	27.0	28.0
M.C.V. (c.μ)	64	65	66	-	-	-	62	67	65	64	66	72	75	78	73	70	66	66
M.C.H.C.	31.5	31.0	29.5	-	-	-	31.0	31.0	30.0	28.0	27.5	23.5	28.5	25.0	27.0	29.0	25.0	18.5
Sedimentation Rate	1.0	0.5	0	-	-	-	0	0.5	0	0.5	0.5	0	0	-	-	-	10.0	3.0
Reticulocytes per 1000 red cells	0	15	5	-	-	-	15	10	8	-	25	91	70	50	100	48	52	98
Coagulation Time (in mins.)	3.5	3.0	4.0	-	-	—	3.0	3.5	3.0	3.5	2.5	3.5	-	2.5	3.0	3.0	3.0	-
White Cell Count (thousands per c.mm.)	12.3	14.8	10.4	8.1	14.7	14.7	8.4	10.7	10.3	11.5	12.6	10.8	10.1	-	8.7	7.6	17.8	18.4

* Noted to have suppurating foot.

[redacted]

TABLE 5. (Contd.)

Rabbit 0703 — 20 F. hepatica at autopsy.

Days after Infection.

	-39	-29	0	7	23	41	51	65	76	86	120	149	173	389	422	481
Red Cell Count (million per c.mm.)	5.9	6.0	5.9	5.8	6.2	-	5.5	4.2	4.1	3.9	3.4	4.7	4.1	3.8	3.3	4.8
Haemoglobin (gm.per 100 ml.)	12.1	12.	11.2	11.2	12.2	-	-	9.3	9.6	9.0	9.0	8.9	7.7	9.0	6.6	11.2
Haematocrit	38.5	38.5	37.5	37.0	40.5	-	40.0	33.0	32.0	32.0	33.0	36.0	31.0	31.0	27.0	37.0
M.C.V. (c.μ)	66	64	63	64	66	-	73	80	79	82	96	77	76	82	82	77
M.C.H.C.	31.5	31.2	30.5	30.5	30.0	-	-	28.0	30.0	28.2	27.5	25.0	25.0	-	24.5	30.5
Sedimentation Rate	0.5	0.5	0	0	0.5	0.5	0.5	0	-	0.5	-	-	-	-	-	-
Reticulocytes per 1000 red cells	28	11	28	24	5	-	35	108	135	108	-	100	130	-	116	358
Coagulation Time (in mins.)	3.0	4.0	3.5	3.5	2.0	2.5	3.5	-	3.0	3.5	3.0	4.0	-	-	-	-
White Cell Count (thousands per c.mm.)	7.4	6.7	6.3	5.8	7	16.5	10.4	8.1	9.8	6.9	5.5	8.9	-	-	-	-

TABLE 5. (Contd.)

Rabbit 0150 — 15 F. hepatica at autopsy.

Days after Infection.

	-25	-16	-4	5	13	17	34	48	59	68	76	87	95	124	128	146	173
Red Cell Count (million per c.mm.)	6.3	6.3	6.0	6.3	6.6	-	6.2	5.7	5.5	4.8	4.4	4.7	-	3.6	3.8	4.4	4.2
Haemoglobin (gm. per 100 ml.)	13.5	13.7	12.5	13.6	12.5		12.3	10.5	9.7	8.7	9.0	10.3		8.8	8.2	8.4	7.8
Haematocrit	43.0	44.0	41.0	41.5	42.0		43.5	38.5	36.5	34.0	33.0	35.5		30.0	31.0	33.5	32.0
M.C.V. (c.μ)	69	70	69	66	64		70	68	67	70	75	76		84	82	76	76
M.C.H.C.	31.0	30.0	30.5	31.0	30.0		28.0	27.0	26.7	25.7	27.5	29.0		29.8	26.7	25.0	24.5
Sedimentation Rate	0.5	0.5	0	-	-		-	1.0	-	0	0	-		-	2.0	1.0	1.5
Reticulocytes per 1000 red cells	13	12	6	0	5		14	-	40	120	82	70		-	68	44	100
Coagulation Time (in mins.)	3.5	4.0	3.5	4.0	4.5		4.0	3.0	4.0	4.5	2.5	3.0		3.0	3.0	3.0	3.0
White Cell Count (thousands per c.mm.)	9.5	8.3	10.1	7.1	9.1	11.7	13.5	14.3	15.1	12.0	9.8	11.1		17.8	8.9	12.5	10.8

TABLE 5. (Contd.)

Rabbit 0469 — 14 F. hepatica at autopsy.

	Days after Infection.																	
	-25	-15	-2	3	6	10	18	32	44	58	66	76	88	100	124	156	173	178
Red Cell Count (million per c.mm.)	6.5	6.1	6.0	–	6.0	–	6.2	5.8	5.5	4.2	3.7	4.2	3.8	4.2	3.4	3.3	2.1	2.4
Haemoglobin (gm. per 100 ml.)	13.2	13.3	13.1	–	13.2	–	13.2	12.4	10.6	7.8	7.4	9.3	9.5	9.5	8.6	7.7	2.9	3.8
Haematocrit	44.0	43.0	42.5	–	41.5	–	42.5	42.0	39.0	29.5	29.0	33.2	35.5	39.5	33.0	27.0	14.0	22.0
M.C.V. (c.μ)	68	70	71	–	70	–	69	72	71	70	78	80	94	95	96	83	68	91
M.C.H.C.	30.0	31.0	31.0	–	33.0	–	31.0	30.0	27.2	26.5	25.8	28.0	27.0	29.8	26.6	28.5	20.0	17.5
Sedimentation Rate	0	0.5	0.5	–	0.5	–	0.5	0.5	0.5	–	0	0	–	2.0	–	0.5	1.5	–
Reticulocytes per 1000 red cells	16	10	14	15	16	–	1	–	–	64	84	154	132	170	–	80	250	328
Coagulation Time (in mins.)	4.0	4.0	4.0	–	3.2	–	4.0	3.0	4.5	4.0	4.5	4.0	4.0	3.0	3.0	3.0	3.0	3.0
White Cell Count (thousands per c.mm.)	10.0	8.8	7.7	14.4	7.4	7.4	8.3	15.2	15.8	18.0	15.0	9.8	8.3	8.2	8.7	11.0	11.1	–

TABLE 5. (Contd.)

Rabbit 0157 — 31 F. hepatica at autopsy

	Days after Infection.										
	-34	-18	0	1	4	11	28	46	56	66	76
Red Cell Count (million per c.mm.)	6.5	6.5	6.0	–	–	6.2	6.1	5.1	4.2	3.1	2.7
Haemoglobin (gm. per 100 ml.)	12.4	13.8	12.0	–	–	12.5	12.1	9.6	7.2	4.5	5.2
Haematocrit	43.0	43.0	40.0	–	–	41.5	42.0	–	28.0	23.5	21.0
M.C.V. (c.μ)	66	66	67	–	–	66	69	–	67	76	77
M.C.H.C.	29.0	32.3	30.0	–	–	29.0	29.0	–	25.8	19.0	25.0
Sedimentation Rate	–	0.5	–	–	–	0.5	0.5	0.5	0.5	0	0
Reticulocytes per 1000 red cells	+	0	–	–	–	5	8	–	56	160	200
Coagulation Time (in mins.)	4.0	3.5	4.0	–	–	1.5	3.0	3.0	–	3.0	–
White Cell Count (thousands per c.mm.)	6.5	7.6	7.9	10.2	9.2	6.0	13.8	21.1	14.9	13.6	11.8

TABLE 5. (Contd.)

Rabbit N217 — 4 F. hepatica at autopsy

	Days after Infection.										
	-21	-16	-11	4	21	38	82	102	155*	205	250
Red Cell Count (million per c.mm.)	5.9	6.1	5.9	5.8	5.8	5.9	6.0	5.8	5.3	5.4	5.2
Haemoglobin (gm. per 100 ml.)	13.0	12.3	13.4	12.6	11.9	13.0	12.6	–	–	10.5	8.8
Haematocrit	42.0	45.0	42.5	39.0	39.0	44.0	44.0	42.0	35.0	38.0	35.0
M.C.V. (c.μ)	71	73	72	67	67	74	73	73	66	70	67
M.C.H.C.	31.0	32.5	32.0	32.0	30.5	30.0	29.0	–	–	28.0	25.5
Sedimentation Rate	0	0	1.0	–	1.0	0.5	0	0.5	1.0	–	–
Reticulocytes per 1000 red cells	11	4	9	9	4	26	–	27	64	30	30
Coagulation Time (in mins.)	4.0	5.0	4.0	–	3.5	2.5	3.5	3.5	–	–	3.5
White Cell Count (thousands per c.mm.)	7.3	7.8	8.4	8.3	7.3	13.4	11.3	9.6	–	–	–

* Plasma greenish.

TABLE 5. (Contd.)

Rabbit H475 — 20 F. hepatica at autopsy

Days after Infection.

	-25	-19	-4	13	29	45*	63	91	128	173	218	368
Red Cell Count (million per c.mm)	5.9	5.7	5.8	5.9	5.8	5.4	5.2	4.2	4.5	4.3	4.0	4.1
Haemoglobin (gm. per 100 ml.)	40.0	39.5	38.0	38.5	37.0	35.0	35.0	33.5	36.0	30.5	30.0	30.0
Haematocrit	11.4	11.2	11.5	11.2	11.2	10.5	9.1	9.8	10.5	8.3	8.2	8.2
M.C.V. (c.μ)	68	70	66	66	64	65	68	80	80	71	75	74
M.C.H.C.	29.0	28.5	30.0	31.0	30.0	30.0	26.0	30.5	29.0	27.0	27.5	27.5
Sedimentation Rate	2.0	0.5	1.0	-	-	1.5	1.5	0.5	0	1.5	-	-
Reticulocytes per 1000 red cells	12	16	16	20	21	25	39	60	125	108	150	90
Coagulation Time (in mins.)	3.5	3.0	3.0	4.0	3.5	4.0	3.0	4.0	3.0	3.0	-	1.0
White Cell Count (thousands per c.mm.)	5.9	6.6	5.7	8.7	8.0	10.2	19.1	11.2	9.8	7.4	-	6.5

* Plasma greenish.

[redacted]

TABLE 5.(Contd.)

Rabbit H318 — 34 F. hepatica at autopsy

Days after Infection.

	-20	-19	-4	13	29	45	63	91	128	178*	180*	182
Red Cell Count (million per c.mm)	6.2	5.9	5.9	5.9	5.8	5.0	3.6	3.5	3.4	3.2	2.8	2.6
Haemoglobin (gm. per 100 ml.)	11.2	11.1	11.2	11.7	11.2	10.5	8.5	8.0	7.9	7.1	-	6.1
Haematocrit	41	37	37	36	37.5	33.5	25.5	26.5	28.0	27.5	24.5	24.5
M.C.V. (c.μ)	66	63	63	61	65	67	71	76	82	85	87	94
M.C.H.C.	27.5	30.0	30.5	32.5	30.0	31.5	35.0	30.0	28.5	26.0	-	25.0
Sedimentation Rate	1.0	1.0	0	-	-	2.0	2.0	1.0	1.0	2.0	1.0	-
Reticulocytes per 1000 red cells	21	20	8	10	20	33	13	40	100	320	-	240
Coagulation Time (in mins.)	3.5	4.5	4.5	3.5	3.5	4.5	3.0	3.5	3.5	8.0	11.0	-
White Cell Count (thousands per c.mm.)	8.8	10.1	10.6	11.3	14.8	14.5	12.8	7.2	71.	7.7	-	9.2

* Plasma greenish.

TABLE 5. (Contd.)

Rabbit H251. — 31 F. hepatica at autopsy.

	Days after Infection.								
	-19	-14	-4	13	16	45	63	91	128
Red Cell Count (million per c.mm)	6.1	5.8	6.1	6.0	6.0	5.6	3.6	3.6	3.3
Haemoglobin (gm. per 100 ml.)	11.4	11.2	11.5	12.6	12.2	11.9	8.5	10.0	8.4
Haematocrit	39.0	40.0	40.0	40.0	40.0	37.5	29.0	30.5	35.0
M.C.V. (c.μ)	64	68	66	66	66	68	80	85	108
M.C.H.C.	29.5	38.5	29.0	28.5	29.5	32.0	29.5	27.0	24.0
Sedimentation Rate	2.0	-	0	-	-	1.0	1.0	0.5	2.0
Reticulocytes per 1000 red cells	12	14	4	-	18	42	92	186	220
Coagulation Time (in mins.)	2.5	4.0	3.5	4	4.5	3.5	3.5	0.75	4.0
White Cell Count (thousands per c.mm.)	9.0	6.3	6.3	8.5	11.2	13.6	11.4	10.6	9.2

The anaemia produced by the ligation of the common bile duct of the Rabbit.

While this investigation of the anaemia associated with fascioliasis was in progress, our attention was drawn to work carried out by Goldberg, Hammarsten, Hellstrom, Lindgren and Plum (1950) who showed that ligation of the common bile duct in rats, mice and guinea-pigs produced a macrocytic anaemia which was accompanied by reticulocytosis. In our rabbits with chronic fascioliasis, the majority of the parasites are usually located in the grossly distended common bile duct, and in view of the Scandinavian work described above, it did not seem unreasonable to assume that in this position they might cause obstruction to the flow of bile and that the anaemia, macrocytosis, and reticulocytosis were sequels of this.

The effect of total obstruction of the bile ducts on the blood picture was, therefore, studied in three rabbits for the purposes of comparison with the anaemia associated with fascioliasis. The results are shown in Table 6.

Similar lesions were found at autopsy in each of these three rabbits (Fig. 17). These included thickening and distension of the gall-bladder and bile ducts proximal to the ligature and extensive fibrinous peritonitis involving the liver and adjacent viscera. In each case, also, the abdominal cavity contained bile-stained fluid and the liver was yellowish in colour with a few greenish wedge-shaped areas at the periphery.

Table 6/

Table 6: The effect of complete ligation of the common bile duct.

Rabbit 33.

Days after ligation	0	3	6	11	18	28
Red Cell Count	5.1	4.1	3.7	5.2	5.7	5.2
Haemoglobin	12.1	8.6	8.9	9.6	11.8	12.6
Haematocrit	35.0	28.0	32.0	37.0	42.0	38.0
M.C.V.	69	68	86	72	74	75
M.C.H.C.	34.5	31	28	26	28.5	33
Plasma	Colour-less	Pale Yellow	Pale Yellow	Pale Yellow	Pale Yellow	Yellow
Bilirubin mgms. per 100 ml. serum	0.3	1.3	3.3	2.6	-	8
Alkaline Phosphatase	2.0	7.8	5.0	4.2	-	9.3
Reticulocytes per 1000red cells	40	54	150	54	15	55

Rabbit 456.

Days after ligation	0	3	6	11
Red Cell Count	5.4	3.7	3.7	4.0
Haemoglobin	11.8	9.4	9.6	9.1
Haematocrit	38.0	26.0	31.3	31.0
M.C.V.	70	70	85	77
M.C.H.C.	31.5	36	30.5	29.5
Plasma	Colour-less	Deep Yellow	Deep Yellow	Deep Yellow
Bilirubin mgms. per 100 ml. serum	0.3	11.4	7.1	7.8
Alkaline Phosphatase	2.34	16.4	5.0	5.0
Reticulocytes per 1000 red cells	16	54	138	84

Rabbit 061.

Days after ligation	0	4	5	14	19
Red Cell Count	5.4	4.1	3.9	4.1	4.8
Haemoglobin	11.8	10.4	9.5	10.1	11.8
Haematocrit	38.5	33.0	30.0	34.0	34.0
M.C.V.	71	80	77	83	71
M.C.H.C.	30.6	31.5	32.0	29.5	34.8
Plasma	Colour-less	Yellow	Yellow	Yellow	Yellow
Reticulocytes per 1000 red cells	13	28	53	30	24

The/

The experimental production of a post-haemorrhagic anaemia.

In this experiment blood was removed from two rabbits by repeated venesection from the marginal ear vein over a period of 106 days. Initially, venesection was carried out every alternate day, but in order to minimise damage to the vein, this was gradually extended until during the last month the blood was removed once weekly. A total of 700 ml. was removed from each rabbit during the course of the experiment, more blood being removed during the latter half of the period in an attempt to increase the severity of the anaemia.

The results in terms of the red cell count, the mean corpuscular volume, the mean corpuscular haemoglobin concentration, and the reticulocyte count are shown in Table 7 and Fig. 18.

At autopsy the marrows of each of the rabbits showed normoblastic hyperplasia and sections of their spleens, stained by Prussian blue, showed that the contained much less iron than is normal.

Table 7/

Table 7: The production of a post-haemorrhagic anaemia by repeated venesection.

Rabbit 0309	Days	-34	0	15	22	44	45	50	62	68	80	87	99	107
	Red Cell Count	6.4	6.3	6.0	5.8	4.7	4.8	4.1	3.8	4.4	3.9	5.1	3.8	3.9
	M.C.V.	64	63	63	64	74	70	76	71	68	90	79	89	84
	M.C.H.C.	30.2	30.8	29.5	30.6	25.5	25.5	24	31	24.5	-	27	21.3	27.8
	Reticulocytes per 1000 red cells.	1	7	32	33	42	-	128	180	100	52	45	156	92

Rabbit 1028	Days	-40	-30	0	15	22	44	50	62	68	80	87	99	107
	Red Cell Count	6.9	6.4	6.7	6.7	5.9	5.5	4.5	4.5	5.3	5.4	5.8	4.8	5.2
	M.C.V.	66	66	67	67	64	69	76	77	72	82	72	77	79
	M.C.H.C.	29.5	31.3	29.5	28.4	28.8	24.0	24.0	27.0	25.4	-	25.8	25.2	30.8
	Reticulocytes per 1000 red cells.	5	1	32	20	55	30	106	106	100	24	114	36	38

[redacted]

The effect on the anaemia of the removal of the flukes by an anthelmintic.

In order to study the course of the anaemia after the fluke infection was terminated, five of the rabbits which had received 50 cercariae some months previously were treated with hexachloroethane. This drug has been shown to be an efficient, specific and relatively non-toxic anthelmintic against adult F. hepatica in cattle and in sheep (Olsen, 1943, 1946), and although there are no reports on the effect of the drug on the parasite in rabbits, it was reasonable to assume a similar action.

The bloods of five infected and anaemic rabbits were studied on several occasions over a few weeks. At the end of this time a single does of hexachloroethane was administered orally to each rabbit in an attempt to eliminate the parasites. Four of the rabbits, each of which weighedd about 6 lbs., received 3 gm., and the fifth rabbit, which weighed 4 lbs., received 2 gm.; the drug appeared to be well tolerated by the rabbits. Table 8 shows the blood counts before and after treatment and the result of the autopsies.

Table 8/

Table 8: The effect of hexachloroethane on the anaemia associated with chronic fascioliasis in the rabbit.

Rabbit 197. Body weight 6 lbs. 3 gm. Hexachloroethane.

Days after treat.	-55	-14	-8	-5	-1	3	7	13	Autopsy.
Red Cell Count	3.9	3.3	3.1	3.5	3.8	-	5.2	6.3	No flukes.
Haemoglobin	-	6.0	-	6.5	8.9	-	11.0	13.0	Common bil
Haematocrit	30	25.5	25	24.5	29	26	38	40	duct almos
M.C.V.	77	78	80	70	76	-	73	64	normal in
M.C.H.C.	-	23.5	-	26.5	31.0	-	29.5	32.5	size

Rabbit 198. Body weight 4 lbs. 2 gm. Hexachloroethane.

Days after treat.	-55	-17	-8	-5	-1	3	7	13	16	Autopsy.
Red Cell Count	2.3	2.3	2.3	2.5	2.5	-	2.9	3.6	3.6	10 necrot
Haemoglobin	-	-	-	-	-	-	-	-	-	flukes +
Haematocrit	20.5	22	20.5	24	24	29	25	30	30	living
M.C.V.	88	95	88	95	95	-	86	83	83	flukes
M.C.H.C.	-	-	-	-	-	-	-	-	-	

Rabbit 199. Body weight 6 lbs. 3 gm. Hexachloroethane.

Days after treat.	-73	-4	-1	2	7	24	Autopsy.
Red Cell Count	4.4	4.5	4.5	4.6	5.0	6.0	No flukes
Haemoglobin	-	7.7	8.6	8.6	11.1	-	Common bi
Haematocrit	28	29	29	31	35	38.5	duct almo
M.C.V.	63	65	65	67	70	64	normal in
M.C.H.C.	-	27.0	30.0	28.0	32.0	-	size.

Rabbit A11. Body weight 6 lbs. 3 gm. Hexachloroethane.

Days after treat.	-10	-5	0	2	7	20	Autopsy.
Red Cell Count	3.4	3.7	3.9	4.5	4.8	5.9	No flukes
Haemoglobin	6.7	6.7	7.0	7.7	10.0	12.2	Common bi
Haematocrit	26.0	25.0	25.0	30.5	34.5	41.0	duct re-
M.C.V.	76	67	64	68	72	70	duced in
M.C.H.C.	26.0	27.0	28.5	25.0	29.0	30.0	size.

Rabbit A14. Body weight 6 lbs. 3 gm. Hexachloroethane.

Days after treat.	-12	-6	0	2	6	10	20	Autopsy.
Red Cell Count	2.9	2.7	3.1	3.3	3.8	4.5	5.8	No fluke
Haemoglobin	8.2	7.4	8.5	8.1	10.0	11.1	12.6	Common b
Haematocrit	27.0	26.0	28.0	28.0	33.0	36.5	41.0	duct re-
M.C.V.	95	96	90	90	86	80	72	duced in
M.C.H.C.	30.5	29.0	31.0	29.0	30.5	30.5	31.0	size.

The/

The effect of intravenous iron on the anaemia associated with fascioliasi

The role of iron in the production of red cells is well known and does not require detailed description here. It is sufficient to state that iron is a vital constituent of haemoglobin, and that, if insufficient iron is available in the diet or cannot be absorbed by the intestine, or if excessive quantities of the element are lost through haemorrhage, anaemia will inevitably result. (Wintrobe, 1946; Whitby and Britton, 1950).

In man, iron-deficiency anaemia is characteristically hypochromic and often microcytic (Whitby and Britton, 1950; Wintrobe, 1946) and this has been shown also to be the case in dogs (Farrar and Sturgis, 1936) and in rabbits (Smith, Medlicott and Ellis, 1944). It is well-known that the treatment of iron-deficiency anaemias with oral or parenteral iron is almost always followed by haemoglobin regeneration and the production of normochromic and normocytic red cells.

The experiment recorded below was carried out on two rabbits only, but is worth recording here in view of this well-known specific effect of iron therapy. Two rabbits infected with 50 cercariae and anaemic for some months were injected intravenously with *"Iviron" on several occasions. The frequency of infection and the results of subsequent blood examination are summarised in Table 9.

* "Iviron" (British Schering, Ltd.) is a sterile solution of saccharated iron oxide suitable for administration by intravenous injection. A parenteral preparation of iron was used because of the ease of administration.

It is interesting that the intravenous iron had no effect on the red cell count, or on the haematocrit or haemoglobin values; the increase in M.C.V. obtained in both animals after iron therapy cannot readily be attributed to the amelioration of the iron deficiency in the absence of any improvement in the red cell count or haemoglobin values.

Table 9: The effect of intravenous iron on the anaemia associated with fascioliasis.

Rabbit 10.

Days after 1st injection	-16	-9	-1	5	17	32	49	54	68
Red Cell Count	4.0	3.5	3.5	3.8	3.6	3.4	3.4	–	–
Haemoglobin	8.1	7.4	7.1	7.6	9.0	7.8	*	*	*
Haematocrit	28.8	25.5	25.0	29	30.5	28.0	29.2	29.2	26.5
M.C.V.	72	73	71	76	84	82	85	–	–
M.C.H.C.	28.5	29	29	26.5	29.8	28	–	–	–
Reticulocytes per 1000	166	190	150	190	152	90	150	–	–

Rabbit 12.

Days after 1st injection	-16	-9	-1	5	17	32	49	54	68
Red Cell Count	4.8	4.8	4.7	4.5	4.0	3.9	4.0	–	–
Haemoglobin	10.6	11.1	10.6	9.5	11.5	10.3	*	*	*
Haematocrit	37	34.5	35	34	36	35	36	37	36
M.C.V.	77	72	74	76	90	90	–	–	–
M.C.H.C.	29	33	30.5	28.3	32.0	29.5	–	–	–
Reticulocytes per 1000	116	112	84	110	124	110	–	–	–

0.2cc. Iviron was injected into each rabbit on the 1st and 5th days and 0.4 c.c. on the 7th, 38th and 40th days.

* Unfortunately these haemoglobin estimations are not available.

DISCUSSION

Anaemia, which may be defined as a deficiency of haemoglobin, may be produced in a number of ways. If, for example, the marrow is hypoplastic or aplastic, red cell production will be markedly diminished and anaemia result; again, with an inadequate supply of materials necessary for the proper production of red cells, e.g. the anti-anaemic principle of the liver, the number and quality of the red cells will be reduced, in spite of the fact that the cellularity of the marrow may be increased. Finally, excessive loss of circulating red cells, either by intravascular destruction or from haemorrhage, will reduce the total number of red cells and thus the haemoglobin.

Although this differentiation of anaemias is based on etiology, it is nevertheless of considerable practical value, as each of these types of anaemia is associated with fairly characteristic changes in the red cells and marrow and, from a study of the laboratory findings associated with an anaemia, it is usually possible to obtain some indication of its etiology.

The anaemia which is associated with Fasciola hepatica infections of rabbits was characterised by oligocytosis, macrocytosis, hypochromia and reticulocytosis. It was not associated with an increase in plasma bilirubin or biliverdin and the marrow picture was at all times that of normoblastic hyperplasia. The degree of erthrophagocytosis in the spleen/

spleen was not increased and the stainable iron in this organ was always very much reduced in amount. On each of three occasions, the red cells of a very anaemic rabbit were shown to be negative to the Coombs' test. Blood or blood products could invariably be demonstrated in the lumen of the main bile ducts or in the gut contents of the parasites.

In each rabbit the anaemia started about 42 days after infection, i.e. soon after the parasites entered the bile ducts, and the degree of anaemia was approximately proportional to the number of adult flukes in the bile ducts. In five infected rabbits which were treated successfully with an anthelmintic the red cell count, haemoglobin and haematocrit values returned to normal, and in a sixth rabbit, in which only some of the flukes were destroyed, there was an improvement in these indices.

Two features of the anaemia, the markedly normoblastic marrow and the increased number of reticulocytes in the peripheral blood, were found at all times during the anaemia; the more severe the anaemia, the more active was erythropoiesis in the marrow and the higher the reticulocytosis. These findings and the absence of megaloblastic erythropoiesis at all times show that neither marrow aplasia nor deficiency of the "anti-anaemic principle" of the liver are responsible for the anaemia.

It is the case that normoblastic hyperplasia and reticulocytosis are common features in haemolytic anaemia. In this condition one might also expect as evidence of increased intravascular destruction an accompanying increased level of plasma bilirubin or biliverdin and increased/

increased deposits of iron in the spleen and bone marrow. Also with this type of anaemia, the spleen is often enlarged, increased erythrophagocytosis may be observed on histological examination, and antibodies, demonstrable by the Coombs' test may be adsorbed on to the red cells. None of these features was present in the anaemia associated with fascioliasis and it is reasonable to conclude that increased haemolysis plays no part in its production.

It is well known that anaemia may be found in association with liver disease both in man (Wintrobe, 1946) and in laboratory animals (Schumacker and Wintrobe, 1936; Higgins and Stasney, 1935) and therefore the severe hepatitis which is associated with experimental fascioliasis in the rabbit must be considered as a possible cause of the anaemia.

If anaemia is present in acute or chronic hepatitis in man, it is usually macrocytic or normocytic (Whity and Britton, 1950). The morphology of the macrocytic type may resemble that seen in pernicious anaemia although it is rarely as severe as may be encountered in that condition (Whitby and Britton, 1950). In view of this and of the response of some of these cases to parenteral liver therapy it has been suggested that the anaemia may on occasions be the result of defective storage or metabolism of the anti-anaemic principle (Wintrobe, 1936; Wintrobe and Schumacker, 1933). Larsen (1949) in a study of 58 cases of acute and chronic hepatitis found that the macrocytic anaemia present in his cases was not associated with a raised/

raised mean corpuscular volume, i.e. the mean corpuscular thickness was decreased. He also concluded that the red cell population in hepatitis was composed of two components, one with a normal M.D.*, and one with an increased M.D.; he considered that the latter component was not due to lack of the anti-anaemic principle nor to the presence of increased numbers of reticulocytes but suggested that it may have been caused by a deficiency of nicotinamide. Lindgren (1949) also found that macrocytosis was common in acute hepatitis in man and that the degree of macrocytosis was proportional to the serum bilirubin. Obstructive jaundice as the cause of macrocytic anaemia in man has been reported by Rosenberg and Walters (1936).

Experimentally produced macrocytic anaemia associated with liver damage has been studied in dogs and rabbits by Schumacker and Wintrobe (1936) who produced cirrhosis by the administration of carbon tetrachloride over a prolonged period. The anaemia which developed was usually normocytic, but where liver damage was severe and of long duration it became macrocytic. In these cases the bone marrow was said to be hyperplastic and "macroblastic" in type. They also described varying degrees of haemosiderosis in the spleen of autopsied dogs and noted that in one dog in which liver therapy was attempted reticulocytosis occurred. These authors concluded that macrocytic anaemia developed as a consequence of a fault in the internal metabolism of the anti-anaemic principle.

Reference has already been made to the work of Goldberg, Hammarsten Hellstrom, Lindgren and Plum (1950). These authors showed that ligation/

* Mean Diameter.

ligation of the bile-ducts of rats, mice and guinea-pigs produced an anaemia which was characterised by an increase in mean corpuscular volume, was hyperchromic, and was accompanied by a reticulocytosis and a macrocytosis which was proportional to the serum bilirubin concentration. As described earlier a similar type of anaemia occurred in our rabbits subjected to ligation of the common bile duct.

In the rabbits which were infected with F. hepatica, a severe and extensive acute hepatitis was produced in the migratory phase of the disease, i.e. up to 6 weeks after infection. The haematological tables show that there is no decrease in the haemoglobin and red cell count, and no increase in the mean corpuscular volume, during this stage in any of the rabbits. Furthermore, measurements of the diameters of the red cells of 4 rabbits at this stage of infection (Fig. 19a,b) showed that while the mean diameter was increased in two of these, it was decreased in the other two.

It appears, therefore, that the liver damage which was associated with acute fascioliasis in these rabbits did not produce any immediate or marked effects on red cell production.

Cirrhosis is a prominent feature of chronic fascioliasis but it, too, was not concerned in the etiology of the anaemia. The removal of the flukes from 4 rabbits by an anthelmintic caused the red cell count and haemoglobin to return to normal in each case in a matter of days although at autopsy the livers of these rabbits were extremely cirrhotic. In the fifth rabbit in which only/

only some of the flukes were killed there was a rise in the red cell count and haemoglobin.

In the anaemic rabbits there was normally no increase in plasma bilirubin except on the few occasions referred to earlier, when a transient bilirubinaemia occurred in a few of the rabbits and was ascribed to severe liver damage; also, as is described later in this thesis (Section 5), the excretion time of bromsulphalein by the liver was not generally increased. These findings are in marked contrast to those experienced in the rabbits subjected to ligation of the common bile duct in which the plasma bilirubin, alkaline phosphatase levels, and bromsulphalein clearance times were raised. It may be concluded, therefore, that obstruction of the common bile duct did not occur in these infected rabbits and, therefore, was not concerned in the pathogenesis of the anaemia. This is supported by the fact that the degree of anaemia produced by the parasites was much more severe than that produced by complete ligation of the bile duct.

The exclusion of marrow hypoplasia or aplasia, of intravascular destruction of blood, of liver lesions, or of a deficiency of the anti-anaemic principle of the liver as possible causes of the anaemia emphasises the possible significance of haemorrhage from the bile ducts as the principal etiological factor. The presence of blood in the contents of the bile ducts and in the caeca of the flukes, and the consistency with which this can be demonstrated clearly indicate that a degree of blood loss is associated with the infection;/

infection; some evidence that this may be responsible for the anaemia is provided by the fact that an anaemia similar in all essential respects was produced by removing approximately 30 - 35 ml. of blood weekly from each of 2 rabbits over a period of 106 days. Similar reductions in the red cell counts of rabbits, subjected to the same degrees of blood-letting as were our rabbits, were obtained by Steele (1933) and by Oberg (1949) when studying blood regeneration in this species.

The similarity between these two anaemias, one produced by the infection of rabbits with F. hepatica, and the other produced by repeated bloodletting, together with the fact that haemorrhage can be shown to occur in the former, provide strong presumptive evidence that the parasitic anaemia is haemorrhagic in origin. Final proof that this is indeed the case depends on the demonstration that the degree of blood loss produced by the flukes is of sufficient severity to produce the degree of anaemia obtained.

This point has been investigated and is described in detail in the next section of this thesis. These experiments, which consisted of trace-labelling the red cells and plasma of parasitised and normal rabbits with radioactive isotopes and estimating the amounts of these substances in the bile-ducts and flukes after a specific period, provided fairly conclusive evidence that considerable blood losses occur in the bile-ducts of infected rabbits, e.g. it was estimated that a rabbit with 30 flukes will lose oever 2,500 ml. of blood during a period of one year. Haemorrhage of this nature in the rabbit/

rabbit is compatible with severe anaemia.

It appears, therefore, that the anaemia which is associated with F. hepatica infections of rabbits may be attributed to the haemorrhage caused by the presence of the parasites in the bile ducts. It is probable that this haemorrhage is produced in one or both of two ways : -

(a) The blood is sucked from blood vessels in the wall of the bile duct by the parasites.

(b) The spines of the adult flukes lacerate the wall of the bile duct to such an extent as to cause severe and constant haemorrhage.

From histological studies of the flukes in the bile ducts there is little doubt that the ducts may be severely lacerated (this thesis; Section 5) and a degree of haemorrhage almost certainly occurs in this way. Furthermore, the fairly frequent presence of fluke eggs in the caeca of the parasites is evidence that ingestion of bile as distinct from active blood-sucking must occur at least on occasions (Fig. 20). The parasites do, however, adhere by their suckers to the mucosa of the bile duct (Fig. 21) and it is not unreasonable to suppose that they do suck blood. This is supported by the fact that red and white blood cells may be demonstrated frequently in the fluke caeca (Fig.16) in the proportions in which they occur in blood and by the strong reaction of material from the fluke caeca to tests for occult blood as compared to bile (although the latter may be due to concentration of the blood in the caeca as has been described by Stephenson (1947). This/

This aspect of the anaemia has been investigated further and is described in the next section of this thesis.

If the parasite does suck blood, it may provide an explanation for the relative longevity of the parasite in the sheep (Taylor, 1949; Durbin 1952) and in rabbits (Taylor, 1949) as compared to cattle (Taylor, 1949). In sheep and rabbits the bile ducts become fibrous, but the connective tissue remains cellular and vascular, while in cattle the bile ducts eventually become sclerotic due to dense acellular connective tissue, or to deposits of calcium (Hutyra et al. 1946) and it is possible that the flukes die when unable to obtain blood.

Two points of considerable interest are the macrocytosis and the reticulocytosis which occurred as constant features of this anaemia. Fig. 19 shows the Price-Jones curves of 4 rabbits before infection, during the acute migratory stage, and during the chronic stage of the infection; in each rabbit the mean diameter was elevated in the last stage, that is, during the course of the anaemia. Also, macrocytosis was almost always observed in a study of stained smears and was responsible for the increase in the mean corpuscular volume. It is probable that the increased M.D. was an index of accelerated erythropoiesis (Whitby and Britton, 1950) and was due in some measure at least to the large number of reticulocytes, cells which are known to be larger than mature red cells (Persons, 1929). This is in marked contrast with chronic haemorrhagic anaemia in man (Whitby and Britton, 1950) and in dogs (Farrar and Sturgis, 1936) in which the anaemia is usually of a normochromic or hypochromic microcytic type and the number/

number of reticulocytes in the peripheral blood is normal or decreased. The explanation for this apparent difference in response is probably the presence in the rabbits of sufficient iron to ensure adequate haemoglobination and thus to maintain the accelerated rate of erythropoiesis. Hynes (1949) has shown that in a man subjected to an artificially produced haemorrhagic anaemia, the mean corpuscular volume and the reticulocyte count rose while haemoglobin regeneration was maximal, but once the iron reserves became exhausted both the mean corpuscular volume and the reticulocyte count dropped sharply. Further evidence that the rate of blood loss and not iron deficiency was the primary cause of the anaemia in these infected rabbits is the fact that a microcytic hypochromic anaemia will develop in rabbits reared on an iron deficient diet (Smith, Medlicott and Ellis, 1944) and that in the preliminary experiment described in this paper the administration of intravenous iron to two infected rabbits did not alter the peripheral blood picture. This aspect is at present being further investigated with particular reference to the serum iron levels and to the iron combining power of the sera.

The fact that the coagulation time was not usually increased even during the time when liver destruction was at its peak, i.e. about 6 or 7 weeks after infection, is of interest in view of the role of fibrinogen in clot formation and the manufacture of this substance by the liver; the coagulation time was increased in only one rabbit/

rabbit, H318, 178 and 180 days after infection.

In man, according to Whitby and Britton (1950), the sedimentation rate is increased in all conditions where there is tissue breakdown or where foreign protein enters the blood. These two conditions are present in fascioliasis and in cattle with this disease, Pustowka (1946) observed an increased sedimentation rate; the greater the liver damage and the more numerous the parasites, the more the sedimentation rate was increased. In our rabbits, the sedimentation rate, which is normally negligible, was not increased during infection.

The fragility of the red cells was examined on so few occasions that it is not possible to draw any definite conclusions; in the few samples of infected blood which were examined, the fragility appeared to be slightly decreased.

It was interesting that oedema was not observed at any time in these infected rabbits since a degree of hydrothorax, hydropericardium, ascites and oedema of the loose connective tissue of the throat and brisket is commonly encountered in chronic fascioliasis in sheep and cattle.

SUMMARY

1. The literature on the anaemia associated with Fasciola hepatica infection in domestic animals is reviewed.

2. An experiment is described in which rabbits were experimentally infected with cercariae of F. hepatica; the anaemia which followed infection was studied.

3./

3. The characteristics of the anaemia were compared to those of other anaemias and it was concluded that the anaemia associated with fascioliasis is similar in all essential respects to that produced in rabbits by chronic haemorrhage.

4. The significance of this finding is considered in relation to the calculated blood loss caused by the presence of the parasites in infected rabbits.

5. Several other aspects of the anaemia are discussed.

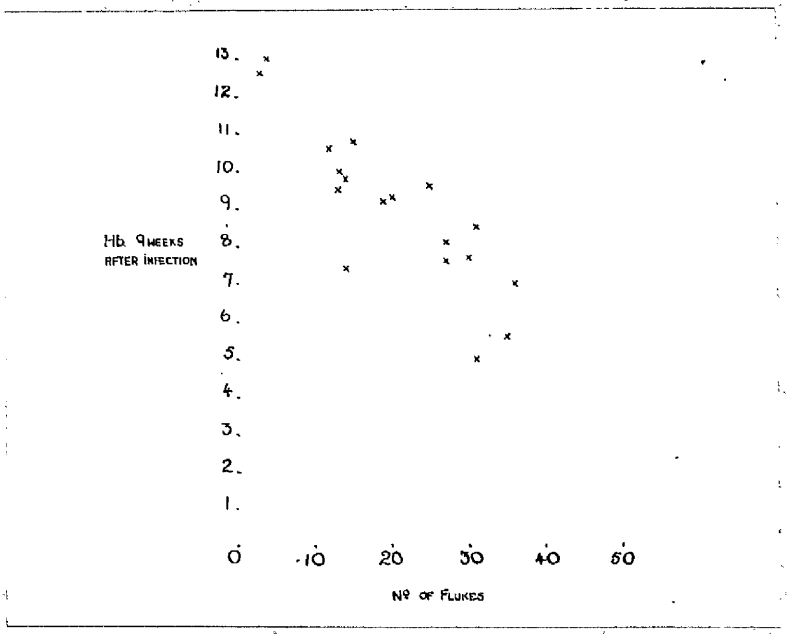
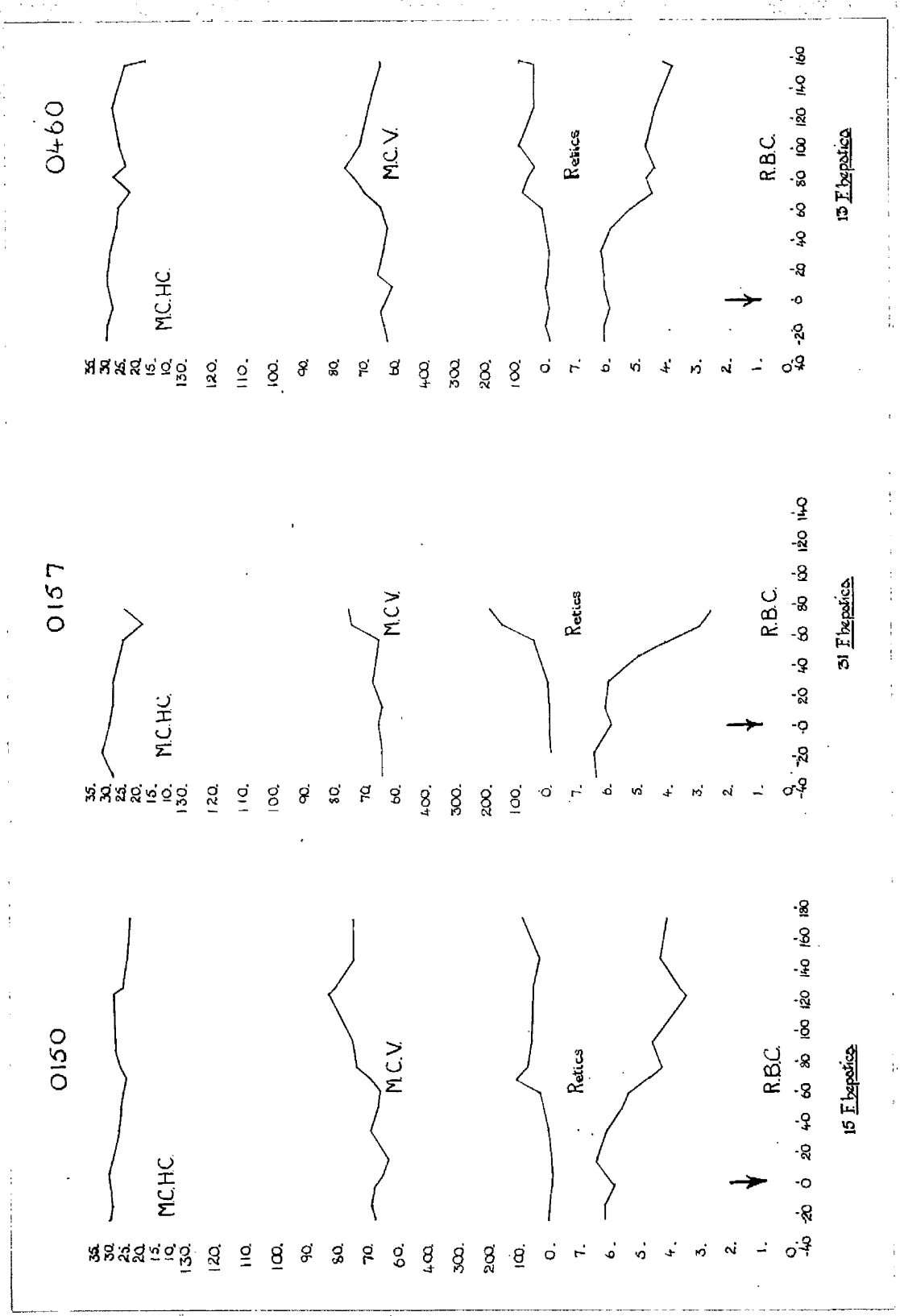


Fig. 8. The relationship between blood haemoglobins and numbers of flukes in rabbits 9 weeks after infection.

Fig.9. The alterations in blood values in infected rabbits.



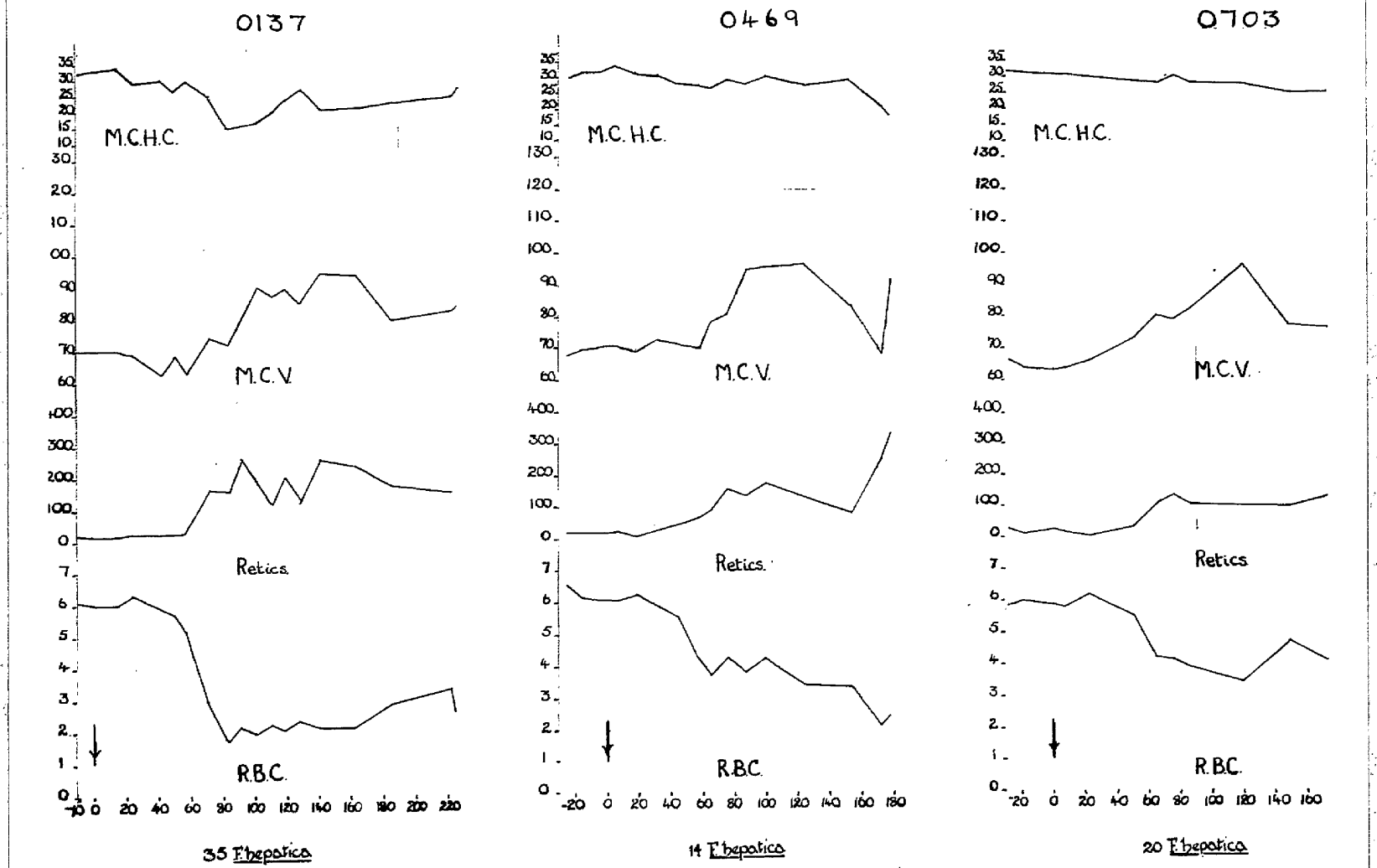


Fig. 9. (Contd.)

VII

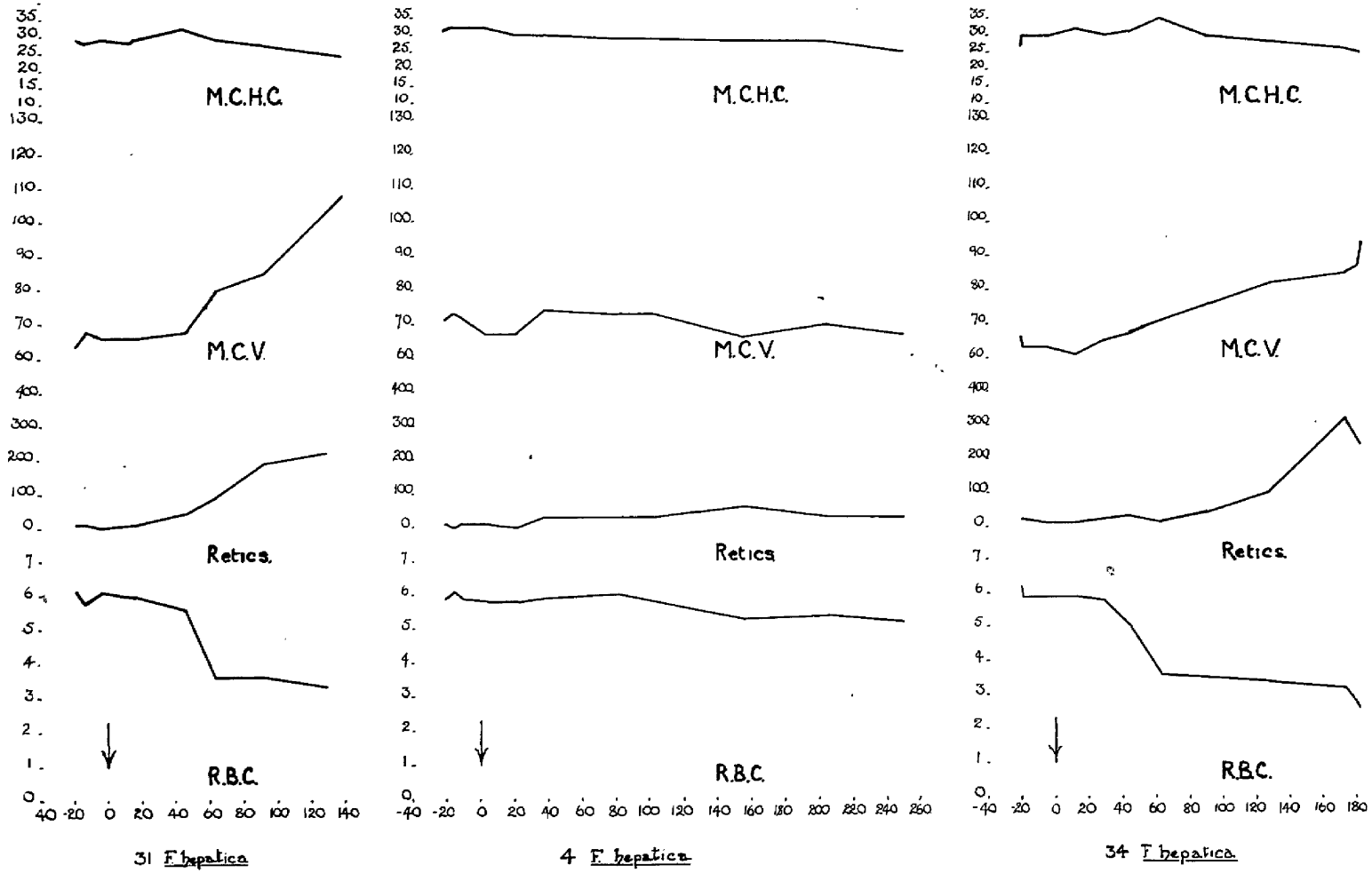
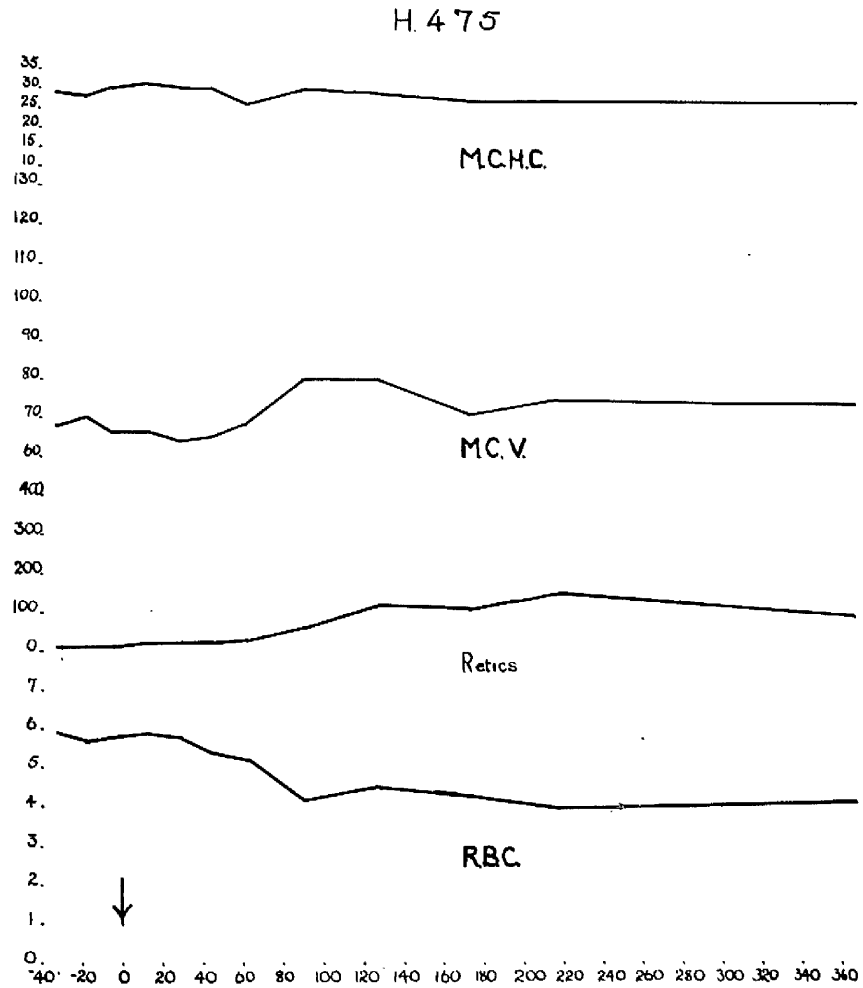

H 251
N 217
H 318
M.C.H.C.
M.C.V.
Retics.
R.B.C.
31 F. hepatica
4 F. hepatica
34 F. hepatica

Fig. 9. (Cont'd).



20 F. hepatica.

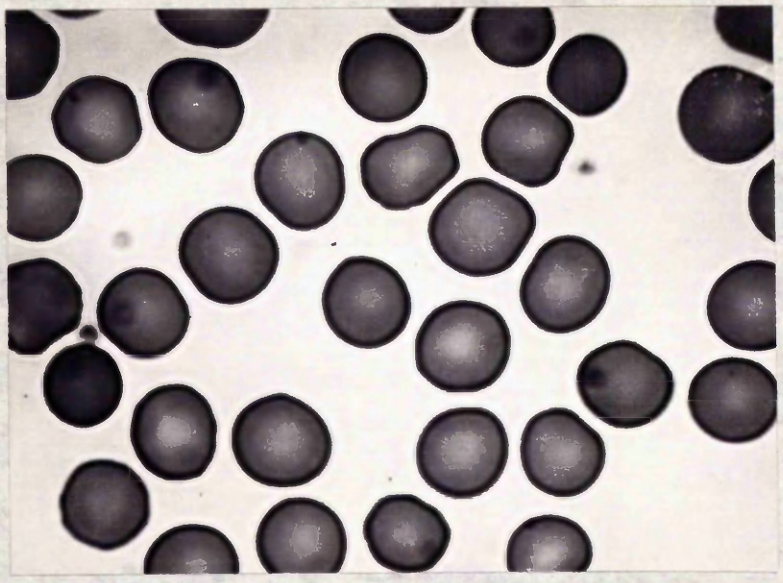


Fig. 10. Normal rabbit red cells. Giemsa x 1300.

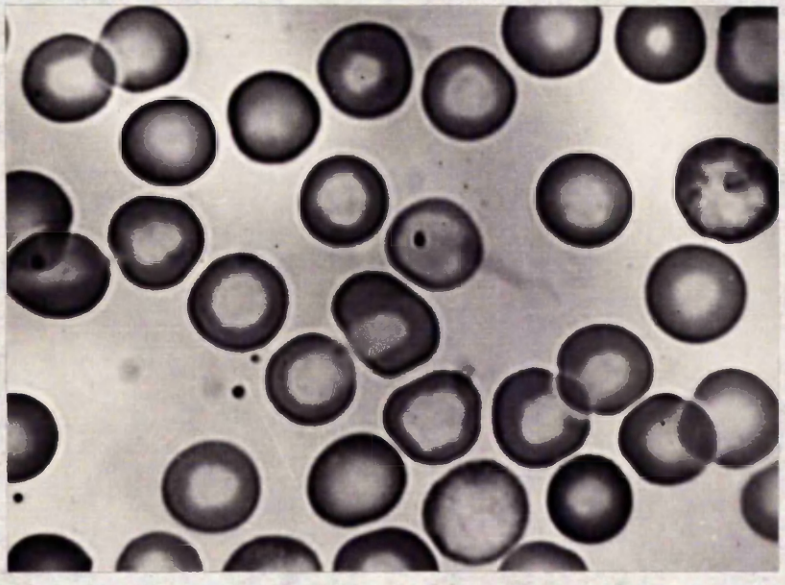


Fig. 11. Red cells from anaemic rabbit. Giemsa x 1300.

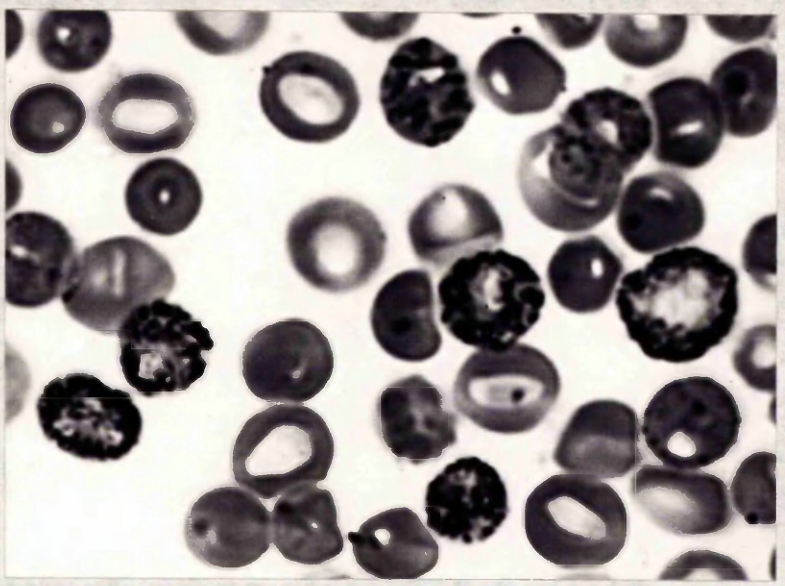


Fig. 12. Reticulocytes in blood film of anaemic rabbit. Brilliant cresyl blue and Giemsa x 1300.

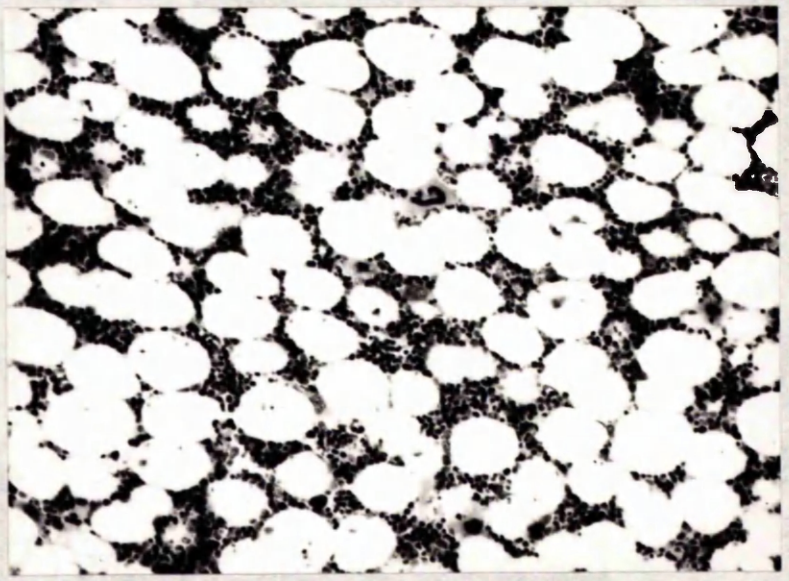


Fig. 13. Section of normal rabbit tibial marrow showing distribution of fat. Giemsa x 400.

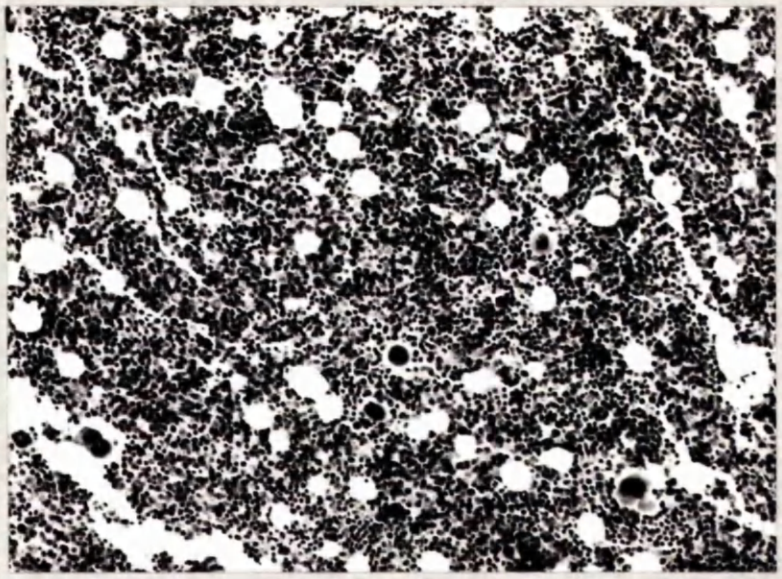


Fig. 14. Section of tibial marrow from anaemic fluke-infected rabbit showing hyperplasia of the haemopoietic tissue. Giemsa x 400.

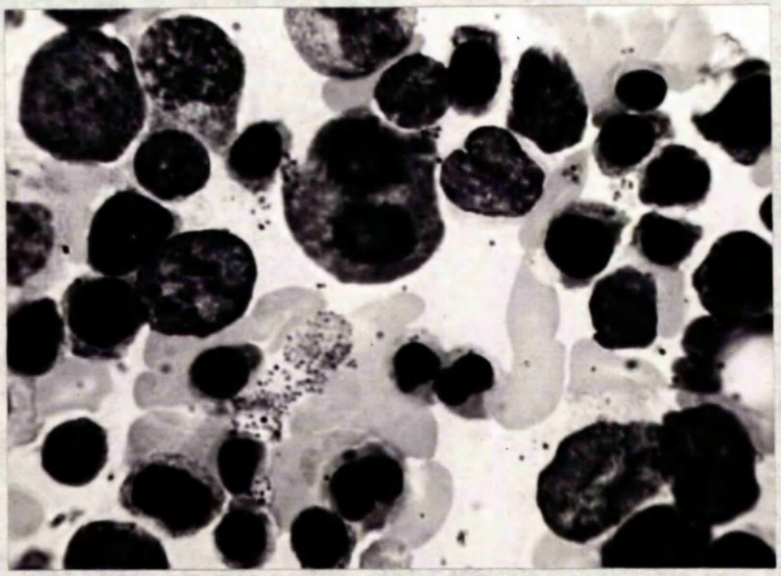


Fig. 15. Film of tibial marrow from anaemic fluke-infected rabbit showing normoblastic hyperplasia. Giemsa x 1000.

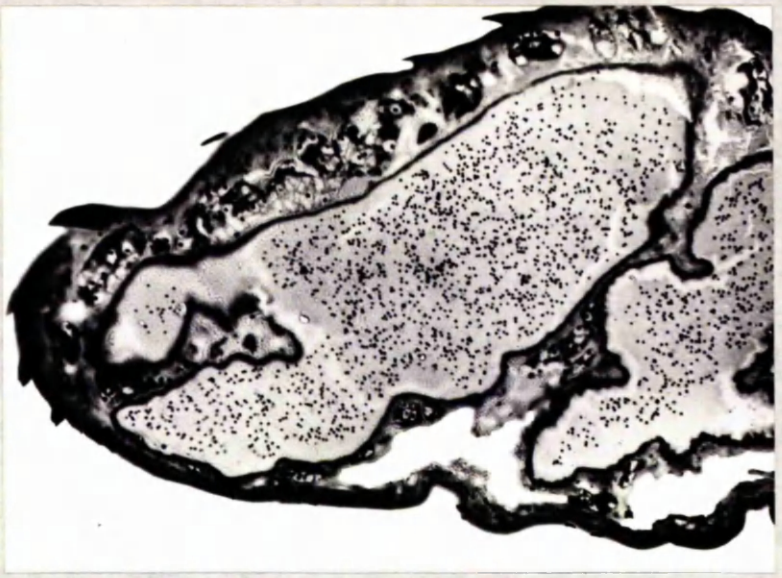


Fig. 16. T.S. of fluke. The lumina of the caeca are filled with intact red and white blood cells. H.& E.x 35.

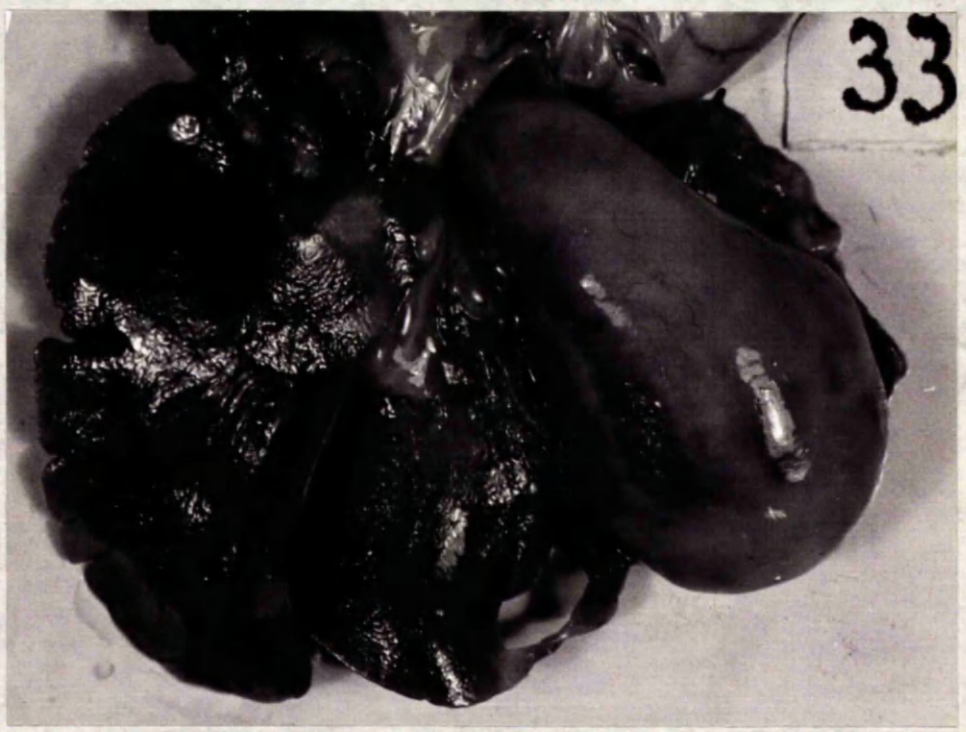


Fig.17. Liver of rabbit subjected to ligation of the common bile duct. The gall-bladder is grossly enlarged and distended.

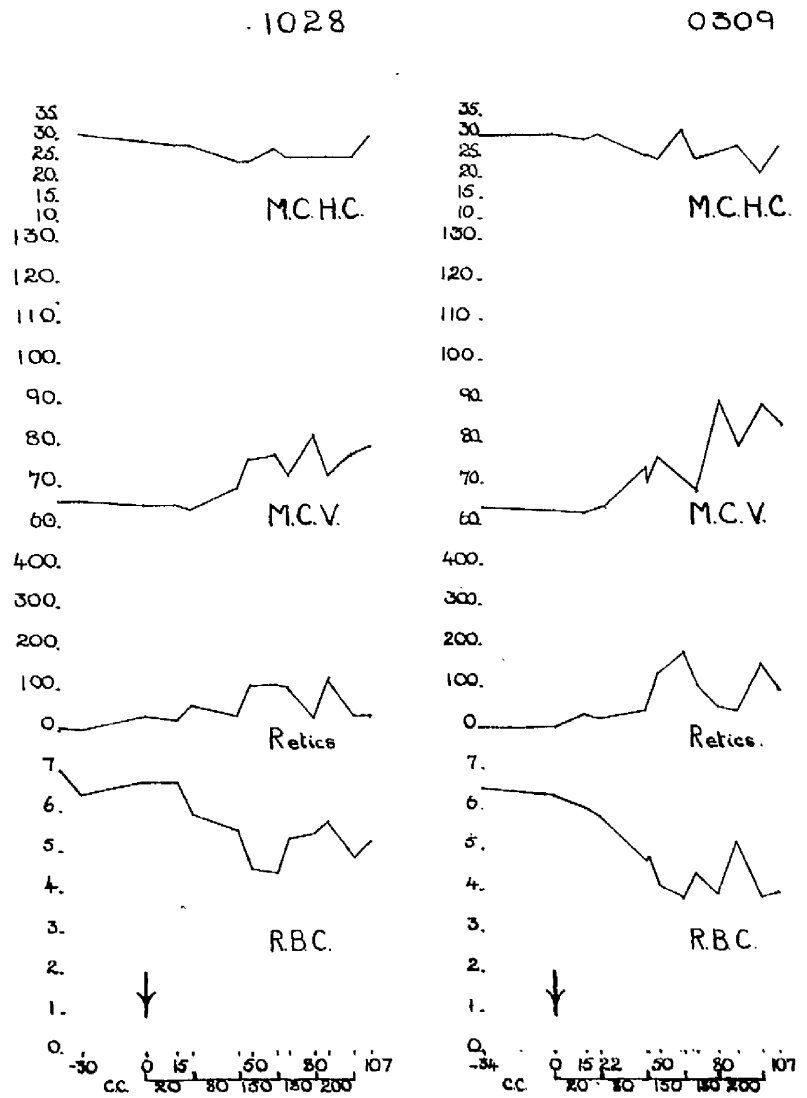


Fig. 18. Blood values of rabbits subjected to repeated venesection.

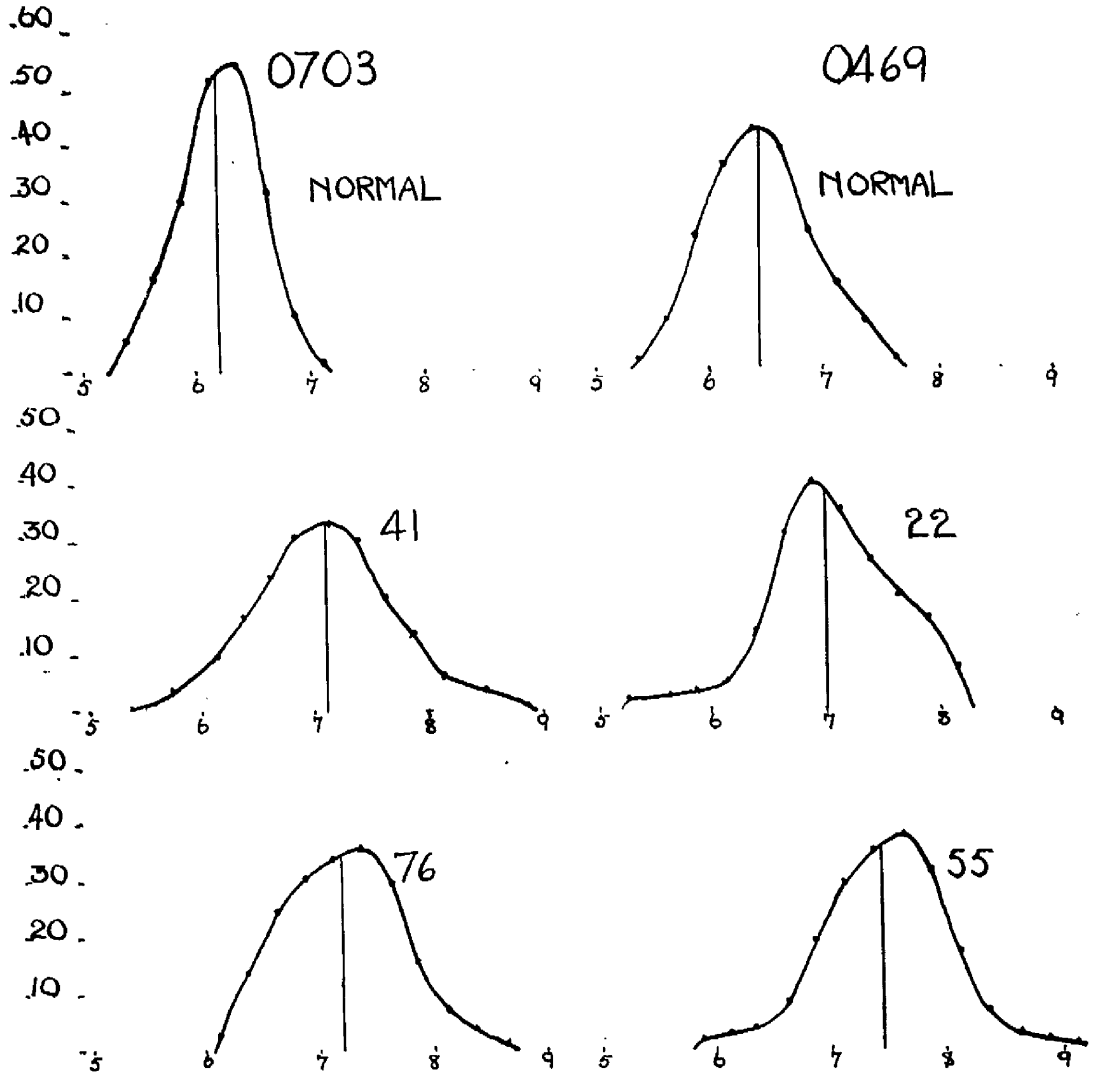


Fig. 19a. Red cell distribution curves of two fluke-infected rabbits, 0703 and 0469.

Figures beside curves refer to days after infection.

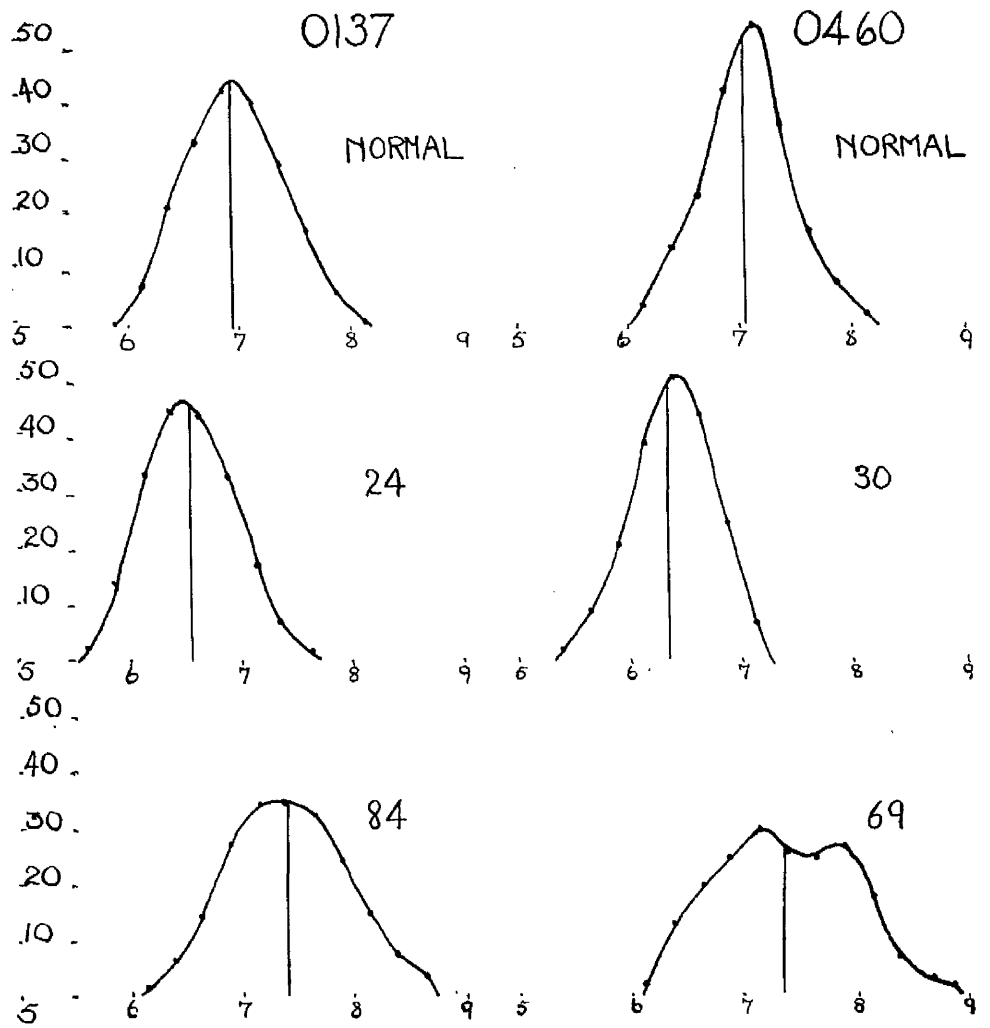


Fig. 19b. Red cell distribution curves of two fluke-infected rabbits. 0137 and 0460.

Figures beside curves refer to days after infection.

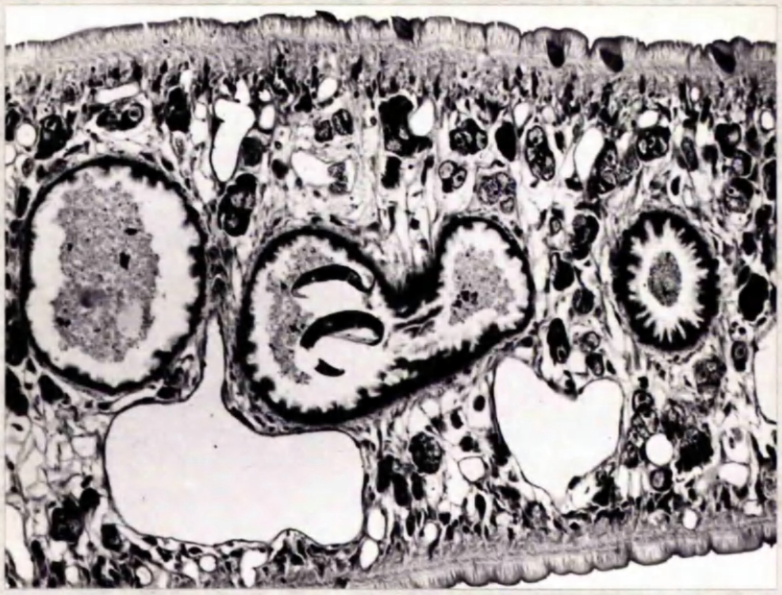


Fig. 20. T.S. of fluke showing fluke egg in caecal lumen. H.&.E. x 110.

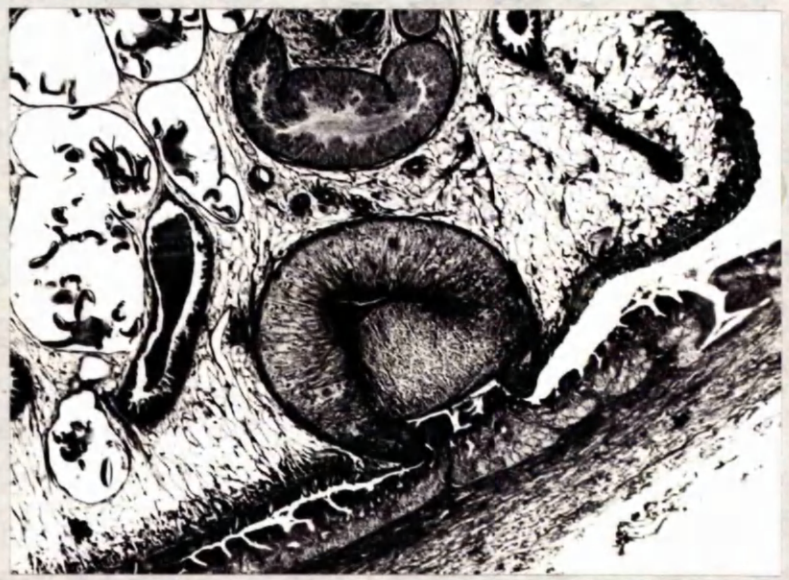


Fig. 21. Sucker of fluke grasping mucosa of common bile duct. Mallory x 30.

SECTION 3

RADIOISOTOPE STUDIES ON THE ANAEMIA PRODUCED BY INFECTION WITH *FASCIOLA HEPATICA*

This section of the work was carried out in collaboration with Dr. W. Mulligan and Mr. F. W. Jennings of the Department of Veterinary Biochemistry of this School.

INTRODUCTION

In the previous section of this thesis it was shown that in the anaemia associated with fascioliasis of rabbits, blood may be consistently demonstrated in the contents of the bile ducts and in the caeca of the flukes. The anaemia was also shown to be similar in all essential respects to the anaemia produced by chronic haemorrhage.

In this section of the work an attempt has been made to show that these observations are connected and that the anaemia associated with fascioliasis is indeed a chronic post-haemorrhagic anaemia produced by the presence of the parasites in the bile ducts. In order to do this the quantitative aspect of blood consumption by the parasite has been studied with the use of isotopically labelled blood; these experiments were carried out initially with ^{32}P-labelled red cells and with ^{131}I-labelled serum albumin used separately. In later experiments labelled red cells and labelled serum albumin were used together.

METHODS AND MATERIALS

^{32}P-labelled red cells

These were prepared essentially as described by Hevesy (1948). Freshly drawn heparinised rabbit blood (5 ml.) was treated with a small volume of radioactive phosphate solution containing about 100 μC ^{32}P and a minute amount of carrier phosphate. After incubation at 37°C. for two hours with frequent mixing, the labelled cells were centrifuged and washed four times with 10 volumes each time of ice cold isotonic saline. Injection of the labelled cells was carried out within a few minutes/

minutes of the final washing.

^{131}I-labelled serum albumin

An 'albumin' solution was prepared from rabbit serum by removal of most of the globulins with 18% sodium sulphate. After dialysis the albumin was trace-labelled with ^{131}I as described by Francis, Mulligan and Wormall (1951).

Kjeldahl analyses

In some of the experiments Kjeldahl analyses on the flukes were carried out in order to correct for differences in the sizes of the flukes, and to obtain a more standard reference in calculating blood loss.

When the flukes were digested with sulphuric acid and catalyst the ammonia from an aliquot was steam distilled into 4% boric acid containing a methyl red-methylene blue indicator (see Kabat and Mayer 1948 p.283). The ammonia was then titrated with N/70 HCl using a microburette.

Radioactivity determinations

All determinations were made using the M6 liquid counter (20th Century Electronics). Each sample was counted for a sufficient time to give at least 2,500 counts i.e. an S.D.* of about 2%. At least two such determinations were carried out on each sample and where the duplicate count rates were not in good agreement, a third determination was made.

Determination of ^{131}I and ^{32}P on the same sample

In the experiment where ^{131}I-labelled serum albumin and ^{32}P-tagged red cells were injected simultaneously, the ^{131}I and ^{32}P activities were determined as follows. The total radioactivity (^{131}I + ^{32}P) of each

* Standard Deviation.

each sample was determined. The ^{131}I was then removed by sulphuric acid digestion, the ^{32}P remaining in the digest; ^{131}I was calculated by difference.

Self-absorption of ^{131}I.

In ^{131}I determinations on homogenised flukes the possibility of self-absorption of the relatively soft -radiations must be considered. An experiment was therefore carried out in which a standard amount of ^{131}I-labelled serum albumin was added to 10 ml. samples of fluke homogenate of varying concentrations as determined by Kjeldahl analysis. The radioactivities of these samples were determined and a self-absorption curve constructed relating counts/min. of sample to mgms. fluke nitrogen. In all our experiments the concentrations of fluke homogenates used were within the range shown in this self-absorption curve and therefore no corrections were necessary.

Infection of rabbits

Infection of rabbits was carried out with 50 7-day-old cercariae encysted on Cellophane and fed on cabbage as described in Section 1 of this thesis. The isotope experiments were carried out at least nine weeks after infection.

EXPERIMENTAL

Experiments with ^{32}P-labelled red cells

Red cells from fluke-infected and normal rabbits were labelled with ^{32}P as described. The labelled cells were then suspended in an equal volume of saline and a 1 ml. sample injected/

injected into the marginal vein of the left ear. Each rabbit was injected with its own red cells. Blood samples (5 ml.) from the marginal vein of the right ear were collected in heparinized bottles, 10 mins. and 1 hr. after injection. Duplicate 1.0 ml. samples from each bleeding were made up to 10 ml. for counting. Each rabbit was killed immediately after the withdrawal of the second blood sample, the abdomen was opened, the common bile duct ligated at its junction with the duodenum, and the liver removed and washed free from superficial blood. The surfaces of the common bile duct and gall bladder were seared with a hot spatula to prevent haemorrhage, a Pasteur pipette was inserted through the seared areas and the bile was withdrawn, transferred to a 10 ml. graduated cylinder and made up to volume for counting.

The large extrahepatic bile ducts which usually contained most of the parasites were then opened, the flukes removed, rinsed in normal saline and transferred to a Kjeldahl incineration flask. The lobes of the liver were carefully dissected and any flukes in the intrahepatic bile ducts also collected, rinsed, and added to the incineration flask. After sulphuric acid digestion of the flukes, the digest was quantitatively transferred to a volumetric flask and made up to 10 ml. for counting. In some of the experiments aliquots of this digest, after radioactivity determination had been carried out, were analysed for nitrogen by the micro-Kjeldahl procedure.

Radioactivity determinations were carried out on the blood samples, bile, and fluke digest. The quantity of 'blood' in the flukes/

flukes was calculated as follows:

$$\text{Vol. of blood in sample (ml.)} = \frac{\text{counts/min. of sample}}{\text{counts/min/ml. blood (60 min. sample}}$$

This figure for the flukes was multiplied by 24 to obtain an approximate measure of the blood loss per day.

The radioactivity of the bile of the normal and infected rabbits was determined in the same way and in all cases was found to be small compared to the activity of the flukes. Some typical results are shown in Table 10.

Table 10: Radioactivity of flukes and bile from infected rabbits and bile from controls.

		Radioactivity c/min.	
Rabbit No.	Method	Flukes (total)	Bile (total)
X	^{32}P cells	474	78
9	"	2311	50
0	"	1293	64
1	"	1994	56
2	"	3985	157
10	"	1698	56
0703	"	732	38
Control	"	-	15
"	"	-	67

Where separate determinations were carried out on gall bladder bile/

bile and bile from the common bile duct of infected animals, it was invariably found that the latter was the more active.

The 10 min. blood sample was taken to ensure that no abnormal loss of label occurred during the experimental period.

Experiments with ^{131}I trace-labelled serum albumin

^{131}I-labelled serum albumin (1 ml.) was injected into the marginal vein of the left ear of a number of normal and infected rabbits. Blood samples from the opposite ear were taken at 10 mins. and again immediately before the rabbits were sacrificed at 1 hr. The bile was collected as before, the flukes were macerated in a Griffith's tube and the homogenate transferred to a 10 ml. graduated cylinder and made up to volume for counting. The blood samples were diluted 1 - 10 for counting, and the amount of radioactivity expressed as ml. blood in the flukes calculated as before. In these experiments the radioactivity of the normal bile was again negligible compared with that of the flukes.

RESULTS

The results obtained from 8 rabbits using ^{32}P-labelled red cells and 3 rabbits using ^{131}I-labelled serum albumin are shown in Table 11.

Table 11/

Table 11: Calculated blood loss of infected rabbits.

Rabbit No.	Method	No. of flukes	Est. blood loss in 24 hrs. (ml.)	Blood loss per mg. fluke protein in 24 hrs. (ml.)
X	^{32}P cells	6	0.67	0.024
9	"	25	4.80	0.024
0	"	18	2.60	0.017
1	"	25	4.82	0.026
2	"	34	7.60	0.031
10	"	13	7.70	"
0703	"	20	4.20	"
5	"	7	3.30	"
A	^{131}I-serum protein	32	3.86	"
13	"	20	2.10	-
P299	"	20	2.13	-

Although there is some degree of correlation between the estimated blood loss in 24 hours and the number of flukes present, this is not marked and the differences are probably due to variations in the feeding activities of the flukes during the period of the experiment and to their relative sizes. A somewhat closer correlation is obtained if estimated blood loss is expressed per mgm. of fluke nitrogen. In this connection, it is interesting that when the caeca of the flukes are filled with a dark brown or red material their radioactivity is particularly high.

In the three rabbits which were injected with ^{131}I-labelled serum albumin the calculated blood loss in 24 hours appears to be less than in those rabbits injected with ^{32}P-labelled cells. This finding is discussed/

discussed later.

To obtain more information on the problem, experiments were carried out in which each rabbit was injected simultaneously with ^{32}P-labelled cells and ^{131}I-labelled serum albumin.

Experiments with ^{32}P-labelled red cells and ^{131}I-labelled serum albumin injected simultaneously

In this experiment the activity of one of the isotopes was determined by difference between total radioactivity and that of the other isotope, (see under methods and materials). The accuracy of such a determination depends to some extent on the amounts of the two isotopes present. It is essential that the activity of the isotope which is determined by 'difference' should not be small compared to the activity of the one determined directly. In these experiments we have found it convenient to work with ^{32}P and ^{131}I activities of the same order.

A solution of ^{131}I-labelled rabbit serum albumin was prepared as described. It was found convenient in practice to prepare labelled red cell suspensions of greater than the required radioactivity and to dilute these with inactive cells from the same animal to give approximately the same count rate/ml. as the labelled albumin solution.

Samples of labelled cells (2.0 ml.) from each rabbit and 2.0 ml. ^{131}I-labelled albumin solution were mixed thoroughly in a small beaker. 2.0 ml. of the mixture were injected into the marginal vein of the left ear. Blood samples (6.0 ml.) were collected in heparinised/

heparinised bottles from the marginal vein of the right ear at 10 min. and immediately before killing the animal at 60 min. Flukes and bile were removed as before. The flukes from each animal were homogenized in a Griffith's tube and made up to 10 ml. The total bile and duplicate samples (1.0 ml.) from the 10 min. and 60 min. bleedings were also diluted to 10 ml. Total radioactivity i.e. (^{32}P + ^{131}I) was determined on the fluke homogenate, bile and blood samples.

Triplicate blood samples (1.0 ml.) from each bleeding, duplicate aliquots (4.0 ml.) of the fluke homogenate, and the total bile were freed from ^{131}I by digestion as described. The digests were each made up to 10 ml. for counting and the ^{32}P activity determined. ^{131}I activity was calculated by difference. It was thus possible to calculate the $^{32}P/^{131}I$ ratio in the blood and flukes. The results are shown in Table 12. Some difficulty was encountered in the digestion of the bile samples (losses occurred due to foaming and spurting) and the results are, therefore, not included in Table 12.

Table 12:/

Table 12: $^{32}P/^{131}I$ ratios in flukes and blood

Rabbit No.	$^{32}P/^{131}I$ ratio 10 min. blood	60 min. blood	flukes	$(^{32}P/^{131}I)$ 60 min. blood / $(^{32}P/^{131}I)$ flukes
6	0.93	0.96	1.84	1.92
9	1.83	1.81	3.47	1.92
10	0.93	0.92	1.62	1.76
16	1.65	1.85	3.39	1.83
17	1.47	1.61	2.95	1.83
18	0.95	0.91	1.68	1.85
19	1.54	1.56	2.17	1.39
51	0.86	0.82	1.52	1.87
54	1.00	1.04	1.58	1.52

Mean = 1.77 $\pm$ 0.17

It can be seen from Table 12 that the $^{32}P/^{131}I$ ratios in the flukes are in all cases greater than those in the 60 min. blood sample. If these ratios had been equal it could have been interpreted as due to the ingestion by the flukes of red cells and plasma in the same proportions as found in whole blood. The $^{32}P/^{131}I$ ratios in the blood at 10 min. and 60 min. show that no significant variation in this ratio took place during the experimental period.

When the $^{32}P/^{131}I$ ratios in the flukes are divided by the corresponding figure for the 60 min. blood sample a remarkably constant value is obtained.

DISCUSSION/

DISCUSSION

In the experiments described in this section an attempt has been made to obtain a measure of the quantity of blood consumed by the flukes and therefore lost to the host animal.

In our experiments with ^{32}P labelled red cells, we have calculated a blood loss of approximately 0.25 ml. per day per fluke, and in the three rabbits using ^{131}I-labelled serum albumin about half this amount.

Using the mean ^{32}P figure a rabbit having 30 flukes in its bile duct would suffer a blood loss of about 7.5 ml. per day. That this amount of daily blood loss is sufficient to produce a marked anaemia is substantiated by the work of Steele (1933) and of Oberg (1949) who removed on an average 4-8 ml. of blood per day from rabbits over a period of several months, with the production of a degree of anaemia of similar order to that found in our experimental rabbits.

The lower figure for blood loss using ^{131}I-labelled serum albumin is substantiated by the results of our experiment with the two isotopes simultaneously. An interesting feature of the simultaneous experiment was the relationship of the $^{32}P/^{131}I$ ratio in blood and in the flukes (see column 5 - Table 12). This could indicate a preferential uptake by the flukes of red cells, or of labelled products from the red cells, compared with that of plasma.

Another possibility is that the turnover of the metabolic products of the labelled plasma proteins is more rapid than that of/

of the labelled materials from the red cells. A finding supporting either of these two hypotheses is that in three cases where analyses were obtained for both isotopes in the bile, the $^{32}P/^{131}I$ ratio was smaller than that in blood and markedly smaller than that in the flukes.

It must be pointed out that in these experiments what one measures is ^{32}P and ^{131}I activities and the assumption is made that these correspond to the red cells and plasma protein originally labelled. However, some loss of 'label' does occur in the body, e.g. ^{32}P escaping from cells in the circulation or by cells being destroyed in the reticulo-endothelium system. Catabolism of the labelled serum albumin also takes place continuously with the liberation of labelled catabolic products.

Normal bile contains phosphate and it is also recognised that the bile is one of the pathways for the excretion of iodine and certain iodinated compounds. It is to be expected, therefore, that after the injection of ^{32}P-labelled cells or ^{131}I-labelled serum albumin some radioactivity will appear in the bile of normal animals. This was found to be the case in our control animals. Our justification for using the radioactivity of the flukes as a measure of 'blood' consumption depends on the large quantitative difference between the radioactivity of flukes and that of the bile of the control animals.

It is felt that further experiments using different labelling techniques for red cells and plasma proteins are indicated, e.g. red cells labelled with radioactive Fe and plasma proteins labelled with ^{35}S. The advantages of multiple labelling in this type of experiment are obvious, e.g. the use of plasma protein labelled simultaneously with/

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SECTION 4

ARTIFICIAL IMMUNITY TO *FASCIOLA HEPATICA* IN RABBITS.

SOME STUDIES WITH PROTEIN ANTIGEN.

with ^{131}I and ^{35}S will enable one to make a more valid assessment of the importance of ^{131}I-labelled catabolic products in the bile. While the estimates of blood loss reported in this section cannot be regarded as a final and absolute measurement, they appear to be of the right order and give additional confirmation of the haemorrhagic nature of the anaemia.

SUMMARY AND CONCLUSIONS

Red cells labelled with ^{32}P and serum albumin labelled with ^{131}I have been used to study the anaemia associated with F. hepatica infections in rabbits and to make an estimate of the amount of the daily blood loss. The blood loss as calculated from ^{32}P experiments is of sufficient magnitude to account for the anaemia. Somewhat smaller values were obtained using ^{131}I-labelled serum albumin.

When ^{131}I-labelled serum albumin and ^{32}P-labelled red cells were used simultaneously it was found that $^{32}P/^{131}I$ ratio in the flukes was consistently higher than in the blood.

The use of radioisotope techniques in the investigation of this type of problem is discussed.

INTRODUCTION

In this part of the thesis an experiment is described in which the protein fractions of F. hepatica were used as immunizing antigens in an attempt to stimulate a resistance to the natural infection. Throughout the experiment the antibody levels of the sera of the immunized and infected rabbits were studied by the quantitative precipitin test. In this introduction the relevant literature on immunity to helminth parasites and on the quantitative precipitin test is reviewed separately.

Immunity to helminth parasites

Resistance of an animal to a helminth infection may take the form of either true acquired immunity or of premunity. In the former, the immunity is essentially a post-infection state in which resistance to reinfection is not dependent on the persistence of the initial infection. In the latter the resistance to reinfection depends on the persistence of an earlier infection and is actually a resistance to superinfection; although this concept of premunity would seem to imply a rather static state of affairs, it has been shown in recent years by various workers (Stewart, 1950a, b, c, d; Taylor and Michel, 1953; Gibson, 1953) that the mechanism of premunity is actually very complex and dynamic.

One may attempt to produce true acquired immunity by two methods. First, by infection of the host with the parasite and ensuring elimination of the latter before reinfection, and second, by the injection of extracts of the parasite.

The first method is preferable if the specific immunity is thought/

thought to be dependent on the stimulus of the excretory or secretory products of the worms and immunity to trematode infection has been studied in this way by a number of workers. One of these was Ozawa (1930) who showed that if dogs are cured of a first infection of schistosomes by an injection of stibnal, their resistance to a second infection of cercariae is greatly increased as judged by the number and size of the parasites which develop. Also working with schistosomes, Fairlie,, Mackie, and Jasudasan (1930) infected monkeys with S. spindale, normally a parasite of man; while they obtained partial development of the parasites in the tissues of previously unifected monkeys, this was followed by spontaneous recovery and subsequent attempts to reinfect these monkeys were almost completely unsuccessful. More recently Kagan and Lee (1953) have demonstrated that acquired immunity towards S. douthitti in mice subsides approximately 3 weeks after the end of effective treatment.

The parasite which has probably been most extensively studied in respect of immune reactions is Nippostrongylus muris, a nematode parasite of the rat. In a susceptible animal, the infective larvae of this species penetrate the skin and are carried by the blood to the lungs, where they break into the air passages; they are then coughed up and swallowed, and settle down in the small intestine where they become adult; normally the bulk of the infection is thrown off several weeks after infection. It has been shown by several workers (Africa, 1931; Schwartz, Alicata and Lucher, 1931; Taliaferro and Sarles, 1939) that if rats are reinfected after this spontaneous recovery/

recovery has occurred, the subsequent infection produces fewer and smaller worms. Repeated reinfections increase this immunity and eventually invading larvae are killed in the lungs and in the skin.

Taliaferro (1940), an authority on helminth immunity has concluded that precipitins of functional importance in immune reactions of the host are formed in response to materials discharged by the worm.

The second method of producing acquired immunity, that is, the injections of extracts of the parasite, is necessary if one wishes to study the immunological properties of specific fractions of the parasite, and to quote Craig and Faust (1951), "As yet very little critical work has been done in animal parasitology to determine the relative specificities of the protein, polysaccharide and cholesterol fractions of whole antigen. This field deserves much more thorough and careful investigation." Work with whole worm extracts has demonstrated the presence of acquired immunity in fascioliasis of rabbits (Kerr and Petkovitch, 1935), schistosomiasis of dogs (Ozawa, 1930), schistosomiasis of mice (Watts, 1949), Cysticercus fasciolaris infections of rats (Miller, 1932), and in haemonchosis of sheep (Stoll, 1942). Probably the most striking of these experiments was the work of Miller (1932) who was able, by the repeated injection into rats of dried adult Taenia taeniaeformis to produce an almost solid immunity to infection with large numbers of onchospheres. This immunity persisted for several months after the last injection of antigen. Stewart (1950b) has reported that he failed to protect sheep against Haemonchus contortus or Trichostrongylus sp. by means of/

of vaccines prepared from ground infective larvae.

The Quantitative precipitin test

When a soluble antigen is mixed in the correct proportions with the corresponding antiserum a precipitate containing both antigen and antibody may be formed. While the precise mechanism of this reaction is still in dispute (Marrack, 1938; Pauling, 1940; Heidelberger, 1949) it is generally agreed that the specific precipitate is formed as a result of union between antigen and antibody molecules, followed by separation from solution of the insoluble antigen-antibody complex. The amount and composition of the precipitate obtained from a given quantity of antiserum depends on the relative amount of specific antibody in the antiserum and on the amount of antigen added. When increasing amounts of protein antigen are added to a fixed amount of antiserum the quantity of precipitate increases up to a maximum and then decreases as shown in Fig. 22. If the supernatants of such a

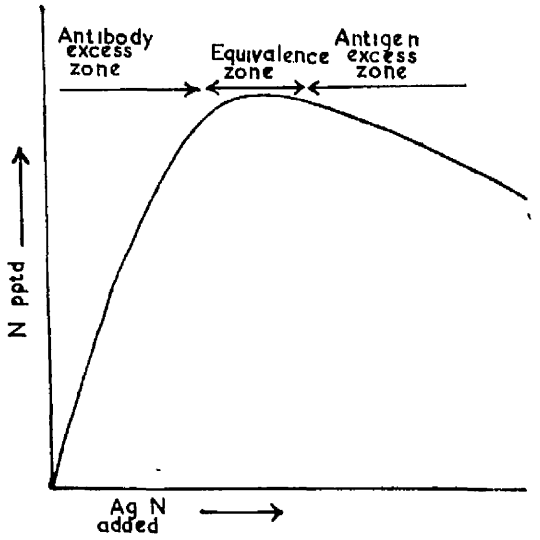

Fig. 22. Typical precipitin curve obtained with homogeneous antigen and homologous antibody.

system are tested for free antigen and antibody, the existance of three zones may be demonstrated:-

(a) A zone of antibody excess.

(b) A region where neither antigen nor antibody are found in the supernatant; this zone corresponds to the region of maximum precipitation and is often referred to as the "equivalence zone".

(c) A zone of antigen excess. In this, the total amount of precipitate is less than in the equivalence zone as some of the antibody forms soluble compounds with the antigen.

The precipitin reaction is commonly used as a test for the presence and amount of antibody in a particular sera and for this purpose it is customary to employ the qualitative precipitin test. This is usually carried out by adding the same amount of antiserum to a series of tubes containing a uniform volume of different dilutions of antigen. The mixture is incubated at 37°C. for 1 - 2 hours and readings are then made of the amount of precipitate in each tube. The disadvantages of this technique are that it is impossible to make an exact comparison of the amount of antibody in different sera and that no information is obtainable about the serological homogeneity of the precipitating antigen.

For these reasons and also because there is no information on the use of quantitative serological techniques in helminth infections, the quantitative precipitin test was used in the present investigation.

The value of the quantitative precipitin test as a method for the estimation of antibody depends largely on the work of Heidelberger/

Heidelberger and Kendall (1929; 1933; 1935) and is based on the analysis of the washed precipitates for nitrogen by the micro-Kjedahl method.

In order to ensure complete precipitation of the antibody, it is necessary to add antigen in slight excess, although still working in the equivalence zone. In practice this may be found by adding successive small volumes of the antigen to a sample of serum with incubation and refrigeration after each addition of precipitate until no further precipitate is obtained. The antibody N may then be obtained by subtracting the antigen N added from the nitrogen of the total precipitate.

One serious limitation of this method is that it is only possible to calculate the antibody N if the precipitating antigen is an immunologically homogeneous protein such as egg albumin. In a system such as this there is complete precipitation of all the antigen in the antibody excess zone and there is a clearly defined equivalence zone. When one is working with an antigen which consists of mixed proteins one cannot assume complete precipitation of the antigen in the antibody excess zone and the region of maximal precipitation is greatly extended, i.e. there is no clearly defined equivalence zone.

It is possible, however, to overcome this difficulty by isotopic labelling of the antibody, e.g. trace-labelling with ^{131}I by the method described by Francis, Mulligan and Wormall (1954). The labelling may be carried out on the whole antiserum or on the separated gamma-globulin fraction without causing any damage to the immunological properties of the antibody (Francis, Mulligan and

Wormall/

Wormall, 1951.) If the antiserum is labelled, the specific precipitates are analysed for both total N and radioactivity. The radioactivity of the precipitates is due to the labelled antibody which has precipitated with the antigen. If a sample of antibody globulin from the labelled serum is analysed for protein N and radioactivity, it is then possible to calculate the antibody N of the precipitates by simple proportion.

METHODS AND MATERIALS

Immunizing antigen

F. hepatica were collected from infected sheep livers. The parasites were freed from adhering bile and other contaminating material by washing in running tap water, were suspended in 0.9% NaCl (1 parasite/ml) and homogenized in a top-drive macerator. The crude homogenate was allowed to stand at 0 to 4 C. for several hours to allow large particles to settle out. The supernatant fluid was then decanted off and further freed from insoluble material by centrifuging at 3000 r.p.m. for 1 hour. This saline extract was usually amber to dark brown in colour, and contained about 1mg. protein N/ml.

Alum-precipitated preparations for immunization were made by treating the saline extract with an equal volume of 10% W/V potash alum (Proom, 1943) and adjusting the pH to 6.5 with 5N NaOH. The precipitate obtained was washed twice with 0.9% NaCl (containing 1:100,000 merthiolate) and finally suspended in 0.9% NaCl (merthiolated) for injection. Kjeldahl analyses showed that the above/

above procedure precipitated the same amount of protein N as did precipitation with 10% trichloroacetic acid, indicating that the alum preparation contained all the proteins in the saline extract.

Precipitating antigen

In order to obtain a more purified protein material for use as a precipitating antigen in qualitative and quantitative precipitin tests, the crude saline extract was subjected to salt fractionation. A protein fraction was obtained which precipitated at half-saturation with $(NH_4)_2SO_4$, and this material, after several reprecipitations, was used as the precipitating antigen in all our experiments.

The saline extract was treated with 1 volume of saturated $(NH_4)_2SO_4$ solution with stirring, and the mixture was allowed to stand at 0 to 4 C. for several hours. The precipitated protein was centrifuged off and treated with 1 volume of 0.1 M phosphate buffer, pH8. Any material which did not redissolve was removed by centrifuging, and the protein fraction was twice reprecipitated with $(NH_4)_2SO_4$ as before. The precipitated material was dialysed against 0.9% NaCl until the dialysate gave no reaction with Nessler's reagent. At each precipitation and during dialysis there was some loss of protein as an insoluble residue, and the yield ultimately obtained was usually about 10% of the total protein in the original saline extract.

Immunization

Rabbits were injected at 8 to 10-day intervals with alum-precipitated antigen prepared as described above. Five ml. of a suspension containing 10 mg. protein N/ml. were injected intramuscularly/

intramuscularly into each hind leg. Samples of sera were collected 9 to 10 days after each injection and were tested for precipitins with the precipitating antigen. Most of the rabbits gave some reaction after one injection, and all gave a good response after two injections.

Seven rabbits (group A) received a total of 3 injections and a further seven (group B) received a total of 6 injections.

Qualitative precipitin tests

A stock 5% solution of the fluke antigen was diluted to 1:20, 1:100, 1:500 and 1:2500 by the addition of 0.9% sodium chloride. 0.25 ml. of each of these concentrations was treated with 0.1 ml. of the serum to be tested in precipitin tubes. The tubes were mixed, incubated at 37°C. for 1 hour and then placed in a refrigerator (4°C) for 2 hours to ensure maximum precipitation. As a control against non-specific precipitation, 0.1 ml. of each sample of serum was incubated with saline, and samples of normal rabbit sera were incubated with antigen on every occasion when sera from infected or immunised animals were under test.

Quantitative precipitin tests

The results of the qualitative precipitin tests gave an approximate idea of the dilution of the antigen and of the volume of serum which would be necessary in order to achieve maximum precipitation.

Duplicate samples (1.0 ml.) of centrifuged serum were pipetted into 15 ml. tapered centrifuge tubes. Successive small volumes of a stock antigen solution (0.2 - 1.0 ml. of antigen containing 0.002 mgm. N/ml.) were added to these with incubation at 37°C. (1 hour) and/

and refrigeration (2 hours) after each addition of antigen, until no further precipitate was obtained. This was taken to correspond to the complete removal of the corresponding antibody from solution. Each of the tubes was then centrifuged for 20 minuts, the supernatant fluids were removed and the precipitates were washed twice with 5 drops of ice-cold saline each time to remove non-specific proteins (Heidelberger and Kendall, 1933) dissolved in a few drops of 0.1 N NaOH and 1.0 ml. of distilled water, and transferred to micro-Kjeldahl incineration flasks. The centrifuge tubes were washed out five times, with 6 drops of distilled water each time, and the washings transferred to the appropriate flask. Total N determinations were then carried out.

As in the qualitative precipitin tests, control tests were performed with immune serum plus saline and with antigen solutions plus normal rabbit serum.

Total nitrogen determinations

These were made by the micro-Kjeldahl method, with the NH_3 distilled into a 4% boric acid solution containing a methyl red-methylene blue indicator (see Kabat and Mayer, 1948, p. 283). The final titration was made with N/70 HCI, using an "Agla" micrometer syringe (Burroughs Wellcome and Co.) or a microburette, according to the amount of protein N present. When the syringe was used, the tip of the syringe needle was kept immersed in the distillate and stirring was effected by a stream of O_2 bubbles.

The determination of antibody nitrogen

The sample which was labelled was one of pooled sera obtained from/

from the infected and immunised rabbits before death. The iodination of the serum was carried out by the method described by Francis, Mulligan and Wormall (1954). Aliquots of the labelled preparation corresponding to 1 ml. of original serum were treated with varying amounts of antigen as before. The mixtures were incubated for 1 hour, refrigerated for 2 hours, and the precipitates spun and washed as described above. The precipitates were then dissolved in a few drops of 0.1 N NaOH, made up to 10 ml. and radioactivity determinations carried out in an M6 liquid counter (20th Century Electronics). Aliquots were then taken for micro-Kjeldahl incineration for total N. The antibody nitrogen in the precipitates was calculated by comparing these radioactivities with that of a gamma-globulin standard prepared from the labelled serum (Keckwick, 1940) and analysed for total N.

Infection of rabbits

Each of the immunized and control rabbits was infected with 50 7-day-old cercariae encysted on cellophane and fed in cabbage as described in Section I of this thesis.

RESULTS

Precipitin tests

Qualitative precipitin tests showed that the sera of infected rabbits contained antibodies which reacted with the precipitating antigen prepared from F. hepatica. Sera from more than 20 normal rabbits were tested with the precipitating antigen and in no case was any positive reaction obtained. Table 13 shows a representative sample of the results obtained from the sera of immunised, immunised and/

and infected, infected, and normal rabbits.

Table 13: Results of qualitative precipitin test on sera of immunised, infected and normal rabbits.

		Antigen Concentrations			
	Rabbit	1:20	1:100	1:500	1:2500
Immunised rabbits after 3 injections of alum-precipitated fluke protein	V1	+	++	+	±
	V2	++	++	++	+
	N856	+++	++	++	Tr.
	N851	±	+	±	Tr.
	0330	++	+	±	Tr.
	0329	Tr.	±	Tr.	F.Tr.
	0371	+	++	±	Tr.
Immunised rabbits 60 days after infection.	V1	++	+++	+	Tr.
	V2	+++++	+++	++	Tr.
	N851	++	+++	++	Tr.
	0330	+	++	+	-
	0329	±	±	F.Tr.	-
Control rabbits 60 days after infection.	C2	+	++	+	Tr.
	C4	+	++	Tr.	-
	C8	+	++	++	Tr.
	C9	++	+++	++	F.Tr.
	C14	+	++	±	-
Normal rabbits		-	-	-	-

- = no trace; F.Tr. = faint trace; Tr. = trace;

±, +, ++, etc. in order of increasing amount of precipitate.

In order to obtain a measure of the amount of circulating precipitin in the experimental animals and to investigate the serological homogeneity of the precipitating antigen used, quantitative precipitin tests were carried out. The type of precipitin curve obtained with the antigen and serum from immunised or infected rabbits was characteristic of that shown by inhomogeneous systems (Fig. 23), i.e., excess antigen was demonstrable in the supernatant in the antibody excess zone and there was thus no clearly defined equivalence zone.

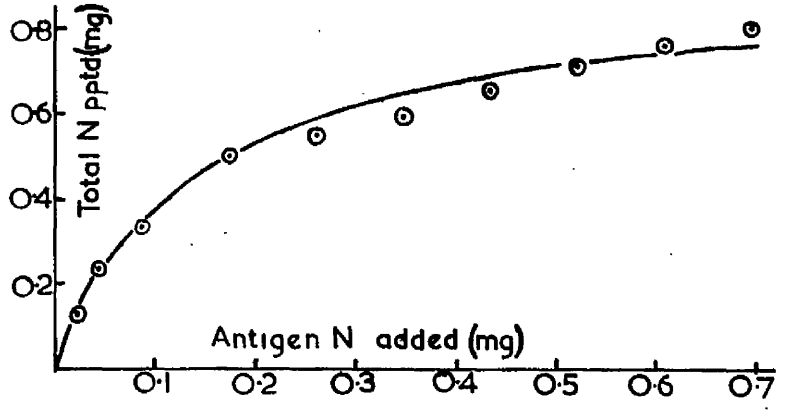

Fig. 23. Precipitin curve obtained by adding increasing amounts of the protein antigen to 1.0 ml. samples of serum from an immunized infected rabbit.

The region of maximal precipitation was also greatly extended and there was no evidence of inhibition by excess antigen.

In a system of this type it is impossible to determine the antigen and anti-body separately in the precipitates unless by some method of labelling one of the reactants, e.g., with a radioactive isotope (Francis, Mulligan and Wormall, 1954). We considered it likely, howeve that/

that the proportion of antibody in the precipitates in the region of maximal precipitation is fairly constant, and thus the figure for total N precipitated in this region probably gives a good measure of the relative amounts of precipitin to the test antigen in the different sera. This was confirmed in an experiment on a pooled sample of sera obtained from the immunized rabbits of Group B just before death. This sample of serum was trace-labelled with radioactive iodine and was then tested quantitatively for precipitins. The amount of antibody N was calculated by comparing the radioactivity of the precipitates with that of a gamma-globulin standard prepared from the labelled serum and analysed for protein N. The results of this preliminary experiment are shown in Fig. 24 and confirm the value of the total N estimations as a measure of the amount of precipitin in these sera.

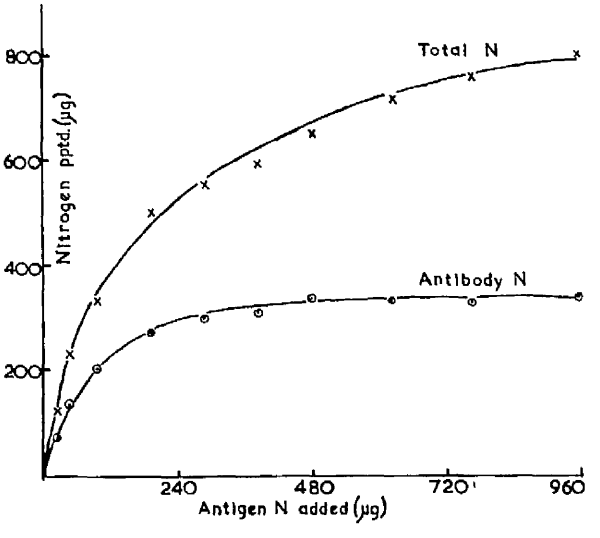

Fig. 24. Total N and antibody N precipitated from 1.0 ml. samples of antiserum by increasing amounts of the protein antigen.

The results of the quantitative tests on the sera of the immunised and control rabbits are shown in Table 14.

Table 14: Quantitative precipitin tests.

	Rabbit No.	Antibody N plus antigen N precipitated from 1.0 ml. serum: Pre-infection (mg. N)	63 days after infection (mg. N)
Immunised rabbits Series A	V1	0.155	0.774
	V2	0.188	1.868
	N856	0.268	0.620
	N851	0.118	0.636
	0330	0.198	0.498
	0329	0.114	0.150
	0371	0.190	
Immunised rabbits Series B	P93	1.280	1.130
	073	1.170	0.922
	049	0.894	0.768
	084	0.844	0.660
	P947	0.846	0.588
	076	0.620	0.656
	G65	0.420	0.968
Control rabbits.	C2		0.265
	C4		0.095
	C8		0.218
	C9		0.330
	C14		0.238
	X		0.148
	9		0.295
	0		0.138
	Z		0.227
	1		0.117

A course of 3 injections of the alum-precipitated preparation (group A) gave a level of precipitin to the test antigen comparable to/

to that obtained in normal rabbits 63 days after infection with 50 cercariae (control group). In group A the precipitin level 63 days after infection was in most cases considerably greater than the level at the time of infection. This increase was presumably due to the stimulus of infection on the immunised animals. No such increase occurred in group B; here the rabbits were subjected to a course of 6 injections prior to infection, and the precipitin level at the time of infection was considerably higher than in group A.

Autopsies on immunised and control rabbits

The experimental rabbits were killed 63 days after infection and the number of flukes recovered from each of the livers on autopsy is recorded in Table 15. In each case the type of lesion found in the liver was noted.

It is evident from the results shown in Table 15 that the average number of flukes in each rabbit in the immunised groups is not significantly lower than the average in the control group. However, the flukes recovered from the immunised groups were invariably smaller than those from the control group. This retarded development was particularly marked in the flukes obtained from rabbits V2, V1, 049 and G65. In order to obtain more definite information on this retardation of development, the flukes obtained from group B and from some of the control rabbits were analysed for total N. The flukes were removed from the liver, rinsed in saline, and analysed for total N by the micro-Kjeldahl method. The results of these determinations are shown in Table 16. The figures obtained confirmed our observations

on/

on the inhibited development of the flukes in the immunised rabbits, i.e., the 125 flukes obtained from group B had an average total N content of 0.41 mg. whereas the 108 flukes from 5 control rabbits had an average total N content of 1.13 mg. Examination of the most retarded of these flukes showed that they were sexually immature.

Table 15: Number of flukes found in immunised and control rabbits on autopsy.

	Rabbit No.	Number of flukes
Immunised rabbits group A	V1	10
	V2	3
	N856	6
	N851	10
	0330	13
	0329	18
	0371	26
	Average,	12.3 ± 7.1
Immunised rabbits group B	P93	14
	073	14
	049	3
	084	31
	P947	17
	076	20
	G65	26
	Average,	17.9 ± 9.1
Control rabbits	C2	6
	C4	18
	C8	20
	C9	25
	C14	25
	C7	6
	X	6
	9	25
	0	18
	Z	34
	1	25
	Average,	18.9 ± 9.4

Table 16/

Table 16: Comparative development of flukes, expressed in terms of their average total nitrogen content, in immunised and control rabbits.

	Rabbit No.	Number of flukes	Total fluke nitrogen (mg.)	Nitrogen per fluke (mg.)
Immunised rabbits, group B	P93	14	7.28	0.520
	073	14	5.96	0.426
	049	3	0.56	0.187
	084	31	13.40	0.432
	P947	17	7.54	0.444
	076	20	14.40	0.720
	G65	26	3.18	0.122
			Average N per fluke (mg.) 0.41 ± 0.20	
Control rabbits	X	6	4.22	0.704
	9	25	32.5	1.30
	0	18	24.2	1.35
	Z	34	38.8	1.14
	1	25	29.0	1.16
			Average N per fluke (mg.) 1.13 ± 0.25	

In general, the livers of the immunised animals showed similar lesions to those of the control group, i.e., moderate fibrinous peritonitis, a degree of fibrosis approximately proportional to the number of flukes present, and a grossly distended common bile duct which usually contained about half of the total number of flukes, the remainder being found in bile ducts in the liver. In rabbits V2, V1, G65 and 049, however, from which the smallest flukes were obtained, there was very extensive fibrinous peritonitis, the livers were very enlarged with numerous large fibrous nodules and showed extensive areas of recent necrosis, caused by damage to blood vessels by migrating/

migrating flukes. In V2 and 049 the common bile duct was normal in size and contained no flukes (figs. 25 and 26) while in V1 and G65 there was a slightly enlarged common bile duct containing a small number of flukes. The majority of flukes from these 4 rabbits were found either migrating through liver tissue or in very fine bile ducts.

DISCUSSION

The immunological relationship between a helminth parasite and its host is likely to be complicated. The worm itself is a complex organism and is certain to contain quite a number of antigenic compounds and complexes. Some of these antigens will be tissue or structural components of the parasite; others may be enzymes or products of its metabolic processes. The presence of these antigens may be demonstrated by using extracts of the parasite in serological tests on infected animals or by using similar extracts as immunising antigens. Difficulties are likely to arise in the attempted integration of the results from these two approaches, e.g., the parasite may contain a number of antigenic compounds which, because of their location, are never effective in the natural infection and, furthermore, antigens which are functional in the normal infection may be destroyed or altered by the processes involved in extracting them from the parasite.

There is now an adequate amount of evidence to demonstrate that an acquired immunity does occur in a number of helminth diseases (see introduction). This acquired immunity has been demonstrated by/

by a resistance to superinfection or to reinfection after the removal of a primary infection, or by the protective action of extracts of the parasite when used as immunising antigens. It has usually been possible in these cases to demonstrate by serological tests the presence of circulating antibodies. There has, however, been little work carried out on helminths, comparable to that on bacteria, on the isolation and purification of the antigenic compounds of the organisms, on a study of their immunological activity and specificity, and on the protective power of the antibodies which they elicit. In this connection it should be noted that there is not necessarily any correlation between the capacity of a helminth antigen to elicit the production of antibodies and the protective power of these antibodies; e.g., Campbell (1939) obtained a purified polysaccharide antigen from the larval form of Taenia taeniaeformis which stimulated the formation of precipitins but which had no protective action against the disease.

From the present investigation, designed largely to study the antigenic properties of proteins from F. hepatica and the protective properties, if any, of the antibodies which they elicit, we have concluded that prior immunisation with fluke proteins has no influence on the number of F. hepatica developing from a challenging infection. The infection of normal rabbits with 50 7-day-old cercariae has been shown to yield an average "take" of 18.6 $\pm$ 8.8. This average is apparently not influenced by the sex of the rabbit nor by the use of cercariae from different snails (Section 1: this thesis). In the/

the experiments described here the control rabbits showed a take of 18.1 ± 9.4, while the immunised groups showed takes of 12.3 ± 7.1 (group A) and 17.9 ± 9.1 (group B), respectively. The reduction in take in group A as compared to that of the control group is not statistically significant. Kerr and Petkovitch (1935) reported that it seemed possible to establish an active immunity in rabbits to F. hepatica by injections of dried fluke material. In their experiments 7 immunised rabbits, each infected with 13 cercariae, yielded 1, 2, 2, 2, 2, 3 and 3 flukes respectively, at autopsy. Several of these flukes were described as "calcified", and repeated faecal examinations failed to demonstrate the presence of fluke eggs. Three control rabbits (also infected with 13 cercariae) had 11, 5 and 9 flukes at autopsy. None of these flukes was calcified and eggs were found in the faeces of all the rabbits. It is difficult to conclude from this experiment how significant is the apparent reduction in take of the immunised group as compared to controls, because of the small number of rabbits (3) in the control group, and the variation in take which occurs in normal rabbits. If this reduction is real it might be due to the influence of antigens other than the proteins.

In our experiment there is no correlation in either the immunised or normal rabbits between the number of flukes found in a rabbit and the level of circulating precipitin to the protein antigen, as shown by the quantitative tests. It must be noted that in the precipitin tests we have measured the level of antibody to only one protein fraction of the parasite and have expressed our results in terms of/

of the total N (antibody N + antigen N) precipitated. The chief difficulty in applying standard immunochemical techniques to the quantitative determination of antibody precipitated by relatively crude antigenic fractions is that one cannot assume complete precipitation of the antigen in regions of antibody excess as is possible with a "pure" protein antigen and its homologous antiserum. This difficulty may be overcome by the labelling of such sera with radioactive iodine and in the preliminary experiment which was described above it was possible to determine the absolute amount of antibody precipitated from a serum by a particular antigen. This isotopic technique simplifies and extends the scope of such analyses and would appear to be particularly suited to the study of the antigenic fractions of helminth parasites.

In the immunised groups there was evidence of inhibited development of the parasites, viz., the smaller size of the flukes (confirmed in group B by micro-Kjeldahl analysis), the sexual immaturity of some of these, and the presence of migratory forms 63 days after infection (in autopsies of over 70 infected rabbits at this stage, we have never encountered migratory forms). This result is comparable to that obtained by Schwartz, Alicata and Lucker (1931) and Chandler (1932) who, working on the resistance of rats to superinfection with Nippostrongylus muris, observed retardation of growth and development without, in most instances, any marked lethal effect.

It/

It is interesting that the four livers in which the most retarded flukes were found also showed by far the most severe inflammatory reactions. This may have been due to the prolonged migratory period of the retarded flukes, but, on the other hand, there were only three flukes each in two of these livers (Table 16). It is possible that a state of specific hypersensitivity had developed as a result of previous immunisation and was responsible for the exaggerated cellular reaction to the presence of the parasites. If this is the case, the possible effect of hypersensitivity should be borne in mind in any attempts to control parasitic disease by means of worm antigens. Similar phenomena have been observed in the study of natural acquired immunity to oesophagostomiasis in sheep (Fourie, 1936; Monnig, 1950), to N. muris infections in rats (Sarles and Taliaferro, 1936; Taliaferro and Sarles, 1939) to ancylystomiasis of mice and ascariasis of guinea pigs (Kerr, 1936 and 1938) and to Cooperia infections in sheep (Andrews, 1939).

SUMMARY

1. Sera from rabbits infected with Fasciola hepatica were found to contain precipitins which reacted with a protein fraction prepared from the adult parasite.

2. Immunisation of rabbits with proteins of F. hepatica was shown to stimulate the production of these precipitins in relatively large amounts, as measured by quantitative precipitin tests.

3. Immunisation of rabbits with these proteins prior to infection was shown to produce inhibition of development of the parasites, but did not reduce their numbers significantly.

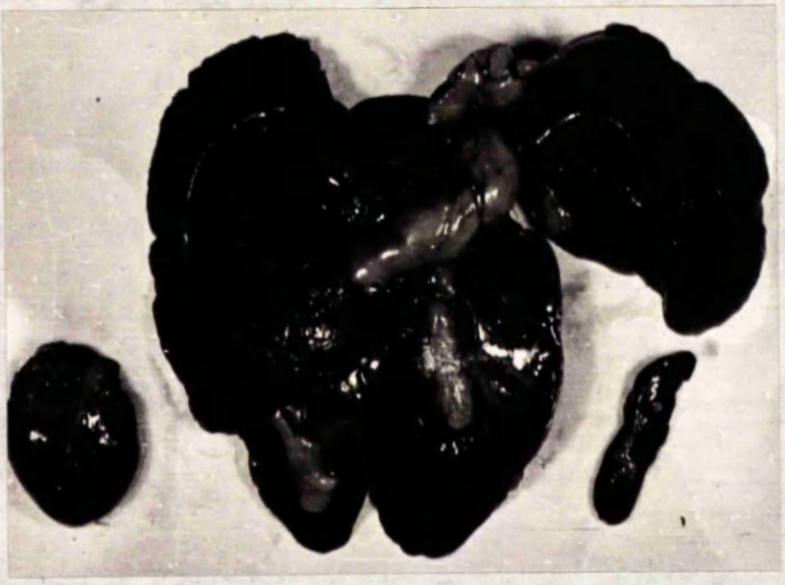

Fig. 25. Typical rabbit liver 9 weeks after infection with cercariae of F. hepatica. The large bile ducts are greatly enlarged and thickened and contain most of the adult parasites.

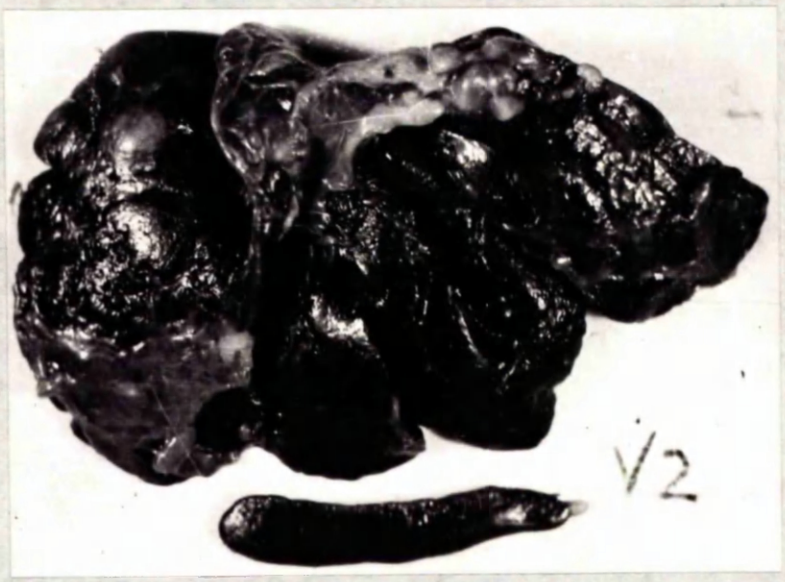

Fig. 26. Liver from immunized rabbit V2, 9 weeks after infection. The extra-hepatic bile ducts are normal in size.

SECTION 5

A STUDY OF THE LIVER LESIONS ASSOCIATED WITH FASCIOLIASIS IN RABBITS.

INTRODUCTION

Most of the literature dealing with the lesions caused by Fasciola hepatica is German in origin (e.g. Joest, 1921; Bugge, 1928; Schumacher, 1939; Ostertag, 1946) and probably for this reason the most complete description of the pathology of fascioliasis is that which is contained in Nieberle and Cohrs' "Lehrbuch der Speziellen Pathologischen Anatomie der Haustiere", (1952).

In their description of the pathological changes in the livers of affected sheep and cattle the authors deal first with the tracts produced by the migrating flukes in the initial stages of the disease. The freshly-formed "Bohrgänge" are dark red and are filled with blood and liver debris. Later, granulocytes, macrophages, and foreign-body giant cells form a palisade around the edges of each tract; these older tracts are greenish in colour due to the presence of large numbers of eosinophils in the inflammatory reaction. The necrotic debris is gradually absorbed and is replaced by granulation tissue which is infiltrated with lymphocytes and large numbers of eosinophils. During this stage thrombosis of branches of the portal vein is frequently observed.

They state that the chronic phase of the disease is characterised by fibrous thickening of the bile-ducts which may be accompanied by stenosis or distension; in cattle the bile-ducts may be calcified. The epithelium of the larger ducts may be necrotic or, on the other hand, be so hyperplastic as to resemble papillomatous tissue. In many/

many places, particularly where the epithelium is eroded, the underlying connective tissue is heavily infiltrated with granulocytes, lymphocytes, macrophages and plasma cells; occasionally there is suppurative cholangitis.

Usually, the left lobe of the liver is cirrhotic and the right lobe is hypertrophied; the authors say that the reason for this is not apparent. The cirrhosis results from the healing of the tracts produced by the migrating flukes, and by the proliferation of connective tissue around the portal tracts.

Cameron (1951) and Monnig (1950) state that jaundice due to biliary obstruction by the adult parasites is a characteristic feature of fascioliasis. On the other hand Hutyra and Marek (1946) state that icterus is usually absent.

The work described in this section of the thesis is a study of the liver lesions produced in the rabbit by infection with F. hepatica. The rabbits were killed at various stages of the infection and the livers were examined macroscopically and histologically. A further small series of rabbits was subjected to bromsulphalein excretion tests in order to confirm that retention of bile was not a feature of chronic fascioliasis in these animals.

MATERIALS AND METHODS

Fifty rabbits were infected, each with 50 7-day old carcariae, by the method described in Section 1 of this thesis. Thirty-six of/

of the rabbits were killed in groups of four at weekly intervals from two to 10 weeks after infection; the remaining 14 were killed at intervals some months later.

At autopsy the liver of each rabbit was examined and the lesions noted. Blocks of tissue removed for histological examination were fixed in 10% corrosive formol and dehydrated in a range of alcohols and finally in amyl acetate; they were then double-embedded using a methyl benzoate-celloidin series followed by paraffin wax. Sections were routinely stained by Mayer's haemalum and eosin; other techniques used in selected cases were picro-Mallory, Gordon and Sweet's reticulin impregnation method, the periodic acid-Schiff method and carbol-chromotrope for eosinphil polymorphonuclears.

During the course of the experiment 20 normal rabbits were also killed and their livers examined as described above.

RESULTS

Normal livers

Macroscopic Findings

The livers of all of the control rabbits were normal in appearance.

Microscopic Findings

Most of the liver lobules and portal tracts in each of the livers are completely normal. Nevertheless, in almost all of the livers two lesions are present. One of these is a lymphocytic/

cytic infiltration of the parenchyma around a minority of the portal tracts; the other is the presence of foci composed primarily of lymphocytes scattered apparently at random throughout the parenchyma. The size of the foci range from a few cells to one-quarter of a lobule and occasionally they incorporate multinucleated liver cells.

The incidence and severity of these lesions in affected livers is invariably low and does not hinder to any great extent the interpretation of the gross pathological changes consequent on infection with F. hepatica.

Infected livers

These are described under three headings:-

(1) Livers from rabbits infected for 2 weeks.

(2) Livers from rabbits infected for 3-7 weeks.

(3) Livers from rabbits infected for 8 or more weeks.

Livers from 2-week infections

Macroscopic Findings

On the surface of the liver were cream-coloured or pink lesions; these appeared as small spots 1-2 mm. in diameter or as streaks which were up to 10 mm. in length and about 2 mm. wide. Similar lesions were seen on the cut surfaces of the organ. The extrahepatic bile ducts were normal in size. There was no gross peritonitis of the visceral surface of the liver.

Microscopical Findings

Examination of the lesions described above show that they are necrotic/

necrotic tracts caused by the migrations of the young flukes through the liver parenchyma.

Although these young flukes (fig. 27) are less than 1 mm. in size, the oral and ventral suckers are easily recognised and the cuticle already possesses small thin spines.

The space left in the wake of the fluke is filled with cell debris, neutrophil leucocytes, lymphocytes and red cells (fig. 28). The columns of liver cells which are immediately adjacent to the tract are atrophied and the nuclei are pyknotic; the sinusoids in this area are distended and are filled with large mononuclear cells, lymphocytes, and granulocytes (fig. 29). The mononuclear cells predominate and although a few of these are typical macrophages with a clearly delineated eosinophilic cytoplasm the majority have a large hyperchromatic nucleus with margination of the chromatin and a cytoplasm which is only faintly visible.

At a later stage, on cross-section the lesion consists of two zones. The inner zone is composed of dead liver cells, erythrocytes, and masses of granulocytes, primarily eosinophils. The outer zone consists principally of the mononuclears already described; phagocytosis by these cells is not marked. On the outside edge of this area a few fibroblasts may be observed; they are particularly in evidence at any point where a portal tract is adjacent to the fluke tract. As the zone of acute inflammation diminishes in size, the fibroblasts become more numerous and small capillaries appear on the outer edge of the tract. Eventually the tract is filled/

filled with a loose connective tissue containing large mononuclear cells and lymphocytes (fig. 30). Also present are a few large multinucleated liver cells and numerous epithelial cells which resemble liver cells but are arranged as small bile ducts.

Livers from 3, 4, 5, 6 and 7-week infections

The changes observed in these livers may be conveniently described together as they differ in degree only. In the older infections the flukes are larger and the lesions are consequently more severe.

Macroscopic Findings

In the heavily infected animals, the greater part of the surface of the liver was covered by patches of fibrin which were fairly firmly adherent to the organ (fig. 31). Adhesions were present between the liver and the stomach, intestine, omentum and diaphragm, and between the individual lobes of the liver. That part of the liver surface which was visible showed numerous tract lesions and similar lesions were seen on cutting into the liver. Wedge-shaped pale necrotic areas with the apex towards the centre of the organ and with sides about 15 mm. in length were common on the edges of the liver lobes. The extrahepatic bile ducts were normal in size. Of the 20 rabbits examined at this stage, six had died of the infection and the livers of each of these six contained 30 or more migrating flukes.

Microscopic Findings

The tracts are similar to those described earlier except that they are larger and more numerous due to the increase in size of the/

the parasites and to their continued wandering. The centre of the tract is composed of cell debris, polymorphonuclears, lymphocytes and larger mononuclears. Erythrocytes may or may not be present in large numbers. Eosinophils predominate among the granulocytes and may be recognised by the poorly lobed nucleus and large red granules characteristic of this cell. Since the cytoplasm of rabbit neutrophils contain red granules which may cause them to be confused with eosinophils in H. and E. stained sections, the carbol-chromotrope method was employed to differentiate the two types of cells since it selectively stains the granules of eosinophile leucocytes. The eosinophils often appear to be degenerate and in these cases the cytoplasm is completely eosinophilic and has lost its granularity.

If the tract is recently formed it is surrounded by a ring of necrotic liver cells and by distended sinusoids which are filled with mononuclears and granulocytes. In older tracts (fig. 32) the zone of necrosis is reduced and the space is filled with large mononuclear cells whose cytoplasm is foamy and presents a syncytial-like appearance (fig. 33) in the meshes of which are present plasma cells, lymphocytes and eosinophils. Fibroblasts, most of which are arranged concentrically, are present on the periphery of the tract (fig. 34). Capillaries with swollen endothelial cells grow in from the periphery of the lesion, but these are not numerous at any time. A few mono- and multinucleated hepatic cells (fig. 35) are commonly present; the latter/

latter may have up to 30 nuclei which may be distributed irregularly or as an overlapping mass. Small ducts lined by cells resembling liver cells are also common.

Individual parasites may settle down in the bile ducts as early as five weeks after infection, when they are about 3 mm. in length, although it is usually about eight weeks before they are all in the bile-ducts. Tracts are, therefore, formed until about the eighth week and in heavy infections the liver may be literally honeycombed.

The bile ducts are also frequently damaged by the parasites and fig. 36 shows a large portal tract adjacent to a fluke tract. The wall of the bile duct has been ruptured at two points and the lumen is filled with necrotic debris and polymorphs.

During the migratory period, particularly in the older infections, it is common to encounter infarcts. The size of these infarcts ranges from part of a lobule to part of a lobe. The large infarcts are produced by flukes tracking across a blood vessel, by thrombus formation in vessels adjacent to a tract, and possibly by inflammatory changes in the walls of vessels.

Fig. 37 shows an infarct near its site of origin in a tract produced by a burrowing fluke. The next illustration (fig. 38) shows the same infarct at its widest point; the blood vessel has divided into three branches and each of these is surrounded by necrotic liver tissue.

Thrombus formation, particularly in veins is commonly encountered/

encountered and this is illustrated by fig. 39 which shows an organising thrombus in a hepatic vein. In some damaged arteries, thrombosis is not found but there occurs separation and disintegration of the muscle fibres of the media and proliferation of the intimal cells; in addition, the wall of the vessel is infiltrated by granulocytes and lymphocytes (fig. 40). In some of these lesions the lumen of the vessel is reduced and appears to be a possible cause of ischaemia.

Small foci of necrosis involving lobules or even parts of lobules are not infrequently seen. While it is possible that these are tangential sections through the necrotic periphery of a fluke tract, they may on the other hand be caused by small emboli originating in vascular damage by the flukes.

In lightly infected liver or in lobes which are not severely damaged by tract formation the portal tracts are unaltered. In areas which are adjacent to, or involved in, fluke tracts three changes are noted. Each portal tract is surrounded by lymphocytes which invade the surrounding liver parenchyma; there is a marked hyperplasia of small bile ducts on the periphery of the portal tract (fig. 41); finally there is extensive oedema of the supporting portal connective tissue, the collagen fibres being widely separated and the lymphatic vessels distended (fig. 42).

At this stage of infection one commonly observes parasites or their tracts immediately below the surface of the liver. During the subsequent inflammatory reaction a thick layer of fibrin is formed/

formed on the peritoneal surface; these foci of fibrinous peritonitis often become confluent and form adhesions between lobes and to the stomach intestine and omentum. Organisation may later supervene on this process.

Livers from infections of 8 weeks and over

Macroscopic Findings

The livers were usually adherent to the stomach, intestine, omentum and diaphragm by strands of connective tissue; the lobes were often inseparable from each other due to similar changes, and were distorted and altered in size. These changes in shape followed no obvious pattern, although the main mass of lobes was usually reduced and the caudate lobe was increased in size (fig. 43). The livers were usually paler than normal and the lobular pattern was clearly demarcated. Multiple nodular hyperplasia as seen in the human subject was not a feature of these cases. The gall-bladder was often increased to twice its normal size and was filled with black or brown bile. The walls of the extrahepatic bile ducts were thickened and the lumen was several times larger than normal; the majority of the parasites were in these ducts and if the liver was examined immediately after death the flukes could be observed moving freely. On section the livers were usually fibrotic; the intrahepatic bile-ducts were distended, their walls were thickened and their lumina sometimes contained flukes.

Microscopic Findings

Numerous areas of fibrous tissue may be observed which represent/

represent advanced stages in the repair of the fluke tracts. The granulation tissue, the formation of which was described earlier, is now more heavily collagenised, there are very few granulocytes and mononuclear cells present, and the tissue is relatively avascular. The small channels, referred to earlier as being lined with cells of parenchymal type, are now indistinguishable from small bile-ducts. The mono- and multi-nucleated liver cells which are characteristic features of the healing tracts are now few in number and appear atrophied; their cytoplasm is filled with fine brown granules and their nuclei are pyknotic (fig. 44). In some areas small hyperplastic foci of liver cells are seen; these may also be found in chronic infections of several months' duration. These hyperplastic foci appear to be short-lived in many instances as degenerating circumscribed areas of liver cells are frequently encountered in the same sections. These fibrous areas formed during the repair of the fluke tracts are frequently surrounded by lymphocytes which may show a tendency to invade the surrounding parenchyma.

In some areas the portal tracts remain unaltered. In others, particularly when the parasites are numerous, the portal tracts are increased in area, are very fibrous, and possess a large number of small bile ducts. Frequently it is impossible to decide if a given structure is a portal tract or a healed fluke tract. The portal tracts may be joined to the areas of residual fibrosis (i.e. the healed fluke tracts) by a network of connective tissue producing the appearance of coarse cirrhosis (fig. 45).

Fluke/

Fluke eggs are commonly found in the smaller bile ducts and a single fluke egg may completely fill the lumen of a small duct (fig. 46) and in larger ducts five or more eggs may produce apparent complete obstruction (fig. 47). The presence of these eggs in intact bile ducts usually provokes no reaction, although occasionally epithelial hyperplasia is noted.

In those ducts which are large enough to contain adult parasites, several lesions are present. There is a great increase in the diameter of these bile ducts and in the thickness of their walls, these changes being most marked in the common bile duct (fig. 48). Most of the parasites are found in this duct, their bodies folded over one another; the majority appear to be free in the lumen, although occasionally their suckers are seen grasping the mucosa (fig. 21). The sharp cuticular spines are prominent features of their anatomy and are often observed embedded in the epithelium of the duct. This tissue is extensively damaged by the presence of these spines and the movements of the flukes. Numerous eggs lie free in the lumen of the duct together with red and white cells and cellular debris.

The state of the bile duct epithelium varies depending on the presence and activity of the parasites. Occasionally it is normal, frequently it is hyperplastic (fig. 49). The tall columnar epithelium may be replaced by a cuboidal or flattened type and in some ducts there is no epithelium at all, the duct consisting entirely of a fibrous tube (fig. 50). In places where the epithelium/

epithelium has been freshly breached by the parasites there is fibrinoid necrosis of the epithelium and underlying tissues; below this necrotic tissue the wall is heavily infiltrated with plasma cells, lymphocytes, eosinophils and fibroblasts (fig. 51). In long-standing cases the walls of the ducts are thickened and fibrous due to the proliferative changes associated with sub-acute and chronic inflammation. Not infrequently necrosis of the complete wall of a thickened and parasitised duct is present (fig. 52); in some of these cases the necrosis involves the surrounding liver tissue.

In some bile ducts, particularly those in which the epithelium is desquamated, the wall is infiltrated with fluke eggs. Occasionally these eggs are grouped together in large numbers (fig. 53), but more often they lie singly in the tissue.

Initially the ova are surrounded and frequently invaded by eosinophils and macrophages (fig. 54); later, each cell is surrounded by concentric whorls of macrophages and fibroblasts and an outer ring of lymphocytes, producing a specific type of focal granuloma (fig. 55).

Areas of necrosis in the liver parenchyma are frequently observed. These range in size from that of a lobule to areas of 1 sq. cm. and may include portal tracts and areas of residual fibrosis; the cause of this lesion is undetermined.

Bromsulphalein Excretion Tests

If a solution of bromsulphalein is injected into the blood stream of the normal human subject, almost all of the drug is excreted in the bile within one half-hour after injection. When hepatic lesions are present grades of retention up to 100% may be encountered. These facts are the basis of the use of bromsulphalein as a liver function test (Rosenthal and White, 1925).

This test was carried out on a number of rabbits; these included a group of three normal rabbits, a group of three rabbits with chronic fascioliasis, and two rabbits whose common bile ducts were ligated. The main object of the experiment was to show that retention of bile was not a characteristic feature of fascioliasis.

Methods and Materials

A prepared solution of bromsulphalein* (5 mg. per kilo.) was injected into the marginal ear vein of each rabbit. Samples of blood were withdrawn 5, 20, and 40 minutes after injection. The serum was allowed to separate and the amount of bromsulphalein in each sample determined as described by Rosenthal and White (1925).

Results

These are detailed in Table 17 and show that the excretion times in both normal and fluke injected rabbits are similar while those/

* obtained from Savoury and Moore, Bond Street, London.

those of the two rabbits whose bile ducts were ligated are greatly increased. It is therefore reasonable to conclude that gross biliary obstruction does not occur in chronic fascioliasis. It is necessary to point out that if the bromsulphalein excretion times of the fluke infected rabbits had been increased it could not have been ascribed as being necessarily due to biliary obstruction.

Table 17

Bromsulphalein excretion times in normal rabbits, rabbits with chronic fascioliasis, and rabbits whose bile ducts were ligated.

Normal Rabbits

	Time after injection in mins.	% retention
No. 11	5	9.6
	20	0.4
	40	0
No. 33	5	6.8
	20	1.1
	40	0
No. N475	5	13.9
	20	1.1
	40	0.3

Fluke-infected Rabbits

	Time after injection in mins.	% retention
No. 0703	5	22.7
	20	1.8
	40	1.8
No. 6	5	10.1
	20	0.7
	40	0
No. 13	5	18.5
	20	0.5
	40	0

Rabbits with Biliary Obstruction

	Time after injection in mins.	% retention
No. 456	5	95.7
	20	75.4
	40	60.9
No. 33	5	97.7
	20	63.2
	40	37.3

DISCUSSION

The lesions which have been described above may be conveniently discussed by taking in turn the abnormalities associated with the normal liver, with the acute or migratory stage of infection and with the chronic or adult stage of infection.

Lymphocytic infiltrates were found in a small percentage of lobules in almost all of the 20 normal livers examined. While hepatic lesions are common in laboratory rabbits due to infection with the protozoan Eimeria stiedae, it seems unlikely that these infiltrates were produced in this way, as this parasite was observed on only one occasion in the epithelial cells of a single bile duct in one of the livers. At autopsy, a few cysticerci of Taenia pisiformis were found in the peritoneal cavity of almost all of the rabbits. These cestodes are carried from the bowel to the liver by the portal stream and gain entry to the peritoneal cavity by intrahepatic migration. Although no necrotic tracts were ever seen in the livers of the control rabbits it is not impossible that the liver lesions which were described resulted from these migrations.

The repair of the necrotic tracts caused by the migratory forms of F. hepatica was followed by proliferation of parenchymal cells and of connective tissue cells; usually the liver cells failed to effect complete repair with the result that the healed tracts were usually represented by areas of fibrous tissue enclosing/

enclosing foci of degenerating liver cells. During the repair of these tracts and on the edges of the oedematous portal tracts the dedifferentiation of liver cells into bile duct epithelium was often observed. Initially, this was characterised by the presence in these areas of numerous channels lined by cells; many of these cells were indistinguishable from hepatic cells while others were transition stages between hepatic cells and bile duct epithelium. Ultimately, the parenchymal identity of these cells was completely lost and the structures were identical with small bile ducts in every respect.

Severe fibrinous peritonitis was a feature of all of the livers which contained young migrating flukes, except those from rabbits killed two weeks after infection; invariably the livers from six and seven week infections were the most severely affected. These two facts show that the gross peritonitis cannot be ascribed to the penetration of the liver capsule by the young flukes from the abdominal cavity, but is associated with the acute hepatitis following the intrahepatic migrations of the parasites.

The frequency with which fluke tracts were replaced by connective tissue has already been noted. If this observation is also applicable to the livers of infected sheep and cattle it is obvious that the known anthelmintics used against fascioliasis, all of which are only effective against flukes in the bile ducts, will not prevent the formation of foci of fibrous tissue in infected livers. It is therefore possible that livers may be rendered/

rendered unfit for human consumption no matter how frequently anthelmintics are administered.

The lesions which were associated with chronic fascioliasis were partly a legacy from the acute stage of the infection and partly caused by the presence of the parasites in the bile ducts. The increase in thickness and in diameter of the intrahepatic bile ducts and the increase in area of the portal tracts was usually accomplished at the expense of the parenchymal tissue; this overgrowth of connective tissue often proceeded somewhat irregularly and lobules or groups of lobules were frequently separated by bands of connective tissue. These outgrowths, together with the fibrous lesions which are characteristic of the healed fluke tracts, produced a coarse cirrhosis.

Jaundice, caused by mechanical obstruction of the bile ducts by the parasites, is described by several writers (Cameron, 1951; Monnig, 1950) as a common sequel of chronic fascioliasis in sheep and cattle. In infected rabbits, jaundice even of a very transient nature is rarely encountered (see Section 9.2) and liver function tests failed to show any evidence of obstruction; histologically, obstruction was only noted in the relatively small number of ducts which were blocked with eggs. In view of the fact that the rabbits which were subjected to ligation of the common bile duct showed intense jaundice, almost complete retention of bromsulphalein, increased alkaline phosphatase levels in the sera, and numerous plugs of inspissated bile in the bile ducts

(see next)

(see Section 2), it is reasonable to conclude that in the rabbit infected with fascioliasis obstruction of the biliary tracts is slight or non-existent and plays no significant part in the pathogenesis of the cirrhosis. It is also of interest to note that ascending suppurative cholangitis frequently associated with obstruction of the large bile ducts in the human subject was not a feature of these livers.

It appears, therefore, that the etiology of the coarse cirrhosis characteristic of chronic fascioliasis in the rabbit is essentially fivefold. First the healing of the necrotic tracts produced by the migrations of young flukes, secondly the infarcts resulting from the vascular damage produced by the latter, and thirdly the chronic cholangitis caused by the presence of the parasites in the ducts. To these may be added the proliferative changes on the periphery of both the portal tracts and the healed fluke tracts and the granulomatous lesions which are formed in response to the presence of fluke eggs in the tissues.

The presence in these livers of numerous granulomata containing fluke eggs has a well-known counterpart in human schistosomiasis. One of the classical lesions of this disease and, according to Craig and Faust (1951) the most important, is the formation of pseudotubercles around eggs lodged in the peri-vascular tissues. The adult worms live in the mesenteric-portal vessels and in the associated vesical and pelvic plexuses and the eggs are discharged into small venules; from there they filter through the wall of the venules and penetrate the mucosa of the bowel or bladder to reach the lumen. According to Shaw and Ghareeb (1938) this migration/

migration is accomplished with the aid of a necrotizing ferment which is produced by the miracidium and diffuses through the pores of the egg-shell. In chronic cases of the disease the eggs are attacked in the perivascular tissues by leucocytes with the eventual production of egg-abscesses and miliary pseudotubercles. In the rabbit livers infected with F. hepatica the eggs are normally discharged directly into the bile-ducts and so reach the exterior; tissue migration is therefore not a necessary part of the life-cycle. No necrosis was observed around the eggs in the periductal tissues and it was probable that they entered these tissues through abrasions produced by the adults in the walls of the ducts. One other feature associated with the eggs of F. hepatica was the frequency with which a small bile duct was observed to be plugged by a single fluke egg. The mechanism whereby this occurred is unknown; it is possible that the genital pore of a fluke was occasionally applied to a small bile duct and that the eggs were shed into this duct and in some way forced up the lumen.

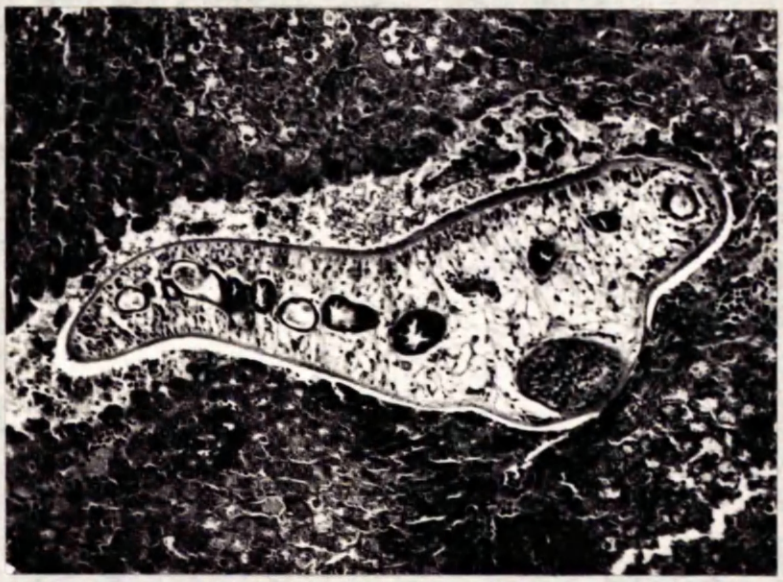

Fig. 27. Young fluke, 2 weeks after infection, migrating through liver parenchyma. H.&.E. x 100.

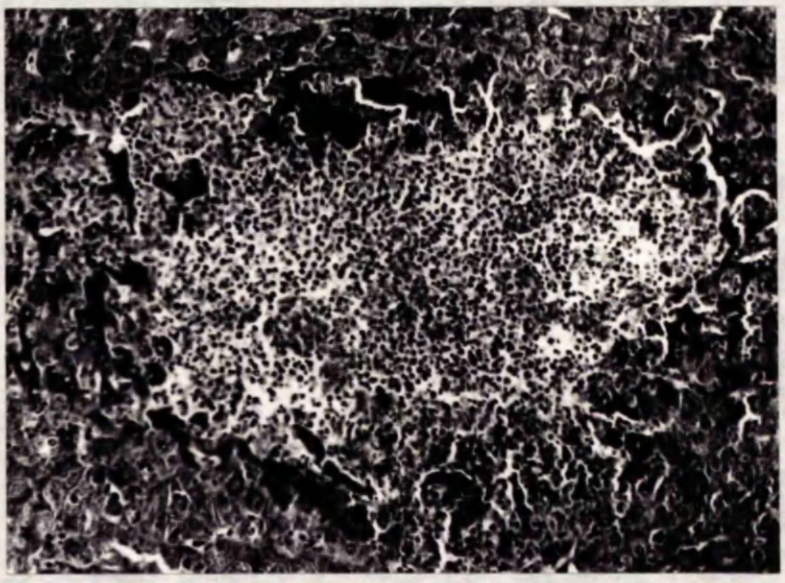

Fig. 28. T.S. of freshly formed fluke tract. The space left by the parasite is filled with polymorphonuclears, lymphocytes, red blood cells, and liver debris. Mallory x 180.

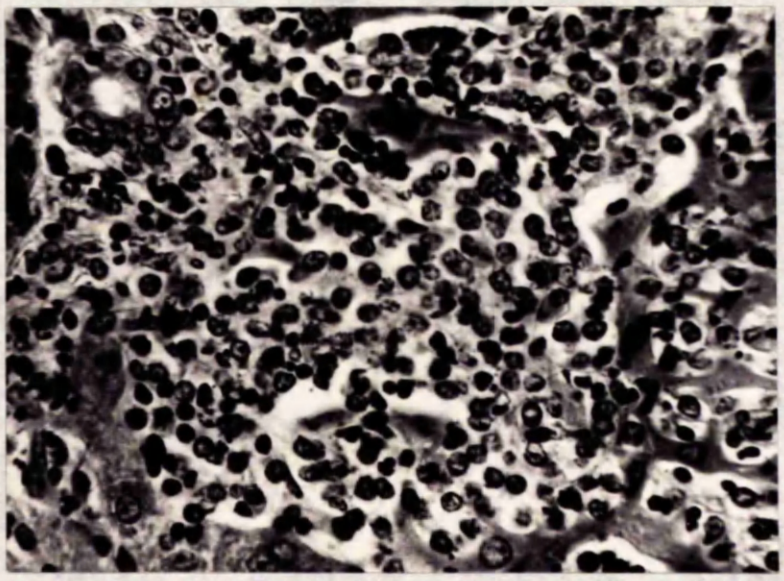

Fig. 29. Degenerating liver cells and distended sinusoids on the periphery of a fluke tract. The sinusoids are filled with large mononuclears and lymphocytes. H.&.E. x 600.

Fig. 30. Freshly healed small fluke tract just below surface of liver. It is composed of loose connective tissue, interspersed with phagocytes, degenerating multi-nucleated liver giant cells, and a few young bile ducts. H.&.E. x 100.

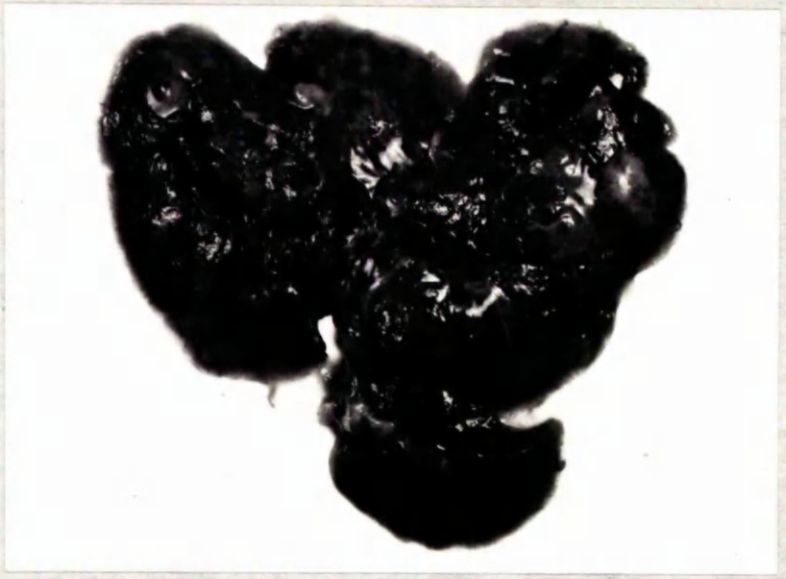

Fig. 31. Liver of rabbit 6 weeks after infection with 50 cercariae. The surface is covered with patches of fibrin.

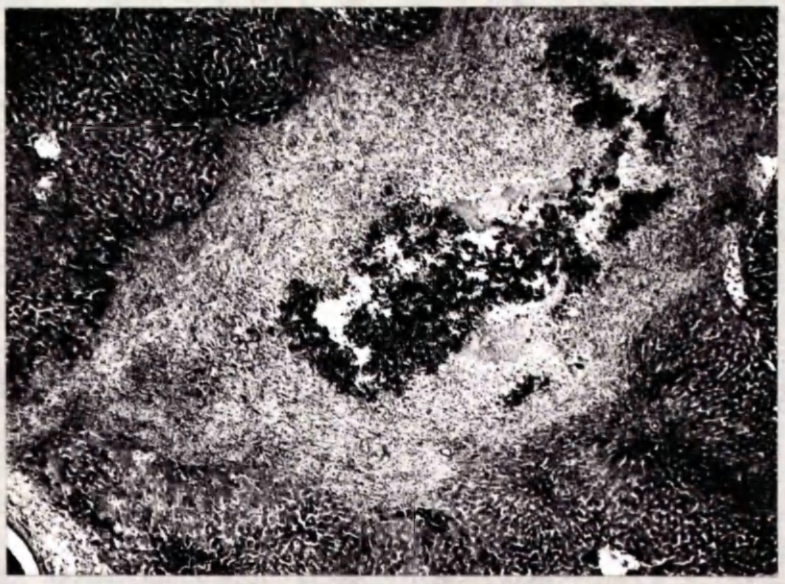

Fig. 32. Partially healed fluke tract showing necrotic debris in centre, surrounded by young connective tissue and mononuclear cells. H.&.E. x 35.

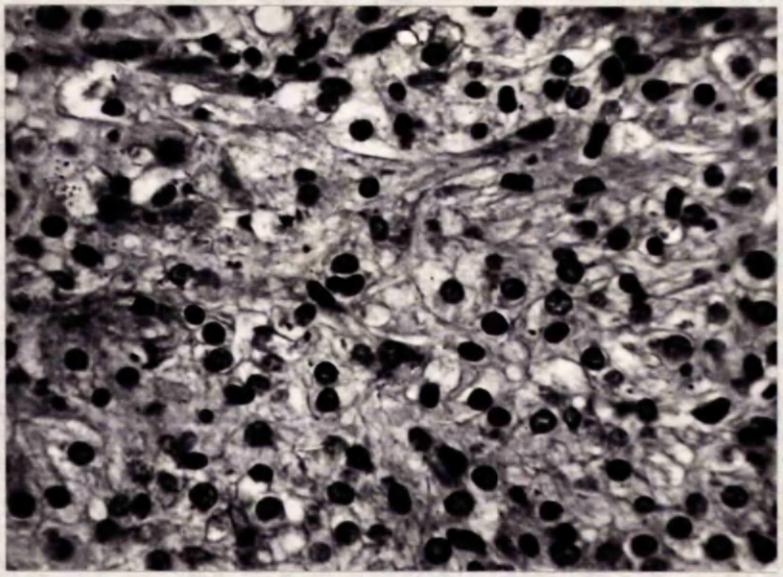

Fig. 33. Appearance of mononuclear cells in healing fluke tract. H.&.E. x 500.

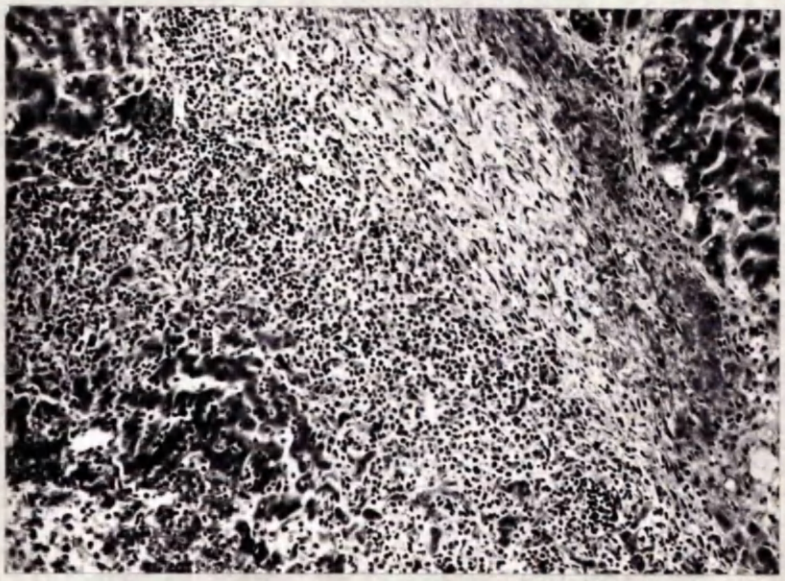

Fig. 34. Part of circumference of healing fluke tract showing concentric arrangement of fibroblasts on periphery. H.&.E. x 110.

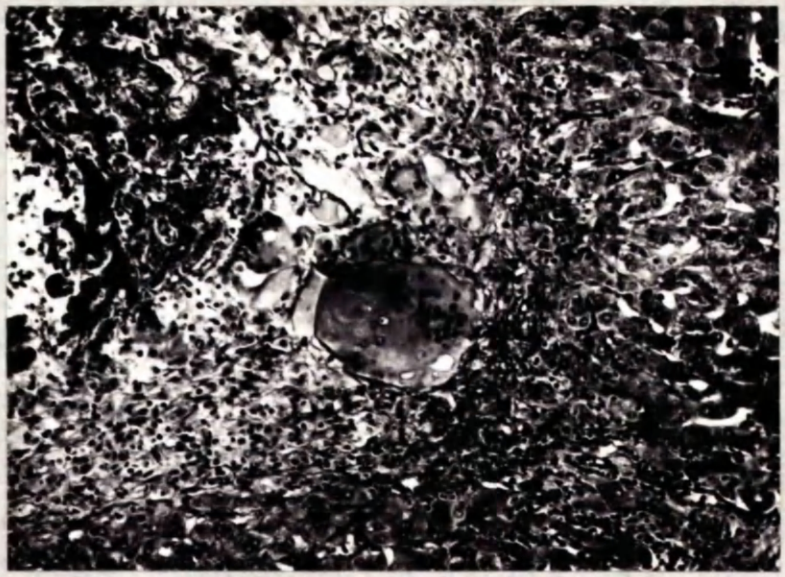

Fig. 35. Multi-nucleated liver giant cell formed during repair of fluke tract. H.&.E. x 130.

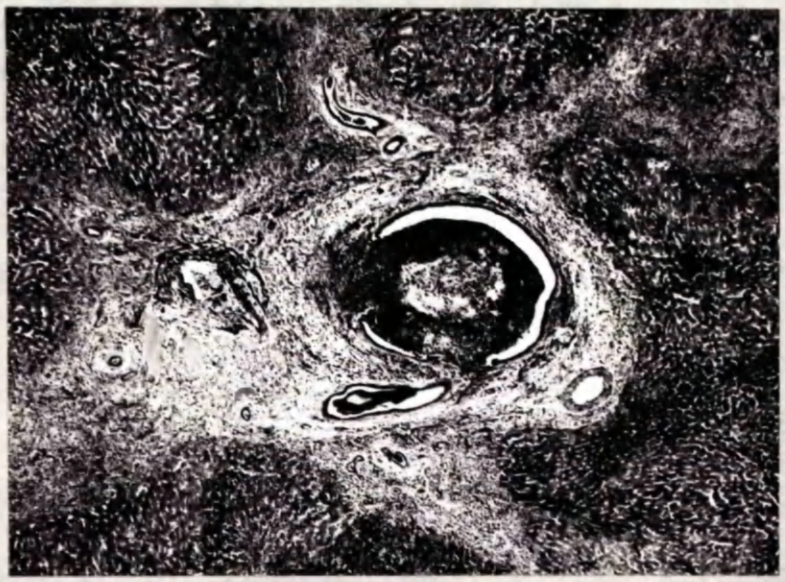

Fig. 36. Portal tract of liver 6 weeks after infection. The bile duct is ruptured at two points and the hepatic artery is damaged; the supporting connective tissue is oedematous and the lymphatic vessels are distended. H.&.E. x 35.

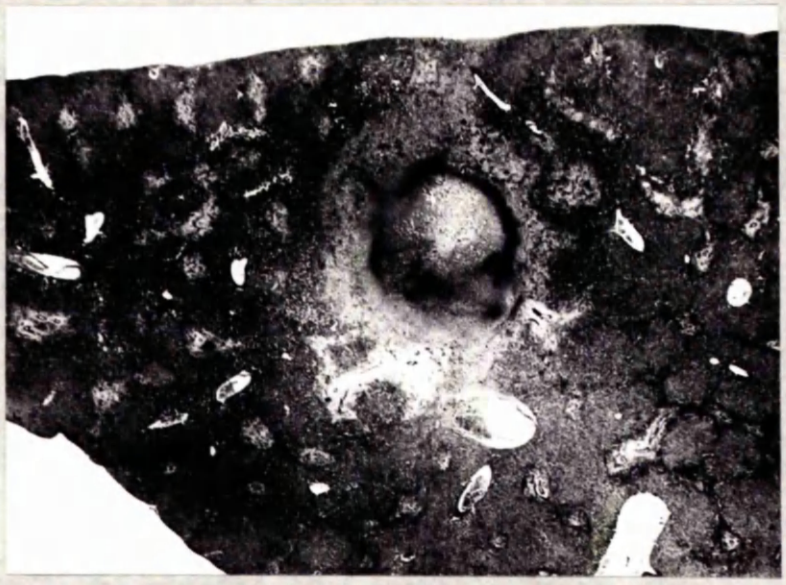

Fig. 37. Infarct of liver of rabbit 6 weeks after infection. H.&.E. x 15.

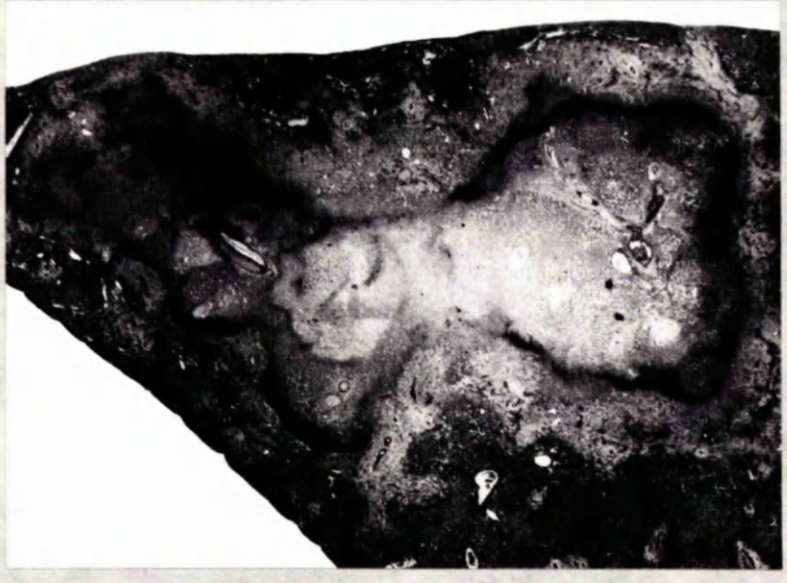

Fig. 38. Same infarct at widest part ; the blood vessel has divided into three branches. H.&.E. x 15.

Fig. 39. Thrombus in hepatic vein. H.&.E. x 60.

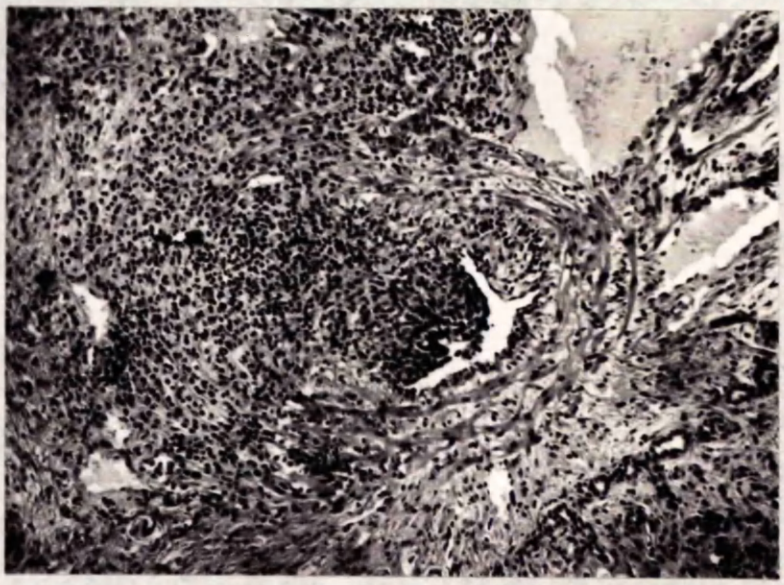

Fig. 40. Hepatic artery showing inflammation and oedema of wall. H.&.E. x 110.

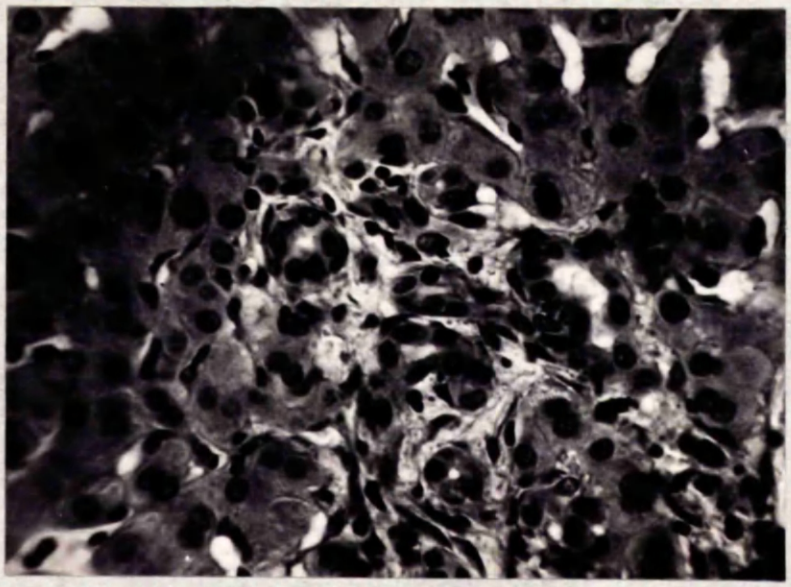

Fig. 41. Hyperplasia of bile ducts on periphery of portal tract. H.&.E. x 400.

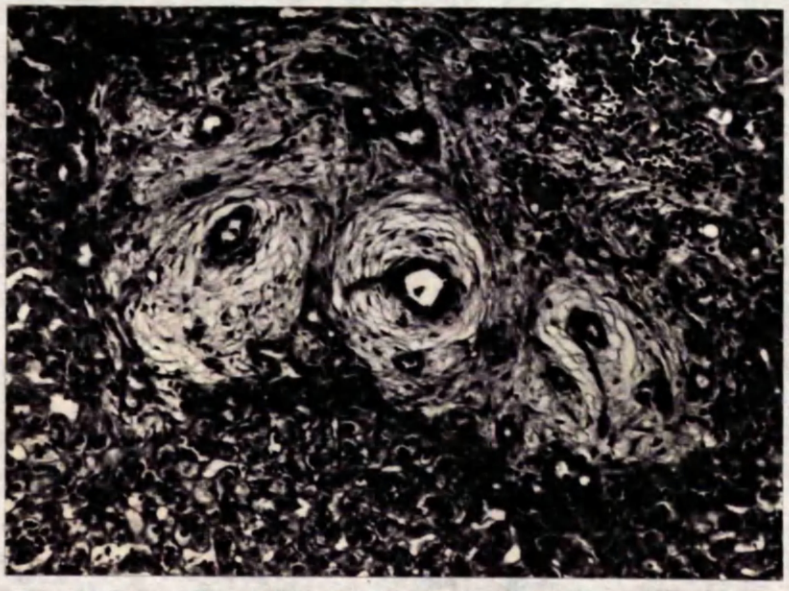

Fig. 42. Oedema of connective tissue of portal tract. H.&.E. x 110.

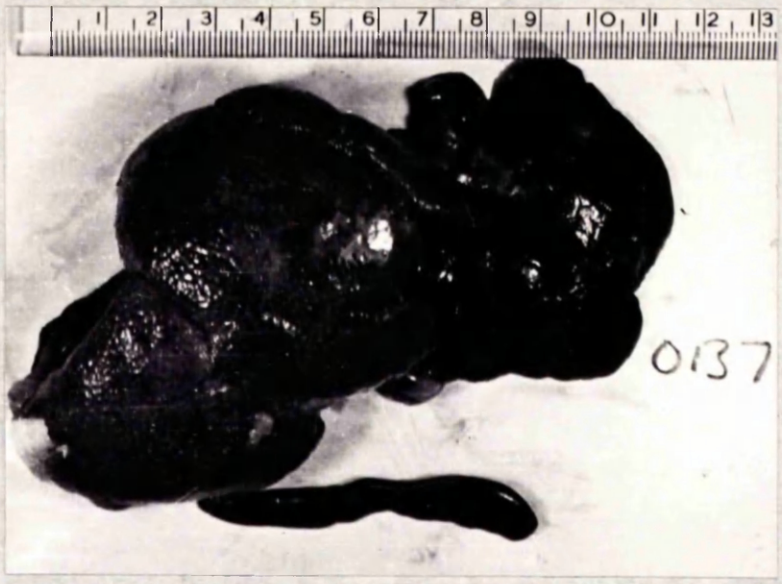

Fig. 43. Liver of rabbit with chronic fascioliasis. The main lobe mass is reduced and the caudate lobe is increased in size.

Fig. 44. Healed fluke tract. H.&.E. x 50.

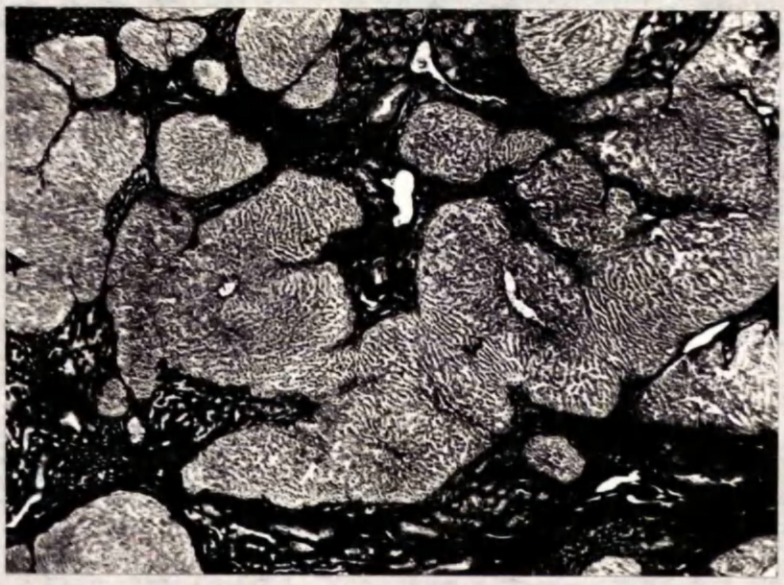

Fig. 45. Coarse cirrhosis of chronic fascioliasis. H.&.E. x 20.

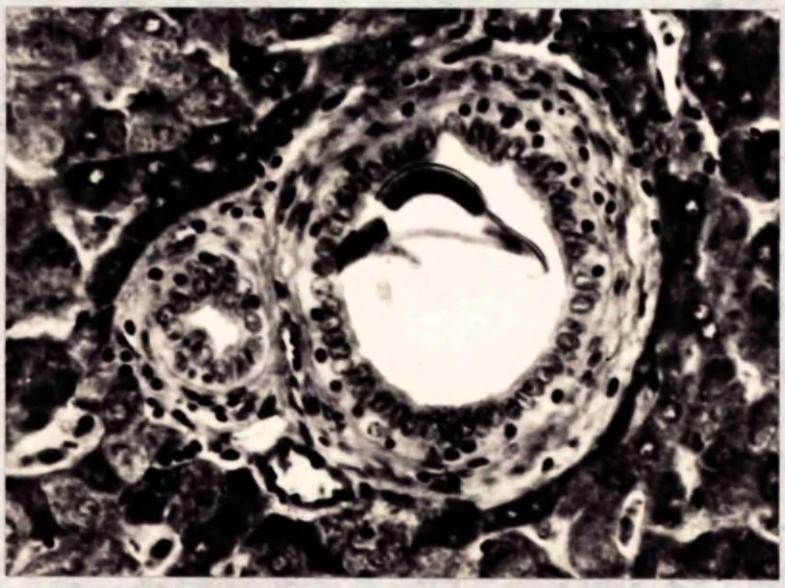

Fig. 46. Fluke egg in bile duct. Mallory x 450.

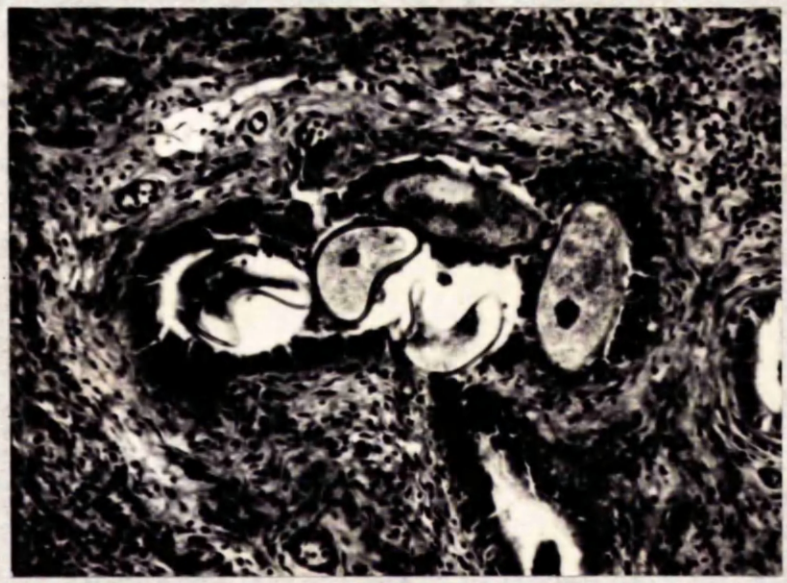

Fig. 47. Fluke eggs blocking lumen of bile duct. Mallory x 110.

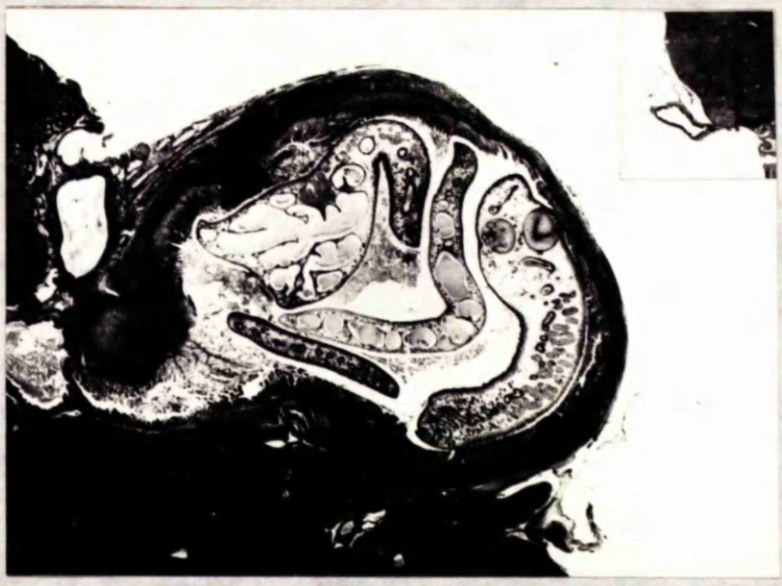

Fig. 48. Increased diameter and thickness of common bile duct in chronic fascioliasis. Insert shows normal common bile duct. Mallory x 8.

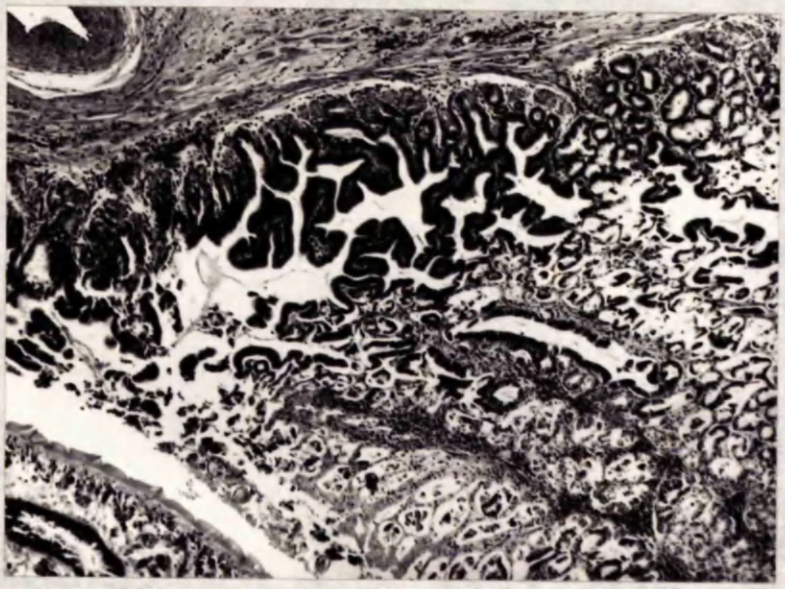

Fig. 49. Hyperplasia of biliary epithelium of common bile duct. H.&.E. x 35.

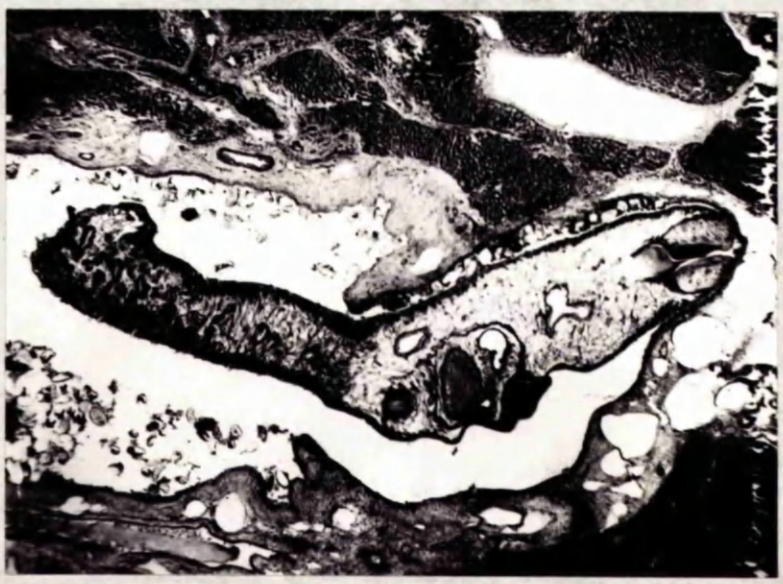

Fig. 50. Common bile duct showing fibrosis of wall. The oral and ventral suckers and the spines of the fluke can be seen. Mallory x 12.

Fig. 51. Ulcer of wall of commom bile duct. H.&.E. x 35.

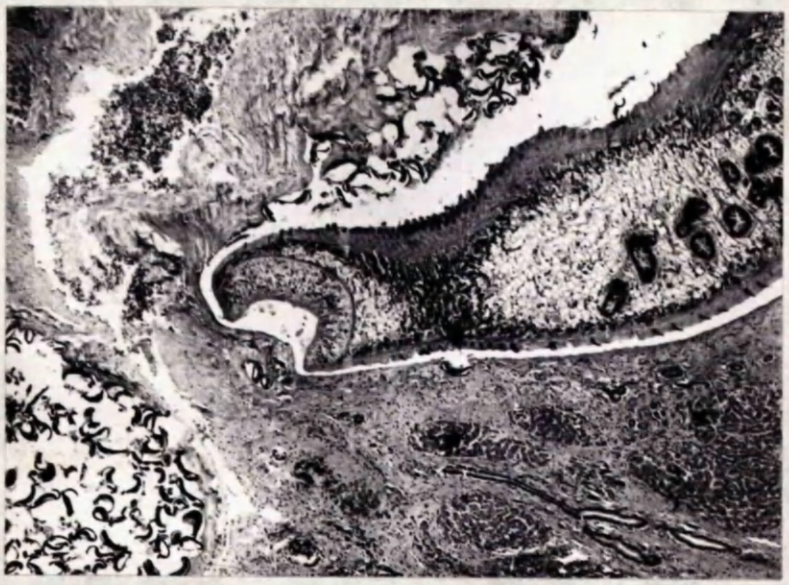

Fig. 52. Necrosis of complete wall of common bile duct. The oral sucker and the spines of the fluke are prominent. H.&.E. x 35.

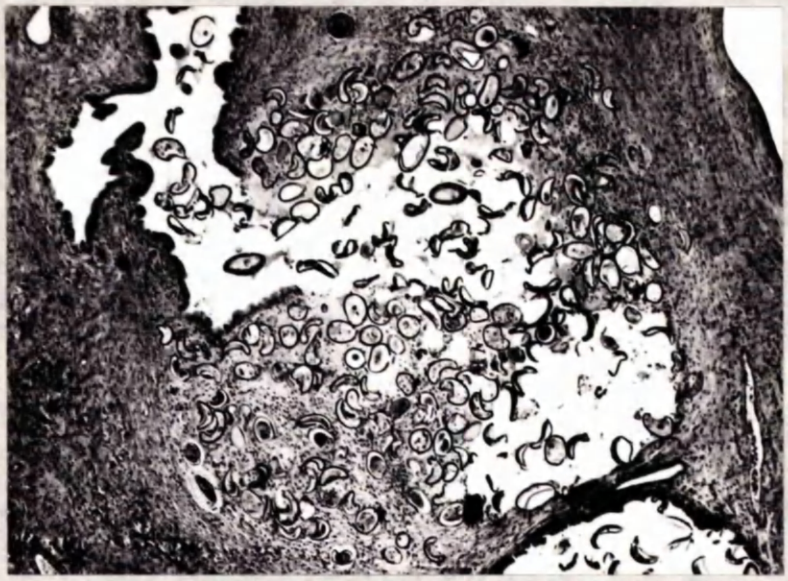

Fig. 53. Seeding of liver tissue with fluke eggs. Mallory x 35.

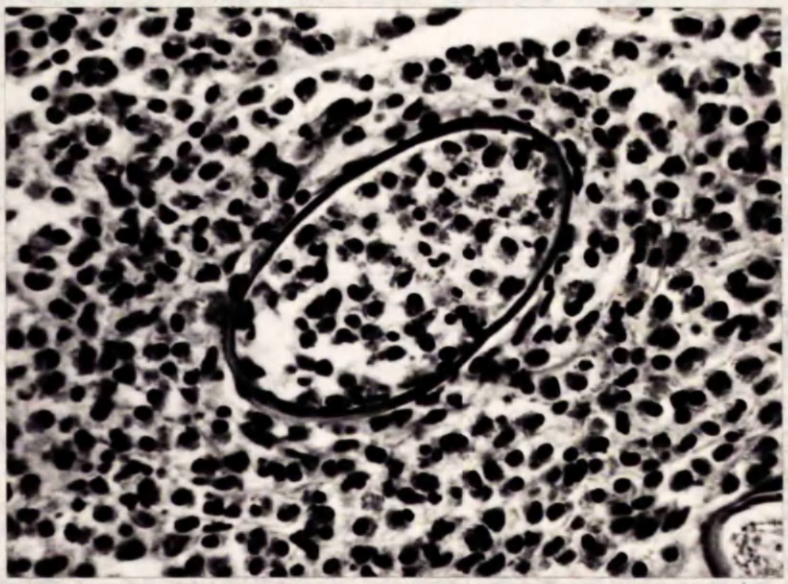

Fig. 54. Fluke egg surrounded and invaded by eosinophils and macrophages. H.&.E. x 420.

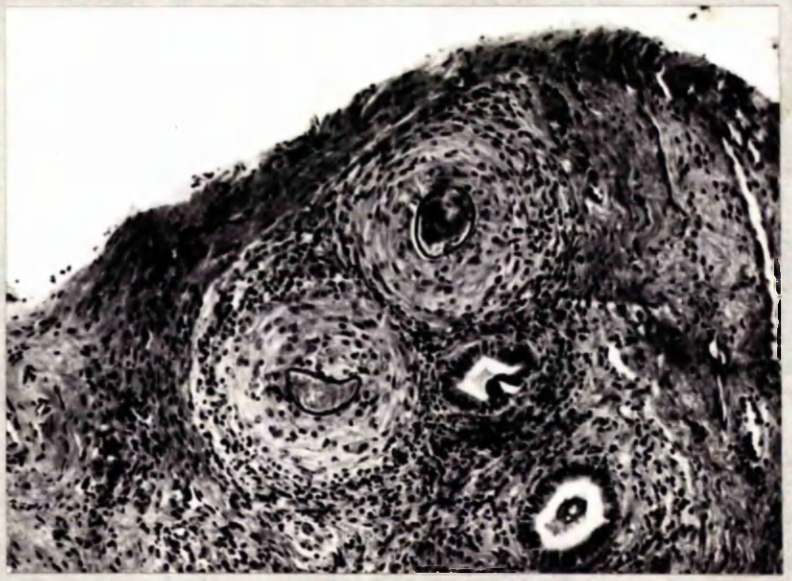

Fig. 55. Egg granulomata. Mallory x 110.

SUMMARY

1. The literature pertaining to the hepatic lesions associated with fascioliasis is discussed.

2. An experiment is described in which the serial pathology of fascioliasis is described in rabbits.

3. These lesions are discussed and it is concluded that the cirrhosis characteristic of fascioliasis is due to:-

(a) the healing of the tracts and infarcts produced by the migrating flukes;

(b) chronic cholangitis caused by the presence of the adults in the large bile-ducts;

(c) hyperplasia of both connective tissue and biliary elements in the portal tracts;

(d) granulomatous lesions formed in response to the presence of fluke eggs in the tissues.

4. Obstruction of the biliary tracts appears to play no part in the pathogenesis of the cirrhosis.

Appendix A

The snails were obtained from a field habitat near Aberfoyle in January, 1949. The farm was one in which fascioliasis was common in the cattle and sheep and the habitat was located during a joint expedition with Mrs. Drummond of the Zoology Department of this University. Snails and mud were removed to the Veterinary School, Glasgow, where the culture has since been maintained.

Appendix B

The spectrophotometric detection of biliverdin in plasma described by Larson, Evans and Watson, (1947), depends on the marked light absorption of biliverdin at wavelengths between 550 mμ and 700 mμ, maximal at 650 mμ. In the method used, 1 ml. of plasma was added to 8 ml. distilled water. The dilution was made up to 10 ml. with a saturated solution of NaCl which has the effect of clearing opacity. The light absorption curves of these dilutions were examined using a Unicam S.P. 500 spectrophotometer. The absorption curve of the plasma of infected rabbits was compared with that of the plasma of normal rabbits similarly diluted. No detectable amount of biliverdin was present in the plasma of either group.

Appendix C

The rabbit red cells were tested for the presence of adsorbed incomplete/

incomplete antibody by the direct antiglobulin test. In this, the red cells were thrice washed with normal saline and diluted to 5% with normal saline. One drop of the 5% suspension of cells was incubated for one hour at 37°C. with one drop of a suitable dilution of an anti-rabbit globulin serum. The cells were then examined for agglutination. The occurrence of agglutination indicated the presence of adsorbed incomplete antibody on the red cells.

The anti-rabbit globulin serum was developed by immunising goats against rabbit serum with intramuscular injections of alum-precipitated rabbit serum (Proom, 1943) followed by intravenous injections of rabbit serum.

The goat serum was then absorbed with well-washed normal rabbit red cells to remove agglutinins and titrated by testing doubling dilutions with rabbit red cells coated with incomplete iso-antibody. Suitable dilutions for tests were determined by this titration.

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The author wishes to express his thanks to Professor J. W. Emslie, who supervised this work, for his help and encouragement. The experiments described in Section 3 and Section 4 were carried out in collaboration with Dr. W. Mulligan and Mr. F. W. Jennings of the Department of Veterinary Biochemistry of this School, and the author is deeply indebted to these two. Thanks are also due to Dr. J. Anderson of the Department of Pathology, The Western Infirmary, Glasgow, who examined the samples of rabbit red cells by the Coombs' test, and to Dr. A. Baikie of the Department of Medicine, The Royal Infirmary, Glasgow, who carried out the work on spectrophotometry; to Mr. J. G. O'Sullivan of the Department of Pathology, for help on frequent occasions and to Mr. W. F. H. Jarrett of the Veterinary Hospital, Garscube, for advice in connection with Section 5; to Professor G. L. Montgomery for permission to work for some months in his Department and to Professor D. F. Cappell, who allowed the author to examine the slides in the micro-museum of the Western Infirmary; to Mr. W. Grant, Divisional Veterinary Officer, Ministry of Agriculture and Fisheries, Stirling, for help in the field; to Mr. W. Penny and the technical staff of the Department of Veterinary Pathology and to Mr. A. Finnie, the School Photographer.

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SUMMARY

This thesis describes some aspects of the experimental infection of rabbits with Fasciola hepatica.

The first part is concerned with the value of the rabbit as host in experimental fascioliasis. To this end, some experiments were carried out to determine the importance of some of the factors which might influence the number of adult flukes developing in a rabbit from a given number of cercariae. It was concluded that two of the most important factors are the technique of infection and the natural resistance of the rabbit. Details are given of a simple, rapid and efficient technique of infection and it was concluded that the natural resistance of the rabbit to infection with F. hepatica was about the same level and within the same limits as that of the sheep.

The work described in the second part of the thesis is concerned with the anaemia associated with fascioliasis. This was shown to be macrocytic and hypochromic and accompanied by a constant reticulocytosis; the plasma contained no excess bilirubin or biliverdin and the spleen was always deficient in iron. The marrow was hyperplastic and normoblastic. Blood or blood products were consistently demonstrated in the common bile ducts of infected rabbits and in the caeca of the flukes. The anaemia disappeared when the flukes were removed by an anthelmintic. It was concluded that the anaemia was similar in all essential respects to that produced in rabbits by repeated bleeding, i.e., that the anaemia was post-haemorrhagic in type.

Attempts were made to estimate the blood losses produced by the adult/

adult flukes in the bile ducts by radioactive isotope techniques in which red cells and plasma were labelled with ^{32}P and ^{131}I respectively, and injected into infected rabbits. These were killed and the amount of blood in the flukes calculated by measuring their radioactivity. It was concluded that each fluke removes 0.25 ml. daily.

A further section describes the results obtained by the immunisation of rabbits with proteins of F. hepatica. This was shown to stimulate the production of precipitins in relatively large amounts as measured by quantitative precipitin tests. Immunisation of rabbits with these proteins prior to infection was shown to produce inhibition of development of the parasites, but did not reduce their numbers significantly.

In the last section of the thesis, the serial pathology of experimental fascioliasis is described in rabbits. These lesions are discussed and it is concluded that the cirrhosis characteristic of fascioliasis is due to:-

(a) the healing of the tracts and infarcts produced by the migrating flukes;

(b) chronic cholangitis caused by the presence of the adults in the large bile ducts;

(c) hyperplasia of both connective tissue and biliary elements in the portal tracts;

(d) granulomatous lesions formed in response to the presence of fluke eggs in the tissues.

REFERENCES/

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