STUDIES ON THE PATHOPHYSIOLOGY OF CHRONIC OVINE FASCIOLIASIS

Summary of a thesis submitted for the degree of Doctor of Philosophy of the University of Glasgow by Peter H. Holmes, B.V.M.S., M.R.C.V.S.

The work described in this thesis is concerned with the pathophysiology of chronic Fasciola hepatica infections of sheep. In this study various isotopic labelling techniques were used to investiga in particular the cause of the anaemia and hypoproteinaemia, which are invariably present in diseased animals. The cause of the blood changes has been the subject of considerable debate over a number of years, and various divergent theories have been put forward. With the advent of satisfactory isotopic methods it finally became possible to study the turnover of both red cells and plasma proteins in the parasitized sheep, and to investigate the routes of excess loss of these blood constituents. The thesis is divided into four sections.

Section I. Studies on the Anaemia Produced in Sheep Chronically Infected by Fasciola hepatica

The first part of this section describes the use of ^{51}Cr-labelled red cells in both experimentally infected and normal sheep. It was found that the parasitized animals showed an increased rate of

disappearance of red cells from the circulation, and that this loss could be accounted for by an increase in the faecal excretion of the isotope. This strongly suggested that the causation of the anaemia was a substantial loss of blood into the gut of the infected animals presumably via the bile. Secondly, the ferrokinetics of infected and normal sheep were studied using ^{59}Fe. The results indicated that erythropoiesis was greatly increased in the infected animals as illustrated by elevation of the ^{59}Fe plasma disappearance rates, and red cell utilisation of ^{59}Fe. In addition, the degree of reabsorption of haemoglobin iron was measured using red cells simultaneously labelled with ^{59}Fe and ^{51}Cr. It was found that only in the most severely infected sheep was significant reabsorption occurring.

Section II. Metabolic Studies of the Plasma Protein Changes Associated with Chronic Ovine Fascioliasis

Firstly, albumin metabolism was studied using ^{131}I-albumin in both infected and normal sheep. It was found that the hypoalbuminaemia in the diseased animals was associated with a significant hypercatabolism of albumin. From indirect evidence it was suggested that the hypoalbuminaemia was caused by an excess loss of plasma proteins into the gut, presumably associated with the red cell loss.

Secondly, three separate techniques for the detection of gastrointestinal protein loss were used in infected and control animals. All the methods, viz ^{131}I-PVP, ^{95}Nb-albumin and $^{51}CrCl_3$, consistently showed that there was a significantly greater loss of plasma into the

gastrointestinal tract in the parasitized sheep. By the use of a double labelling technique it was found that a close correlation existed between the hypoalbuminaemia, hypercatabolism, and increased plasma protein loss in the infected sheep.

Lastly, the metabolism of albumin and of immunoglobulin were stud simultaneously, and it was found that the synthetic rate of immunoglobul was increased to a greater extent than that of the albumin in the infect sheep. It was suggested that the causesof the hypergammaglobulinaemia and hypoalbuminaemia associated with the disease are the results of the inability of albumin synthesis to be increased to the same extent as tha of immunoglobulin, in the face of the substantial plasma losses occurrin in the infected animals.

Section III. Studies of the Onset of the Pathophysiological Changes Following Infection

Red cell and albumin turnover were studied for thirteen weeks following infection of sheep with F. hepatica. The results showed that the commencement of the red cell leak and rise in albumin catabolism wer associated with the arrival of the adult flukes in the bile ducts. In addition it was found that the initial anaemia and hypoproteinaemia sometimes associated with the migratory phase of the infection were probably due to damage to hepatic cells and rupture of small blood vesse in the liver parenchyma by the migrating flukes.

Section IV. Studies of the Changes in Red Cell and Albumin Turnover Following Anthelmintic Treatment of Fluke-infected and Control Sheep

Following anthelmintic treatment of fluke-infected sheep it was found that there was a marked rise in both serum albumin and venous haematocrit. These changes were associated with both a dramatic fall in red cell loss into the gut, and decline in albumin catabolism once the flukes were removed. The latter effect was considered to be in part due to the cessation of the blood loss, and in part to decreased albumin synthesis presumably due to a homeostatic mechanism.

The results of the work described add considerably to the information regarding the pathophysiological mechanisms occurring in fascioliasis, and are all consistent with the theory that the major factor in the aetiology of the blood changes is loss of blood into the gastrointestinal tract.

NAME	ADDRESS AND AFFILIATION	DATE

THESIS
~~XXXXXXXXXXX~~ CONSULTATION CARD

Author: HOLMES, Peter H

Title: Studies on the pathophysiology of chronic ovine fascioliasis. Glasgow, 1969.

Shelfmark: Ph.D. Theses. No. 3317. Copy 2 - VBL

The books on reserve are arranged in alphabetical order by author.

When borrowing: please give your name and the date in the space below. Leave the card on the shelf in place of the book.

When returning: delete your name and the date, and, inserting the card in the book, shelve it in its place.

Borrower's name	date	Borrower's name	date
A. KARUGAH Physiol. Sec.	27/5/69 5/69.		

Borrower's Name	Date	Borrower's Name	Date

STUDIES ON THE PATHOPHYSIOLOGY OF CHRONIC OVINE FASCIOLIASIS

A Thesis

submitted for

The Degree of Doctor of Philosophy

in

The Faculty of Veterinary Medicine

of

The University of Glasgow

by

Peter Henry Holmes, B.V.M.S., M.R.C.V.S.

Department of Veterinary Physiology,

University of Glasgow.

September, 1969.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation for the assistance received during the course of this work.

He is particularly indebted to Professor W. Mulligan, under whose supervision the investigations were carried out, for the expert guidance and support, so freely and ably given during the last three years.

Appreciation is also due to Dr. J.D. Dargie, who is involved in similar investigations of *Fasciola hepatica* infections in rabbits, for helpful discussion and collaboration. To Mr. J.M. McLean for skilled technical assistance and collaboration, and to Mr. E.M. Glancy for competent technical help.

Thanks are also expressed to Dr. J. Armour and Dr. F.W. Jennings for helpful advice.

Finally, the author wishes to express his grateful thanks to Mrs. D.J. McGruer for preparation of the text.

CONTENTS

CONTENTS: GENERAL MATERIALS AND METHODS

CONTENTS: SECTION I

STUDIES ON THE ANAEMIA PRODUCED IN SHEEP CHRONICALLY INFECTED BY FASCIOLA HEPATICA

CONTENTS: SECTION II

CONTENTS: SECTION III

CONTENTS: SECTION IV

SECTION IV (Contd.)

GENERAL INTRODUCTION

Infection of sheep and cattle with the liver fluke Fasciola hepatica is widespread throughout the world, and because of its high morbidity and mortality it is of considerable economic importance, indeed in some areas it may constitute the single most important health hazard in sheep production (Rowlands, 1969).

There is abundant evidence of the serbusness of fascioliasis in both sheep and cattle. From slaughterhouse surveys in Britain it has been found that where climatic and topographic conditions are conducive infection may be very high, e.g. in Northern Ireland Ross (1966a) found as many as 88% of cattle had some degree of fascioliasis, whilst a Glasgow slaughterhouse survey from 1965 - 1967 showed 30 - 42% of cattle livers and 12 - 14% of sheep livers to be affected (Nicolson, 1968, cited by Reid, 1968). In North Wales during the epizootic of 1958/59 the losses due to fascioliasis were estimated to be in the region of £560,000 (Rowlands, 1969).

Similar levels of infection have been reported from many countries; e.g. Norway (Enger and Karbo, 1956), Spain (Colombo de la Villa, 1955), Australia (Gordon, 1956), Japan (Ono, 1958), Russia (Popov and Kalitina, 1965). All these surveys give higher infection rates for cattle than sheep, though this is probably because a high proportion of the sheep population tend to be slaughtered at an early age and have less change of being infected.

When considering economic losses due to this parasite, not only the incidence of liver condemnation must be considered but also other factors associated with fascioliasis such as loss of condition, poor growth rate and greater susceptibility to other diseases.

Ovine fascioliasis was described as early as 1379 by Jean de Brie (cited by Reinhard, 1957). As with other parasites, following the initial descriptions of the organism, the next major advance in the documentation of fascioliasis was the description of the life-history in the late 19th century (Leuckhart, 1881; Thomas, 1881).

The adult parasite is leaf-shaped, broader anteriorly than posteriorly, and may reach 30 x 13 mm in size. It is normally found in the bile ducts of sheep and cattle, though it has been recorded in a great variety of animal species including man. The parasite is hermaphrodite, and the eggs enter the duodenum of the host along with the bile and pass to the exterior in the faeces. Once on the ground the egg develops provided the temperature is suitable, i.e. $>9^{o}C$ and $<30^{o}C$. Generally in about three weeks a motile miracidium emerges from the egg. It is propelled through the water by numerous cilia, until it locates a suitable snail to act as the intermediate host. In Britain Lymnaea truncatula is normally involved. The miracidium penetrates the snail, loses its cilia and becomes a sporocyst. The sporocyst then gives rise to a number of rediae. The next stage is the cercariae, produced from germinal cells within the redia, which emerges

through a birth pore. The cercaria then leaves the intermediate host as a free-swimming form and within a short time finds a suitable object, usually a blade of grass, on which to encyst. Following encystment it is known as a metacercaria, which is the infective stage for the final host. The metacercariae may remain infective for several months, the exact time depending on the climatic conditions.

Once the metacercariae is ingested by a suitable host it escapes from its cyst in the duodenum and burrows into the intestinal wall, from where it makes its way to the liver. During the following weeks the immature flukes burrow through the liver parenchyma and finally end up in the bile ducts where they mature into adult parasites.

Early studies of the pathogenesis were mainly concerned with determining the migratory route from the gut to the liver, and it is now widely accepted that they migrate across the peritoneal cavity. Normally little damage results from this stage, and it is only when the young flukes penetrate the liver capsule that clinical signs begin to become apparent. These clinical signs are variable and largely depend on the numbers of parasites invading the liver.

As in other parasitic diseases, the pathogenicity of the agent can best be studied in carefully controlled experimental infections. For this to be possible it is first necessary that infective material can be produced in sufficient quantity by the development of suitable culture techniques. Once metacercariae could be produced in reasonable

numbers various workers reported on the pathogenicity of experimental infections, though for reasons of economy these were initially confined to small animals, e.g. in the rabbit (Urquhart, 1955) and albino rat (Thorpe, 1963) and later in large animals, e.g. in cattle (Ross, 1966b) and sheep (Sinclair, 1962; Reid, 1968). With the addition of these more recent excellent accounts there is now a wealth of literature on fascioliasis in various species, and extensive reviews of the current knowledge of the disease have been given by Dawes and Hughes (1964), Taylor (1964), Pantelouris (1965) and Sinclair (1967b).

Despite a huge amount of research which has been applied to the problem of fascioliasis there remains, however, large gaps in our knowledge, especially in an understanding of the pathophysiological processes involved in the disease. This thesis is an attempt to explain some of the changes which occur in fluke-infected sheep.

Following studies of both natural and experimental infections it is now established that fascioliasis can be divided into two types, acute and chronic, depending largely on the level of infection. The acute form is a result of a large number of immature flukes migrating through the liver, it is however less common than the chronic type which is associated with lower infections, when the adult parasites are in the bile ducts. The main clinical signs of both acute and chronic fascioliasis are depression, inappetence and weakness, with pallor of the mucous membranes, with sudden death commonly occurring in the acute form.

Previous work on the pathogenesis of the disease has largely centred on the recording of various changes in the haematology and blood biochemistry which occur. The most outstanding features of the chronic form especially are anaemia and hypoproteinaemia. Whilst these changes are universally accepted as being cardinal signs in infected animals there has been a considerable divergence of opinion as to their aetiology. With the advent of suitable isotopic labelling techniques it has now become possible to study directly the turnover of both red cells and plasma proteins, and so enable the pathophysiology of the disease to be investigated. The results of this investigation are presented in this thesis.

Firstly, the haematological changes were investigated using red cells labelled with ^{51}Cr. This technique enables both an estimation of red cell survival and of gastrointestinal blood loss to be made, as well as giving a qualitative estimate of the degree of haemolysis occurring. The simultaneous labelling of red cells with both ^{51}Cr and ^{59}Fe is also described. The double labelling technique permits measurement of both the rate of erythropoiesis and the degree of iron loss, as well as the extent of reabsorption of haemoglobin iron, hence providing essential evidence on the aetiology of the anaemia associated with chronic ovine fascioliasis (Section I).

Secondly, the metabolism of plasma proteins was studied in sheep chronically infected with F. hepatica in an attempt to explain the plasma protein changes associated with the disease. For this albumin labelled

with various isotopic markers was used to measure both the turnover rate and the routes of excess plasma protein loss. In addition the metabolism of immunoglobulin was studied in a parallel with that of albumin (Section II).

Thirdly, a study of the onset and development of the anaemia and hypoproteinaemia in chronic ovine fascioliasis is described, with particular reference to alterations in red cell and albumin turnover. For this ^{51}Cr labelled red cells and ^{125}I albumin were studied simultaneously for the thirteen weeks following infection of sheep with F. hepatica (Section III).

Lastly, the effect of anthelmintic treatment of fluke-infected sheep with respect to changes in albumin and red cell metabolism was investigated in two separate experiments. One involving the use of ^{125}I albumin and the other red cells simultaneously labelled with ^{51}Cr and ^{59}Fe (Section IV).

In each case a more complete account of the relevant literature is given in the introduction to the appropriate section.

GENERAL MATERIALS AND METHODS

A. EXPERIMENTAL SHEEP

I. Rearing and Maintenance

All the sheep used were Blackface wethers. They were obtained when one week old, and fed whole milk for the first four weeks. During the third and fourth weeks, hay and lamb weaner pellets (British Oil and Cake Mills Ltd., Renfrew, Scotland) were introduced. After weaning at four weeks, pelleted concentrate was given at ½ lb per 15 lbs body weight, along with ad lib hay and water. The sheep were given 1% NaCl in the drinking water continuously in order to reduce the incidence of urolithiasis (Weaver, 1966).

At 8 weeks of age the lambs were castrated and docked and also inoculated with a combined clostridial sheep vaccine, with a repeat dose 4 weeks later. The animals were weighed at frequent intervals, using an Avery Spring-balance pig weigher. Weighing was generally carried out in mid-morning and until the animals were transferred to metabolism cages, they were housed throughout in concrete pens with oatstraw bedding.

II. Maintenance during Experiments

The experimental animals were confined in standard sheep metabolism cages. Complete separation of faeces and urine was achieved by each sheep being fitted with a faecal bag. The urine passed through the grid floor and was collected in a bucket behind the cage.

III. Injection of Radioisotopes

All isotopically labelled substances were injected into the jugular vein via a jugular catheter, (Portex Plastics Ltd., Hythe, England), and the catheter flushed out with isotonic saline before being withdrawn. Blood samples were taken from the opposite jugular vein.

B. PARASITOLOGICAL TECHNIQUES

I. Metacercariae

The metacercariae used for the infections were kindly supplied by Dr. J. Armour of the Wellcome Laboratories for Experimental Parasitology, University of Glasgow.

II. Infection of Sheep

The requisite number of metacercariae encysted on cellophane were enclosed in a small filter paper and administered by a simple balling gun to the posterior buccal cavity of the sheep. The inoculum was followed by a drench of water to ensure swallowing of the infective dose.

III. Assessment of Mature Fluke Burdens

The infected sheep were killed by rapid intravenous injection of Euthatal (May and Baker Ltd., Dagenham, Essex). A ventral midline incision was made along the abdomen, and the rumen removed. The liver was then carefully extracted along with the common bile duct and a portion of duodenum. The main bile ducts were opened and the flukes removed. Any flukes remaining in the liver tissue were obtained by slicing the liver and incubating the slices in warm isotonic saline for several hours by which time the remaining flukes would emerge. The total fluke burden was then counted.

C. ANALYTICAL TECHNIQUES

I. Blood Analyses

All blood samples were taken from the jugular vein using evacuated 5 ml glass tubes (Vacutainer, Becton, Dickinson and Co., Rutherford, New Jersey, U.S.A.). If uncoagulated blood was required heparinised tubes were used, containing approximately 100 I.u. of dried heparin.

(a) Packed Cell Volume (PCV)

The packed cell volume percentage was determined by the micro-haematocrit method (Hawksley and Son Ltd., London, England).

(b) Total Serum Protein

Total serum protein concentration was estimated by a biuret method (Weichselbaum, 1946).

(c) Serum Protein Fractionation

Separation of individual serum protein fractions was carried out by electrophoresis on cellulose acetate strips (Oxoid Ltd., London, England), followed by staining with Ponceau S and scanning with a Chromoscan, (Joyce, Loebl and Co. Ltd., Gateshead, England).

II. Radioactivity Determinations

(a) Treatment of Samples

Samples of whole blood and/or plasma and the 24 hour urine collection were pipetted into counting tubes, and made up to a volume of 5 ml with 0.02 N NaOH. Each 24-hour faecal collection was weighed, spread out

on a tray or paper, and random 5 g samples taken and packed to a volume of 5 ml in counting tubes. Radioactivity measurements on blood, urine and faeces were carried out in a well-type scintillation counter, type 50006 (Ekco Electronics Ltd., Southend-on-Sea, Essex, England) unless otherwise stated.

(b) Preparation of Standards

A 2 ml sample of the isotopically labelled preparation was taken into a syringe, the syringe weighed, and the contents expelled into a volumetric flask, the syringe reweighed, and the contents made up to a known volume with 0.02 N NaCl. One ml of this was then taken, made up to 5 ml in a counting tube, to act as the standard for each preparation for each animal, and used for correction against decay, changes in the sensitivity of the counting equipment and calculation of the injected dose.

D. CALCULATIONS AND EXPRESSION OF RESULTS

I. Determination of Injected Activity

The amount of activity injected expressed as counts/sec was calculated from the following formula:

$$\text{Injected Activity} = \frac{\text{Wt injected} \times \text{Radioactivity of Standard (cts/sec)} \times \text{Dilution Factor}}{\text{Wt. Standard}}$$

II. Blood Volume Estimation

The blood volume was calculated from either circulating red cell volume or plasma volume, and corrected by the venous haematocrit to give the whole blood volume (no correction was made for the difference between whole body haematocrit and venous haematocrit as other experiments using double labelling techniques had shown that the venous haematocrit/whole body haematocrit ratio in sheep was not constant).

Blood volume calculations were made according to the dilution principle.

$$\text{Blood Volume} = \frac{\text{Total Injected Activity (cts/sec)}}{\text{Activity 10 min blood sample (cts/sec/ml)}}$$

The 10 minute post injection sample was used since it is the opinion of most workers in this field that the taking of one sample at this time is adequate for all practical purposes, and the value obtained doesn't differ significantly from values calculated by either extra-polating to zero-time, or using other samples obtained in the 6 - 12 minute post-injection interval (Wetterforde, 1965).

III. Presentation of Blood Activity

Whole blood or red cell activities were plotted as a percentage of the 10 minute post-injection sample on semi-logarithmic graph paper. From the disappearance curve the "half-life" or $T\frac{1}{2}$ was obtained as the time taken for the activity to fall 50%.

IV. Calculation of Total Faecal and Urinary Activity

The total radioactivity for each 24 hour collection of urine and faeces was determined by multiplying the counts obtained for the 5 ml sample of urine and the 5 g samples of faeces by the appropriate factors to give the total counts.

5. STATISTICAL METHODS

The statistical methods employed were those described by Snedecor (1956) and Bishop (1966). The exponential phase of the radioactivity disappearance curves were calculated by linear regression analysis, a correlation coefficient $r > 0.95$ being accepted. Deviations from the means are expressed as the Standard Deviations (S.D.) of the means. The "t" values were taken to be significant at $p < 0.05$.

SECTION I

STUDIES ON THE ANAEMIA PRODUCED IN SHEEP CHRONICALLY INFECTED BY FASCIOLA HEPATICA

Anaemia has long been recognised as an outstanding symptom of chronic fascioliasis, and all the standard veterinary textbooks make reference to this feature of the disease (Monnig, 1956; Lapage, 1956; Smith and Jones, 1957; Jubb and Kennedy, 1963; Soulsby, 1968).

Although there is no disagreement on the occurrence of anaemia there is a divergence of opinion as to the cause and type of the anaemia.

The anaemia associated with F. hepatica may be due to one or several of the following:

1. Abnormal destruction of red cells caused by some haemolysing factor produced by the parasite.

2. Abnormal loss of red cells due to feeding activities of the flukes.

3. Impairment of erythropoiesis.

The first was favoured by many workers. Flury and Leeb (1926) found that extracts of flukes haemolysed red blood cells; Manzone (1940) claimed that normal red cells were haemolysed by the sera from fluke-infected sheep; Balian (1940) studied the anaemia in natural infections

of sheep and observed a macrocytosis, and on this basis concluded that a haemolytic factor was present.

The second possibility, that flukes cause blood loss presumably due to their feeding activities, was suggested as early as 1880 by Somaer who concluded from histological evidence that flukes lived on erythrocytes and bile duct epithelium.

Railliet (1890) injected plaster containing a blue dye into infected sheep and recovered both plaster and dye from the caeca of the flukes, but was unable to recover the dye from the bile. From these results he concluded that the flukes fed on blood, though these conclusions were later questioned by Müller (1929), whose own work suggested that the parasite fed on bile and desquamated bile duct epithelium. Weinland and von Brand (1926) demonstrated that flukes would feed on clotted blood in vitro. This was also shown by Stephenson (1947) who concluded, from histological and spectroscopical examination of the intestinal contents of flukes, that blood constituted the main source of food. In similar work, Van Grembergen (1950) also came to the conclusion that F. hepatica was haematophagic.

Using fluke-infected and normal rabbits, Urquhart (1955) was able to show that a similar anaemia to that observed in the infected rabbits could be produced by repeated bleeding of normal rabbits. He also demonstrated the presence of blood in both the contents of the bile ducts, and in the caeca of the mature flukes.

The use of isotopically labelled red cells and plasma in the study of the anaemia of fascioliasis was first attempted by Jennings, Mulligan and Urquhart (1956), who used ^{32}P labelled red cells and ^{131}I labelled serum albumin, both separately and simultaneously. One hour after injecting the isotopically labelled substances into fluke-infected rabbits, the animals were killed and the radioactivity determined in the blood, bile and flukes. From their investigation they concluded that adult flukes caused a blood loss of 0.2 ml per day, though when the labels were used simultaneously they found that the $^{32}P/^{131}I$ ratio in the flukes was consistently higher than the corresponding ratio in the blood. This suggested that there was a preferential uptake and/or retention of red cells relative to the plasma by the flukes.

The authors pointed out several possible sources of error in this work, e.g. the use of ^{32}P as a red cell label was unsatisfactory because of rapid loss of isotope from the erythrocytes; the choice of killing the rabbits one hour post injection was purely arbitrary and may have been too short a time for the flukes to ingest their normal quota of blood; regurgitation of caecal contents by the flukes as they were removed would significantly reduce the radioactivity of the flukes, and hence the calculated blood content; and lastly bile is a normal excretion route for iodine, so any ^{131}I released from the labelled albumin during catabolism may have given erroneously high ^{131}I activities in the bile and flukes.

More recently Jennings (1962) and Pearson (1963) used ^{51}Cr labelled red cells to investigate the aetiology of the anaemia, the former in a small pilot experiment using one infected and two control rabbits was able to show that there was a more rapid disappearance of ^{51}Cr labelled red cells from the circulation of the fluke-infected animal, and that this was due to faecal loss presumably due to blood loss via the bile into the gut; the latter injected ^{51}Cr labelled red cells into fluke-infected guinea pigs and sheep and killed the animals two hours later. Samples of blood, bile and flukes were then assayed for radioactivity. Pearson's work suffered from many of the limitations of the earlier studies by Jennings et al (1956) and the writer came to the conclusion that the method was invalid for estimating blood loss through ingestion by the flukes.

The suggestion that impairment of erythropoiesis was the principle factor involved in causing the anaemia was postulated by Balian (1940) in trying to explain the severity of the anaemia observed in chronic fascioliasis. Sinclair (1964) also held the opinion that the anaemia was of the dyshaemopoiotic type, though examination of his results does not entirely support this view, as he showed for example that the anaemia was of the normochromic, normocytic type, and could be produced by regular bleeding of parasite-free sheep. Impaired erythropoiesis as the major cause of the anaemia was inferred by Sewell (1966) from his studies on Fasciola gigantica infections of zebu cattle. He states that

the anaemia is probably due to the parasite either producing a dyshaemopoietic toxin, or denuding the host of an essential haemopoietic factor.

So it would appear that the true aetiology of the anaemia can only be finally determined if the relative importance of the three possible factors mentioned can be satisfactorily assessed.

Fortunately with the advent of the use of ^{51}Cr as a red cell tag by Gray and Sterling (1950b), and its successful use in the pilot experiment by Jennings (1962), an important new technique was provided for the assessment of both blood loss into the gut and the extent of intravascular haemolysis occurring in fluke-infected animals. With the simultaneous use of ^{59}Fe labelled plasma and red cells, it also became possible to study the rate of erythropoiesis and the extent of intestinal loss and reabsorption of iron in chronic ovine fascioliasis.

This section of the thesis describes (1) the use of ^{51}Cr labelled red cells in the quantitative measurement of blood loss and a qualitative assessment of intravascular haemolysis occurring in sheep with mature infections of F. hepatica and (2) studies of the rate of erythropoiesis, the degree of intestinal iron loss, and the extent of iron reabsorption occurring in chronically infected sheep, using ^{59}Fe and ^{51}Cr labelled red cells simultaneously.

A. USE OF ^{51}CHROMIUM LABELLED RED CELLS IN FLUKE-INFECTED AND NORMAL SHEEP

Introduction

The original work by Gray and Sterling (1950a) showed that erythrocytes had a marked affinity for anionic hexavalent chromium as demonstrated by the rapid uptake of radioactive chromium when $Na_2{}^{51}CrO_4$ was added to saline washes of red cells. They also showed that uptake by red cells suspended in saline was appreciably greater than in whole blood. Fractionation of red cells previously tagged with $Na_2{}^{51}CrO_4$ demonstrated that 78% of the total activity was bound to the globin portion of the haemoglobin molecule, with only a trace in the haemin. These results were confirmed by Necheles, Weinstein and LeRoy (1953), but questioned by more recent studies of Carter, Jackson and Kolber (1967), who found considerably more activity in the haemin fraction, namely 30% of the total activity. The difference in results by the later workers can probably be explained by their different methods of separating the haemin from the haemoglobin molecule.

The method of labelling recommended by Gray and Sterling (1950b) consisted simply of incubating $Na_2{}^{51}CrO_4$ with saline suspensions of washed red cells at room temperature for one hour. The cells were then washed three times with cold sterile saline and resuspended in the original untreated plasma for intravenous injection.

This technique has since been used by most workers with minor modifications, e.g. Cooper, Strocbel, Stickney and Owen (1955) incubated the treated cells for only 15 minutes at 37°C. Others (Necheles et al, 1953; Mollison and Veall, 1955) have used Acid Citrate Dextrose as anticoagulant in place of heparin to obtain slightly better labelling efficiency. The number of washes the labelled cells are given has been shown to have no significant effect on the survival rate of the labelled cells once they are injected, (Hughes, Jones and Mollison, 1956).

The concentration of chromium which would prove toxic to the red cells was investigated by Necheles et al (1953) who showed that levels up to 30 micrograms of chromium per millilitre of whole blood had no significant effect on red cell fragility. Though other work by Ebaugh, Emerson and Ross (1953) suggested that 10 µg/ ml whole blood was a more satisfactory concentration, and this was confirmed by Donohue, Motulsky, Giblett, Pirzio-Biroli and Finch (1955).

Owen, Bollman and Grindlay (1954) were the first to show that ^{51}Cr red cells could be used to quantitate blood loss into the gut since when ^{51}Cr labelled red cells were administered into the stomach of dogs, over 90% could be recovered in the faeces. Similar work by Ebaugh, Clemens, Rodman and Peterson (1956) in human subjects showed that 98.0 ± 9.7% of ^{51}Cr administered orally as ^{51}Cr labelled red cells was recovered in the six-day faecal collection period. They concluded that for practical purposes (in humans) once chromium tagged blood is shed into

the gastrointestinal tract, all the radioactivity will be recovered in the stools and therefore radioactive ^{51}Cr represents an accurate maximum estimate of the amount of shed blood in a patient who has ^{51}Cr tagged red cells. Most investigations into gastrointestinal blood loss using ^{51}Cr have been carried out in man and dogs, especially in the study of hookworm infections. Whether an analagous situation exists in sheep was studied by Clark, Kiesel and Goby (1962) who administered measured amounts of tagged blood cells either into the abomasum or orally. The total amount of radioactivity in the faeces was determined for several days following administration of blood. A total of seven sheep were given blood orally and an average of 86.9% was recovered, and 88.4% when given to four sheep in the abomasum. These workers found that absorption from the alimentary tract could be reduced if cells labelled with inactive chromium were circulating.

Apparently the presence of chromium in the blood inhibited the absorption of more chromium from the gut. So it is possible that determinations of gastrointestinal blood loss in sheep using ^{51}Cr labelled cells may give a slight underestimate of the true leak. Though it should be noted that in the investigations of Clark et al (1962) faeces were only collected for several days and it may be that the presence of a well developed caecum in ruminants, compared to that of the dog, prevented all the administered ^{51}Cr from being recovered within the collection period.

The extent to which ^{51}Cr released from labelled red cells is reutilised by other red cells was investigated by Ebaugh, Emerson and Ross (1953) who incubated haemolysed ^{51}Cr labelled red cells along with an equal volume of washed normal erythrocytes and found that uptake by these cells was negligible. They also injected haemolysed ^{51}Cr labelled erythrocytes into the original donor and found that no ^{51}Cr was fixed to the recipient's cells during the 30 day observation period.

Ebaugh et al (1953) in experiments to compare different methods of estimating red cell survival time found that the ^{51}Cr techniques consistently gave shorter values than the Ashby differential agglutination method. The reason for the shortened times was thought to be because of loss or elution of chromium from surviving red cells. The phenomenon of chromium elution from labelled human red cells has been extensively studied. (Mollison and Veall, 1955; Hughes, Jones and Mollison, 1956; Mollison, 1961; Carby and Hjelm, 1962).

When autologous ^{51}Cr labelled red cells are injected into ruminants there is a rapid loss of activity over the first 18-24 hours. This is especially marked in the bovine (Todd and Ross, 1966). The phenomenon was first observed in sheep by Drury and Tucker (1958) who noted that the activity had fallen to 61% of the initial activity within 24 hours.

After this rapid phase was completed, there was a slower exponential loss of labelled cells but the slope of the disappearance curve is greater than that observed in other species. It seems that the loss of activity

is due to chromium elution from the red cells occurring in two distinct components, an initial rapid phase, followed by a slower one. However the eluted activity is largely excreted by the urine and so does not directly affect measurements of gastrointestinal blood loss.

Thus it has been shown that:

(1) ^{51}Cr can be firmly bound to erythrocytes by a straightforward labelling technique.

(2) ^{51}Cr labelled red cells can be used for the quantitative measurement of gastrointestinal blood loss in sheep.

(3) After an initial rapid phase of elution from sheep erythrocytes, the disappearance is slower and exponential. Eluted activity appears in the urine, and is not reutilised, and therefore ^{51}Cr labelled red cells would prove very useful for investigating the aetiology of the anaemia of fascioliasis.

MATERIALS AND METHODS

I. Experimental Animals

A total of twelve sheep were studied in two separate experiments, both experiments were identical in all respects, except the frequency of blood sampling, and so are presented together. Six of the sheep had been infected with F. hepatica three months earlier, two with 1,000 metacercariae and the others with 500. The remaining six sheep served as parasite-free controls.

II. Labelling of Red Cells

A heparinised blood sample equivalent to approximately 10 ml of red cells was obtained from each sheep. After spinning the blood samples for 20 mins at 1,500 - 2,000 rpm, the plasma was removed and retained. A measured volume of $Na_2{}^{51}CrO_4$ (specific activity 1 mC/ml, chromium content 5.4 µg/ml) was added to each sample of erythrocytes; the red cells of the infected animals receiving 1.2 ml, whilst those of the controls received 0.6 ml. The cells were then incubated at 37°C for 30 mins, during which time they were frequently mixed. After labelling the cells were washed twice with isotonic saline, and finally reconstituted with the retained plasma for injection, each sheep receiving its own red cells and plasma.

III. Injection and Sampling

From each mixed sample of labelled red cells suspended in plasma 7 ml was taken into a syringe, the syringe weighed, and the contents

injected intravenously, as previously described in general materials and methods, and the empty syringe finally reweighed. In sheep 60, 61, 62, 65, 68, and 70 blood samples were taken at 5, 10, 15, 30, 60 min, and 2 and 18 hours post-injection, from thereon sampling was carried out twice daily for 13 days, and once daily for a further 3 days. Whilst in the remaining subjects, viz 48, 51, 55, 64, 74 and 78 samples were taken, less frequently, at 10 min, 20 min and 16 hours post-injection and from there once daily for 13 days.

At each bleeding a 5 ml heparinised sample was taken, and from this a 1 ml sample of whole blood pipetted into a counting tube, made up to 5 ml with 0.02N NaOH, and assayed for radioactivity. The haematocrit was also determined for each blood sample. Faeces and urine were collected every 24 hours and aliquots taken as described previously in general materials and methods.

IV. Calculations and Expression of Results

One ml samples of whole blood were counted, corrected for radioactivity decay, and converted to counts per ml of packed red cells using the formula:

$$\text{cts/ml rbc} = \frac{\text{cts/ml whole blood} \times 100}{\text{PCV}}$$

The red cell volumes and hence the blood volumes were calculated as described in general materials and methods. In two infected and two control sheep the blood volume was recalculated at the end of the experimental period by a further injection of autologous ^{51}Cr-labelled

red cells. The whole blood and red cell activities for the thirteen day experimental period were plotted on semi-logarithmic paper and the half-life determined for each disappearance curve.

The total radioactivity for each 24-hour collection of faeces was divided by the activity per ml of whole blood taken at the beginning of the collection period, to give a daily faecal clearance of whole blood, and likewise by the activity per ml of red cells to give a daily faecal clearance of red cells.

The initial period of rapid elution was ignored, blood loss only being calculated during the exponential phase of the activity disappearance curve.

RESULTS

Examination of the red cell activity disappearance curves showed that the infected animals had a significantly shorter half-life compared to the controls, Fig. I and Table I. The increased loss of ^{51}Cr from the circulation of the infected animals could be due to loss of labelled red cells, increased intravascular breakdown of cells, or increased elution of ^{51}Chromium from the labelled cells. The true explanation for the abnormal loss depends upon a quantitative study of the rate and route of excretion of the isotope.

Faecal and Urinary Excretion of ^{51}Cr in Fluke-infected and Control Sheep

Tables 2 and 3 show the daily faecal red cell clearance and Table 4 the mean clearances for the infected and control sheep. It is apparent

Fig. 1. ^{51}Cr-Red Cell Activity in a Fluke-Infected and Normal Sheep

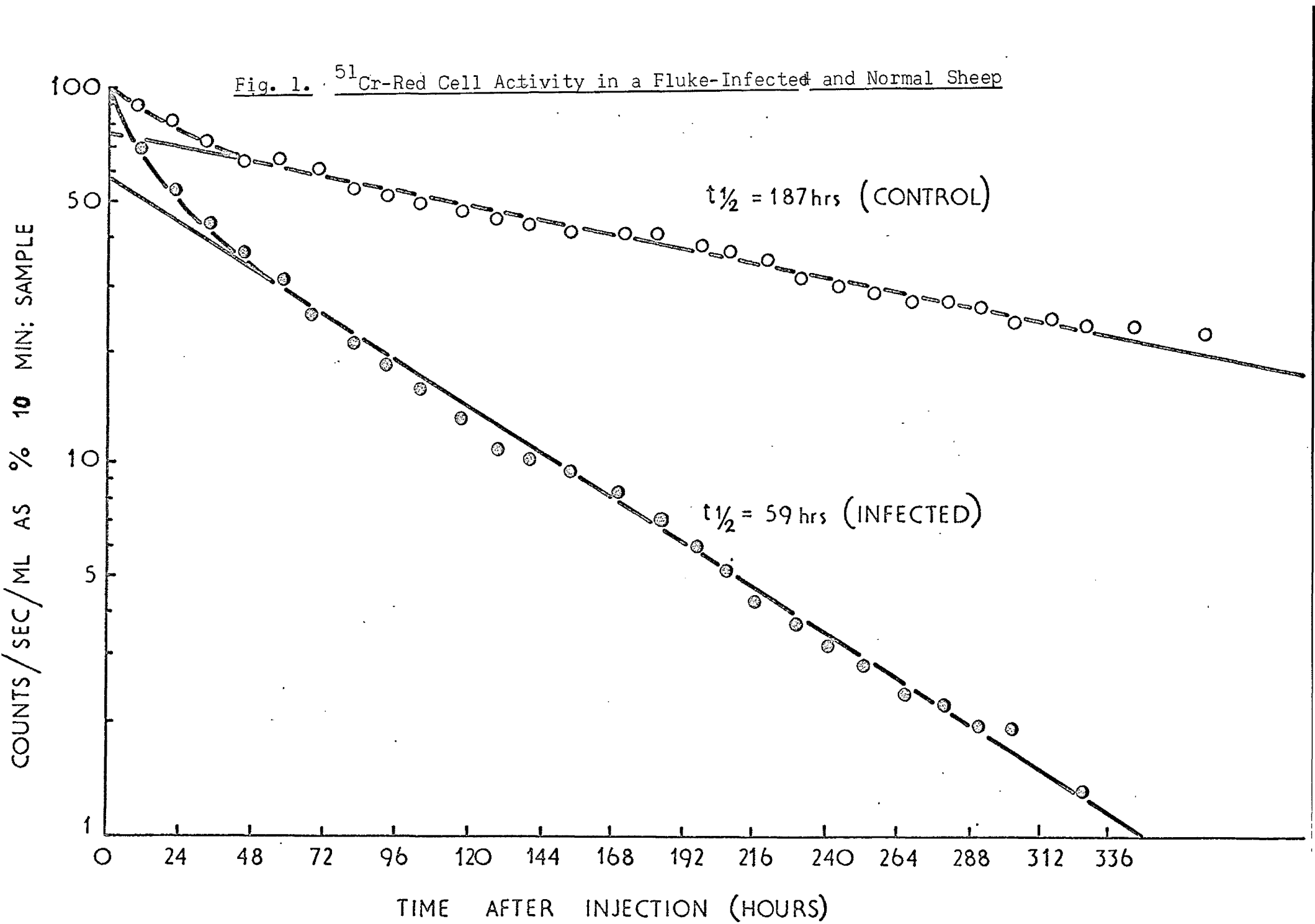


Table 1

Packed Cell Volumes, Red Cell Half-life and Blood Volume in Fluke-infected and Normal Sheep

	Sheep	PCV %	Rbc T½ (hours)	Blood Vol. (ml)	Rbc Vol. (ml/Kg)	Fluke Burden
INFECTED	62	17	59*	894 (811)	6.6 (4.0)	832
	65	27	87*	1320	10.0	450
	68	27	102*	1474 (1596)	11.4 (11.0)	†
	55	26	167	1973	18.6	161
	74	29	160	1745	18.7	†
	78	33	180	1983	23.3	†
	Mean	26	126	1565	14.8	481
	S.D.	5	50	422	6.4	337
CONTROL	60	42	205*	1345	17.1	0
	61	39	193*	1474 (2013)	14.9 (18.2)	0
	70	33	236*	1770 (2151)	18.6 (20.5)	0
	48	30	360	-	-	0
	51	41	305	2137	27.3	0
	64	34	326	1794	21.4	0
	Mean	37	271	1698	19.9	
	S.D.	5	69	318	4.8	
	"t" Test	P<0.01	P<0.01	N.S.	N.S.	

N.S. = Not Significant. * = Bleeding x 2/day. † = Fluke Burden not determined.

Figures in parenthesis = Blood Vol. & Rbc Vol. at end of experiment.

Table 2

Daily Faecal Clearance of Red Cells in Fluke-infected Sheep (ml)

Sheep		62	65	68	55	74	78
Day	1	-	-	-	-	-	-
	2	19.0	15.5	21.1	-	-	-
	3	24.0	22.4	17.6	-	-	-
	4	18.8	24.7	18.0	8.5	16.3	-
	5	45.2	24.6	19.6	8.7	6.2	8.8
	6	28.2	21.1	17.1	8.8	10.1	11.7
	7	23.4	16.9	18.2	8.8	13.5	10.7
	8	32.4	*	26.3	9.1	12.6	14.7
	9	29.7		18.4	7.4	14.2	14.2
	10	22.5		21.9	9.3	12.2	8.9
	11	22.9		17.9	8.9	14.6	17.8
	12	30.2		15.8	13.5	18.0	26.5
	13	26.9		18.9	11.0	15.9	14.6
	Mean	26.9	20.8	19.1	9.4	13.3	14.2
	S.D.	7.2	3.9	2.8	1.7	3.4	5.5

* Developed urolithiasis, killed.

Table 3

Daily Faecal Clearance of Red Cells in Normal Sheep (ml)

Sheep	60	61	70	48	51	64
Day 1	-	-	-	-	-	-
2	0.7	0.6	0.7	-	-	-
3	0.6	0.5	0.5	2.0	-	-
4	0.4	0.5	0.6	-	0.5	-
5	0.5	0.6	0.5	1.3	1.0	1.1
6	0.3	0.4	0.5	1.8	0.6	0.8
7	0.4	0.7	0.9	0.9	0.6	1.1
8	0.6	0.5	0.6	0.7	0.2	0.3
9	0.4	0.6	0.8	0.7	-	1.3
10	0.5	0.5	0.6	-	-	0.9
11	0.5	0.7	0.5	-	-	0.5
12	0.2	0.6	0.2	-	-	0.5
13	0.4	0.2	0.5	-	-	0.8
Mean	0.4	0.5	0.6	1.2	0.6	0.8
S.D.	0.2	0.2	0.2	0.6	0.3	0.3

that there was a significantly higher faecal clearance in the infected animals. The increased faecal loss is reflected in the cumulative faecal activity for the experimental period expressed as a percentage of the injected activity, the infected animals losing 15% and the controls only 0.6% of the injected activity via the faeces. There was no significant difference in the urinary excretion of the isotope between the fluke-infected and control animals, see Table 4.

The blood volumes and circulating red cell volumes were not significantly different between the two groups, though the circulating red cell volume and haematocrit were markedly reduced in the most heavily infected animal, sheep 62.

Excretion of ^{51}Cr after intravenous injection of lysed labelled red cells in fluke-infected and normal sheep

The suggestion has been made that the increase in faecal excretion of ^{51}Cr observed in fluke-infected animals may be due not to loss of red cells but to increased elution of isotope from the cells in infected animals, accompanied by increased bile flow (Sinclair 1967a). To check this hypothesis two infected and one control sheep were injected with a preparation of ^{51}Cr labelled cells which had been lysed by freezing and thawing. Urine and faeces were collected and assayed for radioactivity for a period of seven days after injection. The results are shown in Table 5.

Both the infected and control animals lost about 60% of the injected activity via the urine during the 7 day collection period, whilst the

Table 4

Daily Faecal and Urinary Excretion of ^{51}Cr in Fluke-infected and Control Sheep

	Sheep	Mean Whole Blood Loss (ml)	Mean Blood Loss/Fluke (ml)	Mean Rbc Loss (ml)	Mean Rbc Loss/Fluke (ml)	% Injected Dose Recovered	
						Faeces	Urine
INFECTED	62	225.7	0.271	26.9	0.032	36.2	38
	69	96.7	0.214	20.8	0.046	14.6	47
	68	77.2	-	19.1	-	15.1	30
	55	37.6	0.233	9.4	0.058	6.8	46
	74	42.7	-	13.3	-	10.4	47
	78	43.7	-	14.2	-	7.0	-
Mean		87.3	0.239	17.6	0.045	15.9	42
S.D.		71.7	0.026	6.3	0.010	11.0	8
CONTROL	60	1.5	-	0.4	-	0.6	44
	61	1.8	-	0.5	-	0.6	45
	70	1.5	-	0.5	-	0.9	53
	48	4.0	-	1.2	-	0.5	44
	51	1.5	-	0.6	-	0.2	44
	64	2.5	-	0.8	-	0.6	40
Mean		2.1	-	0.7	-	0.6	45
S.D.		1.0	-	0.3	-	0.2	4
"t" Test		$P < 0.02$		$P < 0.001$		$P < 0.01$	N.S.

Table 5

Urinary and Faecal Excretion of ^{51}Cr over 7 days
in Normal and Fluke-infected Sheep
After Intravenous Injection of Lysed ^{51}Cr-labelled Red Cells

Sheep		% Injected Dose Recovered	
		Urine	Faeces
34	Infected	56.9	2.3
35	"	60.6	2.2
70	Control	60.7	1.5

amounts lost via the faeces were insignificant. The parasitized animals showed a slightly higher faecal loss of ^{51}Cr than the control.

DISCUSSION

Since it can be assumed that ^{51}Cr activity found in the faeces reflects blood loss into the gut it is obvious that a massive loss of red cells is occurring into the gastrointestinal tract of the fluke-infected sheep, particularly if one bears in mind that up to 15% of the ^{51}Cr activity may be reabsorbed from the gut in this species, so that the faecal clearance figures may in fact be a slight underestimate of the total loss.

Examination of the urinary excretion of isotope in the fluke-infected and control animals show no significant difference, indicating that fascioliasis does not increase intravascular breakdown of red cells. Similarly there is no evidence of increased elution of $^{51}Chromium$ from the red cells of the infected sheep, since eluted activity appears in the urine. Bile secretion may be slightly increased in the infected animals as illustrated by the results of the injection of lysed ^{51}Cr labelled red cells, Table 5, since the faecal activity of the infected was slightly higher than that of the control, though the contribution the increased biliary flow makes to the faecal activity in the infected animals from this source is negligible.

From Table 4 it can be seen that the mean whole blood loss per fluke gave similar figures in all three infected sheep whose fluke

burdens were determined, i.e. 0.239 ml whole blood per fluke (± 0.026) which is close to the figure originally suggested for fluke-infected rabbits by Jennings et al (1956), though recent work by Dargie (1969) has given a higher figure in chronically infected rabbits of 0.5 - 0.8 ml whole blood/day/fluke.

When these experiments were carried out, the sheep had three month infections. The earlier work by Kendall and Parfitt (1962) had shown that flukes reach the bile ducts in sheep after 40 days, therefore the infections in the current study could be considered to be mature and of a chronic type. However recent studies on the development of the symptoms of fascioliasis as described in Section III of this thesis have shown that the flukes do not cause a significant red cell leak till ten weeks post infection, and the magnitude of the leak increases up to 20 weeks post infection. In the light of this information it should be noted that the leak of 0.2 ml whole blood/day/fluke obtained in this experiment is probably an underestimate of the final loss occurring in long standing mature infection.

The fluke burden shows a strong positive correlation with the mean daily whole blood faecal clearance ($r = 0.991$, $P < 0.01$) but a poorer correlation with the mean daily red cell faecal loss ($r = 0.968$, $P < 0.05$) which along with evidence given in Section II of the thesis strongly suggests that the loss is of whole blood and not only of red cells.

There was also evidence of an inverse correlation between numbers of flukes in the liver and the daily loss of red cells per fluke ($r = 0.999$, $P < 0.01$), indicating that with greater numbers of flukes the individual parasites cause a reduced loss of red cells. This can probably be explained by the reduced numbers of circulating red cells in the heavily infected sheep being available to the flukes, for as stated earlier the whole blood loss per fluke gave a figure of approximately 0.2 ml regardless of the severity of the infection.

These results have been confirmed by Sewell, Hammond and Dinning (1968) in similar studies on the anaemia of chronic ovine fascioliasis using ^{51}Cr labelled red cells and red cells labelled *in vivo* with $DF^{32}P$. From the ^{51}Cr results they showed that a marked loss of blood was occurring into the gut of the fluke infected sheep. Analysis of the shape of the survival curves of red cells labelled with $DF^{32}P$ indicated a loss of red cells due to either chronic haemorrhage or an haemolysis effect. The estimates of daily random blood loss from the curves were of the same order as those obtained from the ^{51}Cr faecal clearances of the infected animals, however in the most heavily infected sheep, they tended to be higher than the direct ^{51}Cr estimate of blood loss in the faeces, and they concluded that haemolysis was occurring in this animal. However since the urinary activity was not determined they were unable to substantiate this suggestion.

There was no evidence in the present study to suggest significant impairment of haemopoiesis, e.g. circulating red cell volumes determined at the beginning and end of the experimental period showed no significant decrease, though the animal harbouring the heaviest infection did show a slight reduction in red cell volume, along with a fall in haematocrit and loss of weight during the experimental period. Bearing in mind the magnitude of the blood loss into the gut, as much as 20% of the circulating red cell volume per day, see Tables 1 and 4, the infected animals must have been producing red cells at a rate considerably in excess to that of the controls.

This is in contradistinction to the view of Sinclair (1964) who considered dyshaemopoiesis to be the major factor in the aetiology of the anaemia of fascioliasis. He in part based this deduction on the greater severity of the anaemia in his infected animals as compared to that shown by sheep from which he had removed daily 60 ml of blood based on daily blood loss of 0.2 ml/fluke/day (Jennings et al, 1956). This amount would appear to be too low as though the figure of 0.2 ml/day/fluke was found in the present study at 12 weeks post infection, later studies indicate a higher loss of blood/fluke/day, see Section III (Dargie, 1969; Sewell et al, 1968).

In later work by Sinclair (1965) in which larger volumes of blood, namely 130 ml, were removed daily the level of anaemia produced was very similar to that observed in moderate chronic fascioliasis.

Further confirmation of the results presented has come from spectroscopic analysis of the caecal contents of F. hepatica by Todd and Ross (1966), who demonstrated the presence of degradation products of host haemoglobin, and the work of Dargie (1969) using ^{51}Cr labelled red cells in fluke-infected rabbits in which he showed a massive loss of blood was occurring into the gut.

These results lend strong support to the theory that the major factor in the aetiology of the anaemia associated with chronic ovine fascioliasis is blood loss caused by mature flukes in the bile ducts.

SUMMARY

Erythrocytes labelled with ^{51}Cr were used to study the aetiology of the anaemia associated with chronic fascioliasis in six normal and six fluke-infected sheep. The infected animals showed an increased disappearance of isotope from the circulation. Examination of thefaecal and urinary excretion rates revealed the loss to be due to a massive loss of red cells into the gut of the infected animals. There was no evidence of increased haemolysis or excessive elution of ^{51}Chromium from the labelled cells in the infected group. The magnitude of the leak suggests that blood loss caused by the parasites is the principal factor in the causation of the anaemiaassociated with chronic fascioliasis.

B. THE USE OF ^{59}Fe IN THE STUDY OF IRON METABOLISM IN FLUKE-INFECTED AND CONTROL SHEEP

Introduction

Previous studies using ^{51}Cr labelled red cells in fluke-infected and normal sheep showed that:

(a) a massive loss of red cells was occurring into the gut,

(b) there was no evidence of increased haemolysis, and

(c) that erythropoiesis was apparently increased in the infected animals. To investigate further the nature of the red cell leak and the degree of erythropoiesis red cells simultaneously labelled with ^{59}Fe and ^{51}Cr were used.

Measure of Erythropoiesis

Iron is a vital constituent of haemoglobin and hence essential in the production of red cells. Radioactive iron was one of the first isotopic labels to be made available for the investigation of a biological system by the techniques of isotopic trace labelling, and by 1950 ^{59}Fe was prepared in sufficiently high specific activity to allow studies to be made on both normal humans and patients with various haemopoietic disorders (Huff, Hennessy, Austin, Garcia, Roberts and Lawrence, 1950). Following from this initial work the basic techniques have been widely utilized for the study of ferrokinetics in many human conditions.

Iron exchange between body tissues is accomplished by a plasma transport mechanism in which iron is bound to the beta-1-Globulin fraction,

and although plasma iron represents only 0.1% of the total body iron it is nevertheless essential in the transport of iron about the body. Iron entering the plasma is derived from breakdown of haem pigments, body stores and from gastrointestinal absorption, while the major part leaving the plasma is normally directed towards the bone marrow where it is used in haemoglobin synthesis.

From the earlier work of Huff et al (1950) and Wasserman, Rachkoff, Leavitt, Mayer and Port (1952) it appeared that loss of radioiron from the plasma was exponential, suggesting that no appreciable quantities of iron were being returned from the sites of removal, e.g. bone marrow during the observation period. So it was suggested that from the rate of removal of iron from the plasma following injection of ^{59}Fe-plasma, and with a knowledge of the plasma iron concentration and plasma volume, it was possible to calculate the amount of iron passing through the plasma per unit time and so to give a direct index of the rate of blood formation.

However, more recent studies into the ferrokinetics of various haemopoietic disorders have shown that the situation is more complex than that originally conceived. Bothwell, Callender, Mallett and Witts (1956) showed that there was diurnal variations of plasma iron turnover, and also found that the radioiron plasma disappearance could not always be approximated to a single exponential function. Other limitations have been found to be; dilution of the isotope in enlarged iron stores;

synthesis of other iron containing compounds, such as myoglobin and iron containing enzymes; and the early recycling of ^{59}Fe from rapidly destroyed erythrocytes. There is also the risk of error from incorporation of ^{59}Fe into the haemoglobin molecule after the sample has been taken, but before the plasma has been separated from the red cells (London, Shemin, and Rittenburg, 1948).

In spite of these limitations it is well recognised that plasma iron turnover data can yield valuable quantitative information in the study of erythropoiesis (Weinstein and Beutler, 1962).

As the ^{59}Fe enters the marrow it is incorporated into newly formed red cells, and a progressive increase in radioactivity appears in the blood as labelled red cells enter the circulation.

The rate and degree of ^{59}Fe utilisation by the red cells can yield valuable information of effective erythropoiesis, and along with plasma iron turnover, help classify the nature of the anaemia. Since it is known that increased erythropoietic activity is in general distinguished by low or normal serum iron, a rapid ^{59}Fe plasma disappearance rate, high PITR and RbcITR, with increased red cell ^{59}Fe utilisation; whereas reduced erythropoiesis shows high or normal serum iron, normal or reduced PITR and RbcITR, and low red cell ^{59}Fe utilisation.

Estimation of Iron Loss and Intestinal Reabsorption

In addition to studies of erythropoiesis using ^{59}Fe the work was extended to investigate the amount of intestinal loss and reabsorption

of iron. Since the use of ^{51}Cr-labelled red cells in fluke infected sheep had demonstrated that a massive loss of red cells was occurring into the gut, it follows that this represents a significant loss of iron into the gut of the infected animals. Though iron deficiency does not normally occur in adult ruminants, because of the nature of the diet (Blood and Henderson, 1963), it was considered that the degree of blood loss encountered in severely affected animals may be of sufficient magnitude as to increase intestinal reabsorption of haemoglobin iron lost as blood into the gut. Measurements were made on intestinal loss and reabsorption of iron after the method of Roche, Perez-Gimenez and Levy (1957), using red cells simultaneously labelled with ^{59}Fe and ^{51}Cr. The method is based on the fact that ^{51}Cr from the labelled red cells lost into the gut is not significantly reabsorbed and so gives a measure of the total blood loss into the gut. This is compared with ^{59}Fe activity in the faeces, which indicates the amount of iron lost from the animal. The difference between the two gives a measure of the amount of iron reabsorbed.

MATERIALS AND METHODS

I. Experimental Animals

All the sheep used were Blackface wethers aged 12 - 18 months, and reared parasite-free as described in general materials and methods. Two experiments were conducted. The first (Experiment I) involved three control sheep and three sheep infected with 500 metacercariae each 20 weeks previously. The second (Experiment II) contained two controls and two sheep infected 20 weeks previously with 1,000 metacercariae each. Experiment I covered 20 days, and Experiment II 16 days. In the latter case the animals were then treated with anthelmintic and the values obtained pre- and post-treatment are compared in Section IV of this thesis.

II. Labelling of Red Cells

Each sheep was injected intravenously with its own red cells labelled with ^{51}Cr by methods described earlier, each sheep receiving 10 ml of red cells labelled with 6 mc $Na_2{}^{51}CrO_4$, chromium content 37µg. At the same time in the case of Experiment I and 10 days later in Experiment II, ^{59}Fe as ferric citrate in isotonic solution was injected intravenously, each sheep receiving 1 mc (specific activity 1 mc per 88 µg iron).

III. Sampling Methods

Following injection 5 ml blood samples were taken at 10 mins and a further five samples in the following 4½ hours. For the plasma iron

date the blood samples were immediately centrifuged at 2,500 rpm for 5 - 10 mins and 1 ml of plasma carefully pipetted into counting bottles and made up to 15 ml using 0.02 N NaOH. Twice daily blood samples were then taken for three days and from there on only once daily.

Fromthe 24-hour urine collection a 15 ml sample was taken and pipetted into a counting bottle, similarly from the 24-hour faecal collection a random 15 g sample was taken and tightly packed to a volume of 15 ml for counting.

For ^{51}Cr and ^{59}Fe red cell activity determinations a 1 ml sample of whole blood made up to 15 ml with 0.02 N NaOH was used, and the count/rates corrected by the haematocrit.

IV. Analytical Methods

(a) Radioactivity Determinations

Radioactivity determinations were carried out using an Automatic Gamma Counting System (Model 4233, Nuclear Chicago Corporation, 333 E. Howard Avenue, Des Plaines, Illinois, U.S.A.).

(b) Serum Iron

The serum iron concentration was determined by the method of Ramsay (1957).

(c) Haemoglobin Concentration (Hb)

The haemoglobin concentration expressed as grams per 100 ml blood was estimated by the cyanmethaemoglobin method, Stadie (1920); van Kampen

and Zijlstra (1961). (Unicam Instruments Ltd., Cambridge, England.)

(d) Red Cell Counts

Total counts of circulating red cells were made on an electronic particle counter, (Coulter Industrial Sales Co., Elmhurst, Illinois, U.S.A.), and expressed as millions per cu. mm.

V. Calculations and Presentation of Results

(a) Haematological

(i) Mean Corpuscular Volume (MCV)

$$= \frac{PCV\% \times 10}{Rbc \text{ (millions/cu mm)}}$$

expressed as cubic microns (cμ)

(ii) Mean Corpuscular Haemoglobin Concentration (MCHC)

$$= \frac{Hb(gms\%) \times 100}{PCV\%}$$

the results being expressed as a percentage.

(b) Iron Turnover

(i) ^{59}Fe plasma clearance = $T\frac{1}{2}$ (mins)

(ii) Plasma-iron-turnover rate (PITR)

$$= \frac{0.693 \times \text{Plasma Iron Conc (mg/ml)} \times \text{Plasma Vol (ml)} \times 24}{T\frac{1}{2} \text{ (hrs)}}$$

expressed as mg/day and mg/kg/day

(iii) Percentage Iron incorporated into erythrocytes

$$= \frac{100 \times \text{Blood Vol(ml)} \times \text{Max Blood Activity (cts/ml/min)}}{\text{Total Injected Activity cts/min}}$$

(iv) Red Cell-iron-turnover rate (Rbc ITR)

$$= \frac{\text{PITR (mg/day)} \times \%\ ^{59}\text{Fe red cell Incorp}}{100}$$

expressed as mg/day and mg/kg/day.

(c) Iron Absorption

Faecal clearance values were obtained for whole blood and red cells by dividing the total daily faecal activity by the corresponding activity per millilitre of whole blood or packed red cells respectively.

The whole blood clearance value was then converted to milligrams of iron using the formula:

Iron lost/day = Whole blood clearance (ml) x Hb(gm/ml) x 3.4*.

This was done for both ^{51}Cr and ^{59}Fe, and by subtracting the ^{59}Fe values from the ^{51}Cr values the amount of reabsorption occurring was obtained.

RESULTS

Haematological Data

The results are shown in Table 6. The presence of a marked anaemia is illustrated by the significantly lower haematocrit of the infected animals (22 ± 6%) as compared to (32 ± 2%) the controls, similarly the red cell counts of the infected animals were much reduced (6.00 ± 2.51 10^6 cu mm) whilst the control values were (9.66 ± 0.51). The haemoglobin levels of the infected sheep were markedly lower (7.5 ± 3.4) than the normal values (11.2 ± 0.9).

---

* 3.4 mg per gm Hb

Table 6

Mean Haematological Data of Fluke-Infected and Normal Sheep

	Sheep		PCV (%)	Rbc (10^6 cu mm)	Hb(gms%)	MCV (cμ)	MCHC(%)
INFECTED	22	Expt. I	24	7.45	9.2	31.6	39.4
	23		22	5.81	7.6	38.3	31.1
	24		32	9.36	12.1	34.1	38.1
	47	Expt. II	13	4.45	5.4	37.6	32.0
	NT		15	2.93	3.2	39.9	27.5
	Mean		22	6.00	7.5	36.3	34.2
	S.D.		6	2.91	3.4	3.4	4.8
CONTROL	27	Expt. I	31	10.25	11.7	30.6	37.6
	28		34	9.97	12.5	34.3	36.7
	29		31	9.81	10.8	31.7	35.2
	37	Expt. II	29	9.04	10.4	30.4	38.0
	309		33	9.23	10.6	35.1	33.0
	Mean		32	9.66	11.2	32.4	36.1
	S.D.		2	0.51	0.9	2.2	2.0
	"t" test		P < 0.02	P<0.02	N.S.	N.S.	N.S.

Table 7

Mean Results of ^{59}Fe Turnover Studies in Fluke-infected and Normal Sheep

	Sheep		Serum Iron µg%	^{59}Fe Plasma $T\frac{1}{2}$ mins	PIIR mg/day	PIIR mg/kg/day	% Incorpor. by Rbc's	Rbc IIR mg/day	Rbc IIR mg/kg/day
INFECTED	22	Expt. I	170	30.0	26.75	0.71	52.3	14.0	0.37
	23		180	39.9	61.19	1.97	81.9	50.1	1.62
	24		150	78.8	22.49	0.67	65.5	14.7	0.43
	47	Expt. II	-	46.3	-	-	97.4	-	-
	NT		-	31.0	-	-	95.5	-	-
Mean			166	55.2	36.81	1.12	76.5	26.3	0.81
S.D.			15	22.8	21.22	0.74	17.4	20.6	0.70
CONTROL	27	Expt. I	160	200.8	8.74	0.26	56.4	4.9	0.15
	28		180	171.0	9.48	0.27	45.7	4.1	0.12
	29		170	129.2	15.15	0.43	53.8	3.2	0.23
	37	Expt. II	-	121.0	-	-	64.7	-	-
	309		-	150.0	-	-	75.5	-	-
Mean			170	154.4	10.92	0.32	59.2	5.7	0.17
S.D.			10	32.4	3.66	0.10	11.3	2.2	0.06
"t" test			NS	P < 0.001	NS	NS	NS	NS	NS

The MCV values were not significantly different between the two groups though the most anaemic animals had higher values than the controls. Conversely the MCHC results of the most severely affected animals were lower than the control sheep, though again there was no significant difference between the two groups.

Iron Turnover Data

The results of the iron turnover studies are shown in Table 7. As can be seen the serum iron levels of the two groups were identical, however the ^{59}Fe plasma disappearance values show striking differences, see Fig. 2, the $T\frac{1}{2}$ for the infected animals being (55.2 ± 22.8 mins) whilst the control mean half life was (154.4 ± 32.4 mins) showing that the uptake of iron by the marrow was three times as rapid in the infected animals. Similarly the plasma-iron-turnover rate was increased in the fluke-infected sheep to nearly four times the control level, though due to the scatter and size of groups it was not statistically significant.

The percent of the injected dose incorporated into the circulating erythrocytes was higher and more rapid, see Fig. 3, in the infected sheep, especially in the most badly affected cases.

Again the red cell-iron-turnover studies show that the rate was greatly increased in the infected animals to nearly five times the controls.

The infected sheep showed increased loss of both ^{59}Fe-rbc and ^{51}Cr-rbc compared to the controls, Figs. 3 and 4.

Fig. 2. Plasma ^{59}Fe Disappearance Curves in a Fluke-Infected and Normal Sheep

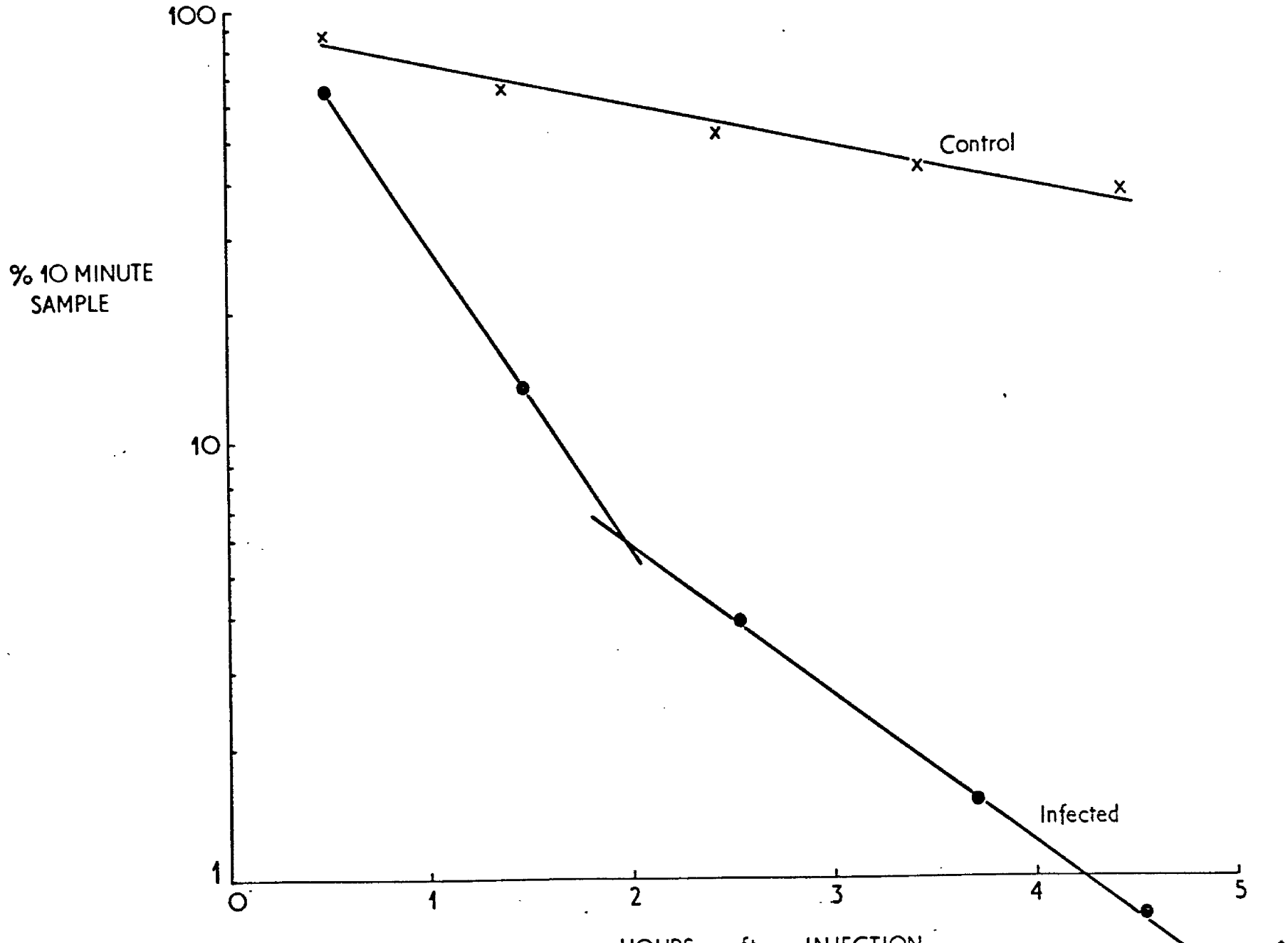
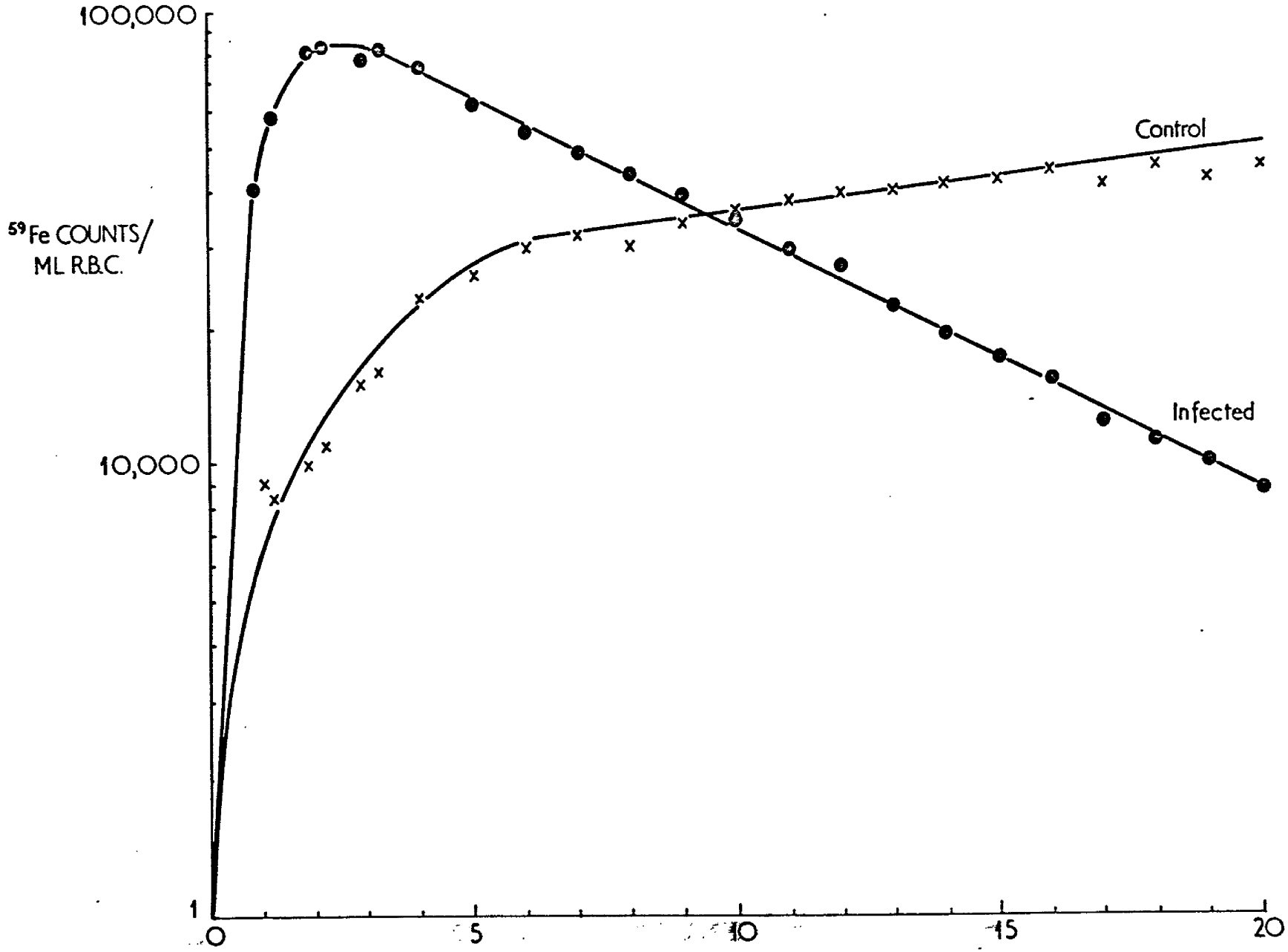


Fig. 3. ^{59}Fe-Red Cell Activity in a Fluke-Infected and Normal Sheep



Intestinal Loss and Reabsorption of Iron

The results are shown in Table 3. They demonstrate that in the control animals a small loss of iron was occurring into the gastro-intestinal tract, as shown by both the ^{51}Cr and ^{59}Fe-rbc results. It should be noted that the loss calculated from the ^{59}Fe data consistently gives a slightly higher intestinal loss than that calculated from the ^{51}Cr data. A similar situation exists in the infected sheep in Experiment I, showing that no reabsorption of haemoglobin iron was occurring. Sheep No. 23 shows an erroneously excessive loss calculated from ^{59}Fe rbc data and is not therefore included in the mean results. However the results from the two most anaemic sheep (Experiment II) did indicate that some reabsorption of haemoglobin iron was taking place, especially in the most severely affected sheep, NT, in which 15 mg iron/day was apparently reabsorbed.

It should be noted that the daily whole blood loss calculated from the ^{51}Cr rbc results and expressed as loss of blood (ml)/fluke gave higher figures than those obtained in the previous ^{51}Cr experiment, namely a mean of 0.489 ml/fluke, consistent with the findings described in Section III of the thesis, that the blood loss increased with time during the 20 weeks following infection.

Fig. 4. ^{51}Cr-Red Cell Activity in a Fluke-Infected and Normal Sheep

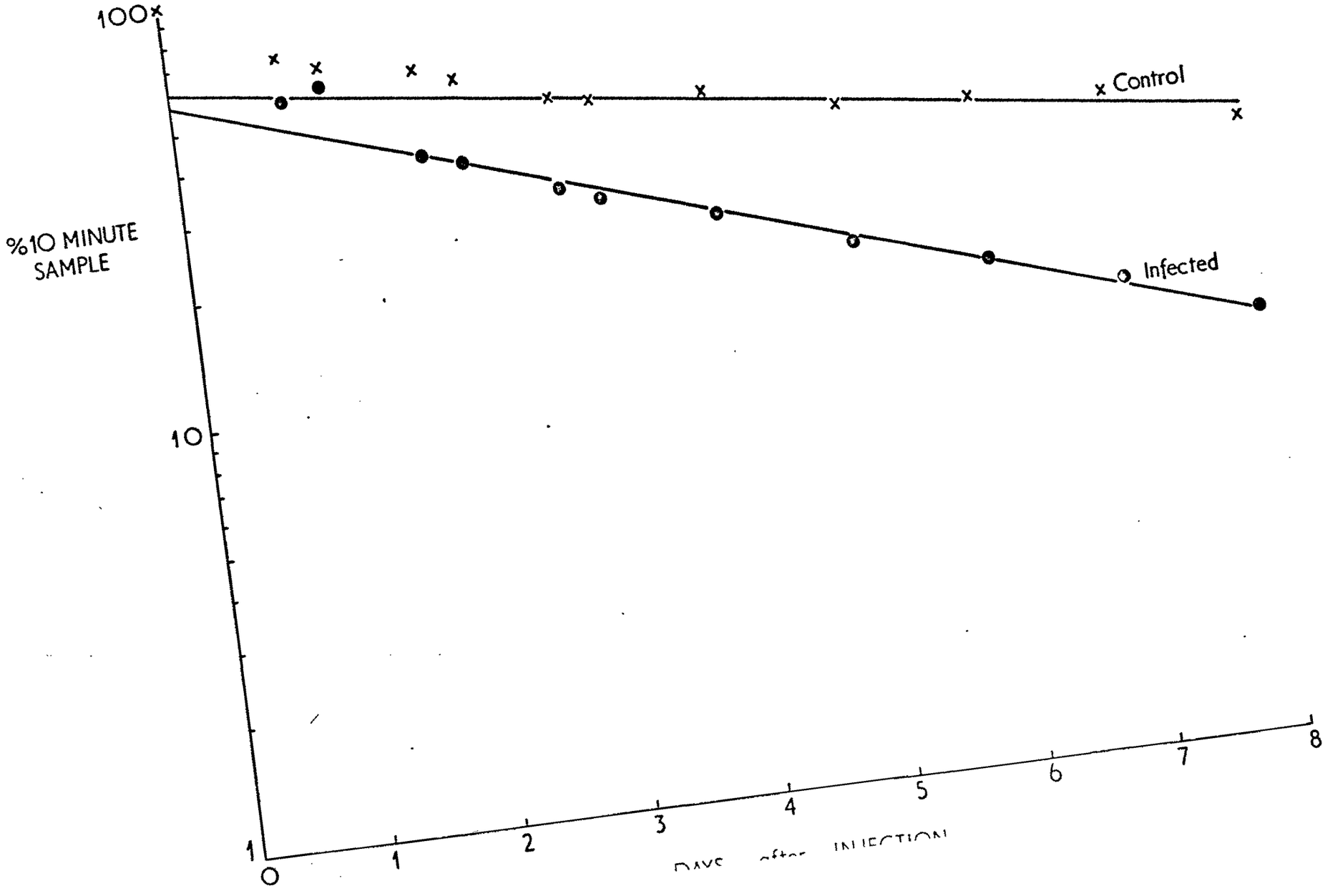


Table 8

Mean Results of Iron Absorption Studies in Fluke-infected and Normal Sheep

	Sheep		^{51}Cr rbc Clearance	^{59}Fe rbc Clearance	Iron Loss mg/day Intestinal	Iron Loss mg/day Faecal	Reabsorption mg/day	Fluke Burden
INFECTED	22	Expt. I	17.4	17.8	23.3	23.9	Nil	125
	23		27.0	(38.5)	31.3	(44.7)	"	283
	24		11.6	12.7	15.2	16.6	"	79
	47	Expt. II	19.1	17.1	17.9	16.0	1.9	-
	NT		33.1	19.1	35.6	20.6	15.0	-
Mean			21.6	76.7	24.7	19.3		
S.D.			9.4	2.8	8.7	3.7		
CONTROL	27	Expt. I	0.6	1.6	0.8	2.1	Nil	Nil
	28		0.6	2.5	0.8	2.4	"	"
	29		0.5	1.2	0.6	1.4	"	"
	37	Expt. II	0.4	1.9	0.3	1.4	"	"
	309		0.9	1.6	0.7	1.3	"	"
Mean			0.6	1.8	0.6	1.7	-	-
S.D.			0.2	0.5	0.2	0.5		
"t" test			P<0.001	P<0.001	P<0.001	P<0.001		

DISCUSSION

The haematological results obtained show that there was a well developed anaemia present in four of the five infected sheep as judged by lowered PCV and red cell counts, and reduced haemoglobin levels. The mean corpuscular volume and mean corpuscular haemoglobin values show the anaemia to be of a normocytic normochromic type, though in the three most severely infected animals there was a trend towards a macrocytic hypochromia. The observations of Sinclair (1962; 1964) showed that the anaemia which developed in experimentally infected sheep was of a normochromic, normocytic type, though no figures for MCV or MCHC were quoted. Work by Reid (1965,) who studied heavier infections in younger animals than those of Sinclair, found that initially the anaemia was of a normocytic, normochromic type but as the disease progressed reticulocytes appeared in the circulation accompanied by a variable macrocytosis, and an occasional hypochromia developed in the longest surviving animals. Similarly recent studies of natural infections in lambs by Ross (1967a and b) showed the anaemia to be macrocytic and hypochromic.

It has been found that a macrocytic hypochromic anaemia is more quick to develop following experimental infections in laboratory animals, (Urquhart, 1955; Thorpe, 1963), than in sheep. Other investigations suggest that the type of anaemia which develops in sheep varies with the age of the host, and the level and stage of the infection, as Gruneall (1955) showed that removal of large quantities of blood from sheep over

a short period of time will produce a macrocytic, hypochromic anaemia, while Sinclair (1964) removing only small quantities of blood over a longer period produced only a normocytic normochromic anaemia.

The serum iron estimations demonstrate that in the sheep examined no iron deficiency was present, however unfortunately the most severely affected sheep's serum iron concentration was not determined and it may be that in this animal an iron deficiency was developing as judged by the mild hypochromia.

In all the infected animals it was shown that the ^{59}Fe plasma disappearance rates were greatly increased indicating an increased requirement for iron by the bone marrow of these sheep. These results agree closely with those of Symons and Boray (1967). In their studies the fate of ^{59}Fe labelled plasma was followed in plasma, bone marrow, spleen, liver, red cells and faeces in 6, 8 and 13 week old fluke infections of sheep. They concluded that the transference to the bone marrow and thence to the erythrocytes was greatly increased probably to a maximum, and only in severe infections of several months standing were plasma iron levels decreased.

From earlier work by Sinclair (1964) using radioiron in investigations on the aetiology of the anaemia associated with ovine fascioliasis in which ^{59}Fe plasma clearances were compared between infected sheep, animals which had been bled daily, and controls, he came to the erroneous conclusion that the anaemia was not caused by blood loss but was due to decreased erythrocyte production, because the bled sheep did not give

similar results to the infected animals. However more recent work has shown that the amounts of blood removed from the bled group in an attempt to simulate blood loss due to the flukes was too small compared to the loss found to be occurring in even moderate infections, and this probably accounts for the discrepancy between the infected and bled groups. In similar later experiments by Sinclair (1965), in which the amount of blood removed daily from the bled group was increased from 60 ml to 130 ml, the ^{59}Fe plasma disappearance rates and ^{59}Fe utilization curves of the infected and bled sheep were very similar.

The values obtained for the red cell-iron-turnover-rate can be shown to be approximately correct by comparing them with values obtained from the red cell life span, by estimating the amount of blood needing to be replaced each day and converting this to milligrams of iron. Unfortunately there is conflicting evidence as to the red cell life span of sheep, the results varying with the methods used. ^{51}Cr studies reported by Hansard and Kincaid (1956) indicated a potential red cell life span of 50 - 60 days, whilst studies using methyene-labelled ^{14}C glycine estimated the life span to be 146 days (Judd and Matrone, 1962). However there is evidence of both breed and age variations (Carter, Matrone and Metzler, 1965; Tucker, 1963). The latter worker used serological, ^{59}Fe and ^{51}Cr techniques in her investigations and concluded that the average red life span for adult sheep ranged from 76 to 133 days (serological method) and from 70 - 153 days (^{59}Fe method). If the red

cell life span then is taken to range from 70 to 150 days this represents in terms of milligrams of iron required per day an amount of 4 - 8 mg, which agrees well with the Rbc-ITR mean value of 5.7 mg/day obtained in the control animals in the present experiment.

Previous studies on ferrokinetics of normal sheep were carried out by Baker and Douglas (1957) in which haempoietic activities in normal sheep and cattle were estimated from ^{59}Fe plasma disappearance rates and red cell utilization curves. Their work included an investigation of one year old lambs, and the mean values they obtained were PITR 0.56 mg/kg/day and Rbc.ITR of 0.39 mg/kg/day as compared to 0.32 and 0.17 respectively obtained for the control animals in the present study. The lower values are probably due to differences in ^{59}Fe plasma disappearance $T\frac{1}{2}$ measurement, since Baker and Douglas (1957) found a two-component clearance curve for most of their animals and assumed the second component to be representative of bone marrow uptake. They obtained a mean $T\frac{1}{2}$ of 94.2 minutes whilst in the present study 154.4 minutes was the mean control $T\frac{1}{2}$.

There are few references in the literature relating to haemoglobin iron absorption in ruminants, though the subject has been extensively studied in man and laboratory animals. The original work by Clark et al (1962) consisted of two separate experiments in sheep, the first to determine the quantity of labelled red cells that would pass through the digestive tract unabsorbed in normal animals, and in the second an attempt was made to quantitate blood loss due to Haemonchus contortus

infections using ^{59}Fe red cells which were given both orally and directly into the abomasum, and during the unspecified collection period 79% of the radioactive iron given in the abomasum was recovered in the faeces, while only 59.5% of the ^{59}Fe administered orally was recovered. In the second experiment on parasitized sheep using doubly labelled red cells it is apparent from their results that in the most heavily infected animals there was some reabsorption of haemoglobin iron occurring.

Studies by Georgi (1964) on the absorption of haemoglobin iron-59 both in normal sheep and animals bled at the rate of 300 ml/week for several months showed that despite the increased requirements for iron by the bled sheep there was no significant absorption of haemoglobin ^{59}Fe and so for practical purposes could be ignored as a source of error in estimating parasitic blood loss in sheep using ^{59}Fe in whole body counting techniques. These results do not contradict the ones presented in this thesis, since in the study by Georgi only 300 ml per week was taken, whilst sheep NT was losing 1,540 ml whole blood per week and would therefore have a much greater demand for iron, hence the re-absorption of approximately 1/3 of the haemoglobin iron lost into the gut. Similar recent investigations by Dargie (1969) in fluke-infected rabbits failed to demonstrate any reabsorption of haemoglobin iron despite the presence of a hypochromia.

It would appear then that in sheep infected with F. hepatica reabsorption of haemoglobin iron can occur but so far there is evidence for it only in the most anaemic animals.

SUMMARY

The ferrokinetics of fluke-infected and normal sheep were studied using ^{59}Fe. The investigations involved estimation of erythropoietic activity by analysis of ^{59}Fe plasma disappearance rates and red cell utilization curves, and estimations of haemoglobin iron reabsorption using red cells simultaneously labelled with ^{51}Cr and ^{59}Fe. It was shown that ^{59}Fe plasma disappearance rates were greatly increased, as were plasma-iron-turnover-rates and red cell incorporation of iron, the results being consistent with increased erythropoietic activity in the infected animals. Only in the most severely infected sheep was there evidence of reabsorption of haemoglobin iron.

SECTION II

METABOLIC STUDIES ON THE PLASMA PROTEIN CHANGES ASSOCIATED WITH CHRONIC OVINE FASCIOLIASIS

Introduction

It is now well established that marked serum protein changes occur in animals suffering from chronic fascioliasis. The most obvious features are a reduction in serum albumin and a rise in gamma globulin concentration. These changes in fluke-infected sheep have been reported by Dalian (1940), Noguchi, Kirisawa, Sugiura and Komine (1958), Ibrovic and Gall-Palla (1959), Nikolic, Nikolic, Novenic, Bugarski, Parlovic, Ciric, Mladenovic and Polic (1962) and in a detailed study on experimental infections by Sinclair (1962). Sinclair showed that there was a rise in serum globulin at five weeks post infection, the gamma globulin contributing the major part of the increase, and as a result the serum protein concentration rose, but not to the extent expected because of a concomitant fall in serum albumin concentration. Later the globulin fraction returned to normal but the pronounced hypo-albuminaemia persisted.

Similarly in later plasma protein studies in sheep Furmaga and Gundlach (1967b) showed significant increases in total protein, β globulins and γ globulins; a significant decrease in albumin; and no marked difference in α globulins and fibrinogen; when infected animals were

compared with a parasite-free control group. A similar picture has been observed in other species infected with *F. hepatica*, e.g. in rabbits (Secretan and Bickel, 1960), in rats (Thorpe, 1965), in cattle (Ross, Todd and Dow, 1966).

Conventional analytical techniques have done little to explain the origin of the hypoalbuminaemia and hypergammaglobulinaemia. Popular theories for the hypoalbuminaemia have been based on the fact that albumin is synthesised in the liver and that therefore liver damage caused by the migrating flukes might interfere with albumin synthesis, plus a possible loss of albumin in the inflammatory exudates in the damaged liver (Sinclair, 1962; Thorpe, 1965).

Since plasma protein levels are dependent on a balance between synthesis and catabolism a true explanation of the altered plasma protein levels can only be obtained if either synthesis or catabolism can be directly determined. With the development of the technique for trace labelling serum proteins with isotopes of iodine it becomes possible to study albumin turnover directly. Hypoalbuminaemia associated with ostertagiasis in cattle has been shown to be due to increased gastro-intestinal loss of albumin (Mulligan, Dalton and Anderson, 1963; Nielsen, 1966; Halliday, Mulligan and Dalton, 1968), and the present investigations were undertaken to see whether an analogous situation existed in fluke-infected sheep. If it could be shown that a loss of albumin was occurring into the gut of parasitized sheep then it would add further weight to the evidence produced in section I of this thesis that the

mature flukes were causing a loss of whole blood.

In the present investigations the use of albumin labelled with various isotopes is described in an attempt to measure the turnover rate and loss of albumin in chronic ovine fascioliasis and hence determine the true aetiology of the hypoalbuminaemia.

The metabolism of IgG was also determined in a parallel study with albumin in an attempt to elucidate the rise in gamma globulin, concomitant with the fall in serum albumin.

A. TURNOVER STUDIES USING ^{131}I-ALBUMIN IN SHEEP CHRONICALLY INFECTED WITH F. HEPATICA

Introduction

The introduction of isotopes in biological investigations provided the opportunity of labelling proteins with isotopes and facilitated a more dynamic approach to the problems of protein metabolism than earlier techniques could supply. Early isotopic techniques involved endogenous labelling with ^{14}C or ^{35}S and while there was no risk of denaturation in these methods, they suffered from the major disadvantage that label released from the catabolised protein was reutilised in the synthesis of new protein molecules, both serum and intracellular (Margen and Tarver, 1956; Goldsworthy and Volwiler, 1957). Other disadvantages were that the technique was time consuming and the final yield of labelled protein poor.

Following the experiments with ^{131}I in 1951 by Sterling a simple technique became available for trace labelling proteins with iodine isotopes, though early attempts showed that the labelled protein could be denatured if too heavily iodinated (Armstrong, Kukral, Hershman, Macleod, Wolter and Bronsky, 1955). This technique does not suffer from the fault of reutilisation of isotope.

With the improved method of McFarlane (1956, 1958) a very useful technique became available for the study of protein metabolism. The absence of denaturation due to iodination was demonstrated in comparative

studies with ^{14}C-preparations (Campbell, Cuthbertson, Matthews and McFarlane, 1956), and in analbuminaemic subjects in which the behaviour of ^{131}I-labelled and of native albumin was found to be identical (Bennhold and Kallee, 1959).

It has been widely applied to situations in which abnormal plasma protein loss occurs such as types of nephritis, severe burns and other serious skin conditions. ^{131}I-albumin has also found extensive application in studies of various abnormalities of the gastrointestinal tract in which excessive protein loss has been shown to be occurring, i.e. the "protein-losing gastroenteropathies" (Jarnum, 1963).

A number of methods have been used in the preparation of ^{131}I-albumin, but the one generally favoured is that of McFarlane (1958) in which the protein in slightly alkaline solution is treated with iodine monochloride to which has been added a solution of radioactive iodine (as carrier-free iodide). Using this method, it is theoretically possible to achieve nearly 100% uptake of the label by the protein. Buffering of the ICI-^{131}I mixture to a weakly alkaline reaction transforms the iodine monochloride to hypoiodite, which is apparently a necessary prerequisite for substitution of iodine in the benzene ring of tyrosine to form mono- and di-iodotyrosine.

In the use of iodine labelled plasma protein for the study of protein turnover certain precautions must be taken if consistent and valid results are to be obtained. These have been outlined by Freeman

(1966). They can be divided into three main categories:- the quality of the injected labelled protein; the state of the subject; the mathematical assumptions about the protein pools of the body and methods of calculation of catabolic rates.

The quality of the injected material should be such that all the activity is protein bound. This can easily be examined by precipitating the protein with trichloroacetic acid and measuring the percentage of the total activity in the precipitate. The activity should be bound to a single protein, and not a mixture of proteins. This can be checked by various in vitro techniques such as electrophoresis or gel filtration. There should be no significant denaturation of the labelled protein. One important cause of denaturation is from over-iodination of the protein. Albumin and IgG can be iodinated to a mean level of 1.5 atoms per molecule without causing denaturation. Self-irradiation of the molecule can also cause denaturation, though this can be minimised by adding unlabelled protein to the preparation once iodination is complete. The major cause of denaturation is in the preparation of the protein prior to labelling. Gross denaturation is easily detected in that the protein is rapidly eliminated from the body. Minor alterations which could significantly affect the turnover results are more difficult to demonstrate though the finding of a faster rate of breakdown at the beginning of the study strongly suggests that the preparation is slightly denatured.

The state of the subjects under investigation also require to be taken into account for various reasons. Iodine remains firmly bound to the protein until catabolism when the iodine is rapidly excreted via the urine, though some iodine is excreted in saliva and gastric juice, due to reabsorption in the small intestine. Faecal excretion is normally negligible. Rapid excretion of ^{131}I following degradation of the labelled protein is essential if the calculation of turnover rate is based on excreted activity. To achieve this it is necessary that uptake of ^{131}I by the thyroid gland has been blocked, for all practical purposes, by the administration of non-radioactive iodine to the subjects four days prior to injection of the ^{131}I-albumin and throughout the observation period. Following injection of ^{131}I-albumin, there is a delay of 1 - 2 days before excretion of ^{131}I released from ^{131}I-albumin by catabolism parallels renal excretion of ^{131}I, but after this initial period, providing the catabolic rate is low the renal excretion of iodine does not affect the turnover rates obtained. However, when the catabolic rate is greatly increased there may be a degree of iodine retention and this may result in an underestimate of the turnover rate of the protein if catabolism is based on excreted activity. Ideally the subjects should be in a state of equilibrium throughout the period of study as judged in practice by a constant body weight and an unchanging plasma protein concentration. This situation is difficult to obtain in some studies particularly those involving diseased subjects in which weight loss and hypoproteinaemia are major characteristics of

the disease, e.g. diarrhoeal disorders and intestinal parasitism. In these cases it has been recommended that weight loss during the study should not exceed 10%, that appetite and general clinical condition should remain good, and that the variations in serum protein concentration should not exceed the variations in the normal control animals (Nielsen and Nansen, 1969).

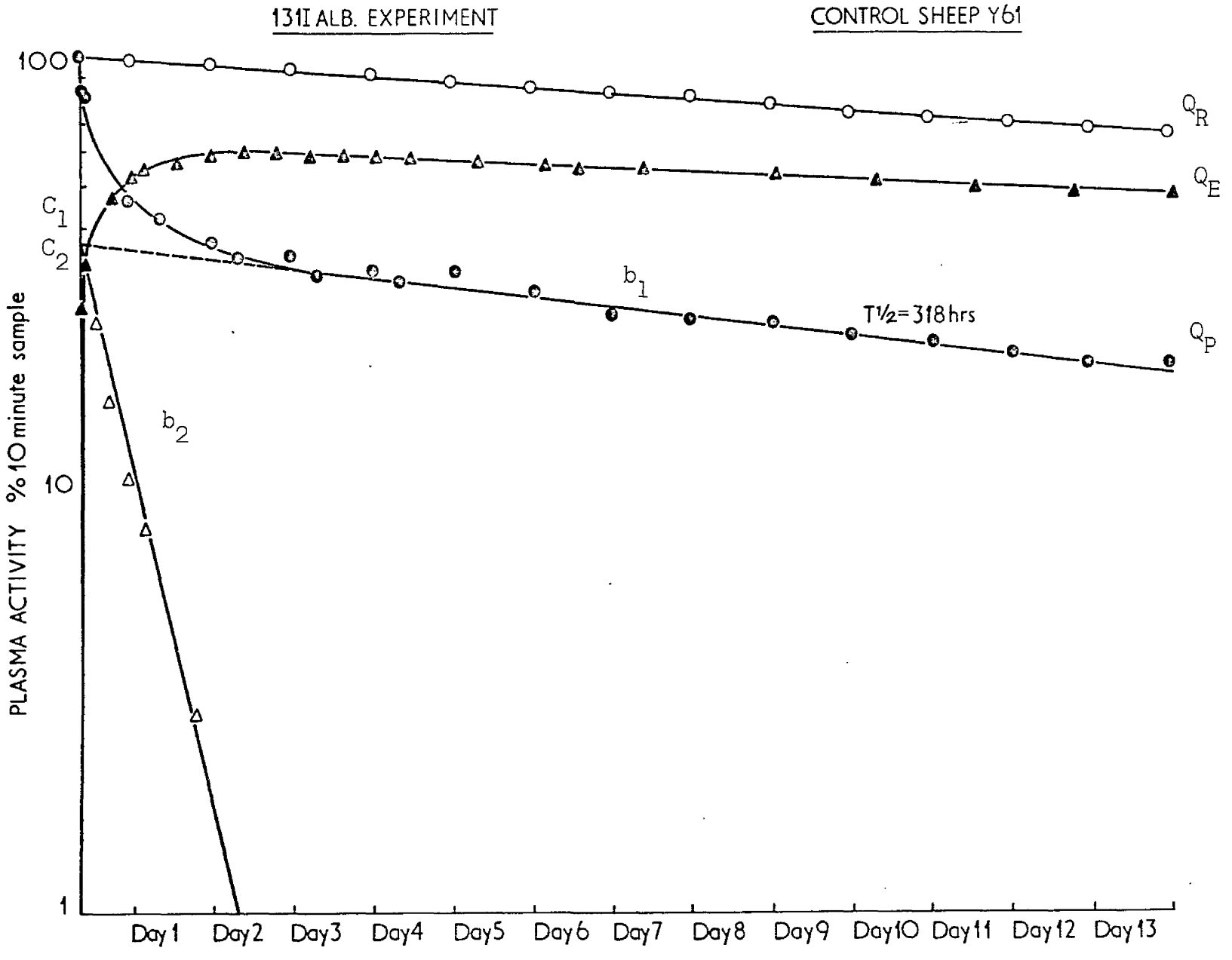
The mathematical methods used in the calculation of the distribution and catabolism of the labelled protein require that various assumptions be made about the sites of synthesis and catabolism as well as the body pools. Most plasma proteins are synthesised in the liver and are discharged into the bloodstream directly,or indirectly via the liver lymph, where they are distributed in the body pools and finally catabolised. The catabolic rate can be determined by two main methods. One depends on the analysis of the plasma disappearance curve, the other on the measurement of excreted activity.

1. Methods which depend on analysis of the plasma activity disappearance curve

(a) Sterling (1951a)

Following injection of ^{131}I-albumin, the plasma activity (Q_p) falls in a characteristic manner, see Fig. 5. Initially there is a rapid fall in activity which is followed later by a more gradual exponential decline. The steep part of the curve is largely due to labelled albumin leaving the circulation to enter the extravascular

Fig. 5. Turnover of Labelled Albumin in a Normal Sheep



pool. The exponential part of the curve, i.e. from day 4 onwards, was considered by Sterling (1951a) to be solely due to catabolism, and suggested that the half life gave a direct measurement of albumin catabolism,

$$K = \frac{0.693}{T_{\frac{1}{2}} \text{ (days)}}$$

This holds only if albumin is catabolised uniformly in both extravascular and intravascular compartments. It is now known that this is not so and that albumin catabolism is essentially intravascular. This being so the final slope in the disappearance curve represents redistribution of albumin as well as catabolism, and so cannot give a true measure of the catabolic rate. It does however give an approximation to it, hence the expression "apparent half life".

(b) Matthews (1957)

This method assumes that body albumin represents a mammillary multicompartment system consisting of the plasma, and small extravascular compartments (lymph, interstitial fluid, etc.) which are in a constant state of flux with albumin in the plasma, where degradation takes place.

The slope constant (b_1) is obtained from the linear portion of the plasma activity curve,

$$b_1 = \frac{0.693}{T_{\frac{1}{2}} \text{ (days)}}$$

and C_1 is the intercept of this line extrapolated to zero time, Fig. 5. Subtraction of this extrapolated portion of the line from the original

steep portion of the curve gives a second line. This line represents the transfer of labelled albumin to the extravascular compartments, and its slope constant (b_2) is obtained in a similar manner to b_1.

The intercept of the second line with the ordinate gives the value C_2, usually only two exponentials are involved.

The fraction of the intravascular pool catabolised per day is obtained from the equation

$$K = \frac{1}{\frac{C_1}{b_1} + \frac{C_2}{b_2}}$$

The slope b_1 and the intercept C_1 can be determined fairly accurately from the graph, but b_2 and C_2 are less accurate since they are obtained by difference. As b_2 is much greater than b_1, K is not grossly influenced by errors in b_2. This method of calculation of turnover rate in general agrees well with methods based on excreted activity. It is particularly useful in studies in which complete collection of urine and faeces are difficult or impossible, e.g. diarrhoeal conditions of large animals, studies in the field, etc.

2. Method which depends on the excreted activity:

Campbell et al. (1956)

This method based on the daily activity excreted in the faeces and urine, assumes that following catabolism of the labelled protein the liberated isotope is rapidly and quantitatively excreted, hence

$$F(CA) = \frac{\text{Total Excreted Activity/Day}}{\text{Plasma Activity}}$$

These workers also devised an equilibrium time method for computation of the total albumin pool. The extravascular activity (Q_E) obtained by subtracting the plasma activity (Q_p) from the retained activity (Q_R) is plotted, see Fig. 5. It was found that Q_E increased to a maximum value and then gradually declined. The method assumes that when Q_E is maximal, an identical amount of ^{131}I-albumin is entering and leaving the extravascular pool and a momentary equilibrium exists, so that the ratio of $Q_E / Q_p = EA/CA$ and hence

$$TA = \frac{CA(Q_p + Q_E)}{Q_p}$$

In the following experiment both the distribution and catabolism of ^{131}I-albumin was studied in fluke-infected and normal sheep.

Materials and Methods

I. Experimental Animals

A total of twelve sheep were studied, six of which had been infected with Fasciola hepatica three months previously. Three of the sheep had been dosed with 1,000 metacercariae each and the remaining three with 500 each. The other six sheep served as parasite free controls. All the animals used were Blackface wethers reared as described in general materials and methods, and aged approximately 8 months. Drinking water containing 0.002% KI and 1% NaCl was given four days prior to the injection of ^{131}I-albumin and from there on continuously throughout the experiment. Any sheep which refused to drink was dosed orally with

concentrated KI and NaCl equivalent to 4 litres of the standard drinking water daily.

II. Preparation of ^{131}I-albumin

Commercial sheep albumin (Cohn Fr. V. Pentex Incorp., Kankakee, Illinois, U.S.A.) was used. Labelling with ^{131}I was carried out by the method of McFarlane (1958).

(a) Iodine monochloride

This was prepared according to the method of Vogel (1951). 5.00 gm KI (AR) + 3.22 gm KIO_3(A.R.) were dissolved in 37.5 ml of distilled H_2O. To this was added 37.5 ml of conc. HCl + 5 ml CCl_4. The mixture was then shaken vigorously to promote the reaction.

$$2HI + HIO_3 + 3HCl = 3ICl + 3H_2O$$

This stock solution contained approximately 147 mg of iodine per ml as iodine monochloride, and by a dilution with saline of 1 to 350 a solution containing 0.42 mg of iodine per ml as iodine monochloride was obtained.

(b) Glycine buffers

Two glycine buffers were required. Buffer A with a pH 8.5 was prepared by adding 9 ml molar glycine in $^{M}/_{4}$ NaCl solution to 1 ml N.NaOH. Buffer B, pH 9.0, was prepared by the addition of 8 ml molar glycine in $^{M}/_{4}$ NaCl solution to 2 ml N.NaOH.

Buffer A is used for conversion of iodine monochloride to hypoiodite, and Buffer B to give the protein an alkaline reaction of pH 9.0 - 9.5.

(c) Iodination of the protein

6.0 ml of ICl solution (containing 0.42 mg I/ml) was taken, and to this was added 10 millicuries carrier-free radioiodide. The iodine monochloride was then converted to hypoiodite by the addition of 15 ml of Buffer A. This was immediately added to the buffered protein solution (30 ml 2% sheep albumin + 15 ml Buffer B), and transferred to a dialysis sac. Carrier protein was then added to reduce the specific activity of the preparation to less than 5 μc/mg protein. The labelled protein preparation was dialysed for 43 hours at 5°C against two changes of 20 lit 0.9% NaCl, and finally spun for 30 minutes at 1,500 rpm before being dispensed for injection.

6.0 ml ICl solution containing 0.42 mg I/ml as ICl used to label 600 mg albumin gives a mean level of 2 atoms of iodine per molecule of protein assuming 100% incorporation. Since in practice incorporation is probably in the order of 50 - 60%, then the number of atoms per molecule is approximately 1:1 in the above preparation.

III. Injection and Sampling

Following dialysis the labelled preparation was divided between six syringes and each weighed, a small amount being retained for the standard. Following intravenous injection and reweighing of the empty syringes blood samples were taken at 10 and 20 mins. post injection and then at frequent intervals throughout the experiment. At each bleeding 4 - 5 ml of heparinized blood was taken, and from this 1 ml of plasma

assayed for radioactivity. Urine and faeces were collected each 24 hours as described in general materials and methods, and aliquots taken for radioactivity determinations. The experiment lasted two weeks.

IV. Calculation and Expression of Results

(a) Albumin pools

(i) Intravascular pool (CA)

From the radioactivity of the plasma sample collected 10 mins post injection, the plasma volume of each animal was determined (dilution principle described in general materials and methods) and this together with the appropriate serum albumin concentration enabled the intravascular pool (CA) to be calculated, and expressed as gms/Kg body weight.

(ii) Extravascular pool (EA)

The determination of extravascular pool was based on the "equilibrium time" method of Campbell et al (1956) described in the introduction, and hence the ratio of EA/CA was obtained.

(b) Catabolic rate

(i) "Apparent half life"

The half-life $T\frac{1}{2}$ of the exponential portion of the plasma activity disappearance curve was taken as an approximate index of catabolic rate.

(ii) Fractional catabolic rate (F(CA))

This more precise method of Campbell et al (1956) determines the fraction of the intravascular pool catabolised/24 hours and is obtained thus:

$$F(CA) = \frac{\text{Total Excreted Radioactivity (cts/sec) / 24 hrs}}{\text{Plasma Activity (cts/sec/ml) x Plasma Vol (ml)}}$$

(iii) Absolute amounts of albumin catabolised

Grams of albumin catabolised per day = F(CA) x intravascular pool (gms) and expressed as gms/kg/day.

(iv) Catabolic rate

The catabolic rate was also determined by the method of Matthews (1957) based exclusively on a mathematical analysis of the plasma activity disappearance curve.

$$K \text{ (catabolic rate)} = \frac{1}{\frac{C_1}{b_1} + \frac{C_2}{b_2}}$$

C = intercept at zero time b = slope constant

(c) Faecal clearance of plasma ml/day

The total faecal activity for each 24 hour collection period was divided by the activity per ml of plasma taken at the beginning of the collection period to give a daily faecal clearance of plasma.

Results

The albumin concentrations and distribution in the two groups of animals are shown in Table 9, along with the body weights and fluke burdens. All the infected animals were hypoalbuminaemic though there was no significant difference between the groups in the size of the intravascular pool of albumin when expressed as ml/kg body weight. This is due to the relative increase in the plasma volume in the infected group.

The distribution of albumin was altered in the parasitized sheep, as shown by the reduced EA/CA ratio.

Table 9

Albumin Concentration and Distribution in Fluke-infected and Control Sheep

	Sheep	Body Wt. (kg)	Plasma Albumin (Gm%)	Plasma Volume (ml/Kg)	CA (gm/Kg)	EA (gm/Kg)	EA/CA	Fluke Burden
INFECTED	79	22.7	1.93	50.9	0.98	1.07	1.09	238
	81	26.1	1.77	43.8	0.77	1.30	1.68	507
	831	15.1	1.83	73.0	0.69	1.35	1.95	*
	832	21.3	2.15	47.2	1.02	1.38	1.35	*
	84	27.0	1.38	56.4	0.76	0.98	1.28	626
	85	20.4	1.32	61.1	0.81	1.04	1.28	*
Mean		22.1	1.73	55.2	0.84	1.19	1.43	
S.D.		4.3	0.32	10.6	0.13	0.17	0.32	
CONTROL	61	34.0	2.70	35.7	0.96	1.69	1.76	nil
	701	26.0	2.27	48.7	1.11	1.78	1.60	nil
	702	30.4	2.38	37.4	0.89	1.25	1.40	nil
	72	36.2	2.39	30.5	0.73	1.24	1.70	nil
	77	31.3	2.76	32.4	0.69	1.53	1.72	nil
	80	31.9	2.91	51.1	1.48	2.40	1.62	nil
Mean		31.6	2.57	39.3	1.01	1.65	1.63	
S.D.		3.5	0.25	8.6	0.26	0.43	0.13	
"t" test		P<0.01	P<0.001	P<0.02	NS	p<0.05	NS	

* Fluke burden not determined.

Table 10

Fraction of the Intravascular Pool of Albumin Catabolised per Day in Fluke-infected Sheep

Sheep		79	81	831	832	84	85
Day	3	0.156	0.072	0.100	0.088	0.238	0.189
	4	0.137	0.081	0.117	0.092	0.316	0.201
	5	0.130	0.095	0.149	0.134	0.142	0.235
	6	0.159	0.112	0.200	0.101	0.255	0.453
	7	0.143	0.123	0.164	0.116	0.276	0.314
	8	0.147	0.127	0.097	0.104	0.347	0.226
	9	0.177	0.177	0.121	0.183	0.365	0.323
	10	0.158	0.241	0.141	0.059	0.397	0.351
	11	0.151	0.264	0.121	0.124	0.361	0.736
	12	0.151	0.117	0.130	0.147	0.426	-
	13	0.196	0.191	0.235	0.108	0.524	0.356
	14	0.095	0	-	0.103	-	0.239
Mean		0.150	0.145	0.143	0.114	0.332	0.329
S.D.		0.022	0.063	0.041	0.032	0.102	0.157

Tables 10 and 11 give the fractional catabolic rates for the individual infected and control sheep throughout the experimental period. Though there was some variation on a day to day basis, there was no outstanding trend of increasing or decreasing catabolic rate relative to time within either group. Figures 5 and 6 illustrate the plasma activity disappearance rate in a control and infected sheep. They illustrate the method of calculation of the catabolic rate by Matthews (1957) method in which the slope b_2 is obtained by subtracting the extrapolated slope b_1 from the initial steep portion of the plasma disappearance curve.

The figures illustrate the greatly increased rate of disappearance of ^{131}I-albumin from the circulation of the infected sheep.

The catabolic rates of albumin for the two groups are given in Table 12. The fraction of the intravascular pool catabolised calculated from the excreted activity in the infected sheep was 0.202 ± 0.100/day compared with 0.081 ± 0.017/day in the controls.

Similarly the catabolic rate calculated by mathematical analysis of the plasma activity showed greatly increased catabolism in the infected sheep (0.239 ± 0.079) compared with the controls (0.093 ± 0.028).

The absolute amount of albumin catabolised per day in the diseased group was 0.165 ± 0.074 gm/kg/day, which was significantly higher than the normal values (0.079 ± 0.014).

Plasma half life values were greatly reduced in the infected animals (161 ± 67 hr) compared with 375 ± 76 hr in the control group.

Table 11

Fraction of the Intravascular Pool of Albumin Catabolised per Day in Normal Sheep

Sheep	61	701	702	72	77	80
Day 3	0.109	0.085	0.098	0.093	0.086	0.024
4	0.114	0.109	0.082	0.103	0.099	0.039
5	0.087	0.093	0.079	0.093	0.093	0.121
6	0.062	0.083	0.064	0.083	0.061	0.045
7	0.107	0.094	0.075	0.082	0.061	0.062
8	0.130	0.110	0.058	0.082	0.094	0.026
9	0.132	0.105	0.090	0.092	0.098	0.056
10	0.135	0.099	0.068	0.094	0.080	0.055
11	0.100	0.043	0.099	0.099	0.048	0.028
12	0.088	0.091	0.056	0.084	0.113	0.025
13	0.079	0.103	0.091	0.100	0.090	0.044
14	-	-	0.047	-	0.046	0.036
Mean	0.104	0.092	0.075	0.089	0.081	0.047
S.D.	0.022	0.017	0.014	0.010	0.020	0.024

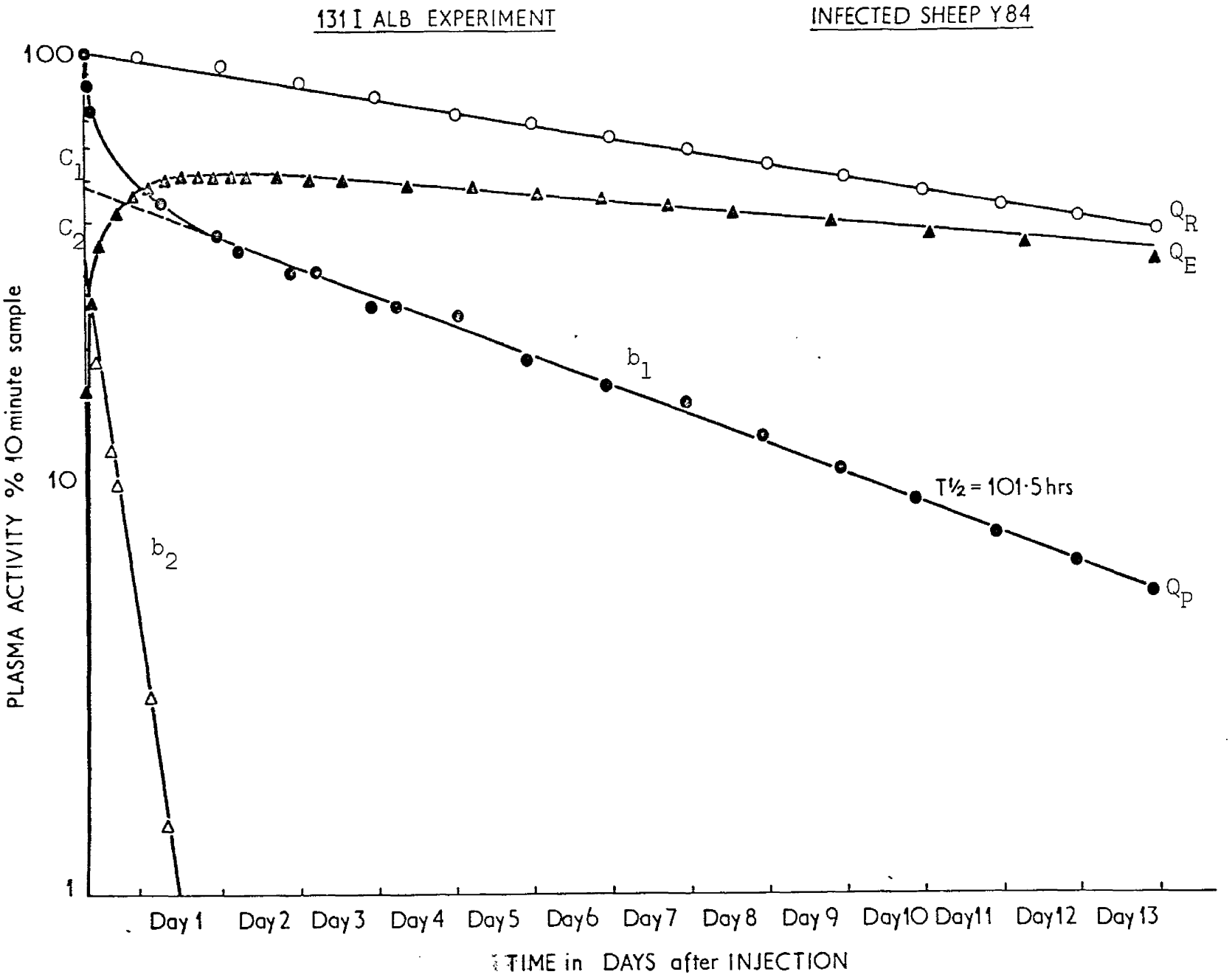
Table 12

Albumin Catabolism in Fluke-Infected and Control Sheep

	Sheep	$T\frac{1}{2}$ (hrs)	F(CA) /day	Gms cat. kg/day	k* /day	Plasma Clearance (ml/day)
INFECTED	79	200	0.150	0.147	0.158	12.6
	81	143	0.145	0.112	0.257	28.5
	831	133	0.143	0.099	0.301	39.5
	832	260	0.114	0.116	0.128	9.6
	84	102	0.332	0.252	0.327	58.7
	85	110	0.329	0.266	0.292	47.2
Mean		161	0.202	0.165	0.239	32.7
S.D.		67	0.100	0.074	0.079	19.4
CONTROL	61	318	0.104	0.100	0.138	7.8
	701	298	0.092	0.102	0.131	8.6
	702	470	0.075	0.067	0.082	5.1
	72	312	0.089	0.065	0.076	5.6
	77	450	0.081	0.072	0.095	3.2
	80	402	0.047	0.070	0.064	5.6
Mean		375	0.081	0.079	0.098	6.0
S.D.		76	0.017	0.014	0.028	1.9
"t" test		P < 0.001	P<0.02	P<0.02	P<0.01	P < 0.01

* K calculated by the method of Matthews (1957).

Fig. 6. Turnover of Labelled Albumin in a Fluke-Infected Sheep



The faecal activity/24 hours expressed as a plasma clearance (ml) was significantly higher in the infected sheep (32.7 ± 19.4 ml/day) compared to 6.0 ± 1.9 ml/day in the control group, suggesting that the increased catabolic rate observed in the parasitized sheep was due to loss of albumin into the gut. The "plasma clearance" is of course a gross under-estimate of plasma actually appearing in the gut, since substantial breakdown and reabsorption of label is known to occur with ^{131}I tagged plasma proteins.

Discussion

The Labelled Preparation

The quality of the labelled preparation was checked in several ways. Precipitation with TCA showed the activity to be approximately 98% protein bound, and electrophoresis showed the preparation to consist only of albumin. There was no evidence of significant denaturation of the protein since there was no significant decrease in catabolic rate over the experimental period. The preparation was injected immediately after removal of unbound activity by dialysis. There is some recent evidence that such preparations may become denatured, and hence give increased catabolic rates, if stored following labelling, probably due to self-irradiation damage (Nielsen and Nansen, 1969). However these authors give no reference to whether the specific activity of the preparation had been reduced, prior to storage, by the addition of carrier protein.

Management of Experimental Animals

The sheep were given 0.0025% KI throughout the experiment in order to prevent ^{131}I uptake by the thyroid. This level is below the one normally accepted in man and laboratory animals as it was found by Campbell, Cuthbertson, Mackie, McFarlane, Phillipson and Sudsaneh (1961), during experiments using ^{131}I-albumin, that sheep are unduly sensitive to levels of 0.01% NaI in the drinking water, the animals refusing to eat and drink and becoming seriously ill. On this basis they recommended 0.0025% NaI as the optimum concentration to be given in the drinking water.

Distribution of Albumin

The results show that all the infected sheep were hypoalbuminaemic which is consistent with the findings of other workers. There was an apparent correlation between the level of plasma albumin and the degree of infection. The body weights of the infected group were significantly lower than those of the control animals despite identical ages and system of management. Weight loss and progressive emaciation are an outstanding clinical sign in both natural and experimental infections (Reid, 1968; Sinclair, 1962).

The extravascular pool of albumin was reduced in the infected sheep, whilst the intravascular pool was similar to that of the controls. A similar situation has been reported in other cases of parasitic hypoalbuminaemia (Halliday, Mulligan and Dalton, 1963; Dargie, 1969).

The exact reason for the alteration in albumin distribution is not clear but it may reflect the inability of synthesis to keep pace completely with the hypercatabolism in the infected animals.

Catabolism and Synthesis

The results show that there was a dramatic increase in albumin catabolism of the infected group relative to the controls, as illustrated by the decreased half-life and increased F(CA), K(Matthews) and absolute amounts of albumin catabolised per day.

These findings have been confirmed in work on rabbits by Dargie (1969) in which fluke-infected rabbits showed elevated albumin catabolic rates of a similar magnitude to those found in the present experiment. Again in investigations on fluke-infected sheep Nansen, Eriksen, Simesen and Nielsen (1968) using ^{131}I-labelled albumin found mean catabolic rates (Matthews) of the infected animals to be 0.139 ± 0.030, which was twice the mean level of the controls.

The fractional catabolic rate calculated from daily excreted activity agreed in general with the catabolic rate determined by mathematical analysis of the plasma disappearance curve, however in two of the infected sheep there was a wide discrepancy between the results of the two methods of calculation. This discrepancy may be in part due to iodine retention which may occur when the catabolic rate is increased. Fig. 5 shows that in normal sheep the plasma curve (Q_p) is approximately parallel to the total activity retained in the body (Q_R)

signifying that renal excretion of ^{131}I is keeping up with ^{131}I-albumin degradation, however when albumin catabolism is greatly increased as occurs in the fluke-infected sheep, Fig. 6, Q_p is not parallel to Q_R because renal excretion of ^{131}I does not proceed at the level of ^{131}I-albumin breakdown and iodine retention occurs. The excreted activity may thus be an underestimate of true catabolism and hence give a lower value than that obtained by mathematical analysis of the plasma disappearance curve.

The catabolic rates for the control animals obtained by the different methods (mean 0.061 and 0.096) agree with other published data on the catabolic rate of albumin in normal sheep(0.073 ± 0.020 obtained by Campbell et al (1961), and 0.077 ± 0.008 by Nansen et al, 1968)). In the study of albumin catabolism in normal sheep Campbell et al (1961) noted that after 12 - 13 days the proportion of non-protein bound activity in the plasma and the catabolic rate calculated from excreted activity began to rise in a manner not previously recorded in other species. In addition the proportion of total excreted activity appearing in the faeces also began to rise. They suggested that this phenomenon was possibly due to re-utilization of iodotyrosine or its breakdown products by the rumenal bacteria. However in the present study there was no evidence of increasing catabolic rates or an increase in the faecal portion of the total excreted activity, though there were quite marked daily fluctuations in the fractional catabolic rate largely as a result of the daily variation in the amounts of urine and faeces excreted.

There seems to be some relationship between the degree of hypoalbuminaemia and the catabolic rate of albumin, in that the infected sheep with the lowest plasma albumin levels had the highest catabolic rate, and the sheep with highest albumin concentrations had the lowest catabolic rates. Since the albumin levels were fairly constant throughout the experimental period, it follows that catabolism was approximately equal to synthesis. Hence the synthetic rate of albumin was greatly increased in all the infected sheep especially in the most heavily infected.

However considerable hyperplasia of hepatic cells occurs in chronic fascioliasis of sheep (Dow, Ross and Todd, 1968), and this probably aids the increased synthesis of albumin observed in the infected sheep. This is supported by the finding that liver function tests are normal in chronic fluke infections of sheep (Simesen, Eriksen, Nansen, Andersen and Nielsen, 1958; Reid, 1969).

Whilst studies using ^{131}I-albumin have given a direct method of assessing the increased turnover rate of albumin in fluke-infected sheep, they give no true explanation as to the route of loss of the protein. The suggestion was made in Section I of this thesis that a loss of whole blood was occurring into the gastrointestinal tract, and so the obvious route for the excessive loss of albumin was thought to be via the bile as a result of the infection. The faecal clearance of plasma did show that a greater loss of albumin was occurring via the faeces in the infected sheep, though this is known to be a gross under-

estimate of the true loss because of substantial reabsorption of ^{131}I from the gut.

In conclusion, the use of ^{131}I-albumin has shown that

(i) in fluke infected sheep there was a reduction in the extravascular pool of albumin;

(ii) hypoalbuminaemia was apparently directly associated with increased albumin turnover rates;

(iii) despite liver damage albumin synthesis was increased at least twofold in the parasitized animals, and therefore the reduced plasma albumin concentration was not due to reduced synthesis but to increased loss of albumin, most probably via the bile into the gut. Further experiments to attempt to quantitate this plasma protein loss are described in the following subsection B.

Summary

Albumin metabolism was studied using ^{131}I-albumin in a group of twelve sheep, six of which were chronically infected with Fasciola hepatica. The results showed that albumin distribution was altered in the parasitized animals with a reduction in the extravascular pool of albumin. Varying degrees of hypoalbuminaemia were observed in all the infected animals and this was accompanied by a significant hypercatabolism of albumin. The elevated albumin turnover rates were shown by reduced albumin half-lives and increases in both fractional catabolic rates and absolute amounts of albumin degraded per day in the fluke-infected sheep. The results suggest

that the cause of the increased albumin turnover rate was excessive loss of plasma proteins into the gut presumably via the bile as a result of the mature Fasciola hepatica infections.

B. THE MEASUREMENT OF GASTROINTESTINAL PROTEIN LOSS USING ^{131}I-PVP, ^{95}Nb-ALBUMIN AND ^{51}Cr Cl_3

Introduction

The ideal substance for the measurement of gastrointestinal protein loss would be a labelled protein which behaved like endogenous protein following intravenous injection, but where the label was not absorbed from the gastrointestinal tract and so could be recovered in the faeces. In addition the label must be readily available, nontoxic, and not reutilised in protein synthesis.

To date no single protein label fulfils all these requirements. Iodine isotopes can be used successfully for measurement of turnover rates but not for quantitative detection of gastrointestinal protein loss because of reabsorption of the label from the gut after protein degradation. Other substances are suitable for assessment of gut loss, e.g. ^{131}I-PVP, ^{95}Nb, and ^{51}Cr-labelled plasma proteins, but cannot be used for measurement of turnover rates. So a combination of protein labels is required if the rates of catabolism and route(s) of excessive loss are to be determined.

The previous subsection described an experiment using ^{131}I-albumin which demonstrated that an increased catabolism of albumin was occurring in the fluke-infected animals, most easily explicable in terms of loss into the gut. The present experiments were an attempt to quantitate this loss.

Gordon in 1959 first demonstrated the use of ^{131}I-polyvinyl-pyrrolidone (PVP) for the measurement of protein loss from the plasma into the gut. PVP which is a biochemically inert macromolecule had been extensively used previously as a plasma expander following its introduction by Hecht and Weese (1943). Early attempts at labelling PVP with isotopes such as ^{131}I and ^{14}C by Ravin, Seligman and Fine (1952) were only partly successful because the preparations were unstable, and it was left to Gordon (1958) to prepare a stable and chemically inert ^{131}I-labelled PVP.

The technique for the detection of gastrointestinal protein loss using PVP consists of injecting the labelled preparation intravenously and then collecting the faeces for several days afterwards. The total faecal activity excreted each 24-hours is expressed as either a "plasma clearance(ml)" or as a percentage of the injected activity.

Extensive studies using Gordon's preparation by Jamum (1961) revealed that though ^{131}I-PVP was a valuable indicator of gastrointestinal protein leakage it gave at best only a semiquantitative estimation of protein loss. Studies in ten normal patients who had been given the preparation orally showed that from 3 - 13.5% of the administered dose appeared in the urine, showing that some ^{131}I had become split off as iodide, absorbed by the gut, and so excreted via the kidneys. Other workers have reported as much as 50% of an oral dose appearing in the urine (Jeejeebhoy, 1965). There are other disadvantages of PVP to be considered. It is not a physiological substance and may behave very

differently from proteins, and it has been found that there are varying amounts of small dialyzable molecules in different batches of PVP and these can affect the estimation of protein leak (Jaznum, 1961), however this disadvantage can, to some extent, be avoided if control and infected animals are compared using the same batch of PVP.

In normal subjects over the average collection period of 4 - 6 days generally less than 1% of the intravenously injected activity appears in the faeces, (Dawson, Williams and Williams, 1961; Jaznum, 1961).

Despite the possible disadvantages discussed above a fair correlation exists between faecal PVP excretion and gastrointestinal protein loss and therefore PVP is a valid marker for the detection of excessive protein loss via the gastrointestinal tract.

In 1961 Waldman introduced ^{51}Cr-labelled albumin as a tool for diagnosing gastrointestinal protein loss, following the demonstration that ^{51}chromic chloride could be used to label albumin (Gray and Sterling, 1951a). ^{51}Cr-labelled albumin was shown by Waldman (1961) to have certain advantages over both ^{131}I-albumin and ^{131}I-PVP since when the labelled preparation was given orally to normal subjects, 93 - 98% appeared in the faeces in the following four days, i.e. no significant absorption occurred, from the gastrointestinal tract of normal subjects. In the original work by Waldman (1961) ^{51}Cr-labelled albumin was prepared by incubating albumin in 10% glucose at pH 4.5 with $^{51}CrCl_3$ for one hour at room temperature. The free and weakly bound ^{51}Cr was removed by passage through resin columns. Labelling efficiency varied between 23 - 50%.

Later studies however revealed that there was rapid transfer of much of the label from the albumin to the globuline, especially the alpha-2 globulins and also it was noted that the plasma activity disappearance curves were considerably shorter than those obtained with ^{131}I-albumin in the same subject, so it became obvious that ^{51}Cr-albumin could not be used for albumin turnover studies (Guillen and Peterson, 1964; Van Tongeren and Majoor, 1966). The latter authors also showed that the disappearance of ^{51}Cr from the plasma after intravenous injection was found to be almost identical irrespective of whether ^{51}Cr-albumin, ^{51}Cr-serum or $^{51}CrCl_3$ were used, so it is preferable and easier to use an in vivo labelling technique by the injection of $^{51}CrCl_3$ (Walker-Smith, Skyring, Mistilis, 1967). More recent studies on the nature of ^{51}Cr-labelling of albumin (Hofer, Schatz and Thumb, 1968) have confirmed that ^{51}Cr labelling of albumin leads to denaturation of the protein molecules and that various subfractions of albumin become labelled with ^{51}Cr. They therefore concluded that ^{51}Cr-labelled albumin was of little if any value for physiological studies of albumin metabolism, though it remains a very useful tool in the detection of excessive gastrointestinal protein loss.

Another approach to the measurement of gastrointestinal protein loss was the use of ^{131}I-albumin in conjunction with oral administration of a resin to prevent ^{131}I being reabsorbed from the gut (Jeejeebhoy and Coghill, 1961). Although the technique seemed promising, later work showed it to be invalid as an accurate method (Freeman and Gordon, 1964; Hoedt-Rasmussen and Kemp, 1964).

In 1964 the successful use of ^{95}Nb-labelled albumin in the study of gastrointestinal protein loss was demonstrated by Jeejeebhoy, Singh, Mani and Sanjana. Their studies showed that ^{95}Nb could be firmly bound to albumin and was non-reabsorbable from the gut. Furthermore, the $T\frac{1}{2}$ and catabolism, calculated by mathematical analysis of the plasma activity curve were found to be similar to those obtained from ^{131}I-albumin data in some subjects. Thus it appeared that ^{95}Nb-albumin might be suitable for study of both gastrointestinal albumin loss and turnover. However, some disadvantages were found in attempting to calculate turnover rates from ^{95}Nb. The ^{95}Nb released by catabolism was excreted slowly so that the concentration of free ^{95}Nb in the plasma became significant, hence urinary excretion of ^{95}Nb cannot be used to measure catabolism. In addition, the 10 minute distribution space was found to be excessively large, making it unsuitable for calculations of plasma volume. Nevertheless, the technique had some advantages over the previously described methods and could provide useful information on gastrointestinal loss of albumin.

More recent techniques which have been developed are those using ^{67}Cu-labelled-ceruloplasmin and ^{59}Fe-labelled iron dextran. Waldman and Wochner (1965) investigated the use of ^{67}Cu-ceruloplasmin and compared it with ^{131}I-ceruloplasmin. Unfortunately the technique though promising, suffered from the disadvantages of high cost of the material and the brief half-life of ^{67}Cu of 62 hours.

The suitability of ^{59}Fe-iron dextran for the detection of gastrointestinal protein loss was demonstrated by Jarnum, Westergaard, Vosing and Jensen (1968), and while the technique suffered from some of the same disadvantages as ^{51}Cr-albumin and ^{131}I-PVP, it was claimed that it had the advantages that a better correlation existed between faecal excretion (as percentage injected dose) and the fractional catabolic rate. It had the additional advantage that no urinary excretion of ^{59}Fe occurred and so the errors involved in urinary contamination of the faeces were removed.

The following studies describe the use in fluke-infected and normal sheep of three of the techniques developed for the measurement of gastrointestinal protein loss, namely using ^{131}PVP, ^{95}Nb-albumin and $^{51}CrCl_3$.

Materials and Methods

Three separate experiments were conducted involving the use of different methods of detecting gastrointestinal protein loss, i.e. ^{131}I-PVP, ^{95}Nb-albumin and $^{51}CrCl_3$-plasma proteins. In addition the relationship of protein loss to red cell loss, and to albumin catabolism was investigated by using ^{51}Cr labelled red cells simultaneously with ^{95}Nb-albumin, and ^{125}I-albumin with $^{51}CrCl_3$-labelled plasma proteins.

Faecal Excretion of ^{131}I-PVP in Fluke-infected and Normal Sheep

I. Experimental Animals

Five infected and six control sheep were studied. Two of the parasitized animals had been infected seventeen weeks previously with

1,000 metacercariae, whilst the remaining three had been dosed with 500 twelve weeks before the experiment. All the sheep were aged 8 - 10 months and had been reared and managed as described in general materials and methods. Drinking water containing 0.0025% KI and 1% NaCl was administered continuously throughout the study and was commenced three days prior to the injection of ^{131}I-PVP.

II. Injection and Sampling

Each sheep received 1 ml (1 mC) of ^{131}I-PVP obtained from the Radiochemical Centre, Amersham, England. Blood samples were taken 23 hours post injection and from there on at regular intervals for six days. From each blood sample 1 ml of plasma was carefully pipetted into a counting-tube. Urine and faeces were collected each 24 hours and 5 ml and 5 gm aliquots taken and assessed for radioactivity.

III. Calculations and Expression of Results

The plasma radioactivity as counts/sec/ml was plotted as a percentage of the 23 hour post injection sample. The daily total faecal activity as cts/sec was expressed as both a percentage of the injected activity and as a daily clearance of plasma.

Results

The results are shown in Tables 13 and 14. It can be seen that all the infected animals were hypoalbuminaemic. The half lives of the ^{131}I-PVP of the infected group were not significantly shorter than those of the controls, (Fig. 8) though it is apparent from the faecal results that a highly significant loss of macromolecules into the gut was

Table 13

Plasma Disappearance and Faecal Excretion of ^{131}I-PVP in Fluke-infected and Control Sheep

	Sheep	Plasma Albumin (gms%)	T½ (hrs)	Faecal Clearance (ml/day)	% Excreted via Faeces	Fluke Infection Nos.	Fluke Infection Duration (weeks)
INFECTED	56	1.83	35	179	9.2	264	17
	62	1.66	35	252	8.9	202	17
	81	1.79	40	109	6.4	507	12
	82	-	49	153	12.4	627	12
	83	1.80	68	156	7.7	*	12
Mean		1.77	45	170	8.9		
S.D.		0.07	13	53	2.2		
CONTROL	49	2.48	44	17.7	1.2		
	54	3.26	40	25.5	1.4		
	58	2.96	45	30.2	1.4		
	61	2.62	63	21.9	2.4		
	70	2.20	58	16.8	1.2		
	72	2.57	62	13.9	1.1		
Mean		2.68	52	21.0	1.5		
S.D.		0.37	10	6.1	0.5		
't' Test		$P<0.01$	N.S.	$P<0.001$	$P<0.001$		

* Fluke burden not determined.

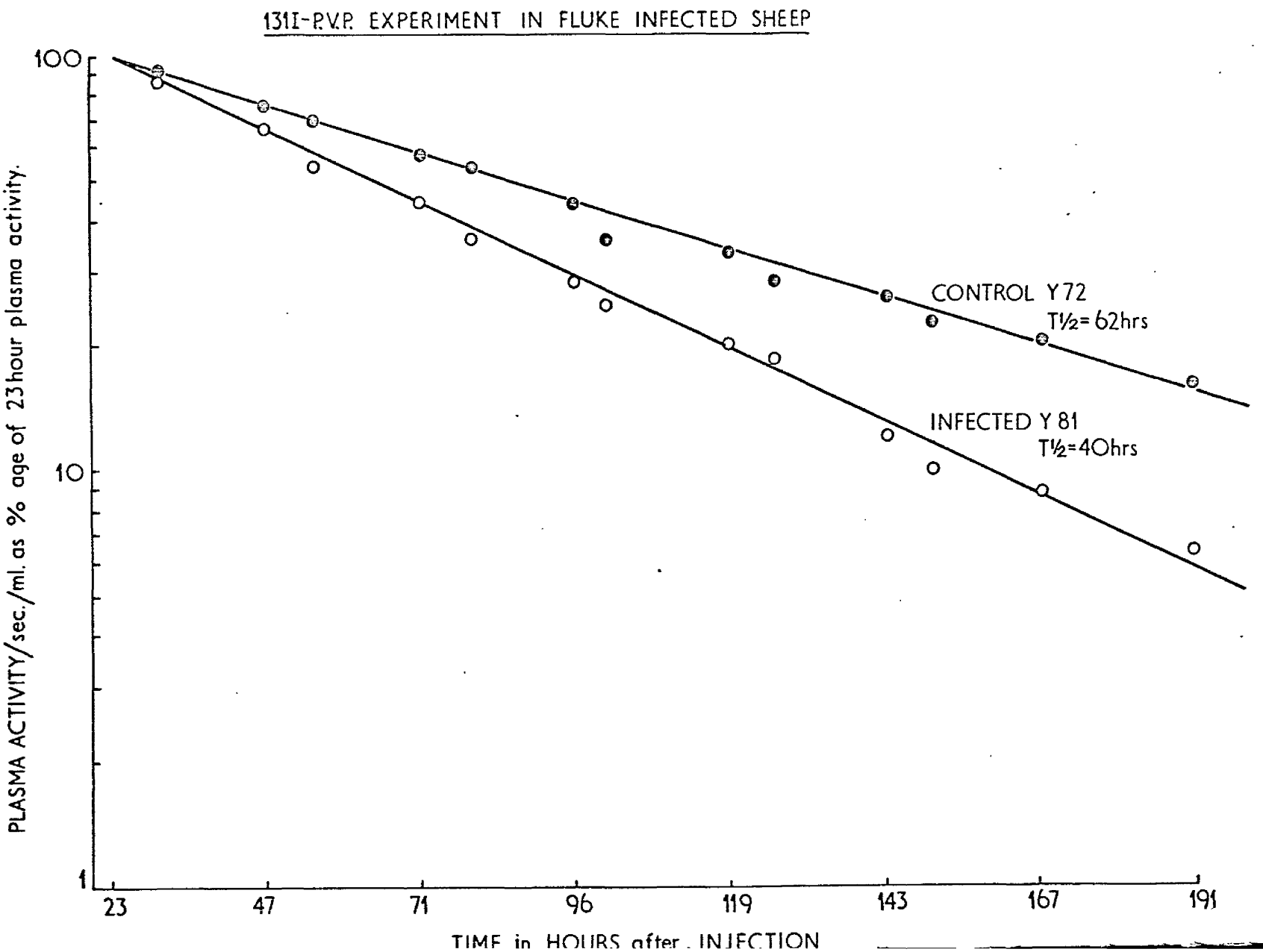
Table 14

Daily Faecal Clearance of ^{131}I-PVP in Control and Infected Sheep(ml)

	Sheep		56	62	81	82	83	
INFECTED	Day	2	131	222	101	50	136	
		3	128	169	105	177	109	
		4	174	255	104	209	158	
		5	176	227	107	225	204	
		6	269	368	105	135	136	
		7	198	272	120	125	194	
	Mean		179	252	109	153	156	
	S.D.		52	67	7	64	37	

	Sheep		49	54	58	61	70	72
CONTROL	Day	2	6.2	13.2	11.5	25.9	11.8	7.1
		3	9.3	13.0	21.0	23.3	13.3	6.5
		4	14.6	16.0	25.0	19.5	17.3	10.2
		5	31.5	19.3	21.0	21.3	18.7	9.9
		6	26.1	29.0	56.6	18.8	19.5	14.3
		7	18.6	62.7	48.0	22.7	20.1	35.3
	Mean		17.7	25.5	30.2	21.9	16.8	13.9
	S.D.		9.7	19.1	17.8	2.6	3.4	10.9

Fig. 8. Plasma Disappearance Curves of ^{131}PVP in a Fluke-Infected and Normal Sheep



occurring in the parasitized animals. The faecal plasma clearances were much greater in the infected animals (170 ± 53 ml/day) than in the controls (21.0 ± 6.1 ml/day). Similarly the percentage of the injected dose recovered in the faeces over the experimental period was far higher in the fluke-infected sheep (8.9 ± 2.2%) compared to the normal animals (1.5 ± 0.5%). There was a negative correlation both between the faecal plasma clearance and the plasma albumin concentration ($r = -0.733$ $p < 0.01$), and between the plasma albumin concentration and the percentage of the injected dose recovered in the faeces ($r = -0.831$ $p < 0.01$).

SIMULTANEOUS STUDIES OF PLASMA AND RED CELL LOSS IN FLUKE-INFECTED AND CONTROL SHEEP USING ^{95}Nb-ALBUMIN AND ^{51}Cr-ERYTHROCYTES

I. Experimental Animals

A total of four sheep were studied, two of which had been infected 7 months previously with 500 metacercariae each. These two were showing signs of infection as indicated by reduced haematocrit levels. All the animals were aged 10 months at the time of the investigation.

II. Preparation of ^{95}Nb-labelled Albumin

Modification of the method of Jeejeebhoy *et al* (1964) was used in which protein buffered at pH 8 is labelled with ^{95}Nb as niobium chloride. Four separate batches were prepared which were bulked after labelling and redivided for dispensing to each animal.

(a) Preparation of Niobium Chloride

Carrier free ^{95}Nb as an oxalate complex was obtained from the Radiochemical Centre, Amersham, England. 1.5 ml of the

^{95}Nb oxalate (containing 1.2 mC) was diluted to a volume of 5 ml with distilled H_2O and evaporated to dryness. It was then redissolved in 5 ml N/100 HCL, evaporated to an approximate volume of 2 ml, and finally diluted to 5 ml by the addition of distilled H_2O.

(b) Labelling of Protein

600 mg (4% solution) of sheep albumin (Cohn. Fr. V, Pentex Incorp. Kankakee, Illinois, U.S.A.) was mixed with 2 ml N/100 NaOH and 10 ml Glycine buffer (pH 9.0). This solution (pH 8) was then raised to 37°C for labelling with niobium chloride. The ^{95}Nb chloride was rapidly jet-sprayed into the buffered albumin solution and the mixture incubated at 37°C for 30 minutes.

(c) Removal of Unbound ^{95}Nb

Unbound ^{95}Nb was removed by passing the labelled protein solution through an alumina column (10 x 0.5 cm) prepared in the hydroxyl form. N/100 NaOH was used to elute the protein from the column.

(d) Efficiency of Labelling

Radioactivity determinations were made on the labelled preparation, prior to removal of the unbound activity, and on aliquots of both the effluent and the alumina following passage of the preparation. From the results it was calculated that 20% of the total activity added to the column remained bound to it, and of the remaining activity in the effluent 73% was protein bound as determined by TCA precipitation. The labelling efficiency was thus in the region of 60%.

The labelled albumin was dialysed for 24 hours against 20 litres 0.9% NaCl, though this did not affect the % activity precipitable with TCA. The preparation was then centrifuged for 30 minutes at 1500 rpm and finally the four preparations were bulked before being dispensed for injection.

III. Labelling of Red Cells

Red cells from each sheep were labelled with ^{51}Cr as previously described in detail in Section I of this thesis.

IV. Injection and Sampling

The ^{95}Nb-albumin and ^{51}Cr-red cells were injected intravenously together, followed by 10 ml warm saline. The first blood sample was taken 10 mins post injection, and further samples daily for the duration of the experiment. From each 5 ml blood sample 1 ml of whole blood and 1 ml of plasma were carefully pipetted into counting tubes and made up to 5 ml with 0.02N NaOH. Faeces and urine were collected each 24 hours, and aliquots taken for radioactivity determinations.

V. Calculations of Presentation of Results

The isotopes ^{95}Nb and ^{51}Cr form a suitable pair for simultaneous measurement of protein and red cell loss in that they can be determined in the same sample because of differences in the energy spectra of their radiations. Each sample was counted twice, once at the "single setting" at which only ^{95}Nb was counted, and once at the "double setting" at which both ^{95}Nb and ^{51}Cr were counted. By comparison of the count rates

Fig. 9. ^{95}Nb-Albumin Disappearance Curve in a Fluke-Infected Sheep

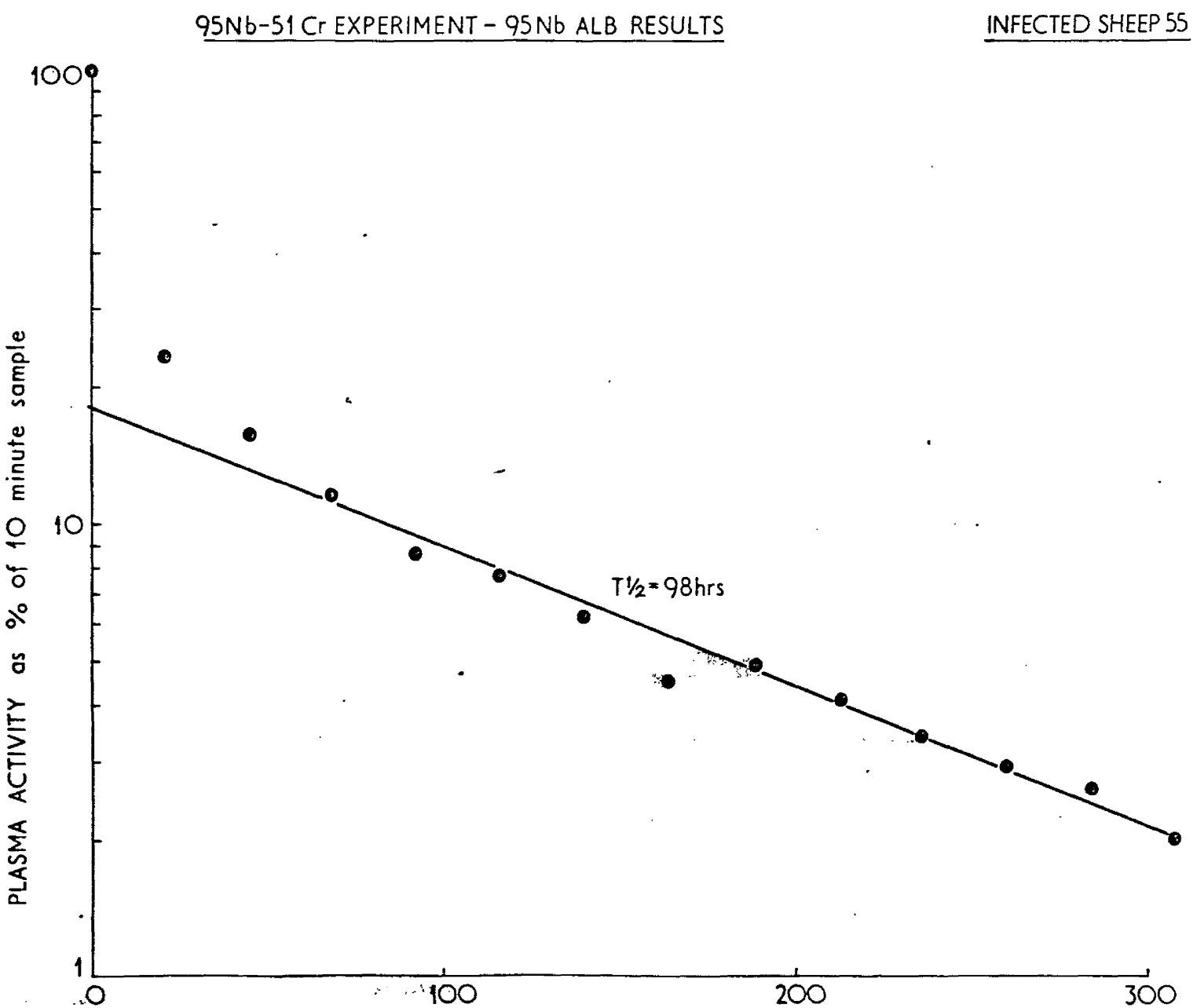
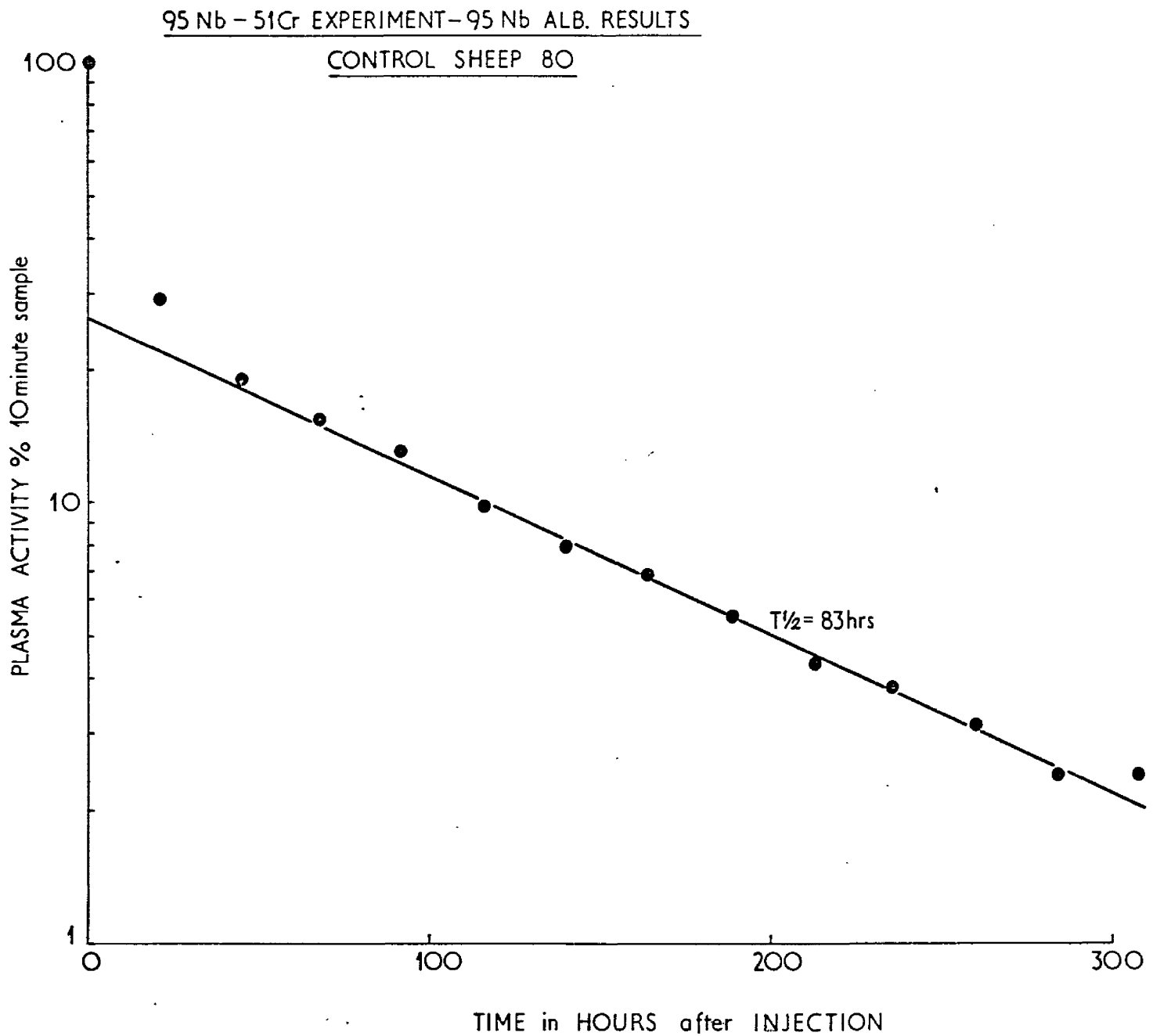


Fig. 10. ^{95}Nb-Albumin Disappearance Curve in a Normal Sheep



for the niobium standard at both the "single" and "double" settings it was possible to obtain a correction factor which enabled the ^{51}Cr and ^{95}Nb activities of each sample to be determined.

The faecal output each 24 hour period was converted to both a blood clearance from the ^{51}Cr data, and a plasma clearance from ^{95}Nb results. Comparisons could then be made of the plasma loss calculated from the red cell clearance corrected by haematocrit, and the actual plasma loss obtained from the ^{95}Nb data.

Results

The plasma activity disappearance curves following injection of ^{95}Nb labelled albumin in a fluke-infected and a control sheep are shown in Figs. 9 and 10. There was little difference between the two in the rate of disappearance of labelled albumin from the circulation, however examination of the faecal activity (Table 15 and 16) showed that there was a much greater loss of plasma into the gastrointestinal tract of the infected sheep (147, 65.5 ml/day) compared to the control sheep (24.3, 16.3 ml/day).

The loss of blood into the gastrointestinal tract was significantly greater in the infected animals (92, 31.3 ml/day) than the controls (<3.0 ml/day). Though accurate determination of the blood loss in the normal sheep was not possible because of the low radioactivity of the faeces.

Faecal clearances of plasma and whole blood were only calculated from days 2 - 5 because after this time the control values became highly variable.

Table 15

Daily Faecal Clearances of Plasma* and Whole Blood† in Fluke-Infected and Control Sheep

			Day 2	3	4	5	Mean	S.D.
Infected	Sheep 55	Plasma (ml)	152	182	95	159	147	40
		Whole Blood (ml)	89	110	60	109	92	23
	Sheep 83	Plasma (ml)	88	66	43	64	66	18
		Whole Blood (ml)	46	32	20	27	31	11
Controls	Sheep 75	Plasma (ml)	15.6	11.7	24.4	45.6	24.3	15.1
		Whole Blood (ml)	<3	<3	<3	<3		
	Sheep 82	Plasma (ml)	12.7	11.1	20.9	20.5	16.3	5.1
		Whole Blood (ml)	<3	<3	<3	<3		

* From ^{95}Nb data

† From ^{51}Cr data

Table 16

Faecal Loss of Albumin and Red Cells in Fluke-Infected and Control Sheep

	INFECTED		CONTROLS	
Sheep	55	83	77	80
PCV (%)	23	23	37	31
Plasma Albumin (gms %)	2.23	2.69	2.79	2.74
Faecal Plasma* Clearance (ml/day)	147	66	24.3	16.3
Faecal Whole† Blood Clearance (ml/day)	92	31.3	< 3	< 3
Cumulative ^{95}Nb Faecal Activity as % Injected	7.0	4.0	0.7	1.0
Cumulative ^{51}Cr Faecal Activity as % Injected	12.6	5.2	< 0.4	< 0.5

* From ^{95}Nb data

† From ^{51}Cr data

SIMULTANEOUS STUDIES OF PLASMA LOSS AND ALBUMIN CATABOLISM IN FLUKE-INFECTED AND NORMAL SHEEP USING $^{51}CrCl_3$ AND ^{125}I-ALBUMIN

I. Experimental Animals

Four sheep which had been infected with 500 metacercariae 6 months previously were studied along with two normal control animals. As in previous experiments 0.0025% KI and 1.0% NaCl was administered in the drinking water throughout the investigation. The experimental animals were aged between 10 - 20 months, and had been raised in conditions which prevented infection with helminth parasites.

II. Preparation of Labelled Proteins

(a) $^{51}CrCl_3$

^{51}Chromic chloride as a sterile solution was obtained from the Radiochemical Centre, Amersham, England. Labelling was carried out by an *in vivo* technique since earlier studies had indicated that similar results were obtained regardless of whether ^{51}Cr-albumin, ^{51}Cr-serum or $^{51}CrCl_3$ was injected (Van Tongeren and Majoor, 1966). Each sheep received 1.5 ml (containing 3 mC ^{51}Chromium activity).

(b) ^{125}I-albumin

Sheep albumin (Cohn Fr. V, Pentex Incorp., Kankakee, Illinois, U.S.A.) was trace labelled with ^{125}I according to the method of Macfarlane (1958). The procedure was identical to that for the labelling of albumin with ^{125}I, which is described fully in the previous subsection.

III. Injection and Sampling

The ^{125}I-albumin and $^{51}CrCl_3$ were injected simultaneously via a jugular catheter. The first blood sample was taken 10 minutes post injection and then further samples daily for the eleven day experimental period. At each bleeding both a heparinized and a clotted sample were taken. From the unclotted sample 1 ml of plasma was carefully pipetted for radioactivity determinations. From each serum sample a portion was taken for electrophoresis in order to ascertain to which plasma proteins the ^{51}Cr was attached. The percentage of protein bound activity was determined by precipitation with TCA.

IV. Calculation and Presentation of Results

The simultaneous use of ^{125}I and ^{51}Cr is made possible because of differences in the energy spectra of their radiations. The methods of calculation were in principle similar to those described for ^{95}Nb and ^{51}Cr except that in the case of ^{125}I and ^{51}Cr it was at the ^{125}I setting of the counter that both ^{125}I and ^{51}Cr count rates were registered, and at the ^{51}Cr setting only ^{51}Cr activity was determined. After suitable correction of the chromium counts/sec these were subtracted from the "double" counts/sec in order to determine the ^{125}I count rate. Urine and faeces were collected each 24 hours and suitable aliquots taken. Both ^{51}Cr and ^{125}I activity of plasma, urine and faeces samples were determined. The faecal clearance of plasma per day was determined from the ^{51}Cr activity of the total daily faeces divided by the radioactivity as cts/sec/ml of the plasma at the beginning of the 24 hour collection

period. From the ^{125}I data the catabolic rate of albumin was determined by the method of Campbell et al (1956) as described in detail in the previous subsection.

Results

A similar picture of increased gastrointestinal protein loss was obtained from the results of the $^{51}CrCl_3$ injection in fluke-infected sheep and control sheep. Tables 17 and 19 show that the infected animals had a greater loss of plasma in the faeces (123 ± 64 ml/day) compared to the two controls (31.2 - 34.4). Similarly the percentage of the dose injected recovered in the faeces over the experimental period was higher in the infected animals (7.9 ± 2.6%) relative to the controls (2.7 - 2.9%).

The results from the ^{125}I-albumin data are given in Tables 17 and 18. They show that there was an apparent increase in albumin turnover rate in the infected animals, as illustrated by the F(CA), (0.109 ± 0.014) compared to the controls (0.089 - 0.087). The difference between the two groups was not statistically significant, largely because two of the infected sheep (34, 35) had low infections (< 100 flukes).

There was a statistically significant correlation between the faecal plasma clearance and the plasma albumin concentration ($r = 0$ 0.805, $p < 0.05$). There appeared to be a positive correlation existing between plasma loss and albumin catabolism. The most hypoalbuminaemic sheep having the highest faecal plasma clearance had also the highest catabolic

Table 17

Gastrointestinal Protein Loss and Albumin Catabolism in Fluke-infected and Control Sheep

	Sheep	Plasma Albumin (gms %)	Faecal* Plasma Clearance (ml/day)	% Excreted * in Faeces	F(CA)†	Gms Albumin † Catabolised (gms/kg/day)
INFECTED	25	2.25	154.3	10.4	0.116	0.111
	26	1.83	196.6	9.8	0.128	0.114
	34	2.27	62.5	4.8	0.099	0.075
	35	2.13	77.2	6.7	0.092	0.080
Mean		2.12	122.7	7.9	0.109	0.095
S.D.		0.20	63.7	2.6	0.014	0.017
CONTROL	70	2.36	31.2	2.7	0.089	0.072
	77	2.31	34.4	2.9	0.087	0.066
Mean		2.34	32.9	2.8	0.088	0.069
S.D.		0.04	2.3	0.1	0.002	0.010
"t" test		N.S.	N.S.	N.S.	N.S.	N.S.

* From ^{51}Cr Results

† From ^{125}I results

Table 19

Fraction of the Intravascular Pool of Albumin Catabolised Per Day in Fluke-infected and Control Sheep

	INFECTED				CONTROL	
Sheep	25	26	34	35	70	77
Day 3	0.089	0.102	0.113	0.094	0.093	0.095
4	0.085	0.107	0.092	0.106	0.111	0.109
5	0.128	0.106	0.090	0.099	0.073	0.085
6	0.119	0.100	0.128	0.101	0.093	0.096
7	0.091	0.111	0.078	0.082	0.099	0.088
8	0.103	0.122	0.086	-	0.083	0.076
9	0.119	0.158	0.107	0.080	0.084	0.077
10	0.168	0.136	0.094	0.082	0.082	0.080
11	0.141	0.148	0.101	0.089	0.082	0.077
Mean	0.116	0.129	0.099	0.092	0.089	0.087
S.D.	0.026	0.022	0.010	0.009	0.000	0.000

Table 19

Daily Faecal Clearance of $^{51}CrCl_3$-labelled Plasma in Fluke-infected and Control Sheep

		INFECTED				CONTROL	
	Sheep	25	26	34	35	70	77
Day	3	141	170	63.2	86.2	29.0	29.9
	4	140	124	58.7	65.7	31.1	34.6
	5	157	173	54.1	82.9	36.8	37.1
	6	148	201	54.5	65.9	26.6	33.9
	7	132	195	60.4	63.4	32.9	32.6
	8	132	194	65.9	55.8	26.9	32.2
	9	176	224	68.5	84.2	25.6	29.2
	10	173	213	61.8	87.2	32.8	29.4
	11	190	275	80.4	103.7	39.1	51.0
Mean		154	197	62.5	77.2	31.2	34.4
S.D.		21	42	7.8	15.3	4.7	6.7

rate of albumin. Conversely the sheep with the lowest clearance had the lowest catabolic rate. However, this correlation was not statistically significant. Electrophoresis and TCA precipitation of the serum sample taken during the experiment revealed that the percentage of ^{51}Chromium activity attached to the serum albumin was 25 ± 4% and that 86 ± 3% of the plasma activity was protein bound.

The ^{51}Cr-plasma half-lives for the infected group (100 ± 14 hrs) was only slightly lower than the controls (136 ± 34 hrs). The ^{125}I-plasma half-lives, however showed a more marked difference between the infected (254 ± 47 hrs) and control animals (312 ± 34 hrs).

Discussion

All the techniques used in the present study for the detection of gastrointestinal protein loss indicated that a significantly greater loss of plasma proteins into the gut was occurring in the fluke-infected animals relative to the normal sheep. The fluke-infected sheep appeared to be losing 100 - 200 ml plasma/day, whilst the controls in general showed a daily loss of 20 - 30 ml.

The PVP results illustrate well the striking difference of plasma loss between the infected and control groups, and in addition show that the duration of the infection significantly affects the plasma loss per fluke, since the 12 week infections showed losses of only 0.21 ml/fluke, whilst the 17 week infections caused a plasma loss of 0.68 - 1.24 ml/fluke.

Although the average molecular weight of PVP (30,000 - 40,000) is much less than that of albumin (70,000) because of the different shapes

of the molecules the diffusion coefficients are not so dissimilar, thus the faecal clearance of PVP may give a reasonable measure of albumin clearance. This would be supported by the fairly good correlation between the PVP results and those obtained with ^{95}Nb-albumin.

If then the ^{131}I-PVP clearance can be taken to indicate approximately the plasma loss per day it means that in the sheep with the lowest serum albumin level a loss of 250 ml plasma per day was occurring into the gut, representing about one fifth of the plasma volume of the animal.

A significant correlation was shown to exist between the plasma albumin concentration and both the faecal PVP plasma clearance and the percentage of the injected dose of PVP recovered in the faeces. This is contrary to the findings of Jarnum et al (1968) who in studies in humans suffering from protein-losing gastroenteropathies were unable to demonstrate any statistically significant correlation between faecal ^{131}I output and serum albumin levels, following injection of ^{131}I-PVP.

The results of the experiment using ^{95}Nb-albumin and ^{51}Cr-red cells indicate the various advantages and disadvantages of this technique. It is obvious from the ^{95}Nb plasma disappearance curve of the control animals that the preparation was considerably denatured, since the ^{95}Nb half-lives of the control animals were less than one third of those obtained in other control sheep using ^{131}I-albumin. Hence no significant turnover data could be obtained from the results. However, the

technique was still valid for the detection of gastrointestinal protein loss.

The ^{95}Nb faecal activity when expressed as a percentage of the injected dose shows that a significantly greater amount was being lost in the faeces of the infected sheep than in those of the controls. Similarly when the ^{95}Nb faecal activity is expressed as a plasma clearance the differences between the infected and control groups are equally obvious. Unfortunately after the fifth day of the experiment the faecal plasma clearance of the sheep began to show marked daily fluctuations. This was particularly pronounced in the control animals in which there was a marked elevation in the clearance rates. One possible explanation of this anomaly is that there was a progressive rise in free ^{95}Nb in the plasma as reported by Jeejeebhoy et al (1964) as free ^{95}Nb released by normal endogenous catabolism was excreted slowly. Unfortunately, TCA precipitation of the plasma samples were not carried out so it is impossible to be sure that a rise in free ^{95}Nb occurred. Recent studies by Jeejeebhoy, Jamum, Singh, Nadkarin, and Westergaard (1969) have indicated that if ^{95}Nb-albumin preparations are purified by two passages through DEAE cellulose columns it is possible to obtain turnover results very similar to those obtained by ^{131}I-albumin when catabolism is calculated by mathematical analysis of the plasma activity disappearance curve.

Results from the ^{51}Cr-red cell studies carried out simultaneously with ^{95}Nb-albumin indicate that in the infected animals a greater loss

of plasma was occurring than can be accounted for by a loss of whole blood, after making allowance for the "background" loss of plasma in the control animals.

This is supported by evidence from electron microscope studies which have demonstrated that breakdown of the "tight junction" between the epithelial cells in parasitized mucosae occurs. These junctions act as a protein seal in normal epithelia, and their breakdown allows passage of protein from the interstitial spaces into the lumen (Murray and Jarrett, 1967).

The results of the ^{125}I $^{51}CrCl_3$ study show that there was a close relationship between the serum albumin level, the enteric loss of plasma and catabolic rate of albumin since the most hypoalbuminaemic subject showed the greatest plasma clearance and the most elevated catabolic rate, and vice versa, the sheep with the highest serum albumin concentration had the lowest plasma clearance and the lowest catabolic rate of albumin. The use of ^{125}I-albumin / ^{51}Cr-albumin was first attempted by Kerr, Du Bois and Holt (1967), who tried to correlate albumin turnover data obtained from the ^{125}I-albumin results, with gastrointestinal catabolism calculated from the faecal excretion of ^{51}Cr-albumin. However this technique is not completely valid since the ^{51}Cr is attached not only to albumin but to other plasma proteins also, especially transferrin. Thus the faecal plasma clearance calculated from the ^{51}Cr data does not solely represent loss of albumin. In the study of Kerr et al (1967) they found 85% of the ^{51}Cr plasma activity was in the albumin fraction, but in the present study only 25% of the activity was attached to albumin.

The rapid loss of ^{51}Cr plasma activity in a similar manner to that observed in the ^{95}Nb experiment serves to emphasize the lack of validity of using $^{51}CrCl_3$ as an accurate technique for measuring albumin catabolism due to both denaturation of the labelled proteins and to the rapid attachment of most of the ^{51}Cr to the betaglobulins. Nevertheless, it has proved to be a very useful and sensitive technique for detecting abnormal gastrointestinal protein loss, especially since a fair correlation has been found to exist between the hypercatabolism of albumin associated with various protein-losing gastroenteropathies and the loss of plasma proteins into the gastrointestinal tract as measured by $^{51}CrCl_3$ (Jamnum et al, 1968). Such a correlation was found in the present study.

In conclusion, the different techniques for detecting gastrointestinal protein loss have all consistently shown that a significantly greater loss of plasma occurs into the gastrointestinal tract of fluke-infected sheep compared to control animals. This loss has been shown to be greater than that expected, purely from a loss of whole blood alone. In addition a close agreement was found to exist between the loss of plasma, the degree of hypoalbuminaemia, and the hypercatabolism observed in the parasitized animals.

Summary

Three separate techniques for the detection of gastrointestinal protein loss were used in a total of ten normal sheep and eleven animals infected with F. hepatica. All the methods, ^{131}I-PVP, ^{95}Nb-

albumin and $^{51}CrCl_3$ showed that there was a significantly greater loss of plasma into the gastrointestinal tract, presumably via the bile, in the parasitized sheep than in the controls. By the additional use of double labelling techniques it was shown that (1) the loss of plasma was greater than could be expected from a loss of whole blood alone, (2) a close correlation existed between the hypoalbuminaemia, hypercatabolism, and plasma loss observed in the fluke-infected sheep.

C. SIMULTANEOUS TURNOVER STUDIES OF ^{131}I-ALBUMIN AND ^{125}I-IMMUNOGLOBULIN

Introduction

Chronic ovine fascioliasis is characterized by a marked hypoalbuminaemia and hyperimmunoglobulinaemia (mostly due to the elevated gammaglobins) which occurs in infected animals (Balian, 1940; Noguchi et al, 1956; Ibrovic and Gall-Palla, 1959; Nikolic et al, 1962; Sinclair, 1962). The aetiology of the hypoalbuminaemia can be explained on the basis that it results from the substantial loss of red cells and plasma which occurs in fluke-infected animals and has been demonstrated earlier in this thesis. However the rise in immunoglobulins which occurs despite the excessive plasma loss is not explained, unless the synthetic rate of immunoglobulin exceeds the loss of plasma protein. The present study was carried out to determine the turnover rate of immunoglobulin relative to that of albumin, in an attempt to assess the rate of synthesis of immunoglobulin in the parasitized sheep.

The sheep, like other mammalian species apparently possesses at least two types of protein with immunological functions, a 19S beta 2M-globulin (IgM) and a 7S gamma globulin (IgG). In the ovine as in the bovine and caprine, the immunoelectrophoretic pattern of IgG is not of a single arc, but as a "gullwing shape" in which the faster gammaglobulin (IgG fast) component shows a spur of partial identity with the slower component (IgG slow), (Silverstein, Thornbecke, Kramer and Lukes, 1963;

Aalund, Osebold and Murphy, 1965). Only IgG slow was used in the present investigation.

Immunoglobulins can be labelled in an identical manner to that used for albumin as devised by McFarlane (1958). As in the study of albumin turnover rates using ^{131}I-albumin, described in the earlier subsection, there are various assumptions which have to be made regarding the preparation, the subject and the mathematical methods used in determining turnover rate.

Preparation

The activity must be shown to be protein-bound. In addition, the preparation must be homogenous, i.e. only contain a single plasma protein. This is more difficult to achieve in the study of immunoglobulins than was the case with albumin, but the purity of the preparation can be tested easily by immunoelectrophoresis.

Denaturation is also an increased hazard in immunoglobulin studies, especially during the preparation of the plasma protein, as well as from over-iodination or by radiation damage. Though denaturation can be demonstrated by physico-chemical methods, these methods usually only reveal severe changes in the structure of the protein molecule. The best way of testing the labelled IgG preparation is with biological methods, which in practice is done by evaluating the results of the turnover study. Denaturation is suspected when the excretion of radioactive iodine excreted in the first 24 hours is greater than on subsequent days; if the fractional catabolic rate calculated from

excreted activity decreases during the study; or if the plasma volume calculated from the IgG data is significantly greater than results using labelled albumin.

Subjects

The animals under study must be in an approximately steady state with respect to plasma proteins as demonstrated by unchanging body weight and plasma protein concentrations.

Mathematical Methods of Determining Turnover Rate

In order to understand changes in metabolism of IgG some assumptions must be made regarding normal sites of synthesis and catabolism. It is now generally accepted that antibody production occurs in the plasmocytic cell lines (White, Coons and Connolly, 1955; Thorbecke, Asofsky, Hochwald and Sicking, 1962; Mellors and Korngold, 1963). It is assumed that from a functional point of view, synthesis can be regarded as intravascular since plasma cells occur predominantly in the bone marrow, spleen, thymus, where lymphatic drainage is insignificant so that the gammaglobulin produced will be removed by the plasma. Production also occurs in the liver and lymph nodes where the lymphatic drainage will remove the gammaglobulin to the blood stream.

There is still considerable controversy regarding the exact sites of IgG degradation, though it is generally accepted that it occurs intravascularly (Cohen and Freeman, 1960). The divergence of opinion is mainly concerning the relative importance of different sites in catabolism.

The following experiment was undertaken to assess the turnover rates of IgG and albumin simultaneously in fluke-infected and normal sheep.

Materials and Methods

I. Experimental Animals

Two sheep which had been infected 6 months previously with 500 metacercariae and showing evidence of infection by reduced haematocrit values were used along with three normal control animals. All the sheep were Blackface wethers aged approximately 10 months, and had been reared as described in general materials and methods. Drinking water containing 0.0025% KI and 1% NaCl was given throughout the experimental period, and commenced four days prior to the injection of the labelled proteins.

II. Preparation of the Proteins

(a) IgG Slow

The immunoglobulin was prepared from pooled sheep serum by means of anion exchange column chromatography using DEAE-Sephadex (A-50) with phosphate buffer (0.01M, pH 8.0) as eluent. IgG-slow was eluted in the first protein peak. Fractions of this peak were pooled and freeze-dried until required. Immunoelectrophoresis of the preparation with rabbit anti-sheep serum was carried out as a check on the purity of the preparation.

(b) Albumin

Commercial sheep albumin was used (Cohn Fr. V, Pentex Incorp., Kanka ee, Illinois, U.S.A.).

III. Labelling of Protein Preparations with Radioiodine

The ^{125}I and ^{131}I labelling were carried out according to the method of McFarlane (1958). The method used was identical to that described in the previous subsection, A. As a check on the labelling procedure the two labelled proteins, i.e. ^{125}I-IgG and ^{131}I-albumin were mixed with ovine serum and separated by gel filtration chromatography using Sephadex G-100.

IV. Injection and Sampling

The two labelled preparations were injected together via a jugular catheter. The first heparinized sample was taken from the opposite vein 10 mins. post injection and further samples twice daily for three days, and once daily for the following twelve days. From each blood sample 1 ml of plasma made up to 5 ml with 0.02N NaOH was taken for radioactivity determinations along with aliquots of urine and faeces collected each 24 hours.

V. Calculations and Presentation of Results

Since the degradation site of gammaglobulin is thought to be intravascular as in the case of albumin, similar methods of calculation of turnover rates were used as previously described for albumin in subsection A.

The following parameters were calculated

(a) Plasma Volume by the dilution principle using the 10 min. post injection sample.

(b) Body Pools

(i) Intravascular pool (CG) (CA)

= Plasma volume x serum concentration gms % of albumin or gammaglobulin.

(ii) Extravascular pool (EG) (EA)

Determined by the "equilibrium time" method of Campbell et al.

(c) Catabolic Rate

(i) "Apparent half-life" obtained from the plasma activity curve.

(ii) Fraction of the Intravascular Pool Catabolised Per Day F(CG) F(CA)

Calculated from the total excreted radioactivity per day, divided by the total plasma activity by the method of Campbell et al (1956).

(iii) K (Catabolic Rate). This was obtained by mathematical analysis of the plasma activity disappearance curve according to the method of Matthews (1957).

(iv) Absolute Amount of Gammaglobulin (or Albumin) Catabolised per day

= F(CG) or F(CA) x Intravascular Pool (CG) or (CA) in gms/Kg.

(d) Faecal Clearance of Plasma ml/day

$$= \frac{\text{Total Daily Faecal Activity (cts/sec)}}{\text{Plasma Activity (cts/sec/ml)}}.$$

Fig. 11. PROTEIN and RADIOACTIVITY DISTRIBUTION FOLLOWING G-100 SEPHADEX GEL FILTRATION of DOUBLE LABELLED OVINE SERUM. COLUMN 85×3 cms BUFFER 0·1M TRIS/HCl pH=8·0

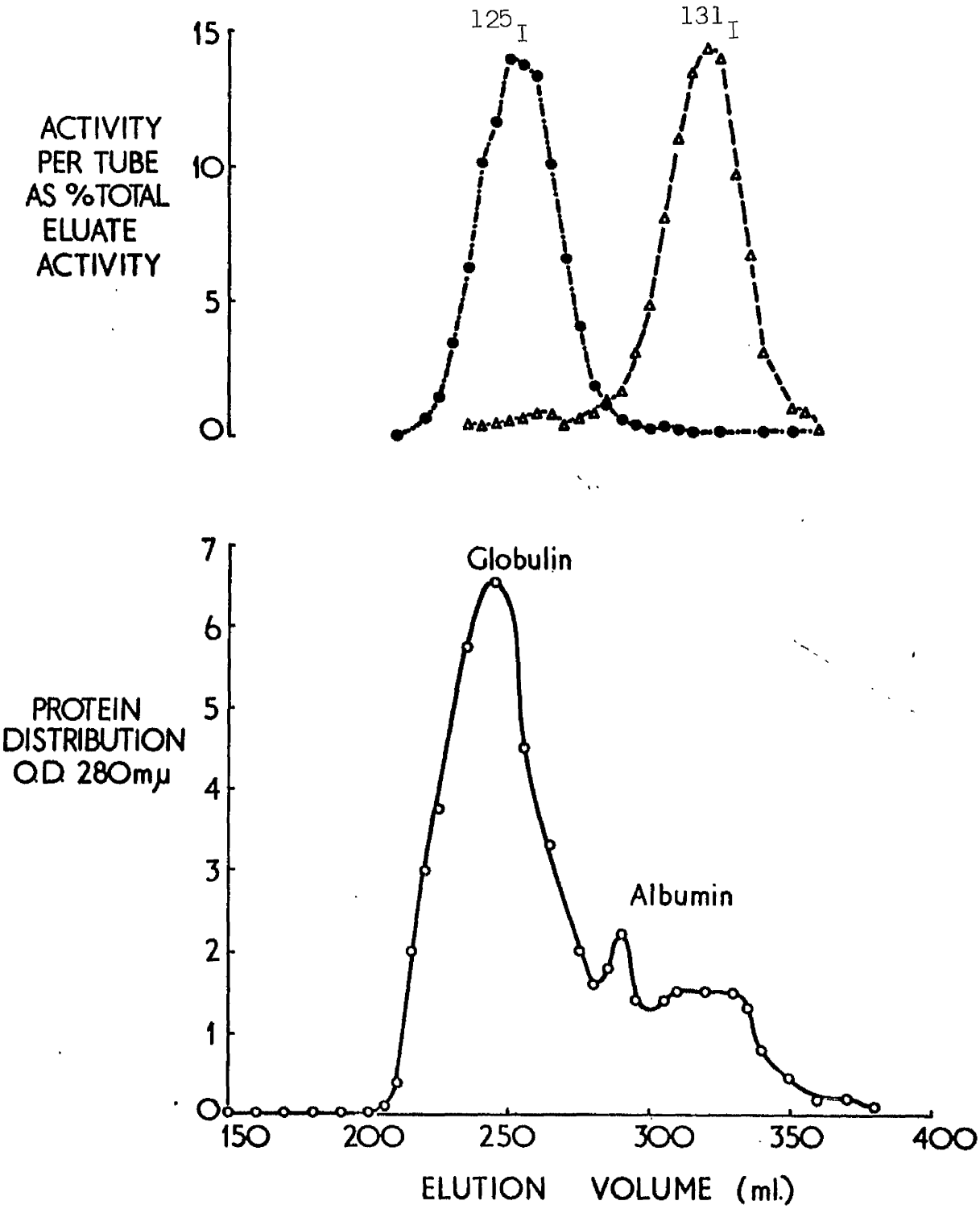


Table 20

IgG Concentration and Distribution in Fluke-Infected and Normal Sheep

	Sheep	Body Weight (kg)	Plasma γ Globulin (gms %)	Plasma Volume (ml/kg)	CG (gm/kg)	EG (gm/kg)	EG/CG
INFECTED	62	29.0	3.36	54.0	1.81	2.19	1.21
	74	30.3	2.32	45.0	1.04	1.12	1.0
	Mean	29.9	2.84	49.5	1.42	1.66	1.18
	S.D.	1.3	0.74	6.4	0.54	0.75	0.69
CONTROL	54	34.9	2.47	33.3	0.82	0.94	1.15
	58	35.4	2.02	43.0	0.86	0.88	1.02
	49	27.7	2.62	38.6	1.01	1.26	1.25
	Mean	32.7	2.37	38.3	0.90	1.03	1.14
	S.D.	4.3	0.31	4.8	0.10	0.20	0.11
"t" test		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 21

Albumin Concentration and Distribution in Fluke-Infected and Control Sheep

	Sheep	Plasma Albumin (gms %)	Plasma Volume (ml/kg)	CA (gm/kg)	EA (gm/kg)	EA/CA
INFECTED	62	1.44	53.6	0.77	0.55	1.57
	74	2.15	43.9	0.95	0.56	1.30
	Mean	1.80	48.8	0.86	0.56	1.44
	S.D.	0.50	6.3	0.13	0.01	0.19
CONTROL	54	3.21	32.6	1.06	0.82	1.71
	58	4.03	41.9	1.70	1.39	1.81
	49	3.10	38.3	1.21	0.96	1.75
	Mean	3.46	37.8	1.32	1.06	1.76
	S.D.	0.53	4.7	0.33	0.30	0.05
		$P < 0.05$	N.S.	N.S.	N.S.	N.S.

Results

(1) Quality of the Labelled Preparation

(a) Protein Binding

TCA precipitation indicated that 97% of the radioactivity in the final preparation was protein bound. Gel filtration using Sephadex G-100 revealed the radioactivity to be confined to two separate peaks which corresponded to the separate protein fractions. (See Fig. 11).

(b) Homogeneity of Immunoglobulin

Immunoelectrophoresis revealed that the preparation of immunoglobulin was pure IgG-slow.

(2) Concentration and Distribution of Immunoglobulin and Albumin

The results are given in Tables 20 and 21. There was no significant difference in body weight between the two groups. Gamma-globulin levels of the infected animals were higher than the controls group whilst the serum albumin levels were reduced in the parasitized sheep compared to the normal animals. The plasma volume calculated from both IgG and Albumin data show that the plasma volume expressed as ml/kg was higher in the infected group than in the control sheep. However, the plasma volumes for the individual animals calculated from the ^{131}I-albumin and ^{125}IgG data show excellent agreement.

The extravascular/intravascular ratio of IgG distribution was very similar in both the infected and control groups, though the albumin distribution ratio indicated a reduced extravascular pool of albumin in the two infected sheep.

Table 22

The Fraction of the Intravascular Pool of IgG Catabolised Per Day in Fluke-infected and Normal Sheep

Sheep	CONTROLS			INFECTED	
	54	58	49	62	74
Day 2	0.114	0.117	0.112	0.182	0.145
3	0.069	0.122	0.049	0.209	0.100
4	0.054	0.055	0.156	0.203	0.200
5	0.068	0.066	0.101	0.146	0.116
6	0.118	0.054	0.131	0.259	0.149
7	0.178	0.147	0.267	0.349	0.301
8	0.184	0.143	0.168	0.272	0.232
9	0.216	0.137	0.093	0.307	0.282
10	0.185	0.193	0.127	0.285	0.215
11	0.192	0.158	0.166	0.351	0.281
12	0.190	0.209	0.172	0.303	0.239
13	0.137	0.169	0.262	0.510	0.266
14	0.259	0.183	0.264	0.345	0.271
Mean	0.151	0.135	0.159	0.286	0.215
S.D.	0.062	0.050	0.069	0.094	0.067

Table 23

The Fraction of the Intravascular Pool of Albumin Catabolised Per Day in Fluke-Infected and Control Sheep

Sheep	CONTROLS			INFECTED	
	54	58	49	62	74
Day 2	0.098	0.117	0.075	0.041	0.117
3	0.056	0.047	0.028	0.136	0.067
4	0.043	0.082	0.078	0.151	0.136
5	0.046	0.053	0.046	0.095	0.069
6	0.074	0.038	0.052	0.158	0.081
7	0.111	0.101	0.104	0.206	0.165
8	0.107	0.093	0.053	0.156	0.129
9	0.124	0.086	0.030	0.160	0.143
10	0.104	0.101	0.041	0.146	0.108
11	0.100	0.075	0.049	0.172	0.120
12	0.101	0.088	0.052	0.144	0.099
13	0.057	0.067	0.079	0.238	0.111
14	0.118	0.079	0.073	0.152	0.116
Mean	0.088	0.079	0.059	0.158	0.112
S.D.	0.026	0.020	0.021	0.033	0.026

Table 24

Mean Results of IgG Catabolism in Fluke-infected and Normal Sheep

	Sheep	$T\frac{1}{2}$ (hrs)	F(CG)	K^	Gms/day/kg Catabolised	Plasma Clearance(ml)
INFECTED	62	116	0.286	0.246	0.517	105.5
	74	143	0.215	0.198	0.224	36.8
	Mean	130	0.251	0.222	0.371	71.2
	S.D.	19	0.049	0.035	0.213	48.6
CONTROL	54	200	0.151	0.158	0.123	18.0
	58	210	0.135	0.146	0.116	24.5
	49	160	0.159	0.186	0.161	16.9
	Mean	190	0.148	0.163	0.133	19.8
	S.D.	26	0.010	0.017	0.024	4.1
	"t" Test	N.S.	$P < 0.05$	N.S.	N.S.	N.S.

(3) Turnover Rates of Immunoglobulin and Albumin

Tables 22 and 23 give the fractional catabolic rates for the individual sheep throughout the experimental period. Though there is some variation on a day to day basis, there is no distinct rise or fall in catabolic rate during the experiment.

Figures 12 - 15 illustrate the radioactivity in the intravascular and extravascular pools of IgG and albumin in a control and infected sheep. They show that the "half-lives" of both IgG and albumin are reduced in the infected animal, and further, that the half-life of the IgG is shorter than that of albumin in both the infected and the control sheep.

The catabolic rates of IgG and albumin are summarised in Tables 24 and 25.

The fractional catabolic rates of both IgG and albumin of the infected group, calculated from both excreted activity and mathematical analysis of the plasma disappearance curves, were significantly higher than the control animals.

There was a significant positive correlation in the five sheep between the plasma gammaglobulin concentration and the F(CG) calculated from the urinary and faecal activities ($r = 0.829$, $P < 0.05$) and a poorer correlation with the fractional catabolic rate calculated from the plasma disappearance curve ($r = 0.776$, $P < 0.1$).

In the case of albumin, there was a negative correlation between the plasma albumin concentration and both F(CA) ($r = 0.865$, $P < 0.05$)

Table 25

Mean Results of Albumin Catabolism in Fluke-infected and Normal Sheep

	Sheep	$T\frac{1}{2}$ (hrs)	F(CA)	K	Gms/day/kg Catabolised	Plasma Clearance(ml)
INFECTED	62	200	0.158	0.175	0.121	58.8
	74	235	0.112	0.148	0.106	17.5
	Mean	217	0.135	0.162	0.113	38.2
	S.D.	25	0.032	0.020	0.010	29.2
CONTROL	54	330	0.088	0.127	0.093	10.5
	58	450	0.079	0.099	0.135	16.4
	49	390	0.059	0.102	0.071	6.4
	Mean	390	0.075	0.109	0.100	11.1
	S.D.	60	0.010	0.014	0.033	5.0
"t" Test			$P < 0.05$	$P < 0.05$	$P < 0.05$	N.S.	N.S.

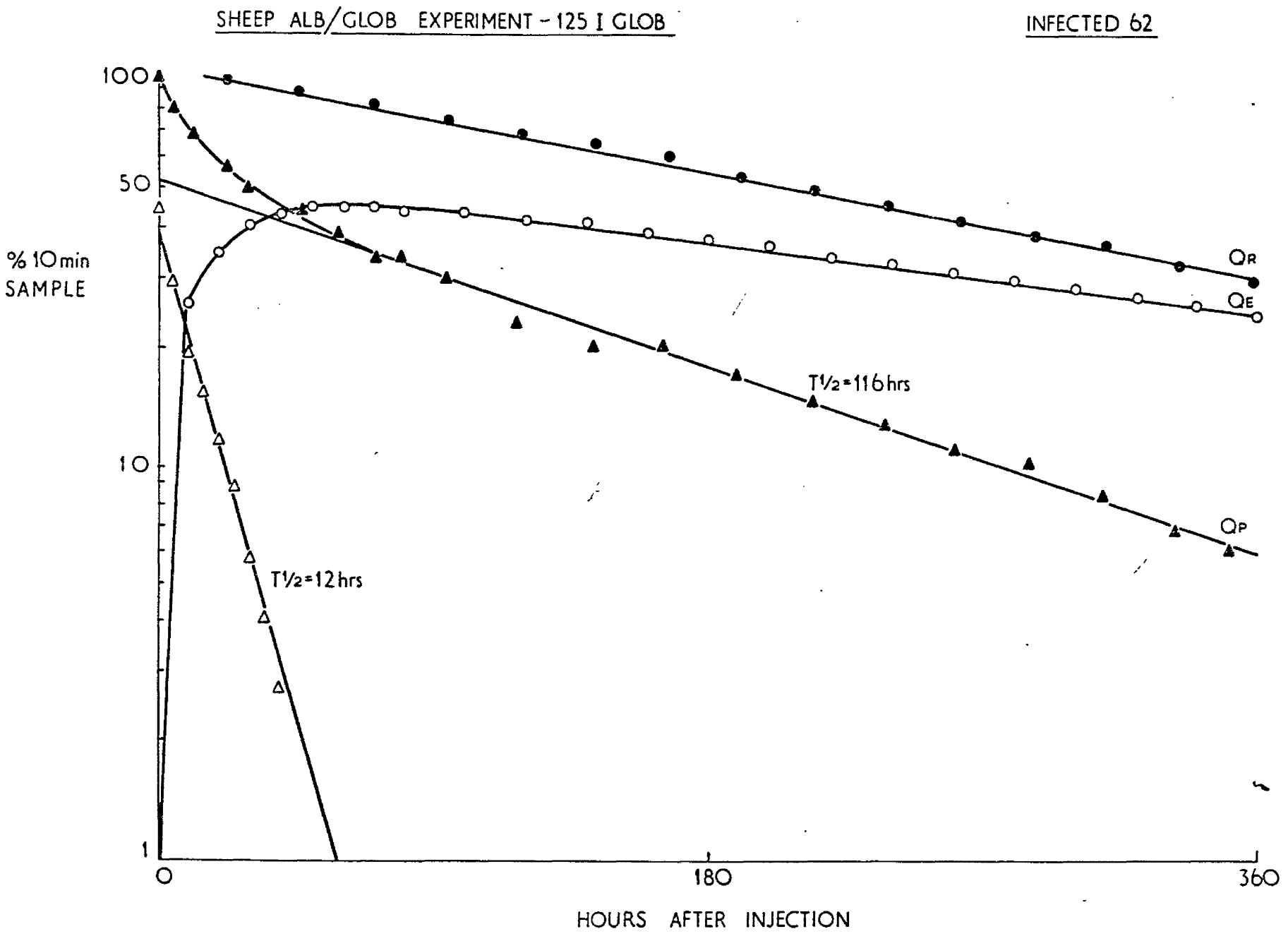


Fig. 12. IgG Turnover in a Fluke-Infected Sheep

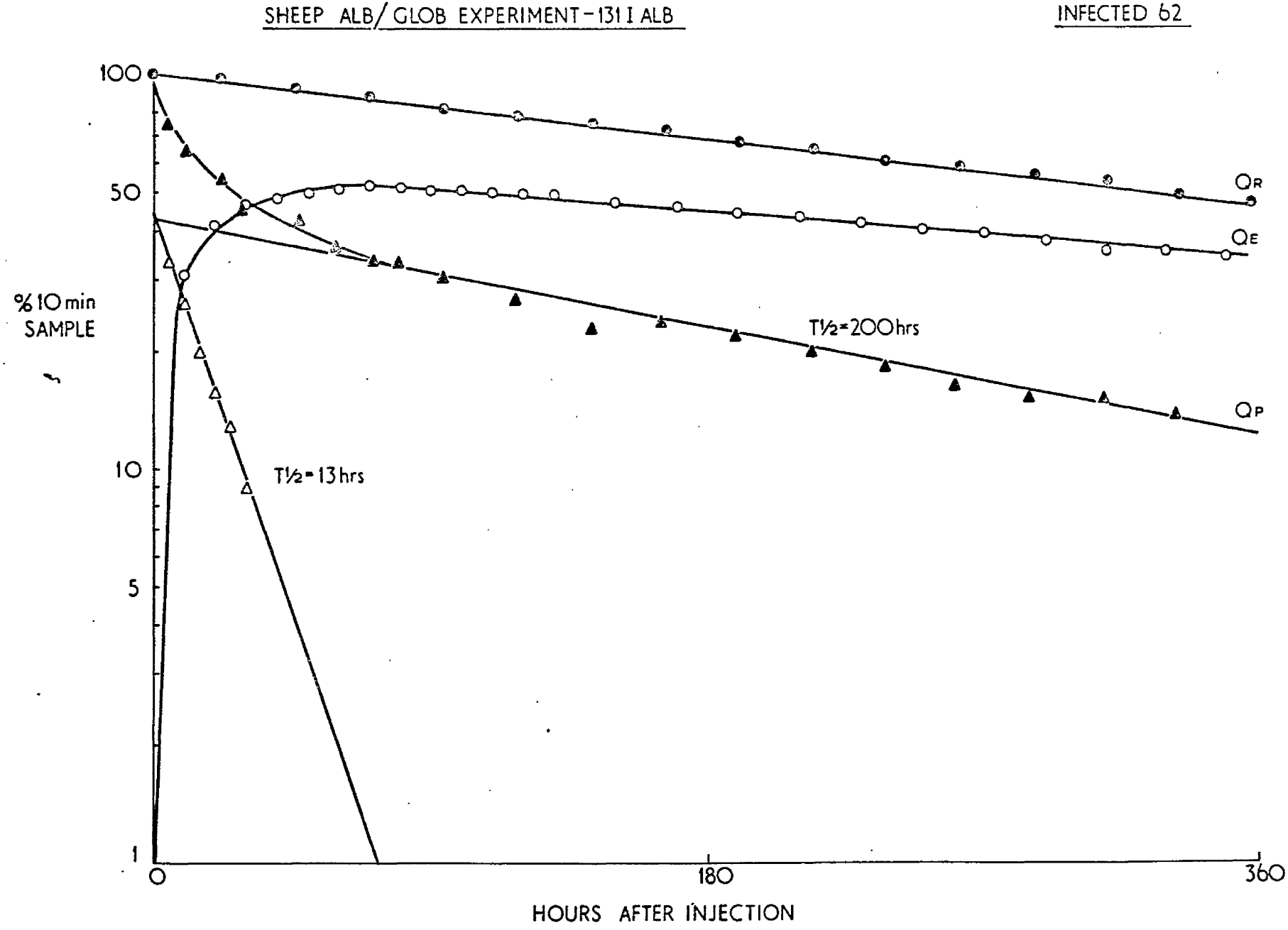


Fig. 13. Albumin Turnover in a Fluke-Infected Sheep

and the fractional catabolic rate calculated from the plasma disappearance curve ($r = -0.942$ $P < 0.01$). The "apparent half-lives" of both immunoglobulin and albumin were significantly reduced in the infected animals relative to the controls, with a positive correlation between plasma albumin levels and albumin half-lives ($r = 0.963$ $P < 0.01$) and a negative correlation in the five sheep between plasma gammaglobulin levels and the IgG half-lives ($r = -0.736$ $P < 0.1$).

The grams of gammaglobulin catabolised per day was apparently increased in the infected sheep, though because of the scatter and small number of animals examined this increase was statistically insignificant.

The faecal clearance of plasma was elevated in the infected animals, as shown by both the ^{131}I-albumin and ^{125}I-IgG data, compared to the controls. In addition, the ^{125}I-IgG results gave a higher clearance value than the ^{131}I-albumin results in both the control and infected sheep. However these values are gross underestimates of true gastro-intestinal plasma loss because of the significant reabsorption of iodine label which takes place in the small intestine.

Discussion

Tests on the quality of the immunoglobulin preparation indicated both that it was homogenous, consisting only of IgG slow and that all the radioactivity was protein bound. Evaluation of the turnover data of both IgG and albumin showed that no significant denaturation of the preparation had occurred since(1) the urinary activity excreted in the

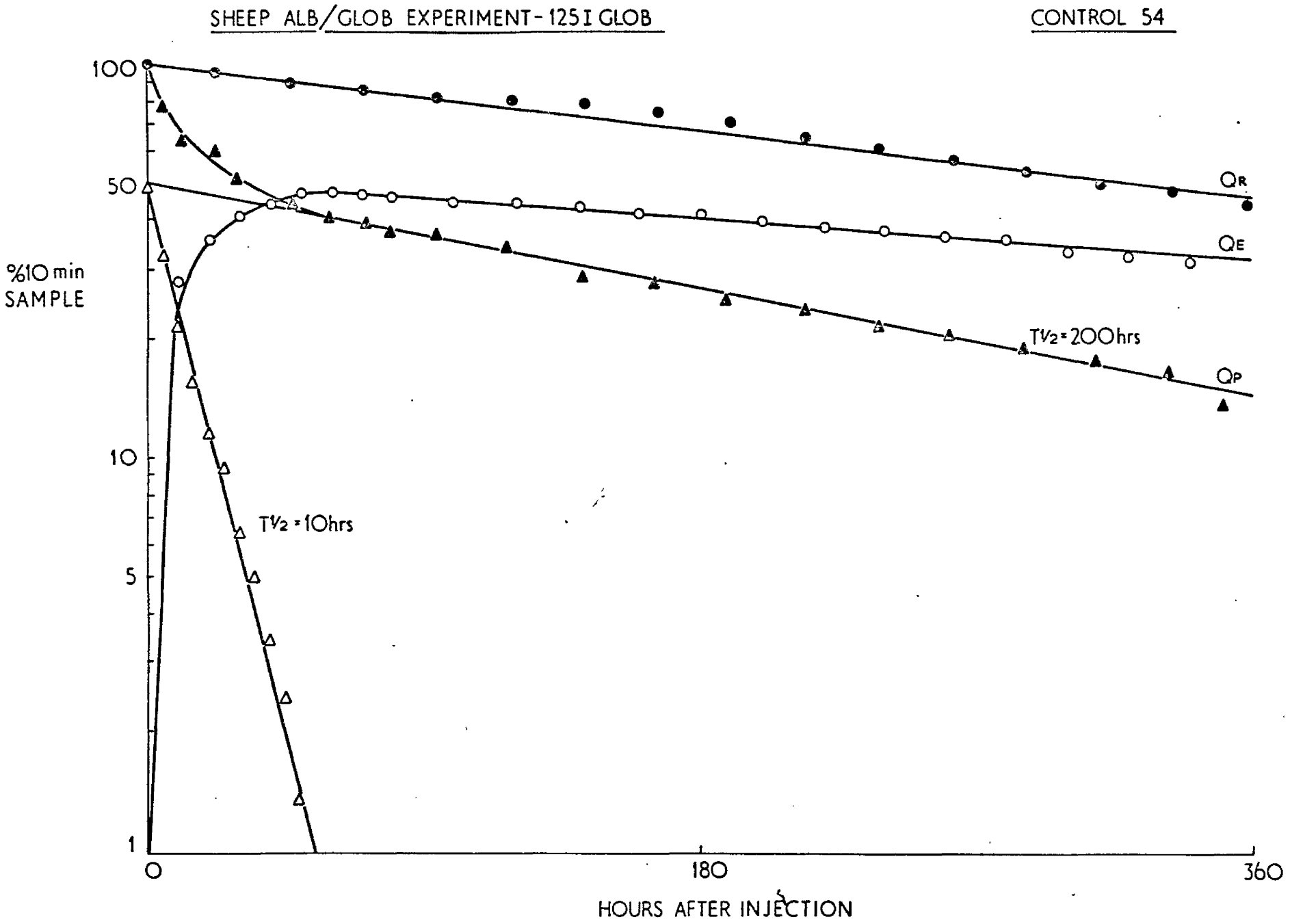


Fig. 14. IgG Turnover in a Normal Sheep

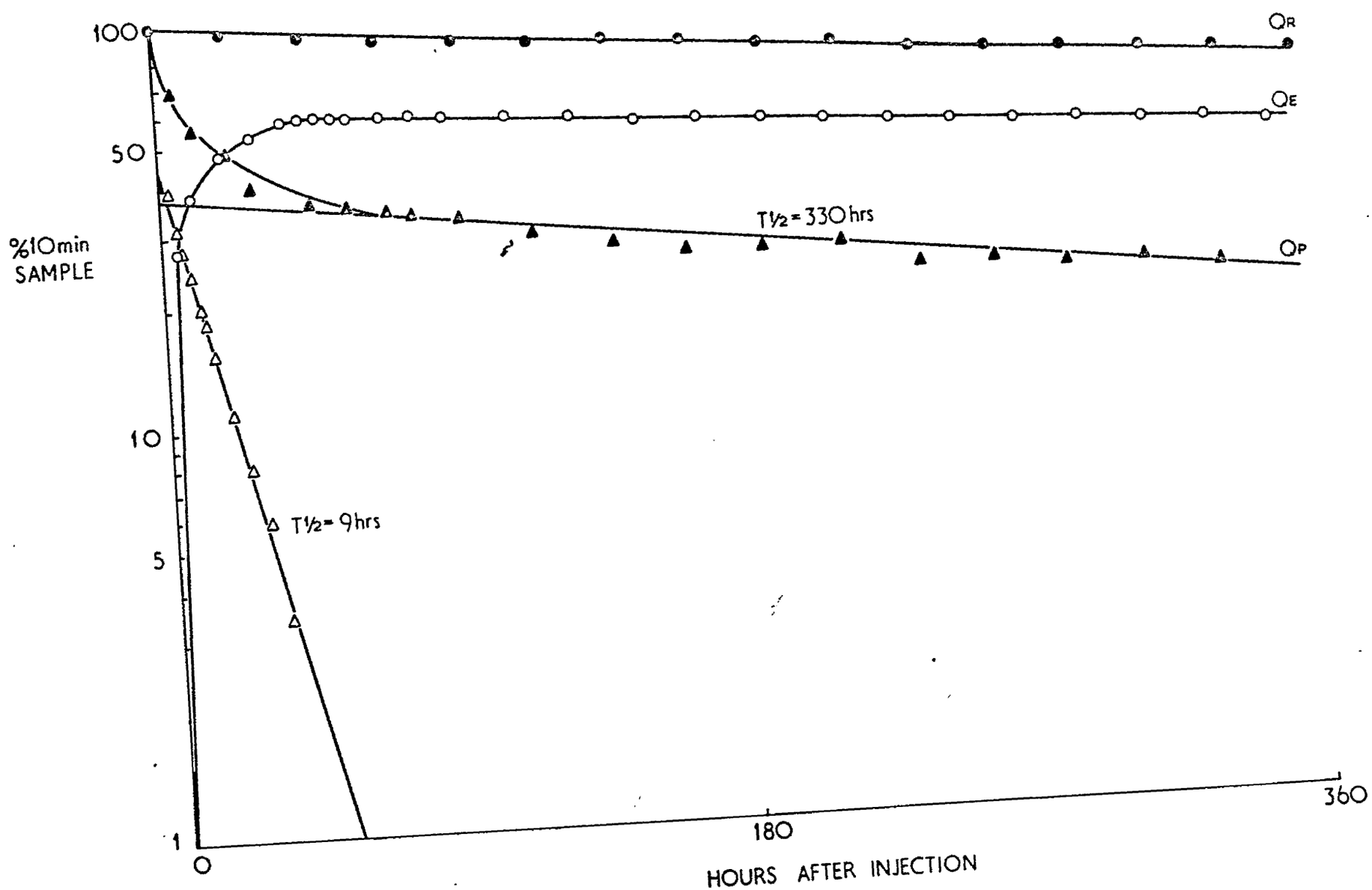


Fig. 15. Albumin Turnover in a Normal Sheep

first 24 hours was not higher than on any subsequent days, (2) there was no decrease in the fractional catabolic rate calculated from excreted activity during the period of observation, (3) the plasma volumes calculated from both the IgG and albumin data showed very close agreement.

The plasma concentrations of gammaglobulin and albumin revealed that there appeared to be an inverse relationship between the two plasma proteins in both the infected and control animals ($r = -0.004$ $P < 0.1$). The sheep with the most pronounced hypergammaglobulinaemia was the most hypoalbuminaemic and vice versa.

In the calculations of IgG plasma concentrations in the two groups of sheep, some explanation is required. The concentration of gammaglobulin was taken as being the same as the IgG concentration. This is not strictly true since although IgG form the greatest part of the protein in the gammaglobulin band, other immunoglobulins are also present and in addition some IgG is found outside the gammaglobulin band. However these factors tend to counterbalance one another so that changes in serum gammaglobulin can in general be taken to represent changes in IgG concentration. Ideally IgG levels should be determined by immuno-precipitation but this was not possible in the present investigation.

The distribution of IgG was quite similar to that of albumin, but there was an indication that a greater proportion of IgG was in the intravascular compartment than in the extravascular. The extravascular/

intravascular ratio of IgG was similar in the two groups of sheep, though the ratio of albumin distribution was reduced in the infected group, as has been shown earlier in subsection A. Though only a small number of sheep were involved in the present experiment it is pertinent that work in normal human subjects and patients with hypergammaglobulinaemia due to various causes, showed that the distribution ratio of IgG was identical in the two groups.

The results clearly show that the catabolic rate of both IgG and albumin were significantly greater in the infected animals. This was demonstrated by the decreased "apparent half-lives" and elevated fractional catabolic rates, calculated both from excreted activity and mathematical analysis of the plasma activity disappearance curves.

It should be noted that even in the normal sheep IgG catabolism was nearly twice the level of the albumin catabolic rate. Early studies in humans to compare degradation rates of IgG and albumin have reported that the rates are similar (Dixon, Talmage, Maurer and Deichmiller, 1952; Gitlin, Janeway and Farr, 1956). More recently, using better preparations it has been shown that the catabolic rate of gammaglobulins is lower than albumin in normal adult human patients (Jarnum, 1963; Andersen, 1964). Studies in rabbits (Dargie, 1969) showed that in this species the turnover rate of immunoglobulin was higher than that of albumin in apparently normal animals.

The faecal plasma clearance rates calculated from IgG and albumin data would seem to suggest that the gastrointestinal tract may be more

important in the catabolism of immunoglobulin than of albumin. However since there is extensive reabsorption of iodine label from the gut it is not possible to be sure on this point.

Since the animals under study were in roughly steady state conditions it follows that synthesis is equal to catabolism. In terms of grams of protein synthesised per kilogram body weight per day, it appears that in the sheep showing the most pronounced hypergammaglobulinaemia IgG was being synthesised at approximately four times the control level, yet albumin synthesis was only slightly increased.

It is interesting to compare these results with similar independent studies in fluke-infected and normal sheep by Nansen, Eriksen, Simesen and Nielsen (1968). They studied simultaneously the turnover rates of albumin and IgG slow, in a group of eight naturally infected sheep and compared these with three normal controls.

The results of the albumin studies were very closely similar to those obtained in the present work. The diseased animals showed decreased half-lives and increased fractional catabolic rates of albumin. Unfortunately their IgG preparation appeared to be significantly denatured since the plasma volumes calculated from the IgG data were grossly in excess of those calculated from the albumin results. It is generally agreed that the albumin plasma volume normally exceeds the gammaglobulin plasma volume by approximately 2.5%, (Andersen, 1962). Since the preparation used by Nansen et al (1968) was denatured it was

not possible to calculate turnover rates for IgG though the half-lives were very similar to those obtained in the present study. The reason for the similar half-lives despite the denaturation would appear to be due to the type of denaturation, since Freeman (1959) showed that overiodinated albumin has an increased breakdown rate throughout the whole experiment so that no deduction on turnover can be made. However heat-denatured protein is rapidly removed from the circulation by the reticulo-endothelial system, where the protein is phagocytized and the label rapidly excreted in the urine, and the protein which remains has a similar catabolic rate to native protein, hence it is possible to obtain normal plasma half-lives despite some denaturation of the injected preparation.

From the reduced IgG half-lives in the infected animals Nansen et al (1968) concluded that IgG synthesis was approximately four times the control level; this agrees well with the present study in which the mean infected level was three times that of the controls.

The most important factor regulating immunoglobulin synthesis is antigenic stimulation. There is extensive evidence that animals produce antibodies following either infection with F. hepatica of the parenteral injection of a range of somatic and metabolic antigens prepared from young and adult flukes. The immunological tests which have had most attention are the precipitin and complement fixing reactions, and a wide range of antigens have been studied, (Urquhart,

Mulligan and Jennings, 1954; Hughes, 1963; Sewell, 1966). Various attempts have been made to induce immunity to fascioliasis by both active and passive immunisation but unfortunately to date they have not achieved great success. So it would seem that the flukes provide the major antigenic stimulus for increased immunoglobulin synthesis observed in chronic fascioliasis.

It is well known that the cellular response around the parasitized bile ducts involves large numbers of both lymphocytes and plasma cells. Both these cell types have been shown to be involved in antibody production. The extent to which the local production of antibody is responsible for the hypergammaglobulinaemia observed in fascioliasis has yet to be elucidated, since IgG synthesis normally occurs in extrahepatic tissues. However, recent studies by Jabbari and Leevy (1967) in rats with CCl_4 - induced liver cirrhosis have shown that immunoglobulins are synthesized in the liver, under these circumstances. Thus it would appear that the liver in addition to being involved in increased albumin synthesis in chronic fascioliasis, is also responsible for the elevated immunoglobulin levels found in this disease. Since IgG synthesis at least in moderate infections is greater than the loss of plasma caused by the flukes, a hypergammaglobulinaemia develops. Whilst albumin synthesis is increased to a lesser amount and so loss of plasma outstrips synthesis, leading to a hypoalbuminaemia.

It appears that reduction in IgG levels per se do not stimulate synthesis,

whereas in the case of albumin a reduction in plasma albumin levels can stimulate synthesis. Studies on rabbits involving daily plasmaphoresis (Matthews, 1961) have indicated that despite significant loss of plasma, albumin levels can be maintained. The extent to which albumin synthesis can be increased is not known, but it is generally elevated in clinical cases of gastrointestinal protein loss in humans, though the highest rate is not normally more than twice the normal level (Gordon, Bartter and Waldman, 1959; Spaertz and Jarnum, 1959; 1961). However IgG synthesis is capable of being increased to as much as 30 times the normal level (Birke, Liljedahl, Olhagen, Plantin and Ahlinder, 1965).

The loss of plasma protein in the gut as a result of the presence of adult flukes in the bile ducts, not only imposes an excessive demand on the synthetic mechanisms but also represents an important loss of antibodies which must be resynthesized from the reabsorbed amino acids. Hence, it is not surprising that fluke-infected animals are found to be extremely susceptible to a variety of other disease conditions.

Summary

The turnover of ^{131}I-albumin and ^{125}I-IgG were studied simultaneously in two fluke-infected sheep, along with three control animals. The synthetic rate of immunoglobulin was increased to a greater extent than that of albumin in the infected animals. It is suggested that the cause of the hypergammaglobulinaemia and hypoalbuminaemia observed in chronic ovine fascioliasis is due to the relative increase

in synthetic rates of IgG and albumin in the face of the substantial plasma loss which has been shown in the previous subsection to occur in fluke-infected animals.

SECTION III

STUDIES OF THE ONSET OF PATHOPHYSIOLOGICAL CHANGES FOLLOWING INFECTION

Introduction

Fascioliasis in sheep is generally considered to occur in two distinct forms, acute and chronic. The acute type results from ingestion of large numbers of metacercariae and death normally occurs during the migration of the immature flukes in the liver parenchyma. Chronic fascioliasis is associated with the adult stage of the parasite in the bile ducts of the sheep. It is characterised by progressive loss of weight, anaemia, hypoproteinaemia and in many cases eventual death, if the animal is not treated.

The experiments described in Sections I and II of this thesis were all carried out during the chronic phase of the disease, i.e. when adult flukes were in the bile ducts.

An attempt was made to investigate the changes in red cell and albumin turnover which occur during the migratory or acute phase of *F. hepatica* infections, and to correlate these changes in turnover with alterations in haematocrit and albumin concentrations.

Many of the initial studies on the pathogenesis of fascioliasis were concerned with the migratory route of the parasite from the alimentary tract to the liver. Largely as a result of the work of Schumacher (1938), it is now accepted that the young flukes migrate across the abdominal

cavity. Earlier reports by Simitsin (1914) and Shirai (1927) had shown that following the infection of rabbits, flukes could be found in the peritoneal cavity before there was evidence of liver damage. The ability of young flukes to penetrate the liver capsule has been demonstrated in rabbits, goats and sheep (Shaw, 1932) and in cattle (Morrill and Shaw, 1942) by injecting immature flukes directly into the peritoneal cavity. Various other studies in laboratory animals have confirmed that this is the common migratory route (Urquhart, 1956; Schumacher, 1956; Dawes, 1961a).

The time taken for migration from the intestine across the peritoneal cavity to the liver appears to vary with different experimental hosts. Dawes (1961a, 1961b, 1962) found that in mice, young flukes could be found in the abdominal cavity less than 24 hours after infection, by 48 hours they had begun to penetrate the liver capsule, and at 24 days they had entered the bile ducts. Little work has been carried out in other species though Kendall and Parfitt (1962) in a report on chemotherapy of fascioliasis in sheep briefly state that young flukes may be found in the peritoneal cavity 18 hours after infection and after a further 72 hours they have penetrated the liver capsule.

More work has been directed to determining the prepatent period of the parasite because of its diagnostic value. Kendall and Parfitt (1962) found flukes in the bile ducts of sheep 40 days post infection, and eggs in the faeces by 55 days. Sinclair (1962) also first

observed eggs in the faeces of sheep 56 days after infection, whilst Dixon (1964) detected eggs 63 days after infecting experimental sheep. In more recent studies Furmaga and Gundlach (1967a) reported the pre-patent period in sheep to be 73 - 88 days. Reid (1968) first observed eggs in the faeces 70 days post infection, but it was 90 days before eggs appeared in the majority of the infected group.

Detailed investigations of the histopathology of the migratory phase of Fasciola hepatica infections have been well documented in experimental infections of laboratory animals (Urquhart, 1956; Dawes, 1963; Thorpe, 1965a), but only recently in cattle and sheep (Ross, Todd and Dow, 1966; Dow, Ross and Todd, 1967; Dow, Ross and Todd, 1968).

Many investigators have recorded changes in haematological and plasma protein values during the acute stage. Sinclair (1962, 1964) observed that a fall in red cell numbers began at about 60 days post infection, whilst Furmaga and Gundlach (1967a) reported a developing anaemia from seven weeks post infection, but Reid (1968) using slightly heavier infections reported a fall during the 5th week of the disease.

There have been several reports of the plasma protein changes following infection. Sinclair (1962) reported a fall in plasma albumin levels commencing approximately 56 days post infection and a rise in the gammaglobulin fraction from 35 days onwards. Furmaga and Gundlach (1967b) described a fall in plasma albumin and a rise in gammaglobulin

from seven days post infection though there were considerable fluctuations during the first 5 weeks. Reid (1968) reported a fall in albumin and a rise in gammaglobulin commencing about 5 weeks post infection.

Though there have been several descriptions of the sequential changes in red cells and albumin levels following infection, there has not been any attempt to explain these changes. Since the fall may be due to decreased production, increased breakdown or altered distribution, it is only by a study of the kinetics of the blood constituents that the true aetiology may be understood.

Studies of the onset and development of the anaemia and hypo-albuminaemia were carried out simultaneously using ^{125}I-albelled albumin and ^{51}Cr-labelled red cells. The investigation covered the thirteen weeks following infection, i.e. the migratory or acute phase of the disease and beyond, with a further study at twenty three weeks, to determine whether there was an increasing demand by the parasites on the host following their development in the bile ducts.

Materials and Methods

I. Experimental Procedure

The experiment was designed to cover the migratory phase of fluke-infection of sheep. The period was subdivided into three separate experiments. 1 - 3 wks, 3 - 6 wks, and 6 - 13 wks post infection. In each subperiod red cell and albumin turnover rates were determined in

three normal sheep and in three animals infected with 1,000 metacercariae each. Two of the sheep studied in the third stage were investigated further at 23 - 25 weeks post infection. In addition two sheep infected with 500 metacercariae each were studied from 10 - 13 weeks post infection.

II. Experimental Animals

The twentyone sheep used in this experiment were adult Blackface wethers aged 12 - 15 months, and had been reared and maintained in conditions precluding the possibility of infection with helminths. Drinking water containing 0.0025% KI and 1% NaCl was given four days prior to the injection of ^{125}I-albumin and from there on continuously throughout the experimental period.

III. Labelling Procedures

(a) Preparation of ^{125}I-albumin

Labelling was carried out by the method of McFarlane (1958) as described fully in Section IIA. In each experiment 30 ml of 2% sheep serum albumin was trace labelled with 10 mc Na ^{125}I. Each sheep received approximately 10 ml of labelled protein containing about 1,200 µc.

(b) Labelling of Red Cells with ^{51}Cr

About 40 ml of heparinized blood was taken from each sheep and the plasma removed and retained. Approximately 10 ml of red cells were labelled with 0.5 ml $Na_2{}^{51}CrO_4$ (specific activity 10 mc ^{51}Cr per ml, chromium content 35 mg/ml) and incubated for 30 mins at 37°C.

The labelled cells were then washed free of unbound isotope and reconstituted with the retained plasma prior to injection.

IV. Injection and Sampling

The labelled red cells and albumin were injected together from separate syringes via a three way tap and jugular catheter. Heparinized blood samples were taken 10 minutes post injection and at frequent intervals during the first three days, and from there on samples were taken every 24 or 48 hours for the duration of the experiment. From each blood sample 1 ml of whole blood and 1 ml of plasma were carefully pipetted for radioactivity determination. The haematocrit was determined for each bleeding. Plasma protein concentrations were determined from serum samples taken each 14 days. Faeces and urine were collected each 24 hours and suitable aliquots taken.

V. Calculations and Expression of Results

^{125}I and ^{51}Cr can be measured in the same sample because of differences in the energy spectra of their radiations.

(a) ^{125}I-albumin Data

(i) Plasma Volume

The plasma volume was determined from the 10 minute sample by the dilution principle.

(ii) Albumin Pools

The intravascular pool (CA) was determined from the plasma volume and the plasma albumin concentration. The extravascular pool (EA) was obtained by application of the "equilibrium time" method

of Campbell et al (1956).

(iii) Catabolic Rate

"Apparent half-life"

The half-life $T\frac{1}{2}$ of the exponential portion of the plasma activity disappearance curve was taken as an approximate index of catabolism (Sterling, 1951a).

Fractional Catabolic Rate F(CA)

Since steady state conditions were not prevailing during this study catabolic rate was only determined by the method of Campbell et al (1956). This method is based on the daily excreted activity.

(b) ^{51}Cr-red Cell Data

(i) "Apparent Red Cell Half-life" $T\frac{1}{2}$

The half-life of the red cell activity was obtained from the activity disappearance curve.

(ii) Faecal Clearance of Red Cells

The radioactivity of each 24-hour faecal collection was expressed as millilitres of red cells lost into the gut.

Results

0 - 3 Weeks Post Infection

Albumin Distribution at Time of Infection

The results are presented in the appendix Table I. It is apparent that there is no difference in albumin concentration and distribution between the two groups.

Albumin Turnover

There were significant differences in albumin turnover (Appendix Table II) in the period 0 - 1 week post infection when the fractional catabolic rate of the infected group was higher than the controls. Similarly the grams of albumin catabolised were increased during 0 - 2weeks in the parasitized sheep.

Red Cell Turnover

No differences were apparent between the two groups (Appendix Table III). Unfortunately the faecal ^{51}Cr activity was too low to allow accurate measurements of red cell clearance, though it is known that the ^{51}Cr red cell loss into the gut was similar in both groups.

3 - 6 Weeks Post Infection

Albumin Distribution at 3 Weeks Post Infection

No marked alterations in albumin distribution were noted (Appendix Table IV), though the serum albumin concentration and extravascular pool size were marginally reduced in the infected sheep. By 5 weeks post infection the albumin concentrations of the infected animals were much reduced,(see Figs. 16, 18.).

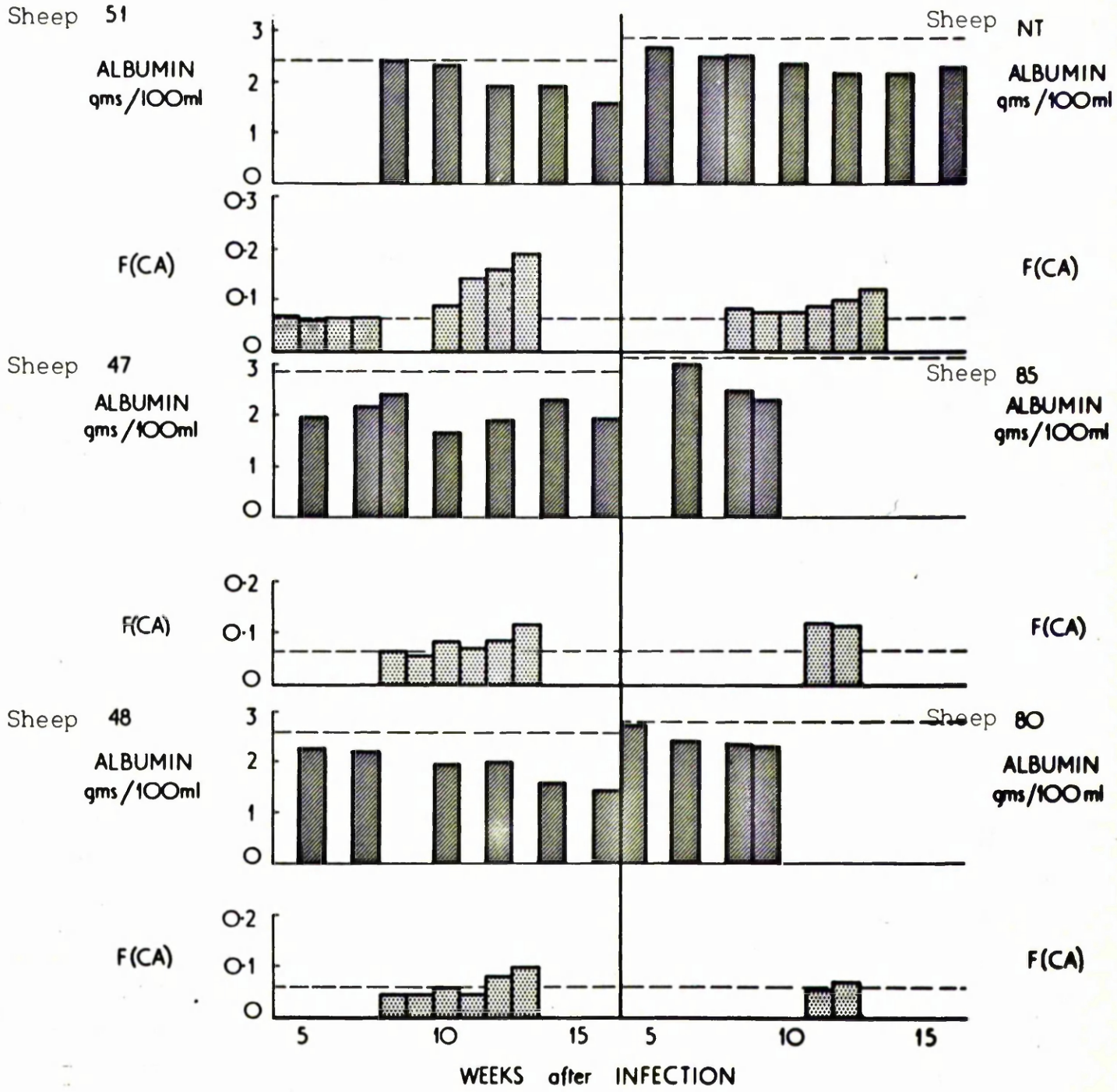
Albumin Turnover

There were no significant differences in turnover rate between the infected and normal sheep (Appendix Table V).

Red Cell Turnover

During the period 3 - 6 weeks post infection, there was a significant reduction in the red cell survival of the infected animals

Fig. 16. ALBUMIN CATABOLISM following INFECTION with F. HEPATICA (0 – 15 weeks)



(— — — Mean Control)

as illustrated by the markedly reduced half-life (Appendix Table VI). This loss of red cells from the circulation could not be explained by either increased haemolysis, since the urinary activity was identical in both groups, or loss of red cells into the gut since there was no rise in faecal activity. By 6 weeks post infection the haematocrit of the parasitized sheep (mean 25% ± 2) was significantly lower than the controls (mean 29% ± 2) $P < 0.05$, (Fig. 18).

6 - 13 Weeks Post Infection

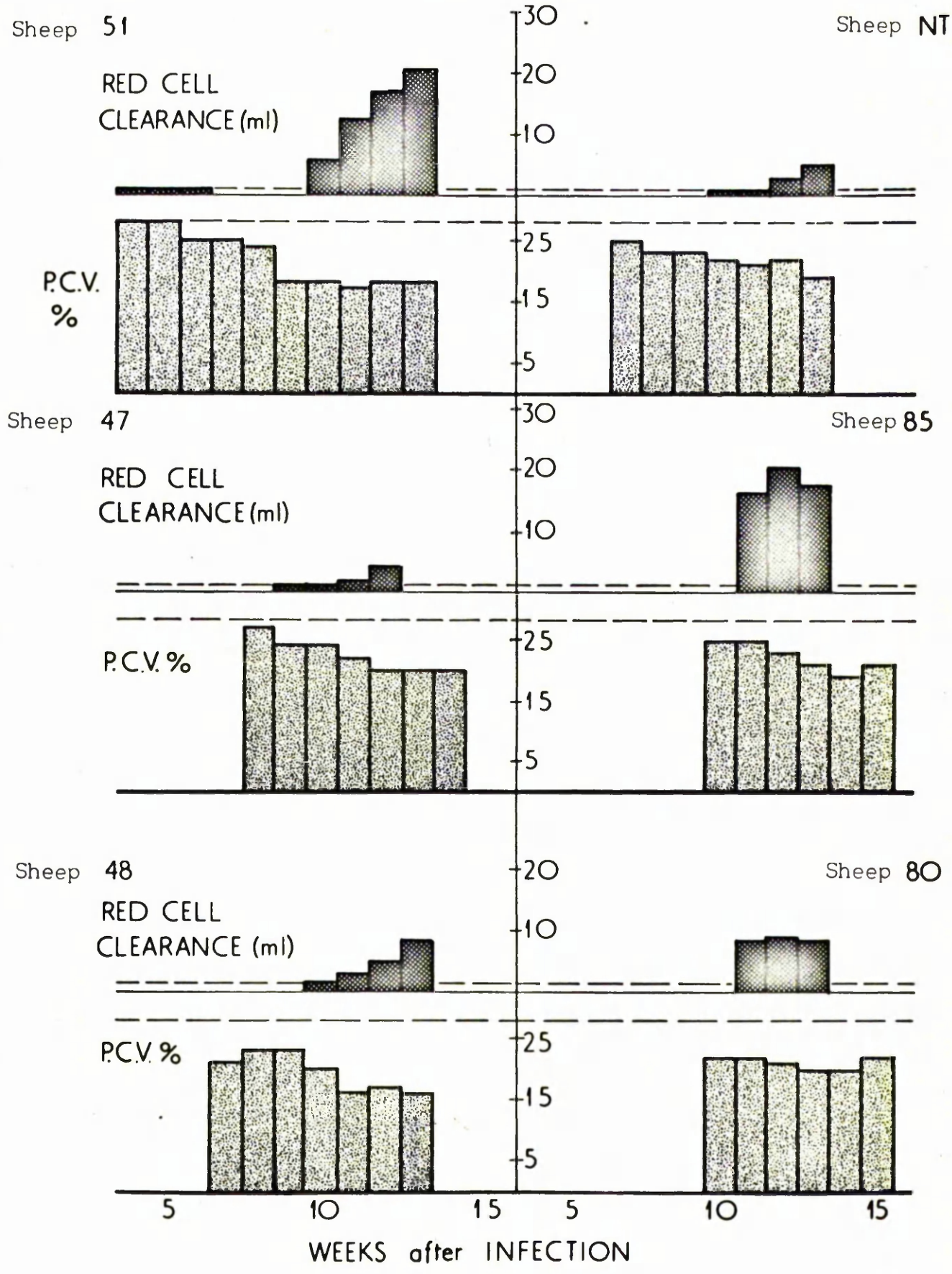
Albumin Distribution at 6 Weeks Post Infection

At this stage of the infection the plasma volume and intravascular albumin pools were significantly greater in the infected sheep (Appendix Table VII). The albumin concentration was significantly lower in the infected animals. Total protein levels were increased in the infected animals (mean 7.47 ± 0.28) relative to the controls (mean 6.77 ± 0.53) as a result of a rise in serum globulin at this time, illustrated by a significantly decreased albumin/globulin ratio ($P < 0.001$) in the infected sheep.

Albumin Turnover 7 - 10 Weeks

At this stage of the infection the "apparent half-life" of albumin in the infected sheep was significantly reduced (440 ± 17 hrs) compared with the controls (527 ± 28 hrs). Though the catabolic rate was not increased until week 9 - 10 when the amount of albumin catabolised in the infected animals was significantly greater than the controls.

Fig. 17. RED CELL LOSS following INFECTION with F. HEPATICA (0-15 weeks)



(— — — Mean Control)

Albumin Turnover 10 - 13 Weeks

Sheep No. 51 was reintroduced into the experiment at this stage, and largely as a result of this the catabolic rate of albumin was much greater in the infected group, so that by week 12 - 13 the turnover rate was twice that of the controls (Appendix Table IV). Because of the wide scatter of the infected group the differences between the diseased and normal animals were not statistically significant except in the case of the "half-lives" when the mean $T\frac{1}{2}$ of the infected group was much shorter ($P < 0.01$) than that of the control group.

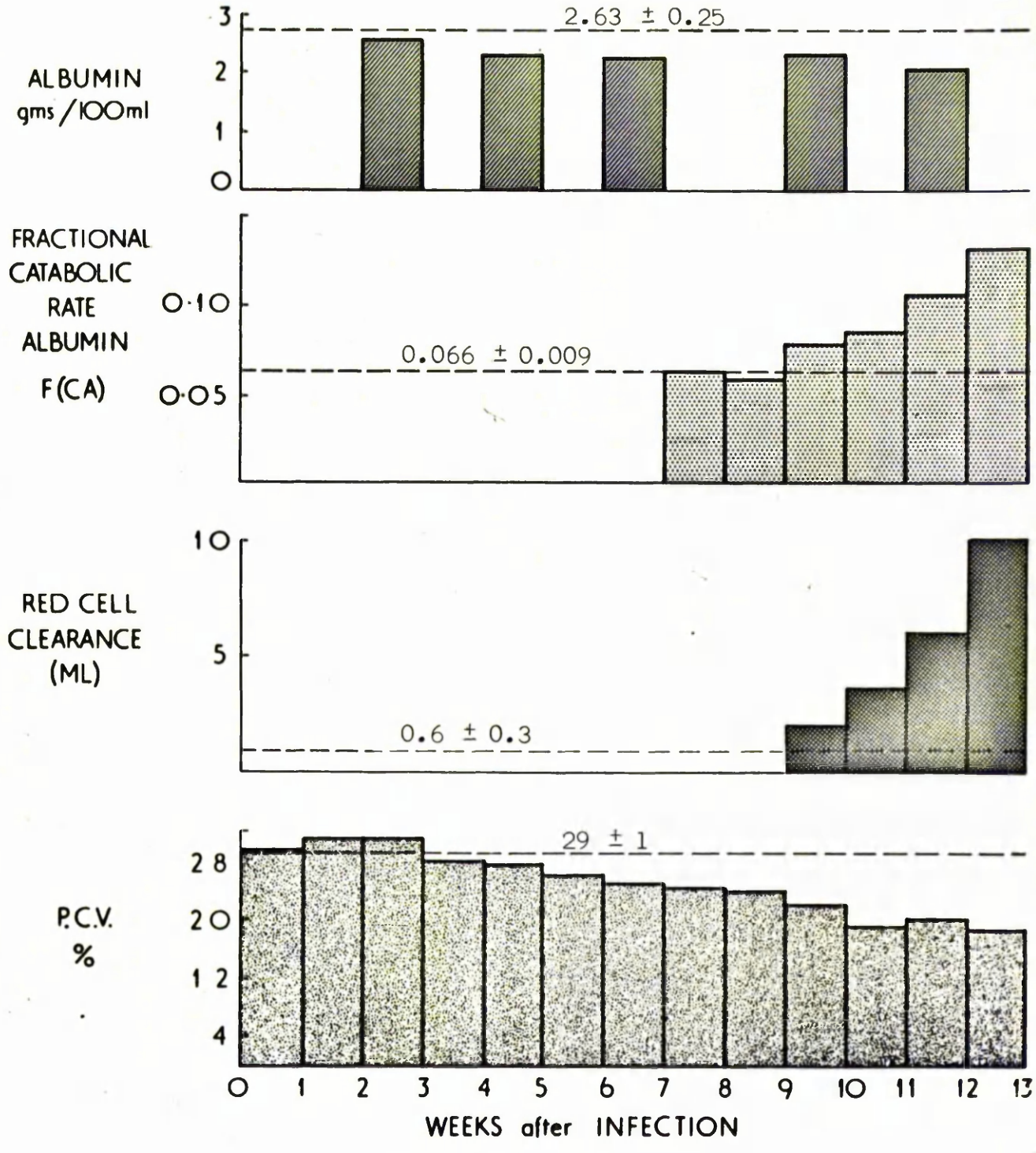
Red Cell Turnover 7 - 10 Weeks

The haematocrit of the infected animals continued to fall during this period, Figs. 17, 18. Unfortunately the ^{51}Cr faecal activity of both groups was too low to allow accurate measurement of red cell loss into the gut. However it was obvious that no significant loss of red cells was occurring by this route in either the infected or normal animals.

Red Cell Turnover 10 - 13 Weeks

The circulating red cell volume as ml/kg was reduced in the infected sheep, as was the haematocrit (Figs. 17, 18. Appendix Table X). The red cell survival time was significantly lower in the parasitized sheep. Examination of the excretory routes of ^{51}Cr showed that the loss of red cells was occurring into the gut, as illustrated by the increasing faecal clearance of red cells, and was not due to increased

Fig. 18. RED CELL LOSS and ALBUMIN CATABOLISM following INFECTION with F. HEPATICA (0-14 weeks) MEAN VALUES



(— — — Mean Control)

haemolysis or elution since the urinary activity was similar in both groups.

Examination of the fluke burden of two of the sheep studied 10 - 13 weeks post infection indicated that the sheep with the lower infection, i.e. sheep No. 51, developed an increased red cell loss and hypercatabolism of albumin earlier than the other sheep. To test the hypothesis that lower burdens lead to more rapid migration to the bile ducts two more sheep given lower doses of metacercariae were studied and the results are shown in the Appendix (Tables XI, XII, XIII).

They show that by 10 weeks these sheep had developed both a significantly higher red cell loss and catabolic rate of albumin, compared to sheep with heavier infections.

23 - 25 Weeks Post Infection

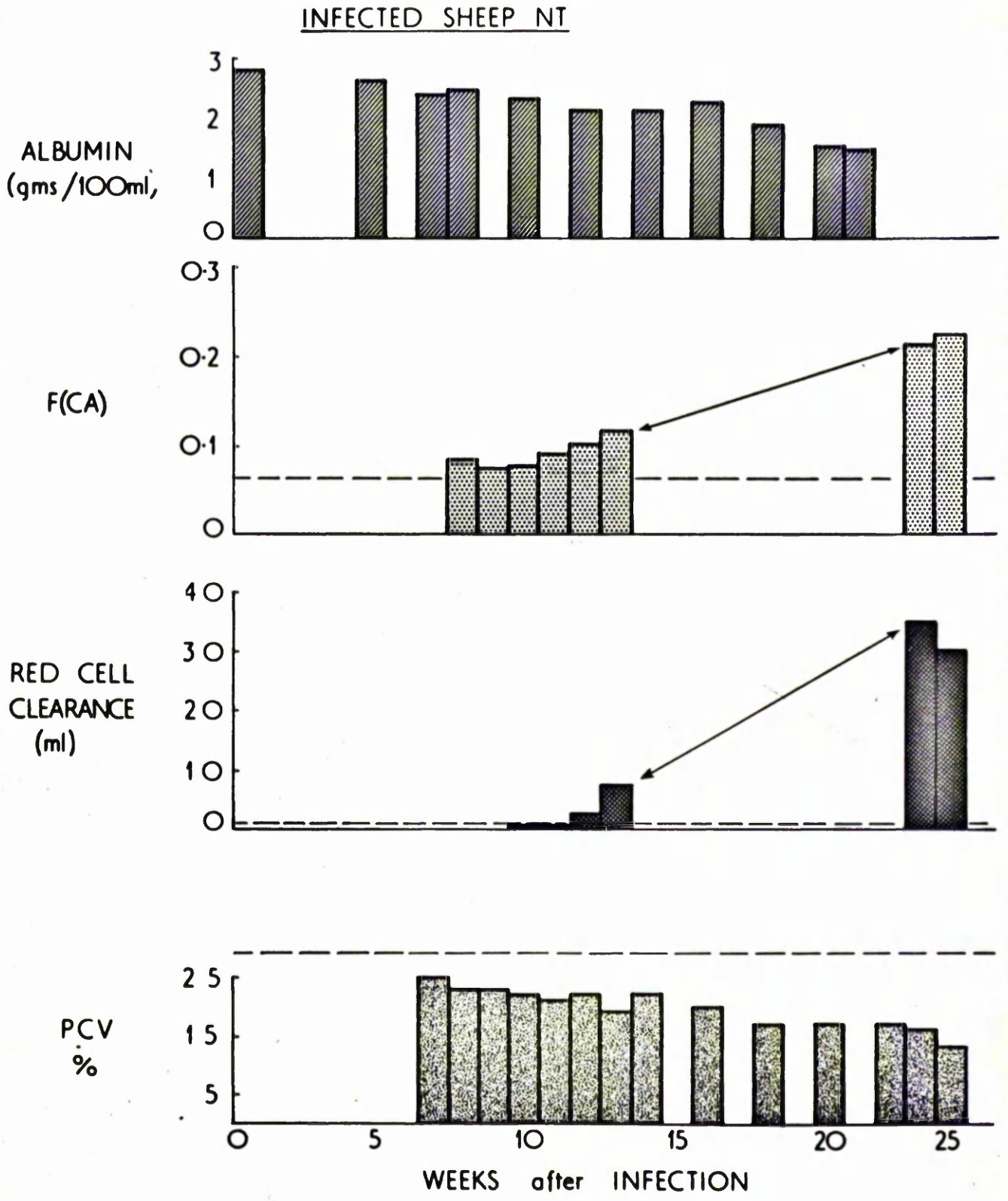
Albumin Distribution at 23 Weeks

Comparison of Appendix Tables VII and XI and Figs. 19 and 20 show that by 23 weeks the sheep had become progressively more hypoalbuminaemic with a marked reduction in the intravascular albumin pool and an even greater drop in the extravascular pool, so that the EA/CA ratio was reduced.

Albumin Turnover

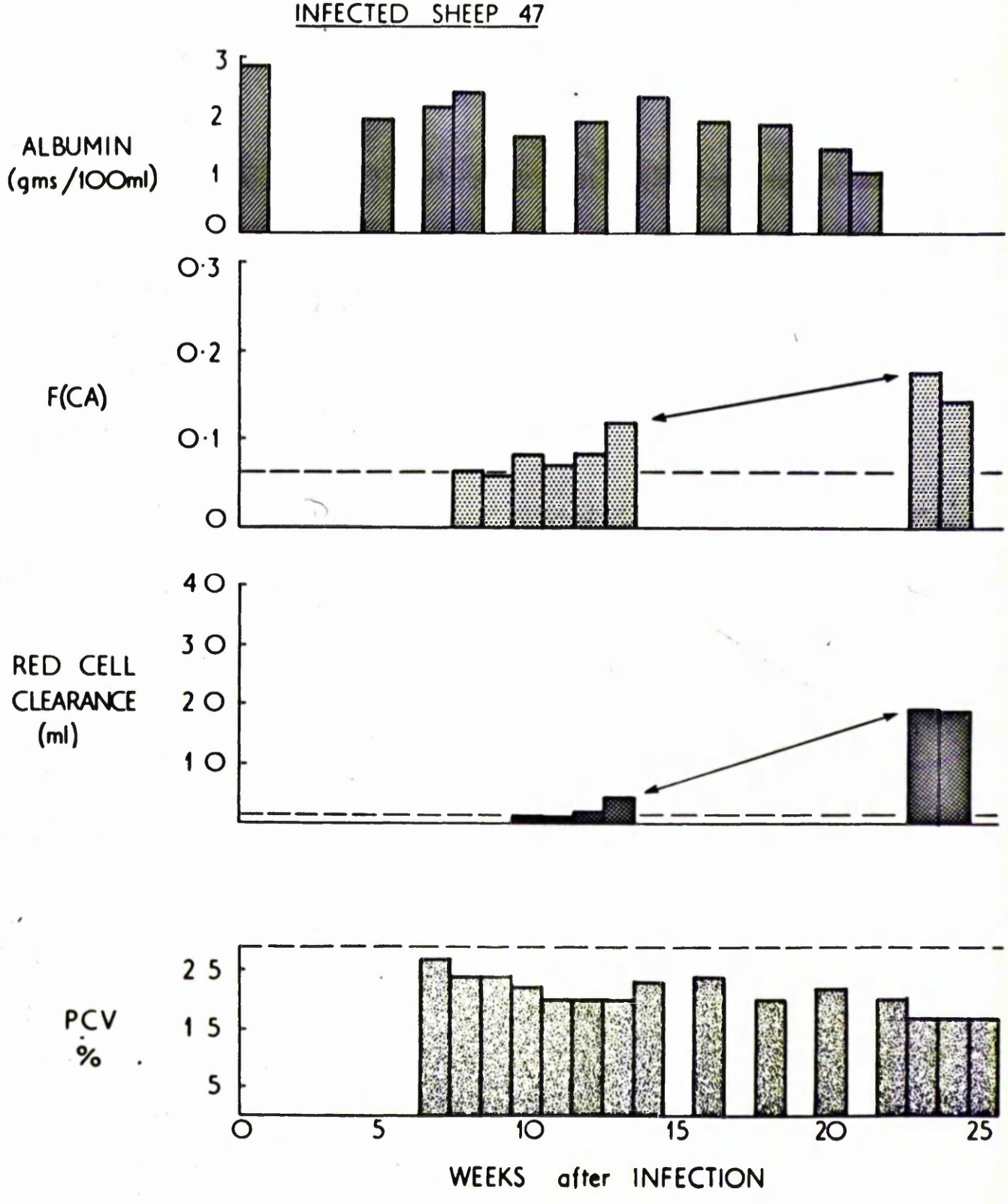
The turnover rate of albumin was significantly increased above the rate observed at 13 weeks post infection, Appendix Table XII, with the fractional catabolic rate three times the control level. Similarly

Fig. 19. Red Cell Loss and Albumin Catabolism following Infection



(— — — Mean Control)

Fig. 20. Red Cell Loss and Albumin Catabolism following Infection



(/ — — Mean Control)

the "apparent half-life" was greatly reduced to a third of the control level.

Red Cell Turnover

By 25 weeks the haematocrits were further reduced, and the circulatory red cell volumes were significantly lower than those of the controls. There was tremendous loss of red cells from the circulation as shown by the reduced red cell "half-life". This loss was shown to be due to a grossly elevated faecal red cell clearance, (Appendix Table XIII).

Discussion

The experiment covering the first three weeks following infection showed that there was no significant difference between the infected and normal groups. The only exception to this was that the fractional rate and grams of albumin degraded by the infected animals was increased marginally in the two weeks following infection. Whether this was caused by the flukes migrating through the gut wall and penetrating the liver is doubtful, but it remains a possibility.

During the 3 - 6 week period a fall in serum albumin was observed as previously reported by other workers, (Fumaga and Gundlach, 1967b; Reid, 1968). This reduction may be due to decreased synthesis or increased catabolism or to a combination of both. Decreased albumin synthesis as occurs for example in cirrhosis, is characterised by increased plasma half-lives and low turnover rates (Sterling, 1951b;

Wilkinson and Mendenhall, 1963), whereas increased catabolism is distinguished by decreased plasma half-lives and elevated turnover rates. Under "steady state" conditions catabolism = synthesis, and although iodine-labelled albumin gives only a direct measure of catabolism, it is possible to speculate on synthesis under these conditions. However in the present study "steady state" conditions were not prevailing as shown by the altering body pools of albumin, so it is not possible to deduce exactly the state of synthesis in these animals. The catabolic rate is not markedly different from the controls although the plasma half-lives are slightly reduced. This suggests that the decrease in albumin is most probably due to a combination of decreased synthesis and normal or slightly elevated catabolism caused by the mechanical damage by the flukes during their migration through the liver parenchyma. Whether the decreased synthesis if it occurs, is due to damage to the hepatic cells or to other causes has yet to be elucidated; e.g. it has been shown that a decrease in albumin synthesis can occur in situations of hypergammaglobulinaemia as a regulatory mechanism to maintain the colloid osmotic pressure of the plasma (Bjorneboe and Schwartz, 1959).

A progressive anaemia also developes at 5 weeks post infection as previously reported by Reid (1968) although examination of faecal excretion of ^{51}Cr showed that it was not until 10 weeks post infection that a significant loss of red cells into the gut occurred. The fall in haematocrit was accompanied by a shortening of the red cell survival

time of the infected sheep, indicating that red cells were being lost from the circulation in excess of normal breakdown. One possible explanation for the reduction in both haematocrit and red cell $T\frac{1}{2}$ at this stage is not a loss of red cells but a haemodilution effect related to the hyperproteinaemia present at this time. However this is unlikely since the plasma volume and albumin $T\frac{1}{2}$ of the parasitized sheep were in the normal range. Nor can the red cell loss be explained by either increased haemolysis or elution, since the urinary activity was identical in the two groups. Dow et al, (1968) have described the histopathology of Fasciola hepatica infections of sheep at different stages of migration through the liver. They report that by 6 weeks after infection many of the tracts caused by the burrowing flukes were filled with blood. It is during this time that the growth rate of the young flukes is greatly accelerated (Boray, 1967), so it seems possible that the haemorrhagic lesions caused by the rapidly growing young flukes may be sufficient to cause a fall in both haematocrit and red cell $T\frac{1}{2}$, and for reasons which are not fully understood the ^{51}Cr does not appear quantitatively in the urine.

By about 10 weeks when the flukes are entering the bile ducts in large numbers (Dow et al, 1968) a progressively increasing loss of red cells into the gut commences which rapidly becomes of such a magnitude as to account for the anaemia which continues to develop.

The loss of red cells is presumed to be a loss of whole blood. This hypothesis is supported by the rise in catabolic rate of albumin which commences at about the same time and is accompanied by a further reduction in serum albumin levels.

It is apparent from the results that there was a marked variation in the time taken by the flukes to migrate to the bile ducts as judged by the elevated red cell loss into the gut and rise in albumin catabolism. The sheep with the lighter infections showed loss of red cells and albumin much earlier than those with heavier infections. This may be due to an inhibition in development of the young flukes in heavier infections due to a crowding effect similar to that seen in some parasitic nematode conditions in which massive infections lead to inhibition of development of the larvae and reduced body size of the adults. A similar phenomenon has been shown to occur in experimental fascioliasis of the rat (Thorpe, 1965a) and in calves (Ross, 1965). More recently Boray (1967) reported that in light fluke infections of sheep most flukes were already in the bile ducts from week 8, but in heavier infections large numbers of flukes were still in the parenchyma up to 12 weeks post infection. It is not known whether retardation is brought about by an immune response or mechanical obstruction to the migrating flukes from the severe tissue reaction caused by the massive invasion of the liver by the young flukes.

By 23 weeks post infection there had been a progressive decrease

in both serum albumin and red cells and a commensurate increase in both the catabolic rate of albumin and in the loss of red cells into the gut. These were presumably both due to an increasing blood loss caused by the maturing parasites in the bile ducts. Previous work by Dixon (1964) showed that flukes continued growing in size until at least twenty weeks post infection.

As a result of the present studies and those of other workers it is apparent that the onset and development of the anaemia and hypoalbuminaemia associated with fascioliasis are governed by the size of the infecting dose of metacercariae. When sheep are harbouring infections of less than 200 flukes the fall in haematocrit and serum albumin occurs at approximately nine weeks post infection, when the flukes are entering the bile ducts, (Sinclair, 1962). In heavier infections of 250 - 650 parasites (Reid, 1966) and in the present study a moderate anaemia and hypoalbuminaemia developed during the migratory (acute) stage at approximately 5 weeks afterinfection. If massive infections of 4,000 - 5,000 metacercariae are given, then a decrease in PCV and serum albumin can be detected as early as 1 - 2 weeks post infection (Boray, 1967; Roberts, 1963).

In conclusion the present study has shown that the anaemia and hypoalbuminaemia observed in moderate chronic infections of sheep with Fasciola hepatica are due to two mechanisms. The earlier minor anaemia and hypoalbuminaemia which occur during the migratory (acute) phase are

brought about by the immature flukes. These young parasites cause an anaemia due to haemorrhagic lesions brought about by their burrowing through the liver parenchyma. This was demonstrated by the decreased ^{51}Cr red cell half-lives observed during this stage. The hypoalbuminaemia is probably caused by a combination of decreased synthesis and slightly increased degradation of albumin due to hepatic cell damage. The later and much more pronounced anaemia and hypoalbuminaemia seen from 10 weeks onwards and normally associated with chronic fascioliasis are due to the activities of the flukes once in the bile ducts. The present evidence suggests that it is in this situation that the flukes cause a progressive blood loss as illustrated by the accelerating faecal red cell clearances and increasing albumin catabolism seen in the infected animals.

Summary

Red cell and albumin turnover were studied in eleven sheep following infection with F. hepatica using ^{51}Cr cells and ^{125}I-albumin. The study covered the thirteen weeks after infection with a further study at 23 - 25 weeks.

The results showed:

(1) The anaemia and hypoalbuminaemia associated with ovine fascioliasis were due to two distinct mechanisms, the first occurring during the migratory phase as a result of liver damage, and the second more important mechanism commencing once the parasites were in the bile ducts and brought about by blood loss into the intestine, presumably via the bile.

(2) The severity of the infection had a marked effect on migration of the young flukes through the liver. Lighter infections migrated to the bile ducts quicker than heavier infections.

(3) Once in the bile ducts the blood loss increased in severity with maturation of the adult flukes.

SECTION IV

STUDIES OF THE CHANGES IN ALBUMIN AND RED CELL TURNOVER OF FLUKE-INFECTED AND CONTROL SHEEP FOLLOWING ANTHELMINTIC TREATMENT

Introduction

The experiments described in the preceding sections of this thesis showed that sheep chronically infected with F. hepatica lose large amounts of both red cells and plasma proteins into the gastro-intestinal tract. The magnitude of these losses is probably sufficient to account for the anaemia and hypoalbuminaemia associated with the chronic form of this disease.

In the previous section it was shown that these losses only begin once the migrating flukesreach the bile-ducts of the definitive host. The present study was undertaken to test further the hypothesis that the flukes directly cause a loss of both whole blood and plasma proteins by comparin g the red cell and albumin turnover before and after removal of the parasites by anthelmintic treatment.

If it can be shown that the red cell loss and hypercatabolism of albumin are significantly reduced once the flukes are killed, it would finally confirm the idea that it is activities of the live flukes themselves in the bile ducts which are the cause of the anaemia and hypoalbuminaemia.

Following the discovery of the anthelmintic properties of carbon tetrachloride in 1926, many different compounds which are active against F. hepatica have been described. Reviews of the various fasciolicides have been given by Gibson (1965) and Pugh (1965) and comparative therapeutic tests in sheep with various anthelmintics have been reported by Boray, Heppich and Andrews (1967).

Most workers have assessed the efficiency of fasciolicides by both reduction of fluke numbers at autopsy and elimination of faecal egg output and few have reported other effects of treatment on the host. However, changes in haematological and biochemical levels following treatment of fluke-infected sheep have been reported by Sinclair (1962) and Reid (1968).

Sinclair (1962) followed haematological and blood biochemical changes in a group of experimentally infected sheep which were treated with carbon tetrachloride thirty one weeks after infection. Reid (1968) studied similar parameters following treatment with nitroxynil ('Trodax', May and Baker Ltd., Dagenham, England), in a severe natural outbreak of chronic fascioliasis.

In the present study the fasciolicide, oxyclozanide (Zanil', I.C.I. Ltd., Alderley Park, Nr. Macclesfield, England), was used. This drug, introduced in 1966, was shown by Broome and Jones (1966) to be at least as effective as other drugs against F. hepatica and to have a wider margin of safety in laboratory animals. Shortly after these

results were confirmed in sheep and cattle by Walley (1966).

Two experiments are described in this section. The first was a study of albumin catabolism using ^{125}I-albumin, in fluke-infected and normal sheep before and after treatment. The second was a continuation of an experiment described in the first section of this thesis. In this study red cell and iron loss into the gastrointestinal tract were measured both pre- and post-treatment, using ^{51}Cr red cells and ^{59}Fe red cells simultaneously.

Albumin Catabolism in Fluke-infected and Normal Sheep Before and After Treatment

Materials and Methods

I. Experimental Animals

Three infected sheep (500 metacercariae 8 months previously) and two normal sheep were studied for a period of three weeks following the injection of ^{125}I-albumin. All the sheep used were Blackface wethers aged approximately one year, and had been reared and maintained under parasite-free conditions. Drinking water containing 0.0025% KI and 1% NaCl was given four days prior to the injection of the ^{125}I-albumin, and from there on continuously throughout the experimental period.

II. Labelling Procedure

Labelling of the albumin with ^{125}I was carried out by the method of McFarlane (1958) as described fully in Section IIA of this thesis. 30 ml of sheep albumin (Cohn Fr. V, Pentex Incorp., Kankakee, Illinois, U.S.A.) was trace labelled with 10 mc Na^{125}I.

III. Injection and Sampling

The labelled albumin was injected intravenously via a jugular catheter. Heparinized blood samples were taken 10 mins, 6, 18 and 24 hrs post injection, and then every 24 hrs throughout the three week experimental period. From each blood sample 1 ml of plasma was carefully pipetted into a counting tube for radioactivity determination. Aliquots of faeces and urine were taken from each 24-hour collection and assayed for radioactivity.

IV. Anthelmintic Treatment

Treatment was given on the eleventh day of the experiment. All the sheep were dosed orally with oxyclozanide ('Zanil', I.C.I. Ltd., Alderley Park, Nr. Macclesfield, England) at the rate of 17 mg/kg live weight.

V. Calculations and Expression of Results

(a) Plasma Volume

The plasma volume was determined from the 10 minute sample by application of the dilution principle.

(b) Albumin Pools

The intravascular pool (CA) was determined from the plasma volume and the serum albumin concentration. The extravascular pool (EA) was obtained by application of the "equilibrium time" method of Campbell et al, (1956).

(c) Catabolic Rate

"Apparent Half-life"

The half-life ($T\frac{1}{2}$) of the exponential portion of the plasma activity curves were taken as an approximate index of catabolism (Sterling 1961a).

Fractional Catabolic Rate

Since alterations in the plasma disappearance curve following treatment were expected, the catabolic rate was only determined by the method of Campbell et al (1956), which is based on the daily excreted activity.

Amount of Albumin Catabolised Per Day

The amount of albumin broken down daily was obtained my multiplying the intravascular pool (in grams/kilogram) by the fractional catabolic rate.

Results

The concentration and distribution of albumin of the infected and control animals prior to treatment are shown in Table 26. All three infected animals were hypoalbuminaemic (2.12 ± 0.24 gms %) relative to the controls (2.93 ± 0.11 gms %), at the beginning of the experiment. However, seven days post treatment the albumin concentration of the infected group had risen (2.31 ± 0.08 gms %) whilst those of the controls were reduced slightly (2.74 ± 0.20 gms %). This trend was continued, so that by fourteen days post-treatment the infected animals'

Table 26

Albumin Concentration and Distribution of Fluke-Infected and Control Sheep Prior to Treatment

	Sheep	Plasma Volume (ml/kg)	Serum Albumin (gms %)	CA (gm/kg)	EA (gm/kg)	EA/CA
INFECTED	61	60.5	1.93	0.97	0.86	0.89
	74	45.4	2.05	0.93	1.02	1.10
	78	40.5	2.39	0.97	1.36	1.40
	Mean	45.4	2.12	0.96	1.08	1.13
	S.D.	5.0	0.24	0.02	0.26	0.26
CONTROL	309	40.0	3.00	1.20	2.06	1.72
	514	41.1	2.85	1.17	2.02	1.73
	Mean	40.6	2.93	1.19	2.04	1.73
	S.D.	0.8	0.11	0.02	0.03	0.01
"t" Test		N.S.	$P < 0.05$	$P < 0.01$	$P < 0.02$	N.S.

Table 27

Albumin Catabolism in Fluke-infected and Control Sheep Before and After Treatment

	Sheep	F(CA)			Grams of Albumin Catabolised/day		
		Pre-Treat.	Post-treat. Mean 2-5 day	Post-treat. Mean 6-9 day	Pre-Treat.	Post-treat. Mean 2-5day	Post-treat. Mean 6-9 day
INFECTED	61	0.086	0.077	0.051	0.083	0.075	0.053
	74	0.105	0.084	0.060	0.098	0.078	0.061
	78	0.094	0.066	0.055	0.091	0.064	0.053
	Mean	0.095	0.076	0.055	0.091	0.072	0.057
	S.D.	0.010	0.009	0.004	0.008	0.007	0.004
CONTROL	309	0.084	0.076	0.073	0.101	0.091	0.084
	514	0.076	0.070	0.074	0.089	0.082	0.078
	Mean	0.080	0.073	0.074	0.095	0.087	0.081
	S.D.	0.005	0.004	0.001	0.008	0.006	0.004
	"t" Test	N.S.	N.S.	$P < 0.02$	N.S.	N.S.	$P < 0.01$

albumin concentration had risen to 2.69 ± 0.59 gms % and the controls' had fallen to 2.50 ± 0.09 gms %.

At the beginning of the study the distribution of albumin was not significantly different between the two groups, though both the intravascular and extravascular pools were significantly reduced in the infected animals.

The effects of the anthelmintic treatment on albumin catabolism are summarised in Table 27 and Figs. 21 and 22.

Unfortunately the results are complicated by two factors. Firstly, the level of infection in the parasitized group was rather low, and secondly, it is apparent from the results that anthelmintic treatment had an effect on albumin metabolism in the control animals as well as in the infected animals. Nevertheless it is apparent that the fractional catabolic rate of albumin in the infected animals was markedly decreased after treatment.

Studies on Red Cell and Iron Loss Before and After Treatment of Fluke-infected and Control Sheep Using ^{51}Cr-red Cells and ^{59}Fe-red Cells Simultaneously

This was a continuation of Experiment II described in Section IB of this thesis.

Fig. 21. Albumin Turnover in a Normal Sheep before and after Treatment

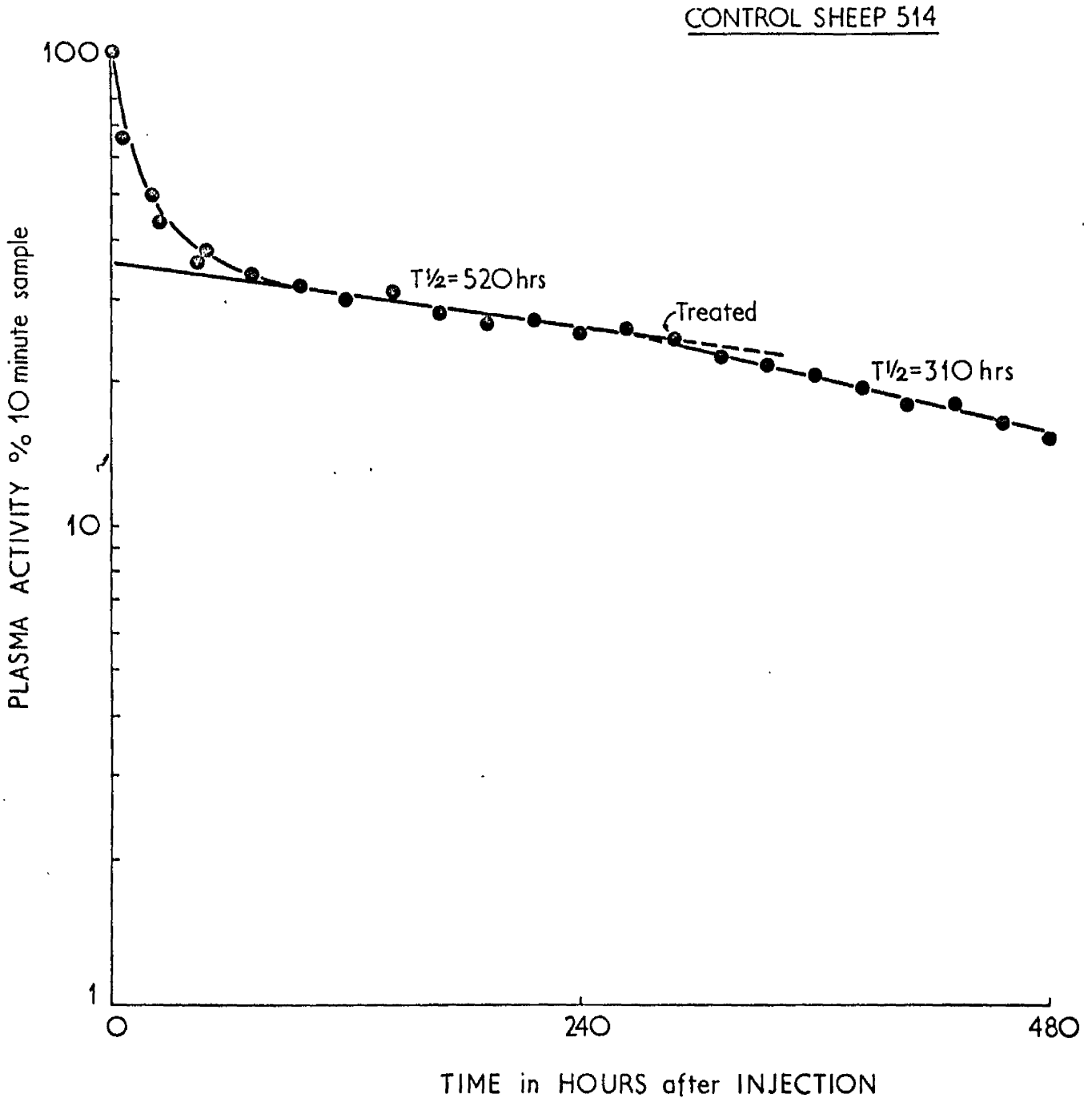
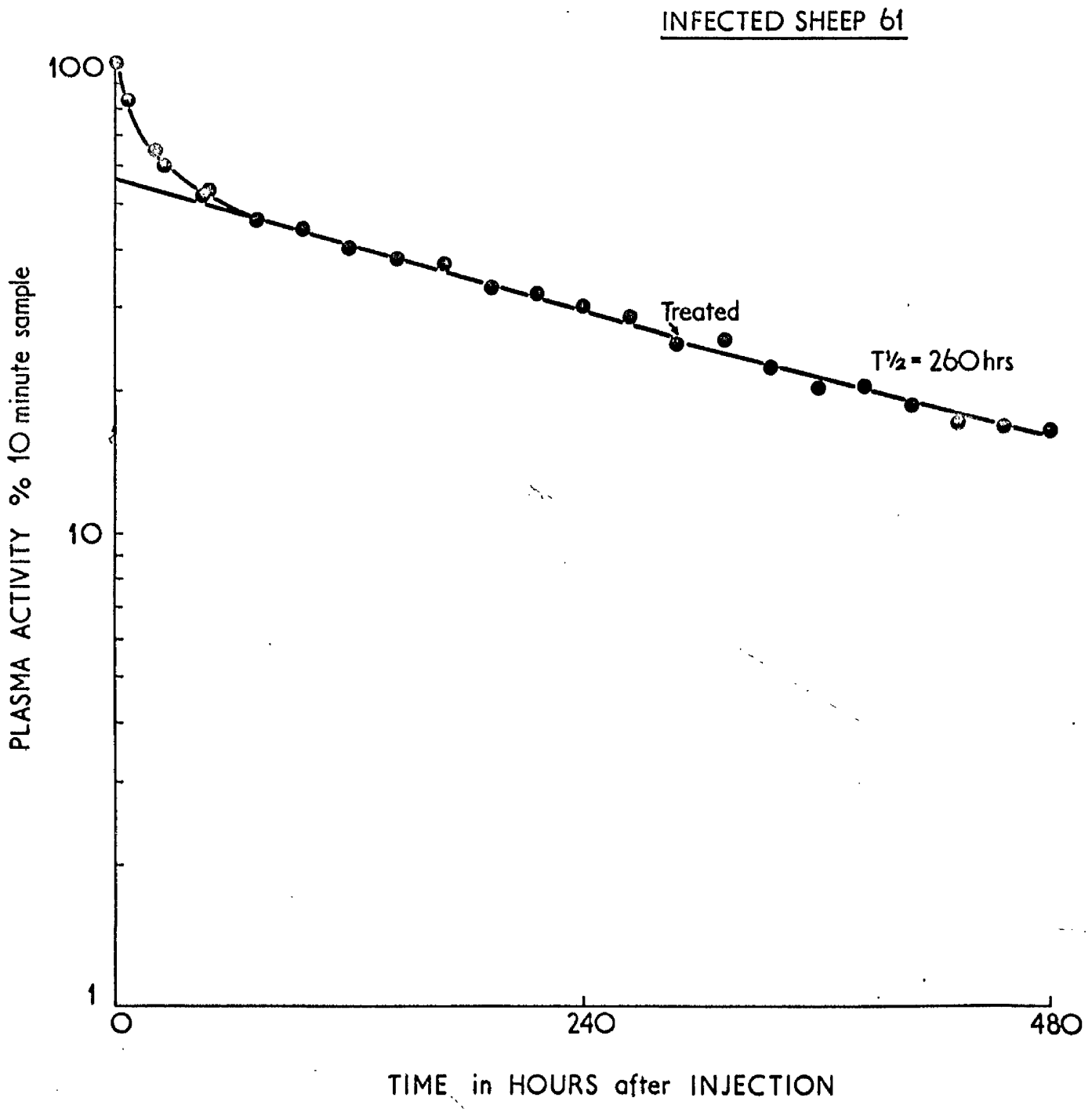


Fig. 22. Albumin Turnover in a Fluke-Infected Sheep before and after Treatment



Materials and Methods

I. Experimental Animals

Four Blackface wethers aged 18 months were used, two of which had been infected 20 weeks previously with 1,000 metacercariae each. The experiment lasted 28 days.

II. Labelling of Red Cells

Each sheep was injected intravenously with its own red cells labelled with ^{51}Cr (Approx. 6 mc ^{51}Cr). A further identical injection of ^{51}Cr-labelled red cells was given one day prior to treatment, i.e. on day 17.

^{59}Fe as ferric citrate as an isotonic solution was injected on day 10 of the experiment.

III. Injection and Sampling

These were as described in Section IB.

IV. Analytical Methods

As previously described in Section IB.

V. Anthelmintic Treatment

All the sheep were dosed orally with oxyclozanide ('Zanil', I.C.I. Ltd., Alderley Park, Nr. Macclesfield, England) on day 18 of the experiment, at the rate of 17 mg/kg body weight.

VI. Calculations and Presentation of Results

(a) Haematological

(i) Mean Corpuscular Volume(MCV)

$$= \frac{PCV\% \times 10}{Rbc\ (millions/cumm)}$$ expressed as cubic microns (cμ)

(ii) Mean Corpuscular Haemoglobin Concentration (MCHC)

$$= \frac{\text{Hb (gms \%)} \times 100}{\text{PCV\%}}$$

(b) Red Cell Loss

The faecal red cell clearance was obtained for each 24 hour period by dividing the total daily faecal activity by the activity per ml of red cells.

(c) Iron Loss and Intestinal Reabsorption

The red cell clearance expressed as a loss of whole blood was converted to milligrams of iron using the formula:-

Iron Lost/day = Whole Blood Clearance x Hb (gm/ml) x 3.4

This was done for both ^{51}Cr and ^{59}Fe, and by subtracting the ^{59}Fe values from the ^{51}Cr, the amount of iron reabsorbed was obtained as described fully in Section IB.

Results

Haematological Results

The alterations in the various blood parameters are shown in Table 28. It is readily apparent that following treatment there was a progressive rise in the level of circulating red cells, as illustrated by the increasing haematocrit, red cell counts and haemoglobin levels. The MCV values of the infected animals showed a progressive fall, from a state of macrocytosis before treatment to a normocytosis afterwards. Conversely the MCHC values in the infected animals showed a progressive

Table 28

Haematological Changes Following Anthelmintic Treatment of Fluke-infected and Control Sheep

Sheep	Infected		Control	
	47	NT	37	309
PCV% Pre-treatment	18	15	29	33
1 - 7 day Post-treatment	20	17	28	32
8 -14 day Post-treatment	24	20	29	35
Rbc (10^6 cu.mm)				
Pre-treatment	4.45	2.93	9.04	9.23
1 - 7 day Post-treatment	5.96	4.29	8.71	9.42
8 -14 day Post-treatment	6.73	5.89	9.38	10.35
Hb (gms %)				
Pretreatment	5.4	3.2	10.4	10.6
1 - 7 day Post-treatment	7.2	5.4	10.4	12.5
8 -14 day Post-treatment	9.1	7.8	13.2	13.7
MCV (cu)				
Pre-treatment	37.6	39.9	30.4	35.1
1 - 7 day Post-treatment	34.4	39.1	31.0	35.6
8 -14 day Post-treatment	38.2	34.6	34.9	33.9
MCHC (%)				
Pre-treatment	32.0	27.5	38.0	33.0
1 - 7 day Post-treatment	35.0	32.3	38.5	37.3
8 -14 day Post-treatment	38.5	38.2	42.5	40.0

increase after treatment, from a hypochromia to a normochromic level.

Red Cell Loss

The faecal loss of red cells in the infected and control sheep are shown in Table 29. Both the ^{51}Cr red cell and the ^{59}Fe red cell data show that prior to treatment a massive loss of red cells was occurring into the gastrointestinal tract of the infected sheep. However, after removal of the flukes by anthelmintic treatment the red cell loss dropped very rapidly to the control level. The alteration in red cell loss was reflected in the altered rate of disappearance of red cells from the circulation in the infected animals after treatment. Prior to therapy the red cell half-lives of the parasitized sheep were 120 and 72 hr and the controls' 384 and 320 hr. After treatment the red cell half-lives of the infected sheep had risen to 260 and 180 hr respectively, and the control values were 320 and 290 hr.

Iron Loss

Iron loss into the gastrointestinal tract reflected the changes in red cell loss following treatment. Before therapy some reabsorption of iron from the gut was occurring in the most anaemic animal, Sheep NT, yet after treatment the iron losses dropped to control level with no reabsorption of haemoglobin iron taking place.

Discussion

These results show in a very dramatic manner the effect of removal of the flukes on albumin and red cell turnover in sheep chronically infected with F. hepatica.

Table 29

Faecal Red Cell Clearances and Iron Absorption
in Fluke-infected and Control Sheep
Before and After Anthelmintic Treatment

Sheep	Infected		Control	
	47	NT	37	309
^{51}Cr Red Cell Loss (ml/day)				
Pre-treatment	19.1	33.1	0.4	0.9
3 - 7 day Post-treatment	0.2	0.4	0.5	0.9
8 -12 day Post-treatment	x	0.4	0.3	x
^{59}Fe Red Cell Loss (ml/day)				
Pre-treatment	17.1	19.1	1.9	1.6
3 - 7 day Post-treatment	0.4	0.5	2.3	2.0
8 -12 day Post-treatment	0.3	0.6	1.2	0.9
Intestinal Iron Loss (mg/day)				
Pre-treatment	17.9	35.6	0.3	0.7
3 - 7 day Post-treatment	0.2	0.4	0.6	1.2
8 - 12 day Post-treatment	x	0.5	0.5	x
Faecal Iron Loss (mg/day)				
Pre-treatment	16.0	20.6	1.4	1.3
3 - 7 day Post-treatment	0.5	0.5	2.9	2.7
8 -12 day Post-treatment	0.4	0.8	1.9	1.2
Iron Absorption mg/day)				
Pre-treatment	1.9	15.0	Nil	Nil
3 - 7 day Post-treatment	Nil	Nil	Nil	Nil
8 -12 day Post-treatment	Nil	Nil	Nil	Nil

x Activity too low to allow accurate calculations.

The observations on albumin metabolism before and after treatment revealed several interesting points. The infected sheep prior to therapy were all hypoalbuminaemic to varying degrees, but following treatment there was a progressive rise in the serum albumin level, so that by fourteen days post-treatment the albumin levels of the infected and control animals were very similar. These findings are similar to those of Sinclair (1962), who observed that after administration of carbon tetrachloride to fluke-infected sheep there was an immediate rise in serum albumin back to the control level. Similarly, in observations on the treatment of a natural outbreak of chronic fascioliasis, Reid (1966) reported a rapid increase in serum albumin levels during the three weeks following therapy.

The removal of the flukes has also a marked effect on the catabolism of albumin. The results of the present experiment show that following treatment there was a progressive fall in the catabolic rate of albumin until it was lower than the control level. It would be expected that along with the decrease in the fractional catabolic rate there would be an alteration in the apparent half-life in the infected group. As it was, no alteration was seen.

The reason why the falls in albumin catabolism were not matched by a concurrent increase in the apparent half-lives in the infected animals is difficult to explain. A comparison of results for the infected and control groups is further complicated by alterations which occurred in the albumin metabolism of the normal animals after

treatment. In this group a fall in the plasma apparent half-life was not accompanied by a simultaneous rise in catabolic rate. So it would appear that the change in albumin turnover in the parasitized sheep after treatment was not only due to removal of the flukes, but also to the direct effects of the anthelmintic as well.

In this connection it is pertinent that the anthelmintic used caused a mild diarrhoea, which was most evident in the control animals, though all the animals were dosed at the same rate. Scouring has been reported as a not uncommon side-effect of oxyclozanide, though it is usually of a mild and transient nature (Froyd, 1968).

Decreased synthesis of albumin as observed in some human conditions is characterised by decreased catabolic rates and extended plasma half-lives (Sterling, 1961b; Wilkinson and Mendenhall, 1963). It therefore seems possible that following treatment there is a reduction in the catabolic rate of albumin firstly down to the level of the controls, as a direct result of cessation of the leak of plasma once the flukes are removed from the bile ducts. This is then followed by a further reduction in catabolic rate caused by reduced synthesis due to a homoeostatic mechanism preventing the serum albumin concentration rising above the control level. Studies by Rothschild, Oratz, Evans and Schreiber (1960) showed that albumin synthesis does not respond to changes in plasma albumin concentration per se. More recent studies have shown that an indirect system probably exists

which is sensitive to changes in colloid osmotic pressure of the plasma. Though the exact mechanism has not been elucidated it appears that hepatic interstitial albumin is involved, and an inverse relationship between hepatic interstitial albumin and albumin production has been reported (Rothschild, Oratz and Schreiber, 1966).

Another possible explanation for the apparent decrease in albumin synthesis in the infected animals following treatment, is that liver damage already present as a result of the fluke infection may have been exacerbated by the fasciolicide. Deaths among fluke-infected rats after administration of antholmintics have been reported by Thorpe (1965b), and Boray, Happich and Andrews (1967) have recorded increased liver damage, after therapy, in experimentally infected sheep and rats. However, in a detailed histochemical study of the effects of some antholmintics in experimental fascioliasis in the rat (Thorpe, 1968) oxyclozanide was not found to cause any detectable changes in the host liver, though this does not necessarily mean that there were not functional changes.

Red cell changes following treatment were even more striking than those of albumin. The alterations in the haematological values all showed a fairly rapid return to normal levels. The PCV of the infected sheep rose by 25% of the pre-treatment value in the fourteen days following therapy, and the red cell counts and haemoglobin values showed a similar dramatic rise. This rapid and marked response to

treatment by the red blood cells confirms the findings of Sinclair (1962) and Reid (1968). The marked reduction in mean corpuscular volume following treatment was also similar to previous findings (Sinclair, 1962; Reid, 1968). The change in MCHC after therapy was less marked. This feature was reported by Reid (1968), who found that it was ten days before any increase was noted. Unfortunately in the present study the rise in MCHC values in the infected animals was obscured by a concurrent rise in the control animals. Thus it is clearly established that following treatment there is a rapid and marked alteration of the various blood indices to control levels.

Since the anaemia present in fluke-infected sheep can be explained by a massive loss of red cells into the gut in these animals it is obvious that changes in gastrointestinal loss would most readily account for the haematological changes. This was borne out by the results obtained from both the ^{51}Cr red cell and ^{59}Fe red cell studies, as a most dramatic drop in faecal red cell clearance was observed following therapy of the infected sheep. In the most severely infected sheep, the red cell loss dropped from a mean daily loss of 33 ml rbc/day to the control level of <1 ml/day almost immediately. A similar change was observed in the intestinal reabsorption of haemoglobin iron, as prior to treatment the most anaemic animal was reabsorbing approximately 15 mg Fe/day, yet after treatment reabsorption fell to nil.

Following this abrupt cessation of red cell loss into the gastrointestinal tract in any significant amounts, red cell survival was prolonged to a much greater extent than before treatment although the half-lives of the infected animals did not achieve the level of the controls. A possible explanation for this is that the red cell volume of the infected sheep was presumably rising after treatment. Indirect evidence of such an increase is the striking rise in PCV and red cell counts observed after therapy. This would mean that new unlabelled cells were probably diluting the labelled red cellsalready present in the circulation at a more rapid rate in the infected sheep compared to the controls, so that as a result the red cell half-lives of the infected sheep were underestimated. So it would appear that the increased rate of disappearance of the labelled cells in the infected animals post-treatment is not due to a continuing loss of cells, but to a diluting effect of the labelled erythrocytes by unlabelled cells.

Summary

The effect of antholmintic treatment on albumin and red cell turnover was studied in two separate experiments. The first involved three infected and two control sheep injected with ^{125}I-albumin. Following treatment there was a marked rise in serum albumin levels, and a fall in the catabolic rate of albumin. It was suggested that there are two separate causes for the drop in catabolism, one due to

removal of the flukes and a cessation of the whole blood loss, and two, due to reduced synthesis of albumin. In the second experiment red cell turnover was studied in two infected and two control sheep. After therapy there was an almost immediate fall in the magnitude of the red cell loss down to control level, and a return of the haematological parameters back to normal values.

These results add further evidence to the theory that the anaemia and hypoproteinaemia associated with chronic fascioliasis are a direct result of the feeding activities of the adult flukes in the bile ducts.

GENERAL DISCUSSION

The results of the work described in this thesis leave little doubt that associated with chronic fluke infection of sheep there is a massive loss of both red cells and plasma proteins into the gastro-intestinal tract. In addition the extent of the loss is so great as to account for the anaemia and hypoproteinaemia associated with the disease. The series of experiments described have illustrated the successful application of isotopic labelling techniques to understanding the underlying pathophysiological mechanisms involved in a specific parasitic disease. These techniques are likely to find increasing application in the study of parasitic diseases in the next decade.

The use of ^{51}Cr red cells has shown how valuable information can be obtained not only on red cell survival and the extent of red cell loss into the gut, but also on possible haemolysis or increased elution which might occur in the infected animals. This technique, however, gives only indirect evidence on the rate of erythropoiesis. This can be remedied by the use of radioiron which provides a useful method for not only measuring erythropoiesis but also the extent of red cell loss into the gut. In addition by the double labelling of red cells with both ^{51}Cr and ^{59}Fe it becomes possible to measure the extent of haemoglobin iron absorption from the gut.

The measurement of erythropoiesis with radioiron also serves to

emphasize the importance of carrying out standard biochemical techniques along with the isotopic studies since, e.g. an increased rate of disappearance of ^{59}Fe from the plasma does not mean *per se* that erythropoiesis is increased, as plasma iron turnover rates may be considerably elevated in conditions associated with hyperplastic states of the marrow such as pernicious anaemia, though erythropoiesis is decreased, this being characterized by a high serum iron level and low red cell utilisation.

By the use of this double labelling technique valuable information can be gained on both the turnover of red cells and the routes of excess loss. There are numerous parasitic diseases to which these techniques could readily be applied. To date isotopic red cell studies have only been carried out on known blood sucking parasites, i.e. *Haemonchus contortus* (Clark, Kiesel and Goby, 1962; Brambell, Charleston and Tothill, 1964; Georgi and Whitlock, 1965) and in canine hookworm infections (Clark, Kling, Woodley and Sharp, 1961; Miller, 1966a and b). There are, however, numerous other parasitic conditions in which anaemia is known to occur, with only conjecture as to the likely aetiology, e.g. in infections with *Ostertagia ostertagi*, *Trichostrongylus axei*, *Nematodirus spp*, *Cooperia spp*, etc. (Lepage, 1956; Blood and Henderson, 1963). It is in these cases that isotopic techniques similar to the ones described in this thesis could be most useful.

The use of ^{125}I and ^{131}I plasma proteins in fluke-infected sheep serves to illustrate the type of information which can be obtained

from this kind of study. From standard biochemical methods it was found that the plasma albumin levels were reduced in the diseased sheep, but that plasma gammaglobulin levels were slightly elevated. From this information it might appear that opposing mechanisms were operating to bring about this difference. However when the kinetics of albumin and immunoglobulin are studied, along with suitable techniques for the measurement of gastrointestinal protein loss, it was found that the effects were in fact due to the apparent inability of albumin synthesis to increase to the same extent as immunoglobulin in the face of an excessive loss of plasma proteins into the gastrointestinal tract. Albumin synthesis was found to be increased only to approximately twice normal, whilst immunoglobulin synthesis was apparently increased by a factor of four.

The use of $^{51}CrCl_3$ and ^{125}I-albumin simultaneously was shown to be an excellent technique for the concurrent study of gastrointestinal protein loss and albumin turnover, and it is a method which could be readily applied to many gastrointestinal parasitic infections which are characterised by hypoproteinaemia. To date, however, the only parasitic condition which has been successfully investigated with trace-labelled albumin is ostertagiasis (Halliday, Mulligan and Dalton, 1968), though various diarrhoeal disorders of cattle, including ostertagiasis, have been investigated by Nielsen (1966).

The demonstration of a significant blood loss in the parasitized sheep serves to illustrate the great demand placed on the synthetic

mechanisms of the host, since not only must the infected animal maintain normal synthesis, but the rate must be increased if it is to maintain normal levels. Though it must be admitted that many of the breakdown products of the red cells and plasma proteins lost into the gut will be reabsorbed, it nevertheless requires that these "building blocks" have to be reconstructed by the erythropoietic and plasma protein producing cells of the animal. The exact effect of this constant protein loss and resynthesis on the host has not yet been elucidated, though it is obvious that since protein synthesis needs to be greatly increased, the nutrition of the host will have an important bearing on the state of the animal. Indeed many of the symptoms, such as weight loss, may be aggrevated by fluke-infected sheep being maintained on poor planes of nutrition, especially in the winter months when the most severe infections occur. At this level of nutrition non-essential protein synthesis is probably reduced so that red cells and plasma protein synthesis can be increased in an attempt to maintain normal levels. In this context it is of interest that in mice infected with Nematospiroides dubius; measurement of protein synthesis using ^{14}C-leucine uptake has shown that skeletal muscle uptake was reduced whilst liver protein uptake was elevated (Symons, 1969).

Attention must also be given not only to the nutrition but also the immunological consequences of gastrointestinal protein loss, since greatly increased losses of immunoglobulins, despite the elevated

synthesis, may possibly bring about a significant impairment of protective mechanisms. This has already been shown to be true for young animals. For example puppies which are normally resistant to distemper virus infection, due to maternal antibody, for 8 - 10 weeks post-partum lose their resistance much earlier when infected with hookworms.

In addition to highlighting the valuable information which can be obtained from the application of isotopic labelling techniques to the study of the pathophysiology of parasitic disease, attention must also be given to some of the limitations in the present study. There are two disappointing aspects of many of the experiments described in this thesis. Firstly, the number of sheep which could be used in any given experiment was often lower than would have been desired. This was almost entirely due to economic factors restricting the number of parasite-free sheep available for this type of study. Leading on from this, it would have also been desirable to have killed all the sheep after each experiment in order to confirm the individual fluke burdens of the sheep, so that the results could be expressed as ml/fluke etc, but because of the low numbers of sheep available this could not be done. Secondly, the great variation in the "take" of the infective doses of metacercariae was regretable and occurred despite giving the same number of metacercariae from the same batch to sheep of a similar status, though variations in "take" are a common feature of many parasitic infections. The effect of this variation in "take" was that there was a range in the severity of the pathogenic

effects in any given experiment. This meant that the application of the Student "t" test often showed the difference between the control and infected animals to be insignificant, largely because of the variation in the parasitized group, coupled with the small numbers of sheep involved.

Other practical difficulties which also must be overcome for the successful use of isotopic techniques in this type of study include; the preparation of suitable labelled preparations which are not denatured, the use of suitable animals which are under relative steady state conditions and a system which enablescomplete separation and collection of urine and faeces. However, most of these difficulties can be overcome, and the isotopic techniques described can be used to yield much valuable information on the pathophysiological mechanisms involved in the host/parasite relationship.

REFERENCES

Aalund, O., Osebold, J.W., Murphy, E.A. (1965). Archs Biochem. Biophys., 109, 143.

Andersen, S.B. (1962). Clin. Sci., 23, 221.

Andersen, S.B. (1964). "Metabolism of Human Gamma Globulin", Blackwell Scientific Publications, Oxford.

Armstrong, S.H., Kukral, J., Herchman, J., McLeod, K., Wolter, J., Bronsky, D. (1956). J. Lab. clin. Med., 45, 51.

Baker, N.F., Douglas, J.R. (1957). Am. J. vet. Res., 18, 295.

Balian, B. (1940). Nuova Vet., 18, 154.

Bennhold, H., Kallee, E. (1959). J. clin. Invest., 38, 863.

Birke, G., Liljedahl, S.O., Olhagen, B., Plantin, L.O., Ahlinder, S. (1965). Acta Med. scand., 177, 227.

Bishop, O.N. (1966). "Statistics for Biology", Longmans, Green & Co. Ltd., London.

Bjorneboe, M., Schwartz, M. (1959). J. exp. Med., 110, 259.

Blood, D.C., Henderson, J.A. (1963). "Veterinary Medicine", 2nd Edition. Bailliere, Tindall & Cox., London.

Boray, J.C., Happich, F.A., Andrews, J.C. (1965). Vet. Rec., 77, 175.

Boray, J.C. (1967). Ann. trop. Med. Parasit., 61, 439.

Boray, J.C., Happich, F.A., Andrews, J.C. (1967). Vet. Rec., 80, 216.

Bothwell, T.H., Callender, S., Mallett, B., Witts, L.J. (1956). Br. J. Haemat., 2, 1.

Brambell, M.R., Charleston, W.A.G., Tothill, P. (1964). J. comp. Path. Ther., 74, 338.

Broome, A.W., Jones, W.G.M. (1966). Nature, Lond., 210, 744.

Campbell, R.M., Cuthbertson, D.P., Matthews, C.M., McFarlane, A.S. (1956). Int. J. appl. Radiat., 1, 66.

Campbell, R.M., Cuthbertson, D.P., Mackie, W., McFarlane, A.S., Phillipson, A.T., Sudeaneh, S. (1961). J. Physiol., Lond., 158, 113.

Carter, M.W., Matrono, G., Metzler, C. (1965). J. gen. Physiol., 49, 57.

Carter, B.B., Jackson, D.F., Kolber, A.R. (1967). Int. J. appl. Radiat. Isotopes, 18, 615.

Clark, C.H., Kling, J.M., Woodley, C.H, Sharp, N. (1961). Am. J. vet. Res., 22, 370.

Clark, C.H., Kissel, G.J., Goby, C.H. (1962). Am. J. vet. Res., 22, 977.

Cohen, S., Freeman, T. (1960). Biochem. J., 76, 475.

Colombo de la Villa, G. (1955). Bull. off. int. Epizoot., 43, 63.

Cooper, M., Stroebel, C.F., Stickney, J.M., Owen, C.A. (1955). Fed. Proc., 14, 31.

Dargie, J.D. (1969). Personal communication.

Dawes, B. (1961a). J. Helminth. R.T. Leiper Suppl., 41.

Dawes, B. (1961b). Nature, Lond., 190, 646.

Dawes, B.(1962.) J. Helminth., 26, 11.

Dawes, B. (1963). Parasitology, 53, 135.

Dawes, B., Hughes, D.L. (1964). "Advances in Parasitology", Academic Press, London and New York, 2, 97.

Dawson, A.M., Williams, R., Williams, H.S. (1961). Br. med. J., 2, 667.

Dixon, F.J., Talmage, D.W., Maurer, P.H., Deichmiller, M. (1952). J. exp. Med., 96, 313.

Dixon, K.E. (1964). J. Helminth., 38, 203.

Donohue, D.M., Matulsky, A.G., Giblett, E.R., Pizzio-Bixoli, G., Vivanuvatti, V., Finch, C.A. (1955). Br. J. Haemat., 1, 249.

Dow, C., Ross, J.G., Todd, J.R. (1967). J. comp. Path. Ther., 77, 377.

Dow, C., Ross, J.G., Todd, J.R. (1968). Parasitology, 58, 129.

Drury, A.N., Tucker, H.M. (1958). Immunology, 1, 204.

Ebaugh, F.G., Emerson, C.P., Ross, J.F. (1953). J. clin. Invest., 32, 1958.

Ebaugh, F.G., Clemens, T., Rodnan, G., Peterson, R.E. (1958). Am. J. Med., 25, 169.

Enger, O., Karbo, A. (1956). Norsk Landbr., 22, 60.

Flury, F., Leeb, F. (1926). Klin. Wschr., 5, 2054.

Freeman, T. (1959). Clinica chim. Acta, 4, 788.

Freeman, T., Gordon, A.H. (1964). Gut, 5, 155.

Freeman, T., (1966). "Protides of the Biological Fluids", 14, Elsevier Pub. Co., Amsterdam.

Froyd, G. (1968). Br. vet. J., 124, 116.

Furmaga, S., Gundlach, J.L. (1967a). Acta Parasit. pol., 14, 313.

Furmaga, S., Gundlach, J.L. (1967b). Acta Parasit. pol., 15, 93.

Garby, L., Hjelm, M. (1962). Scand. J. clin. Lab. Invest., 14, 581.

Georgi, J.R. (1964). Am. J. vet. Res., 25, 246.

Georgi, J.R., Whitlock, J.H. (1965). Am. J. vet. Res., 26, 310.

Gibson, T.E. (1965). "Veterinary Anthelmintic Medication", Commonwealth Agricultural Bureaux.

Gitlin, D., Janeway, C.A., Farr, L.E. (1956). J. clin. Invest., 35, 44.

Goldsworthy, P.D., Volwiler, W. (1957). Ann. N.Y. Acad. Sci., 70, 26.

Gordon, H. McL. (1955). Aust. vet. J., 31, 182.

Gordon, R.S. Jnr. (1958). J. Polym.Sci., 31, 191.

Gordon, R.S. Jnr., Bartter, F.C., Waldman, T. (1959). Ann. intern. Med., 51, 559.

Gordon, R.S. Jnr., (1959). Lancet, 1, 325.

Gray, S.J., Sterling, K. (1950a). J. clin. Invest., 29, 1604.

Gray, S.J., Sterling, K. (1950b). J. clin. Invest., 29, 1614.

Grunsell, C.S.G. (1955). Br. vet. J., 111, 473.

Guillen, R.T., Peterson, M.L. (1964). J. Lab. clin. Med., 64, 865.

Halliday, G.M., Mulligan, W., Dalton, R.G. (1968). Res. vet. Sci., 9, 224.

Hansard, S.L., Kincaid, E. (1956). J. Anim. Sci., 15, 1200.

Hecht, G., Weese, H. (1948). Münch. med. Wschr., 90, 11.

Hoedt-Rasmussen, K., Kampo, E. (1964). Gut, 5, 153.

Hofer, R., Schatz, H., Thumb, B. (1968). Clinica chim. Acta, 22, 135.

Huff, R.L., Hennessy, T.G., Austin, R.E., Garcia, J.F., Roberts, B.M., Lawrence, J.H. (1950). J. clin. Invest., 29, 1041.

Hughes Jones, N.C., Mollison, P.L. (1956). Clin. Sci., 15, 207.

Hughes, D.L. (1963). Thesis. University of London.

Ibrovic, M., Galla-Palla, V. (1959). Veterinaria, Sarai., 8, 591.

Jabbari, M., Leevy, C.M. (1967). Proc. Soc. exp. Biol. Med., 126, 692.

Jarnum, S. (1961). Scand. J. clin. Lab. Invest., 13, 447.

Jarnum, S. (1963). "Protein-losing Gastroenteropathy", Blackwell Scientific Publ., Oxford.

Jarnum, S., Westergaad, H., Vasing, M., Jensen, H. (1968). Gastroenterology, 55, 229.

Jeejeebhoy, K.N., Coghill, N.F. (1961). Gut, 2, 123.

Jeejeebhoy, K.N., Singh, B., Mani, R.S., Sanjana, S.M. (1964). "Physiology and Pathophysiology of Plasma Protein Metabolism". Hans Huber Publ., Berne & Stuttgart.

Jeejeebhoy, K.N. (1965). "Radioisotope Techniques in the Study of Protein Metabolism. Technical Report Series 45, 169. International Atomic Energy Agency, Vienna.

Jeejeebhoy, K.N., Jarnum, S., Singh, B., Nadkarni, G.D., Westergaard, H. (1968). Scand. J. Gastroent, 3, 449.

Jennings, F.W., Mulligan, W., Urquhart, G.M. (1956). Expl. Parasit., 5, 488.

Jennings, F.W. (1962). Ph.D. Thesis, University of Glasgow.

Jubb, K.V.F., Kennedy, P.C. (1963). "Pathology of Domestic Animals", Academic Press, New York and London.

Judd, J.T., Matrono, G. (1962). J. Nutr., 77, 264.

Kendall, S.B., Parfitt, J.W. (1962). Br. vet. J., 118, 1.

Kerr, R.M., Du Bois, J.J., Holt, P.R. (1967). J. clin. Invest., 64, 2064.

Lapage, G. (1956). "Veterinary Parasitology", 1st Edition. Oliver and Boyd, Edinburgh, Scotland.

Leuckhart, R. (1881). Zool. Anz., 99, 641.

London, I.M., Shemin, D., Rittenberg, D. (1948). J. biol. Chem., 173, 797.

Marcone, A. (1940). See Ballen, B. (1940). Nuovva Vet., 18, 164.

Morgen, S., Tarver, H. (1956). J. clin. Invest., 35, 1161.

Matthews, C.M.E. (1957). Physics Med. Biol., 2, 36.

Matthews, C.M.E. (1961). J. clin. Invest., 40, 608.

McFarlane, A.S. (1956). Biochem. J., 62, 135.

McFarlane, A.S. (1957). Ann. N.Y. Acad. Sci., 70, 19.

McFarlane, A.S. (1958). Nature, Lond., 182, 53.

Mellors, R.C., Korngold, L. (1963). J. exp. Med., 118, 387.

Miller, T.A. (1966a). J. Parasit., 52, 844.

Miller, T.A. (1966b). J. Parasit., 52, 856.

Mollison, P.L., Veall, N. (1955). Br. J. Haemat., 1, 62.

Mollison, P.L. (1961). Clin. Sci., 21, 21.

Monnig, H.O. (1956). "Veterinary Helminthology and Entomology", Balliere, Tindall & Cox, London.

Morrill, D.R., Shaw, J.N. (1942). Bull. Ore. Agric. Exp. Stn, 408, 30.

Müller, W. (1923). Zool. Anz., 57, 273.

Mulligan, W., Dalton, R.G., Anderson, N. (1963). Vet. Rec., 75, 1014.

Murray, M., Jarrett, W.F.H., (1967). Personal communication.

Nansen, P., Eriksen, L., Simesen, M.G., Nielsen, K. (1968). Nord. Vet Med., 20, 651.

Nicholes, T.F., Weinstein, I.M., LeRoy, G.V. (1953). J. Lab. clin. Med., 42, 358.

Nielsen, K. (1966). "Gastrointestinal Protein Loss in Cattle. A Clinical and Pathophysiological Study". Thesis. C. Fr. Mortensen, Copenhagen.

Nielsen, K., Nansen, P. (1969). "Isotopes and Radiation in Parasitology. Proceedings of a Research Coordination Meeting, International Atomic Energy Agency, Vienna.

Nikolic, B., Nikolic, V., Nevenic, V., Bugarski, M., Pavlovic, O., Ciric, V., Mladenovic, Z., Polic, F. (1962). Acta Vet., Beogr., 12, 53.

Noguchi, I., Kirisawa, T., Suguira, K., Komine S. (1958). J. Jap. vet. med. Ass., 11 (3), 113.

Ono, Y. (1958). Bull. Off. Int. Epizoot, 46, 555.

Owen, C.A. Hollman, J.L., Grindlay, J.H. (1954). J. Lab. clin. Med., 44, 238.

Pantelouris, E.M. (1965). "The Common Liver Fluke Fasciola Hepatica L." Pergamon Press Ltd., Oxford.

Pearson, I.G. (1963). Exp. Parasit., 13, 186.

Popov, K.K., Kalitina, Z.I. (1962). Zool. Zh. Ukr., 41, 1793.

Pugh, D.M. (1965). Ir. vet. J., 19, 223.

Railliet, A. (1890). See Stephenson, W. (1947).

Ramsay, W.N.M. (1957). Clinica chim. Acta, 2, 214.

Ravin, H.A., Seligman, A.M., Fine, J. (1952). New Engl. J. Med., 247, 921.

Reid, J.F.S. (1968). Ph.D. Thesis, University of Glasgow.

Reinhard, E.G. (1957). Expl Parasit., 6, 209.

Roberts, H.E. (1968). Br. vet. J., 124, 483.

Rocho, M., Perez-Gimines, M.E., Levy, A. (1957). Nature, Lond., 162, 1278.

Ross, J.G. (1965). Nature, Lond., 208, 907.

Ross, J.G. (1966a). Br. vet. J., 122, 489.

Ross, J.G. (1966b). Ph.D. Thesis. Queen's University, Belfast.

Ross, J.G., Todd, J.R., Dow, C. (1966). J. comp. Path. Ther., 76, 67.

Ross, J.G. (1967a). Vet. Rec., 80, 214.

Ross, J.G. (1967b). Vet. Rec., 80, 368.

Rothschild, M., Oratz, M., Evans, C., Schreiber, S. (1960). Am. J. Physiol., 210, 57.

Rothschild, M., Oratz, M., Schreiber, S. (1966). "Protides of the Biological Fluids," 14. Elsevier Pub. co. Amsterdam.

Rowlands, W.T. (1969). Vet. Rec., 84, 154.

Schumacher, W. (1938). Z. Parasit Kde., 10, 608.

Schumacher, W. (1966). Z. Parasit Kde., 17, 276.

Schwartz, M., Jaenum, S. (1959). Lancet, 1, 327.

Schwartz, M., Jaenum, S. (1961). Dan med. Bull., 8, 1.

Secretan, P.H., Bickel, G. (1960). Arch. Mal. Appar. dig., 49, 642.

Sewell, M.M.H. (1966). Vet. Rec., 78, 98.

Sewell, M.M.H., Hammond, J.A., Dinning, D.C. (1968). Br. vet. J., 124, 160.

Shaw, J.N. (1932). J. Am. vet. med. Ass., 6, 511.

Shiral, M. (1927). Sci. Rep. Inst. infect. Dis. Tokyo Univ., 6, 511.

Silverstein, A.M., Thorbecke, G.J., Kraner, K.L., Lukes, R.J. (1963). J. Immun., 91, 384.

Simesen, M.G., Eriksen, L., Nansen, P., Andersen, S., Nielsen, K. (1968). Nord. Vet. Med., 20, 688.

Simistin, D.F. (1914). Zentbl. Bakt. Parasit Kde, 74, 280.

Sinclair, K.B. (1962). Br. vet. J., 118, 37.

Sinclair, K.B. (1964). Br. vet. J., 120, 212.

Sinclair, K.B. (1965). Br. vet. J., 121, 451.

Sinclair, K.B. (1967a). Vet. Rec., 80, 363.

Sinclair, K.B. (1967b). Helminth. Abstr., 36, 115.

Smith, H.A., Jones, T.C. (1957). "Veterinary Pathology", Henry Kimpton, London.

Snedecor, G.W. (1956). "Statistical Methods", 5th Edition, Iowa State University Press.

Sommer, (1880). Z. wiss. Zool., 34, 539.

Soulsby, E.J.L. (1968). "Helminths, Arthropods and Protozoa of Domesticated Animals". Balliere, Tindall & Cassell, London.

Stadie, W.C. (1920). J. biol. Chem., 41, 237.

Stephenson, W. (1947). Parasitology, 38, 123.

Sterling, K. (1951a). J. clin. Invest., 30, 1228.

Sterling, K. (1951b). J. clin. Invest., 30, 1238.

Symons, L.E.A., Boray, J.C. (1967). Vet. Rec., 81, 130.

Symons, L.E.A. (1969). Personal communication.

Taylor, E.L. (1964). "Fascioliasis and the Liver Fluke". F.A.O. Agic. Stud., No. 64.

Thomas, A.P. (1881). J. Roy. Agric. Soc., England, 17, 1.

Thorbecke, G.J., Asofsky, R.M., Hochwald, G.M., Siskind, G.W. (1962). J. exp. Med., 116, 295.

Thorpe, E. (1963). D.V.M. Thesis, University of Glasgow.

Thorpe, E. (1965a). Res. vet. Sci., 6, 498.

Thorpe, E. (1965b). J. comp. Path. Ther., 75, 45.

Thorpe, E. (1968). Ann. trop. Med. Parasit., 62, 361.

Todd, J.R., Rose, J.G. (1966a). Clinica chim. Acta, 14, 27.

Todd, J.R., Rose, J.G. (1966b). Expl. Parasit., 19, 151.

Tucker, E. (1963). Res. vet. Sci., 4, 11.

Urquhart, G.M., Mulligan, W., Jennings, F.W. (1954). J. infect. Dis., 94, 126.

Urquhart, G.M. (1955). Ph.D. Thesis, University of Glasgow.

Urquhart, G.M. (1956). J. Path. Bact., 71, 301.

van Grembergen, G. (1950). Annls Soc. R. Zool. Belg., 81, 15.

van Kampen, E.J., Zijlstra, W.C. (1961). Clinica chim. Acta, 6, 538.

van Tongeren, J.H.M., Majoor, C.L.H. (1956). Clinica chim. Acta, 14, 31.

Vogel, A. (1951). "Quantitative Inorganic Analysis", 2nd Edition, p. 366. Longmans, Green & Co., London.

Waldman, T.A. (1961). Lancet, 2, 121.

Waldman, T.A., Wochner, R.D. (1965). "Radioisotope Techniques in the Study of Protein Metabolism". Technical Report Series 45, 171. International Atomic Energy Agency, Vienna.

Walker-Smith, J.A., Skyring, A.P., Mistilis, S.P. (1967). Gut, 8, 166.

Walley, J.K. (1966). Vet. Rec. 78, 267.

Wasserman, L.R., Rashkoff, I.A., Leavitt, D., Mayer, J., Port, S. (1952). J. clin. Invest., 31, 32.

Weaver, A.D. (1966). Personal communication.

Weichselbaum, T.E. (1946). Am. J. clin. Path., 10, 40.

Weinland, E., von Brand, T.O. (1926). Z. vergl. Physiol., 4, 212.

Weinstein, I.M., Beutler, E. (1962). "Mechanisms of Anaemia". McGraw-Hill Book Co. Inc., London.

Wetterfords, J. (1965). Acta med. scand., suppl. 430, 177.

White, R.G., Coons, A.H., Connolly, J.M. (1955). J. exp. Med., 102, 83.

Wilkinson, P., Mendenhall, C.L. (1963). Clin. Sci., 25, 281.

A P P E N D I X

Table I

Albumin Concentration and Distribution in Fluke-infected and Control Sheep: at the Time of Infection

	Sheep	Plasma Volume (ml/kg)	Serum Albumin (gms %)	CA (gm/kg)	EA (gm/kg)	EA/CA
INFECTED	42	42.5	2.60	1.18	1.60	1.36
	49	40.7	2.96	1.20	1.85	1.54
	73	42.8	2.30	0.99	1.47	1.48
	Mean	42.0	2.62	1.12	1.64	1.46
	S.D.	1.1	0.33	0.11	0.19	0.09
CONTROL	77	41.2	2.32	0.96	1.34	1.40
	80	40.3	2.13	0.86	1.17	1.36
	285	37.9	2.54	0.96	1.47	1.53
	Mean	39.8	2.33	0.93	1.33	1.43
	S.D.	1.7	0.21	0.06	0.15	0.09
"t" Test		N.S.	N.S.	N.S.	N.S.	N.S.

Table II

Albumin Catabolism in Fluke-infected and Control Sheep: 1 - 3 Weeks Post-infection

	Sheep	F(CA)			Absolute Amounts Albumin Catabolised gms/day		
		0 - 1 week	1 - 2 week	2 - 3 week	0 - 1 week	1 - 2 week	2 - 3 week
INFECTED	42	0.065	0.064	0.069	0.077	0.076	0.081
	49	0.069	0.064	0.057	0.083	0.077	0.068
	73	0.076	0.067	0.063	0.075	0.066	0.062
	Mean	0.070	0.065	0.063	0.078	0.073	0.070
	S.D.	0.006	0.001	0.006	0.004	0.005	0.009
CONTROL	77	0.055	0.048	0.053	0.053	0.046	0.051
	80	0.056	0.054	0.057	0.048	0.046	0.049
	285	0.061	0.057	0.070	0.058	0.064	0.067
	Mean	0.057	0.056	0.060	0.053	0.052	0.056
	S.D.	0.003	0.009	0.009	0.005	0.010	0.010
"t" Test		P<0.05	N.S.	N.S.	P <0.01	P <0.05	N.S.

Table III

^{51}Cr Red Cell Turnover in Fluke-Infected and Control Sheep: 1 - 3 Wecket Post-infection

	Sheep	Red Cell Volume (ml/kg)	Red Cell $T_{\frac{1}{2}}$ (hrs)	Faecal Red Cell Clearance (ml/day)	Faecal Loss as % Injected	Urinary Loss as % Injected
INFECTED	42	15.4	360	<5	<5	85
	49	14.8	430	"	"	79
	73	16.3	500	"	"	63
	Mean	15.5	430			76
	S.D.	0.8	70			11
CONTROL	77	11.7	420	<5	<5	59
	80	14.5	380	"	"	69
	285	17.3	430	"	"	66
	Mean	14.5	410			65
	S.D.	2.8	26			5
"t" Test		N.S.	N.S.			N.S.

Table IV

Albumin Concentration and Distribution in Fluke-infected and Control Sheep: 3 Weeks Post-infection

	Sheep	Plasma Volume (ml/kg)	Serum Albumin (gms %)	CA (gm/kg)	EA (gm/kg)	EA/CA
INFECTED	51	47.1	2.46	1.16	1.56	1.34
	53	41.7	2.36	0.98	1.47	1.50
	81	43.3	2.76	1.20	1.78	1.49
	Mean	44.0	2.53	1.11	1.60	1.44
	S.D.	2.8	0.21	0.12	0.16	0.09
CONTROL	64	35.7	2.82	1.00	1.39	1.39
	86	41.9	2.84	1.19	1.79	1.50
	35	41.1	2.82	1.16	1.91	1.65
	Mean	39.6	2.83	1.12	1.70	1.51
	S.D.	3.4	0.01	0.10	0.27	0.13
"t" Test		N.S.	N.S.	N.S.	N.S.	N.S.

Table V

Albumin Catabolism in Fluke-infected and Control Sheep 4 - 7 Weeks Post-infection

	Sheep	F(CA)			Absolute Amounts Albumin Catabolised gms/day)		
		4 - 5 week	5 - 6 week	6 - 7 week	4 - 5 week	5 - 6 week	6 - 7 week
INFECTED	51	0.060	0.065	0.067	0.070	0.075	0.078
	53	0.075	0.074	0.070	0.073	0.073	0.069
	81	0.071	0.070	0.066	0.085	0.084	0.079
	Mean	0.069	0.070	0.068	0.076	0.077	0.075
	S.D.	0.007	0.005	0.002	0.008	0.006	0.006
CONTROL	64	0.076	0.071	0.066	0.076	0.071	0.066
	86	0.061	0.069	0.064	0.073	0.082	0.076
	35	0.064	0.066	0.062	0.074	0.076	0.072
	Mean	0.067	0.069	0.064	0.074	0.076	0.071
	S.D.	0.003	0.002	0.002	0.002	0.006	0.005
	"t" Test	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table VI

^{51}Cr Red Cell Turnover in Fluke-infected and Control Sheep: 5 - 6 Weeks Post-infection

	Sheep	Red Cell Volume (ml/kg)	Red Cell $T\frac{1}{2}$ (hrs)	Faecal Red Cell Clearance (ml/day)	Faecal Loss as % Inj.	Urinary Loss as % Inj.
INFECTED	51	11.7	326	0.9	1.3	58
	53	9.3	400	1.1	1.5	56
	81	10.3	350	0.6	1.2	60
	Mean	10.4	359	0.9	1.3	58
	S.D.	1.2	38	0.3	0.2	2
CONTROL	64	8.7	470	1.2	1.5	59
	86	10.7	460	0.7	0.8	51
	35	11.0	500	1.0	1.2	62
	Mean	10.1	477	1.0	1.2	57
	S.D.	1.3	21	0.3	0.4	6
"t" Test		N.S.	$P < 0.01$	N.S.	N.S.	N.S.

Table VII

Albumin Concentration and Distribution in Fluke-infected and Control sheep: 6 Weeks Post-infection

	Sheep	Plasma Volume (ml/kg)	Serum Albumin (gms %)	CA (gm/kg)	EA (gm/kg)	EA/CA
INFECTED	47	49.2	2.16	1.06	1.53	1.44
	48	50.8	2.22	1.13	1.50	1.33
	NT	46.4	2.12	1.12	1.76	1.57
	Mean	48.8	2.16	1.10	1.60	1.45
	S.D.	2.2	0.05	0.04	0.14	0.12
CONTROLS	37	35.5	2.78	0.99	1.98	2.00
	98	32.8	2.70	0.79	1.80	2.28
	84	35.8	2.68	0.96	1.56	1.63
	Mean	34.7	2.72	0.91	1.78	1.97
	S.D.	1.7	0.05	0.11	0.21	0.33
"t" Test		$P < 0.001$	$P < 0.001$	$P < 0.05$	N.S.	N.S.

Table VIII

Albumin Catabolism in Fluke-infected and Control Sheep: 7 - 10 Weeks Post-infection

	Sheep	F(CA)			Absolute Amounts Albumin Catabolised cms/day		
		7 - 8 week	8 - 9 week	9 - 10 week	7 - 8 week	8 - 9 week	9 - 10 week
INFECTED	47	0.067	0.058	0.083	0.071	0.061	0.088
	48	0.042	0.044	0.060	0.047	0.050	0.068
	N.T.	0.083	0.073	0.076	0.093	0.082	0.085
	Mean	0.064	0.058	0.073	0.070	0.064	0.080
	S.D.	0.020	0.014	0.012	0.023	0.016	0.010
CONTROL	37	0.084	0.078	0.067	0.083	0.077	0.066
	58	0.077	0.068	0.073	0.061	0.054	0.058
	84	0.082	0.083	0.062	0.079	0.080	0.060
	Mean	0.081	0.076	0.067	0.074	0.070	0.061
	S.D.	0.004	0.007	0.005	0.011	0.014	0.004
"t" Test		N.S.	N.S.	N.S.	N.S.	N.S.	$P < 0.05$

Table IX

Albumin Catabolism in Fluke-infected and Control Sheep: 10 - 13 Weeks Post-infection

	Sheep	F(CA)			Absolute Amounts Albumin Catabolised gms/day		
		10 - 11 week	11 - 12 week	12 - 13 week	10 - 11 week	11 - 12 week	12 - 13 week
INFECTED	47	0.072	0.084	0.119	0.076	0.089	0.126
	48	0.040	0.083	0.096	0.045	0.094	0.108
	NT	0.087	0.100	0.121	0.097	0.112	0.136
	51	0.141	0.168	0.188	0.162	0.182	0.216
	Mean	0.085	0.106	0.131	0.095	0.119	0.147
	S.D.	0.042	0.035	0.039	0.049	0.042	0.048
CONTROL	37	0.072	0.069	-	0.071	0.068	-
	58	0.062	0.063	-	0.049	0.050	-
	84	0.058	0.054	-	0.056	0.052	-
	Mean	0.064	0.062	-	0.059	0.057	-
	S.D.	0.007	0.008	-	0.011	0.010	-
	"t" Test	NS	N.S.	N.S.	N.S.	N.S.	

Table X

^{51}Cr Red Cell Turnover in Fluke-infected and Control Sheep 10 - 13 Weeks Post-infection

	Sheep	Red Cell Volume (ml/kg)	Red Cell $T_{\frac{1}{2}}$ (hrs)	Faecal Red Cell Clearances 10 - 11 weeks	11 - 12 weeks	12 - 13 weeks	Fluke Burden
INFECTED	47	9.5	288	1.3	1.9	4.0	-
	48	8.7	270	3.3	4.5	8.0	518
	NT	6.6	276	0.7	2.4	7.4	-
	51	9.8	180	9.1	15.4	21.0	363
	Mean	8.7	254	3.6	6.1	10.1	
	S.D.	1.4	50	3.8	6.3	7.5	
CONTROL	37	9.3	480	0.3	0.4	-	
	58	12.7	500	0.8	0.6	-	
	84	10.9	510	0.3	0.3	-	
	Mean	11.0	497	0.5	0.4		
	S.D.	1.7	15	0.3	0.2		
	"t" Test	NS	$P < 0.01$	NS	NS		

Table XI

Albumin Concentration and Distribution
in Fluke-infected and Control Sheep

	Sheep	Time Infected (weeks)	Plasma Volume (ml/kg)	Serum Albumin (gms %)	CA (gm/kg)	EA (gm/kg)	EA/CA
INFECTED	47	23	45.1	1.07	0.48	0.53	1.10
	MT	"	50.5	1.48	0.75	0.81	1.09
	80	10	50.2	2.30	1.15	1.65	1.43
	285	"	39.3	2.33	0.92	1.02	1.11
CONTROL	37	-	42.5	2.80	1.19	1.36	1.14
	514	-	44.2	3.10	1.37	1.70	1.24

Table XII

Albumin Catabolism in Fluke-infected and Control Sheep:

	Sheep	Albumin $T_{\frac{1}{2}}$ (hrs)	F(CA)		Absolute Amounts Albumin Catabolised gms/day	
			23 - 24 week	24 - 25 week	23 - 24 week	24 - 25 week
infected 23 wks	47	151	0.177	0.142	0.084	0.068
	NT	132	0.221	0.232	0.166	0.174
			10 - 11 week	11 - 12 week	10 - 11 week	11 - 12 week
infected 10 wks	80	408	0.059	0.070	0.068	0.081
	285	331	0.121	0.113	0.111	0.105
controls	37	480	0.066	0.058	0.079	0.069
	514	510	0.057	-	0.078	-

Table XIII

^{51}Cr Red Cell Turnover in Fluke-infected and Control Sheep

	Sheep	Red Cell Volume (ml/kg)	Red Cell $T_{\frac{1}{2}}$ (hrs)	Faecal Red Cell Clearance ml/day			Fluke Burden
Infected 23 wks				22 - 23 week		23 - 24 week	
	47	7.6	173	19.3		18.9	-
	NT	6.2	84	35.1		30.0	-
Infected 10 wks				10 - 11 week	11 - 12 week	12 - 13 week	
	80	10.8	261	8.2	9.2	8.8	190
	285	10.2	211	16.3	18.5	18.2	170
Controls	37	17.7	384	0.5	0.8	-	-
	514	20.4	348	1.1	-	-	-