Biochemical and Immunological Studies on some Helminth Diseases of Domestic Animals

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by

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Biochemical and Immunological Studies on some Helminth Diseases of Domestic Animals

A summary of a Thesis submitted for the Degree of Doctor of Philosophy of the University of Glasgow by Francis William Jennings, B.Sc., M.Agr.

The work carried out in this thesis was concerned primarily with immunological studies on Fasciola hepatica infections using the rabbit as an experimental animal, and Dictyocaulus viviparus infections in the bovine. Experiments on the anaemia associated with F. hepatica infections in the rabbit are also reported. The thesis is divided into four sections and the main results are summarised as follows:

Section I Immunity studies on Fasciola hepatica.

1. Prior immunization of rabbits with the proteins of Fasciola hepatica resulted in a retardation in development of the parasites without a significant reduction in the numbers which developed from a challenge with 50 cercariae. The F. hepatica proteins were highly immunogenic as they elicited a marked antibody response in the sera of the immunized rabbits.

2. Prior immunization of rabbits with a polysaccharide fraction of F. hepatica resulted in one experiment in a significant reduction in the numbers of flukes but in a second experiment only in a retardation of development of the flukes. In both experiments the rabbits were challenged with 50 cercariae.

Section II Studies on the anaemia produced in rabbits by *Fasciola hepatica* infections

3. A measurement of the amount of blood lost by fluke-infected rabbits was made using ^{32}P-labelled red cells and ^{131}I-labelled plasma albumin. The amount of blood present in the flukes was calculated by a comparison of their radioactivity with that of the circulating blood at the time of autopsy. The calculated blood loss per day was consistent with the observed degree of anaemia.

4. The simultaneous use of ^{32}P-labelled red cells and ^{131}I-labelled plasma albumin suggested that the flukes preferentially absorbed and/or retained ^{32}P (labelled red cells) relative to ^{131}I (labelled plasma albumin).

5. A preliminary experiment on the use of ^{51}Cr-labelled red cells in estimating blood loss gave a higher value for blood loss per fluke than either ^{32}P-labelled red cells or ^{131}I-labelled plasma albumin.

Section III Studies on *Dictyocaulus viviparus* infection in the bovine.

6. A study was made of the serological response and the degree of immunity in calves resulting from primary and subsequent infections with the bovine lungworm, *Dictyocaulus viviparus*. During the primary infection the level of complement fixing antibodies in the serum rose slowly and reached a peak after the bulk of the infection had been

thrown off. At the second and third infection a typical secondary response was observed. Administration of the primary infection as a series of divided doses did not materially alter the serological response. The resulting immunity of both single and divided primary infections was very good.

7. Passive immunization of calves with serum from 'hyperimmune' donor animals resulted in a high degree of immunity when the calves were challenged with 4000 normal infective D. viviparus larvae.

8. Prior immunization of calves with whole worm antigens of D. viviparus did not produce a significant reduction in the numbers of worms which developed from a challenge infection with normal infective larvae.

Section IV Studies on Dictyocaulus viviparus infection in the bovine.

9. Prior immunization of calves with D. viviparus larvae treated with a suitable dose of x-rays resulted in a very high degree of immunity. An immunization dose of 4000 larvae treated with 40,000r enabled calves to withstand a challenge of 4000 normal larvae.

10. Prior immunization of calves with 1000 D. viviparus larvae irradiated with 40,000r resulted in an acceptable degree of immunity without any marked clinical reaction during immunization.

11. The potential value of the irradiation method both in immunization against worm diseases and as an experimental tool in the study of helminth immunity is discussed.

12. An extension of this work has led to the first field vaccine against a parasitic worm.

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GENERAL INTRODUCTION

The study of immunity to helminth diseases received little attention until the beginning of the 20th Century, and for the next 30 years immunological studies were largely confined to diagnostic tests (see Culbertson, 1941). A great impetus to work on helminth immunity came when Taliaferro in 1929 published his book, "The Immunology of Parasitic Infections", and after the studies of Sarles and Taliaferro (1936) on 'The local points of defence and the passive transfer of acquired immunity to Nippostrongylus muris in rats'.

In many parasitic diseases natural resistance exists which is exhibited in the host specificity of many parasites, i.e. many parasites will infect only one particular species of animal. Although little is known of the mechanism of natural resistance, serum may play a significant role, since the cercariae of many species of flukes are affected by the sera of a wide range of animals, (Standen, 1952), and in many cases animals possessing such a normal serum action against particular trematode cercariae fail to become infected by these cercariae (Culbertson, 1936).

An acquired immunity to many parasites may be developed by the host as a result of one or more infections with the parasite. This acquired immunity or immunity to reinfection has been demonstrated in diseases caused by Trichinella spiralis (McCoy, 1931; Roth, 1939; Oliver-Gonzalez, 1940), Nippostrongylus muris

(Africa, 1931; Schwartz, Alicata and Lucker, 1931; Taliaferro and Sarles, 1939), Ascaris lumbricoides (Kerr, 1938a, b; Fallis, 1948; Sprent and Chen, 1949; Oliver-Gonzalez, 1956), Ascaridia galli (Sadun, 1948), Ancylostoma caninum (Scott, 1930; Otto and Kerr, 1939), Toxocara felis (Sarles and Stoll, 1935), Cysticercus fasciolaris (Miller, 1931a, b; Miller and Massie, 1932), Cysticercus bovis (Kerr, 1935; Penfold, Penfold and Phillips, 1936), Strongyloides ratti (Sheldon, 1937), Strongyloides papillosus (Turner, 1956), Haemonchus contortus (Stoll, 1929; Lisenko, 1956), Trichostrongylus calcaratus (Sarles, 1932), T. retortaeformis (Michel, 1953), T. colubriformis (Stewart, 1950c), T. axei (Gibson, 1953).

Immunity in some parasitic diseases may be transferred passively by the injection of hyperimmune serum collected from animals which have undergone one or more infection of the parasite (i.e. have developed an acquired immunity). Significant reductions in the numbers of worms developing from a challenge infection in passively immunized animals as compared to susceptible controls have been reported in Cysticercus fasciolaris infections of rats (Miller and Gardiner, 1932; Miller, 1934; Campbell, 1938), in Cysticercus pisiformis infections of rabbits (Kerr, 1935), in Nippostrongylus muris infections of rats (Sarles and Taliaferro, 1936, 1938; Chandler, 1938; Sarles, 1939), in Strongyloides ratti infections of rats (Lawlor, 1940) and in Ascaridia galli infections of chickens (Sadun, 1949). Relatively less successful results have been reported in the case of Ancylostoma caninum infections of dogs (Otto, 1938). In schistosomiasis conflicting results have been

recorded, Kawamura (1932) reported protection in dogs and rabbits, whereas Vogel and Minning (1953), Stirewalt and Evans (1953), Kagan (1958) and Levine and Kagan (1960) failed to transfer immunity passively.

Because a strong acquired immunity is developed by the host as a result of natural or experimental infections with many helminth parasites, several workers have attempted to stimulate an active immunity against these worms by artificial means. The results of artificial immunization by the injection of worm homogenates or extracts of dead worm materials have, in general, been disappointing and in many cases conflicting results have been obtained. Kerr and Petkovitch (1935), with F. hepatica infections in rabbits, claimed to have produced immunity by injecting dried fluke material when measured by the numbers of adult parasites developing from a standard challenge infection. In schistosomiasis some workers have reported positive results in inducing resistance by vaccination with worm antigens of S. japonicum (Ozawa, 1930; Kawamura, 1932; Lin, Ritchie and Hunter, 1954). Watts (1949) reported successful immunization with worm antigens in mice, but Thompson (1954) failed to verify her results. Kagan (1958) also failed in his vaccination experiments using antigens of S. douthitti.

In Ascaris lumbricoides infections of mice Wagner (1933) reported successful results but Oliver-Gonzalez (1956) and Soulsby (1957) failed to produce immunity

in the guinea pig by parenteral administration of worm materials.

Some degree of protection has been reported in Trichinella spiralis infections (McCoy 1935) but Bachman and Molina (1933) failed to protect animals using dead worm materials, and Ewert and Olson (1960) also failed using larval materials pulverised by ultrasonic waves as the immunizing antigen.

In Haemonchus contortus infections Stoll (1942) and Lisenko (1956) reported that there was 'some evidence' of immunity after parenteral administration of worm materials, although Stewart (1950b) was unable to obtain evidence that sheep could be protected by this method.

In this approach to artificial immunization by the injection of worm materials, the most successful results have been achieved with Cysticercus fasciolaris (Miller, 1930, 1931c, 1932).

More recently it has been shown that a degree of acquired immunity can be produced in guinea pigs by the subcutaneous injection of embryonated eggs of Ascaris lumbricoides (Oliver-Gonzalez, 1956; Soulsby, 1957) and against Haemonchus contortus infections in sheep by the parenteral injection of exsheathed living larvae (Stoll, 1958; Lisenko, 1956). Attempts to reproduce the latter results with H. contortus in Australian sheep have failed (Stewart, 1959). These experiments using actively metabolising eggs or larvae as immunizing agent are an extension of the work of Thorson (1954) who showed that

the 'excretions and secretions' of living Nippostrongylus muris larvae were able to absorb out most of the protective properties of hyperimmune serum, but that the dead larval tissues did not have this effect.

The work of Thorson emphasises the complexity of the antigenic stimulus encountered by the host in parasitic infections in that one can get numbers of antibodies directed towards somatic antigens and also antibodies to substances present in the so-called 'excretions and secretions'. These latter antibodies may include anti-enzyme antibodies, e.g. it has been shown by Thorson (1953) that immune serum inhibits the lipolytic activity of the secretions and excretions of N. muris larvae. Thorson (1956a) has also shown that the secretions of the oesophogeal glands of dog hookworms contain proteolytic enzymes which can be inhibited by immune serum, although attempts to utilize extracts of these oesophogeal glands as an immunizing antigen failed to produce any immunity to a challenge infection, (Thorson, 1956b).

In general, the injection of dead worm materials fails to produce a good immunity to parasitic diseases. On the other hand the presentation of actively metabolising larvae or embryonated eggs by an abnormal route results in a greater degree of acquired immunity, but the immunity produced is generally not as good as that of an animal which has recovered from a natural infection. It seems likely that the ideal immunization procedure

would be to present the host with living, actively metabolising larvae by the normal route of infection if such larvae could be 'attenuated', or 'inactivated' by some means so that they were unable to exert their full pathogenic effects. By this means the animal would be presented with all the antigenic stimuli of the disease agent and also the sites of stimulation would exactly parallel those of the natural infection.

The work described in this thesis is primarily concerned with immunological studies on two important diseases of domestic animals. The first is liver fluke infections caused by the trematode Fasciola hepatica . In this disease attempts to produce artificially acquired immunity are described using extracts of flukes (Section I) and also some experiments using radioactive isotopes in studies of the anaemia associated with F. hepatica infections, (Section II). The second disease studied was parasitic bronchitis of cattle caused by the nematode Dictyocaulus viviparus. The immunity produced by natural infection, passive transfer of immune sera, and injection of whole worm materials was studied (Section III). Section IV reports experiments on the immunity produced by the administration of partially inactivated larvae.

In each case a fuller résumé of the relevant literature is given in the introduction to the appropriate sections.

Section I

Immunity Studies on *Fasciola hepatica*.

A. The use of protein fractions of *F. hepatica* as immunizing antigen.

B. The use of polysaccharide fractions of *F. hepatica* as immunizing antigen.

Introduction.

Fasciola hepatica was the first trematode to have its life cycle elucidated (Leuckart, 1882; Thomas, 1883). The adult parasite is found in the bile ducts of the host's liver, and cases of infection have been reported in all herbivorous and omnivorous animals including man. The eggs, produced in large numbers by the parasite, enter the intestine with the bile and are evacuated with the faeces. After maturing in water for 9 - 15 days (optimum temperature 22 - 26°C.) the ciliated miracidia emerge. These miracidia move actively in water and can survive up to 24 hours, and if during this time a suitable snail host (mainly Lymnaea truncatula in Britain) is encountered the miracidia actively penetrate the snail tissues, where they develop into sporocysts, rediae, daughter rediae and cercariae. The mature cercariae emerge from the snail in considerable numbers and can be seen visually as minute tadpole-like organisms which swim freely in water. After a short interval, the cercariae cast off their tails and encyst as minute white spherules on aquatic vegetation or blades of grass. These cysts are viable for long periods in a moist atmosphere, but die quickly when desiccated. The time which elapses between the infection of the snail and the shedding of cercariae occupies about 6 - 7 weeks under optimal conditions.

The encysted cercariae when ingested by an animal

excyst in the intestine, penetrate the wall of the gut and migrate across the peritoneum to the liver which they enter by boring through the capsule (Schumacher, 1939). They traverse the liver parenchyma to the biliary passages, where they settle down and grow to maturity. The time which elapses between infection of the host and the mature liver fluke being established is usually between 8 and 9 weeks.

If the numbers of ingested cercariae are excessive the host may succumb during the acute or migratory stage of the infection due to extensive destruction of liver tissue and haemorrhage. The chronic form of the disease, associated with the presence of the adult parasites in the bile ducts, is much more common and is characterised by progressive emaciation, a degree of anaemia and in some instances by oedema of the intermandibular space.

The adult parasite can survive for a long period in the bile ducts, one report by Egorov (1954) stating that a sheep infected when 2 years old had 22 adult flukes in the bile ducts when slaughtered 8 years later, the possibility of another infection occurring during this period having been excluded.

The importance of this disease in Great Britain may be assessed from a survey carried out by Peters and Clapham (1942) who reported that the annual loss due to condemned bovine livers at the slaughterhouse was probably in the order of 1500 tons. Enger and

Karbo (1956) reported that in Norway 4910 (80%) of 6137 cattle examined in 1954 were infected with liver fluke, and that infection was so severe in 3101 of these, that the whole liver was condemned, resulting in a financial loss on the livers alone of approximately £5,500. In addition the average weight of the infected animals was 14Kg less than that of the uninfected animals.

The possibility that animals may develop a resistance to reinfection by F. hepatica does not appear to have been studied experimentally. Culbertson (1941) states that rabbits show immunity to reinfection but quotes no references.

On the basis of field studies in East Africa Coyle (1958) concluded that there was some evidence of resistance to reinfection with F. gigantica in cattle.

When the work reported in this thesis was carried out the only experimental results on artificial immunity to F. hepatica infections in rabbits were those of Kerr and Petkovitch (1935). After immunization of rabbits by injection of dried fluke material and challenge with 13 cercariae, these workers recovered 1, 2, 2, 2, 2, 3 and 3 flukes respectively as compared to 11, 5 and 9 from their three unimmunized controls.

The following experiments were carried out in an attempt to immunize rabbits against F. hepatica using (A) protein antigens and (B) polysaccharide antigens.

A. The use of protein fractions of *F. hepatica* as immunizing antigens.

An experiment to test the efficacy of *F. hepatica* proteins as immunizing antigens was carried out as follows.

Group A

Number in group: 7 rabbits.
Immunization schedule: 3 injections of alum-precipitated *F. hepatica* proteins (100mg/injection) at approx. 2 week intervals.
Challenge: 50 cercariae 2 weeks after the last injection of *F. hepatica* proteins.
Autopsy: 63 days after challenged with cercariae.

Group B

Number in group: 7 rabbits.
Immunization schedule: 6 injections of alum-precipitated *F. hepatica* proteins (100mg/injection) at approx. 2 week intervals.
Challenge: as group A.
Autopsy: as group A.

Controls

Number in group: 10 rabbits.
Immunization schedule: none.
Challenge: as groups A and B.
Autopsy: as groups A and B.

Methods and Materials.

Immunizing Antigen.

Sheep livers which were infected with *Fasciola hepatica* were collected at the local slaughterhouse and as soon as possible the bile ducts were opened. The flukes were removed and freed from adhering bile and other contaminating material by washing under running tap water. These washed parasites were then suspended in 0.85% NaCl (1 parasite/ml) and homogenised in a top-drive macerator. The crude homogenate was allowed to stand at 0 - 4°C for several hours to allow the larger particles to settle out. The supernatant fluid was decanted off and further freed from insoluble material by centrifugation at 1500g for 1 hour. The resultant saline extract was usually amber to dark brown in colour, and contained about 1mg protein N/ml.

Alum-precipitated preparations for immunization were made by treating the saline extract with an equal volume of 10% potash alum, (Proom, 1943) and adjusting the pH to 6.5 with 5N NaOH. The alum-precipitated proteins were then washed twice with 0.85% NaCl (containing 1:100,000 merthiolate) and finally suspended in 0.85% NaCl (merthiolated) for injection. Kjeldahl analyses showed that the above procedure precipitated the same amount of protein N as did precipitation with 10% trichloroacetic acid, indicating

that the alum-precipitated preparation contained all the proteins in the saline extract.

Precipitating antigen.

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

The saline extract was treated with an equal volume of saturated $(NH_4)_2SO_4$ solution with constant stirring and the mixture allowed to stand at 0 - 4°C for several hours. The precipitated protein was centrifuged off and treated with 1 volume of 0.1M phosphate buffer pH8.0. Any material which did not redissolve was removed by centrifugation, and the protein fraction was twice reprecipitated with $(NH_4)_2SO_4$ as before. The precipitated protein fraction was finally transferred to a dialysis sac and dialysed against repeated large volumes of 0.85% NaCl, until the dialysate gave no reaction to Nessler's reagent. At each precipitation and during dialysis there was some loss of protein as an insoluble residue, and the yield ultimately obtained was usually about 10% of the total protein in the original saline extract.

Immunization.

Rabbits were injected at 8 - 10 day intervals with alum-precipitated antigen prepared as described above. Five ml of a suspension containing 10mg protein N/ml were injected intramuscularly into each hind leg. Seven rabbits (group A) received a total of 3 injections and a further seven (group B) received a total of 6 injections.

Qualitative precipitin tests.

A stock solution of the precipitating antigen (1.2% protein N) was diluted to 1:20, 1:100, 1:500, 1:2,500 by the addition of 0.85% NaCl. 0.25ml of each of these dilutions of antigen was treated with 0.1ml of the serum to be tested in precipitin tubes. The contents of the tubes were mixed, incubated at 37°C for 1 hour and then placed in a refrigerator (0 - 4°C) for 2 hours to ensure maximal precipitation. As a control against non-specific precipitation, 0.1ml of each sample of serum was incubated with 0.25ml of saline, and samples of normal rabbit serum were incubated with antigen on every occasion when sera from infected or immunized animals were being tested.

Quantitative Precipitin Tests.

As antibodies are protein in nature, they can be measured in terms of protein N. In a precipitin test where the antigen is a nitrogen-free carbohydrate micro-Kjeldahl analysis of the specific precipitates gives the antibody N. content directly. Where the antigen is also a protein, the precipitate will contain both antigen N. and antibody N. and in such

circumstances it is necessary to obtain an independent measure of one or other of the reactants. In the precipitation reaction between a simple homogenous antigen and its homologous rabbit antiserum the precipitin curve obtained (Fig.1) falls into three fairly well defined zones (a) an antibody excess zone, where by conventional qualitative tests it can be shown that all the added antigen has been precipitated, (b) an equivalence zone corresponding to complete precipitation of both antibody and antigen N. and (c) an antigen excess zone or antigen inhibition zone. In the equivalence zone, subtraction of antigen N. added from total N. precipitated (micro-Kjeldahl analysis) gives the amount of antibody N. precipitated. The use of a more precise method for the determination of antigen in the antibody excess and equivalence zones, will, in many systems, indicate that even in the region of antibody excess, all the added antigen has not been precipitated, and therefore even in this simple type of antibody-antigen system, the results obtained by subtraction of antigen N. added from total N. precipitated leads to slightly erroneous results.

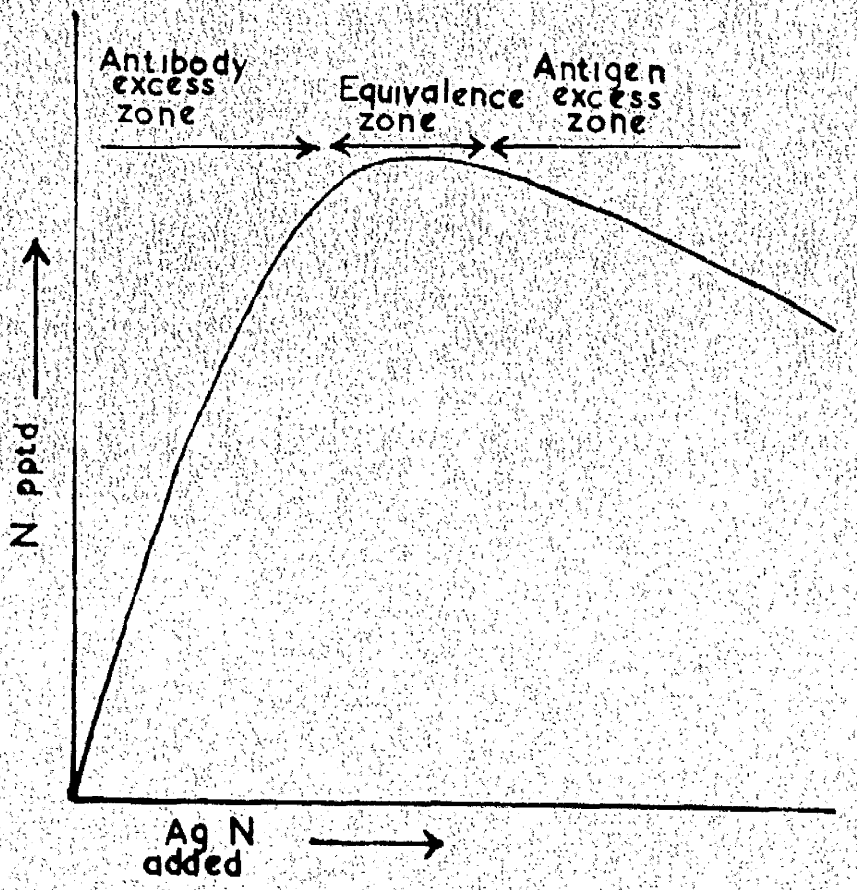
In the case of inhomogenous antigen preparations such as one is likely to obtain from extracts of helminths the added antigen is even less completely precipitated in the region of antibody excess and there is no clearly defined equivalence zone (Fig.2). In experimental work in systems of this type there would appear to be two alternative procedures.

(a) Analysis of the specific precipitates obtained as far as possible in the region of maximal precipitation for total protein N. This will give a measure of antibody N. plus antigen N. and as the ratio of antibody N. to antigen N. in the specific precipitate in this region is fairly constant the total N. precipitated should be proportional to the antibody N. content of the serum.

(b) The employment of some method which enables one to make an independent assessment of either antibody or antigen in the specific

Fig. 1

Precipitin curve obtained between a simple homogenous antigen and its homologous rabbit antiserum. The figure is obtained by adding increasing amounts of the antigen to a fixed amount of antiserum.



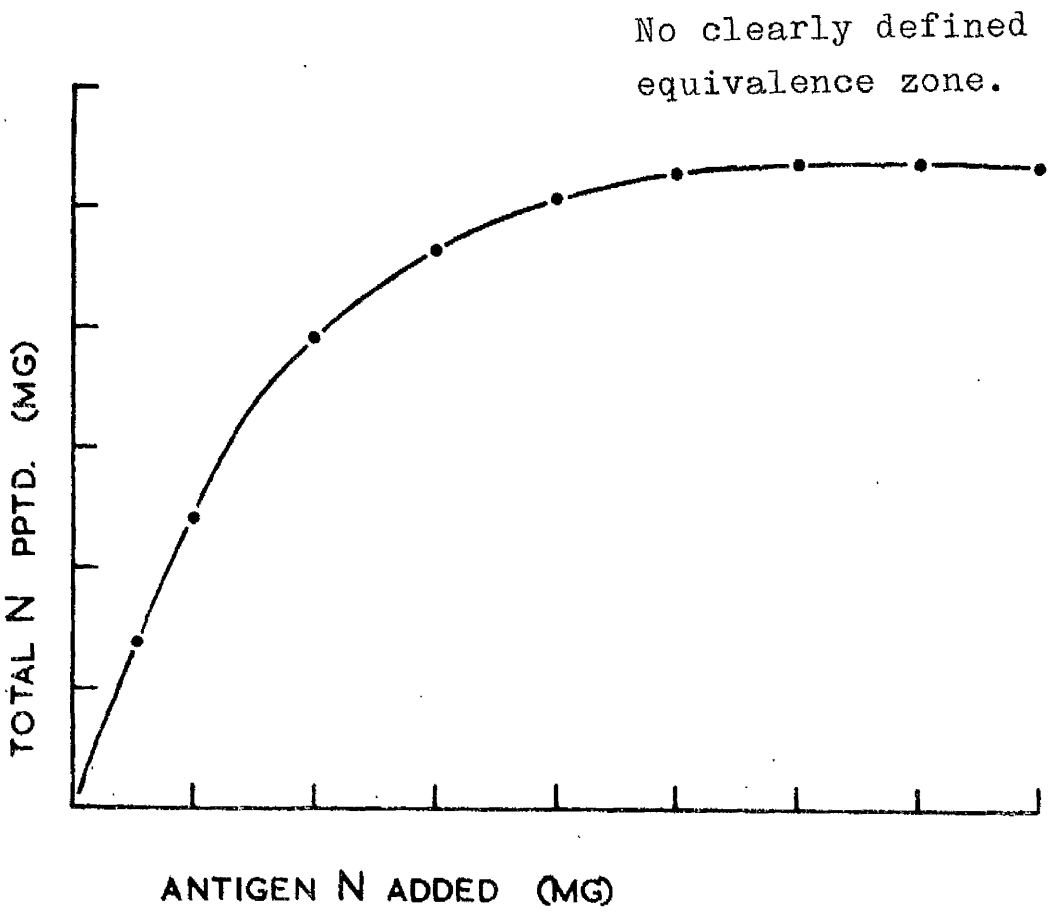


Fig. 2 Precipitin curve obtained between an inhomogenous protein antigen and its homologous rabbit antiserum.

precipitate, e.g. by the isotopic labelling of one of the reactants (see review by Wormall, 1952). When this investigation was being carried out no isotope equipment was available, hence method (a) was employed. However, later on in the work it became possible to carry out isotopic work and some experiments with Iodine-131 labelled antibody were performed.

Method (a) was largely used in the present experiment and was carried out as follows:- The results of the qualitative precipitin test gave some indication of the amount of antigen N. which would be necessary to achieve maximal precipitation.

Duplicate samples (1.0ml) of centrifuged serum were pipetted into 15ml tapered centrifuge tubes. Successive small volumes of stock antigen solution were added to these with incubation (1 hr. at 37°C) and refrigeration (1 - 2 hrs.) after each addition of antigen, until no further precipitate was obtained. This was taken to correspond to complete removal of the corresponding antibody from solution.

In the case of the rabbits in group B where sufficient serum was available increasing amounts of antigen were added to separate 1.0ml volumes of serum, followed by incubation and refrigeration. By analysis of each precipitate a precipitin curve can be constructed.

Each precipitate was centrifuged off, washed twice with 5 drops of ice-cold saline each time to remove nonspecific proteins (Heidelberger and Kendall, 1932), dissolved in a few drops of 0.1 N NaOH and 1.0 ml of distilled water, and transferred to micro-Kjeldahl incineration flasks for digestion and subsequent total

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

Total nitrogen determinations.

Total N. determinations were carried out on the specific precipitates and in some cases on the flukes recovered from the infected rabbits using the micro-Kjeldahl procedure. The digestion mixture consisted of 1 - 2ml sulphuric acid (M.A.R.) and a small quantity of catalyst (8 parts K_2SO_4, 1 part $CuSO_4.5H_2O$, 1 part SeO_2), after digestion the NH_3 was distilled into a 4% boric acid solution containing a methyl red-methylene blue indicator (see Kabat and Mayor, 1948 p. 283).

The final titration was made with $^N/_{70}$ HCl, using an "Agla" micrometer syringe (Burroughs Wellcome and Co.) or a microburette, according to the amount of protein N. present. The titration assembly used with the "Agla" micrometer syringe is shown in Fig. 3.

Paper electrophoresis

Paper electrophoresis was carried out on the sera from some of the rabbits during the experiment. The sera were separated on 7mm. wide strips (Whatman 3 MM) and the developed strips were sectioned in 0.5cm widths, the dye extracted and electrophoretic diagrams constructed as described by Flynn and de Mayo, (1951).

RESULTS.

Precipitin Serology.

Qualitative precipitin tests showed that the sera

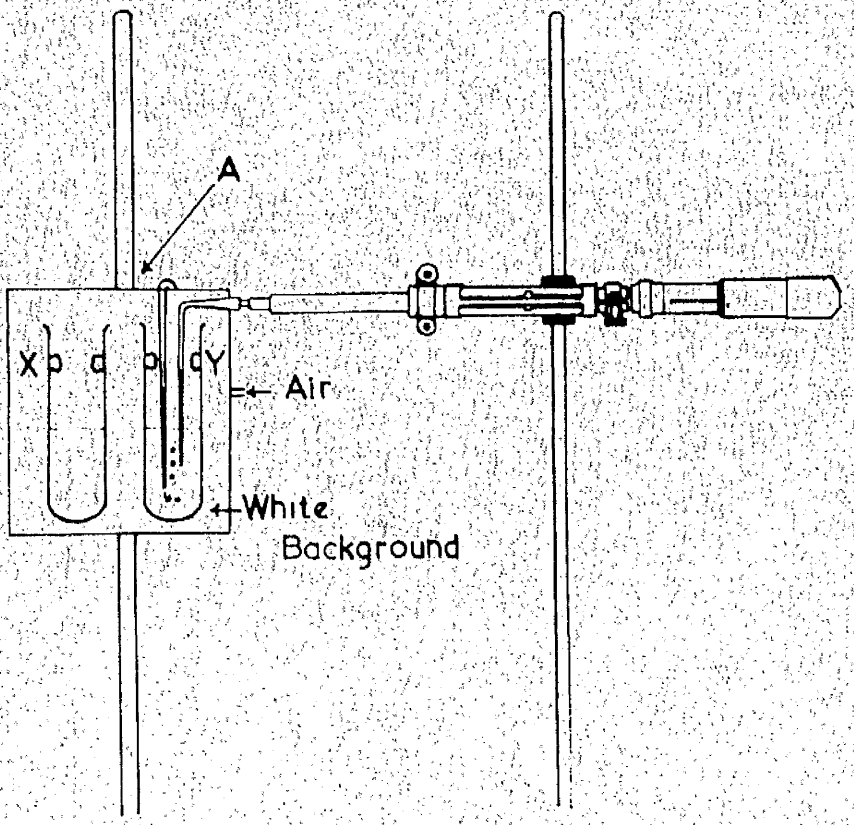


Fig. 2 Titration assembly for 'Agla' micrometer syringe used in micro-Kjeldahl estimation of protein nitrogen.

of infected rabbits contained antibodies which reacted with the precipitating antigen prepared from F. hepatica. Sera from more than 20 normal rabbits were tested with the precipitating antigen and in no case was any positive reaction obtained. Table 1 shows a representative sample of the results obtained from the sera of immunized, immunized and infected, and infected normal rabbits.

The individual precipitin curves for seven of the experimental rabbits are shown in Fig. 4 and from the shapes of the curves obtained there is a strong indication that the precipitating antigen used is composed of more than one component. In a system of this type it is impossible to determine antigen and antibody separately in the precipitates unless by some method of labelling one of the reactants. In a later experiment when isotopic equipment became available, an analysis was carried out on a pooled sample of serum from the immunized infected rabbits. The trace iodination of the serum was carried out as described by Francis, Mulligan and Wormall, (1951), and after freeing the ^{131}I-labelled serum from unbound iodine, aliquots corresponding to 1ml of the original serum were treated with varying amounts of antigen as before. The mixtures were incubated for 1 hour, refrigerated for 2 hours, and the precipitates centrifuged and washed as described previously. The precipitates were then dissolved in a few drops of 0.1 N NaOH, made up to 10 ml and radioactivity determinations carried out in a M6 liquid counter (20th Century Electronics). Aliquots were then taken for micro-Kjeldahl incineration for total N. The antibody N. content of the precipitates was calculated by comparing these radioactivities with that of a globulin preparation separated from the labelled serum, (see

TABLE I

Results of Qualitative Precipitin Tests on the Sera of Immunized, Immunized and Infected, and Infected Normal Rabbits.

	Rabbit No.	Antigen concentrations 1:20	1:100	1:500	1:2500
Immunized	V1	+	++	+	±
Rabbits after	V2	+±	++	+±	+
3 injections of	N856	+++	++	+±	Tr.
alum-precipitated	N851	±	+	±	Tr.
fluke protein	0330	+±	+	±	Tr.
	0329	Tr.	±	Tr.	F.Tr.
	0371	+	+±	±	Tr.
Immunized	V1	++	++±	+	Tr.
rabbits 60 days	V2	+++++	+++	++	Tr.
after	N851	+±	++±	+±	Tr.
infection	0330	+	++	+	-
	0329	±	±	F.Tr.	-
Control	C2	+	++	+	Tr.
Rabbits 60	C4	+	+±	Tr.	-
days after	C8	+	++	+±	Tr.
infection	C9	+±	+++	++	F.Tr.
	C14	+	++	±	-

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

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

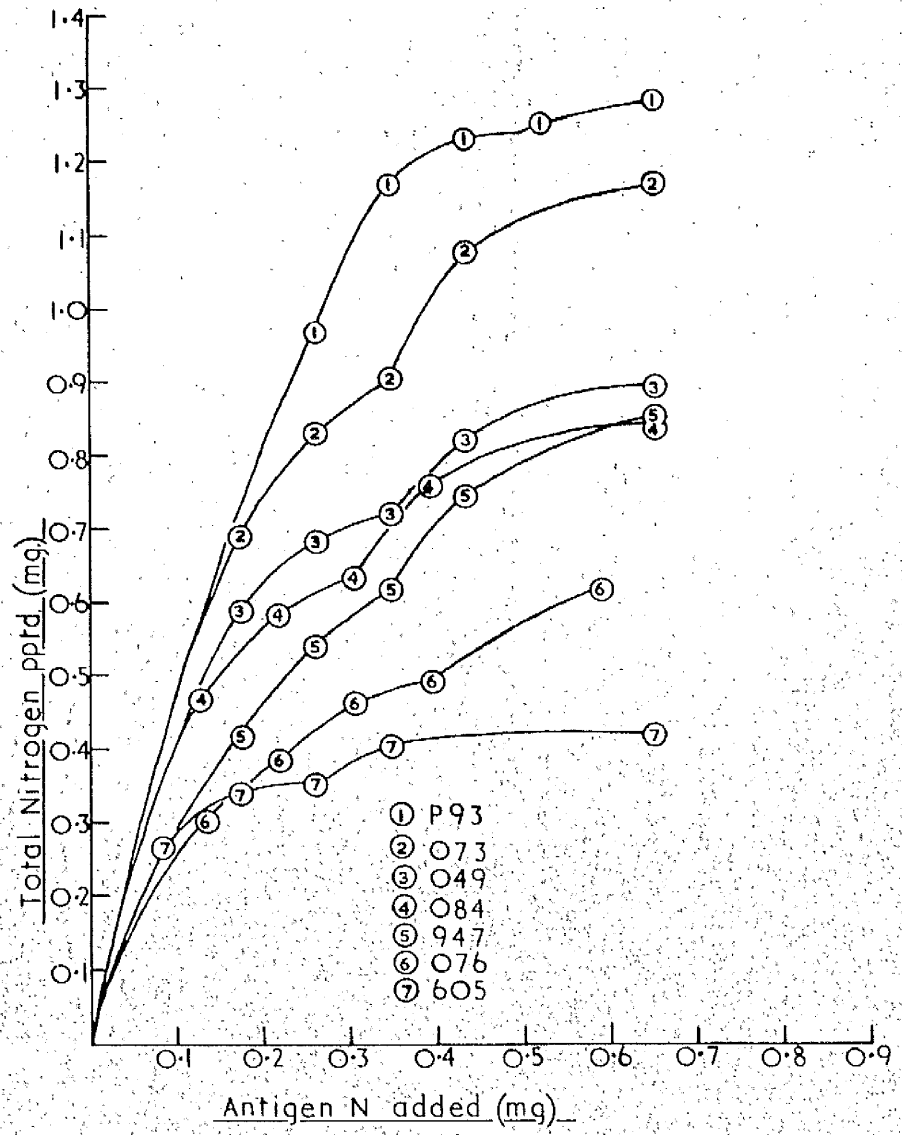


Fig. 4 Individual precipitin curves obtained with the antiserum of immunized, infected rabbits and the protein 'precipitating antigen' of F. hepatica.

Pressman and Sternberger, 1950).

The result of the preliminary experiment is shown in Fig. 5 and it can be seen that whereas the total N. curve continues to rise the antibody N. curve flattens off giving an absolute measure of the antibody N. which is precipitated from the serum by the precipitating antigen.

The results of the quantitative precipitin tests on the sera of the immunized and control rabbits are shown in Table 2.

From Table 2 it can be seen that a course of 3 injections with alum-precipitated proteins from F. hepatica gave a level of precipitin to the test antigen comparable to that obtained in normal rabbits 63 days after infection with 50 cercariae (control group). In group A the precipitin level 63 days after infection was in most cases considerably greater than the level at the time of infection. This increase was presumably due to the stimulus of infection on the immunized animals. No such increase occurred in the rabbits of group B; here the rabbits were subjected to a course of 6 injections prior to infection, and the precipitin level at the time of infection was considerably higher than in group A.

Paper electrophoretic diagrams of the sera of some of the rabbits in group B are shown in Figs. 6 and 7. A large increase in the antibody containing fraction of the sera when rabbits have been subjected to an immunization schedule and infection is apparent.

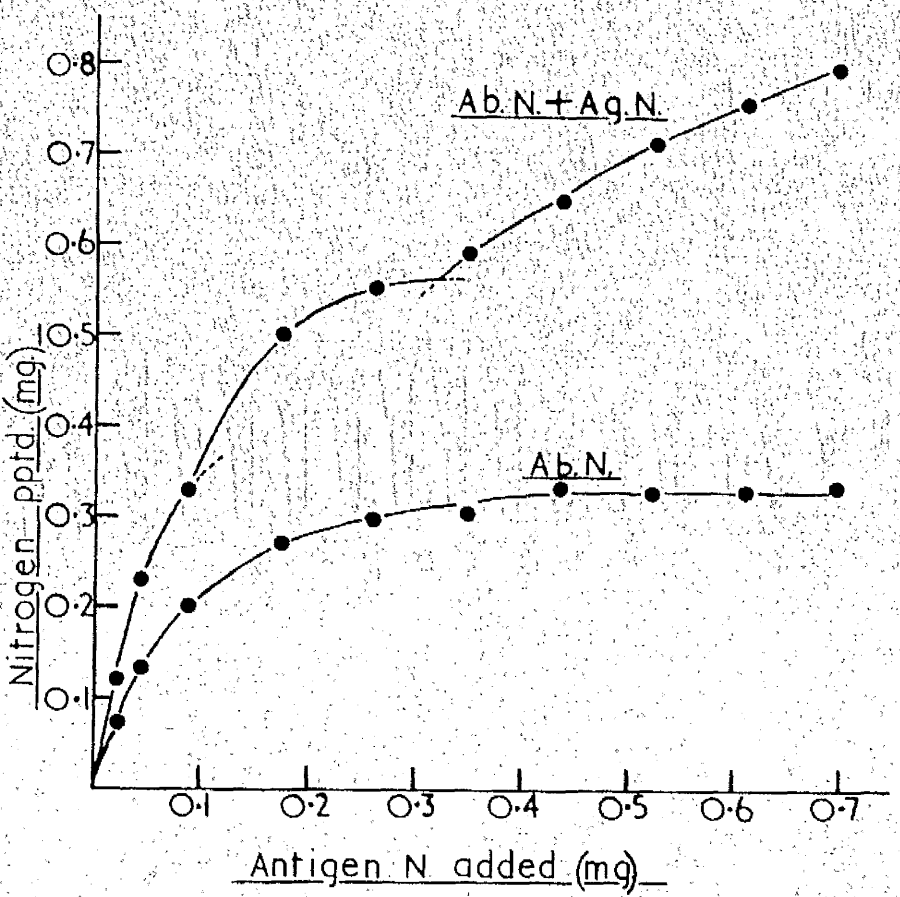


Fig.5 Precipitin curve obtained with the protein precipitating antigen of F. hepatica and pooled antiserum trace-labelled with ^{131}I.

TABLE 2

Quantitative precipitin tests.

	Rabbit No.	Antibody N. + Antigen N. precipitated from 1.0ml serum	
		Pre-infection 63 (mg.N)	days after infection (mg.N)
Immunized rabbits (group A) (3 injections)	V1	0.16	0.77
	V2	0.19	1.87
	N856	0.27	0.62
	N851	0.22	0.64
	0330	0.20	0.50
	0329	0.11	0.15
	0371	0.19	—
Immunized rabbits (group B) (6 injections)	P93	1.28	1.13
	073	1.17	0.92
	049	0.89	0.77
	084	0.84	0.66
	947	0.85	0.59
	076	0.62	0.66
	605	0.42	0.97
Control rabbits	C2	—	0.27
	C4	—	0.10
	C8	—	0.22
	C9	—	0.33
	C14	—	0.24
	X	—	0.15
	9	—	0.30
	0	—	0.14
	Z	—	0.23
	1	—	0.12

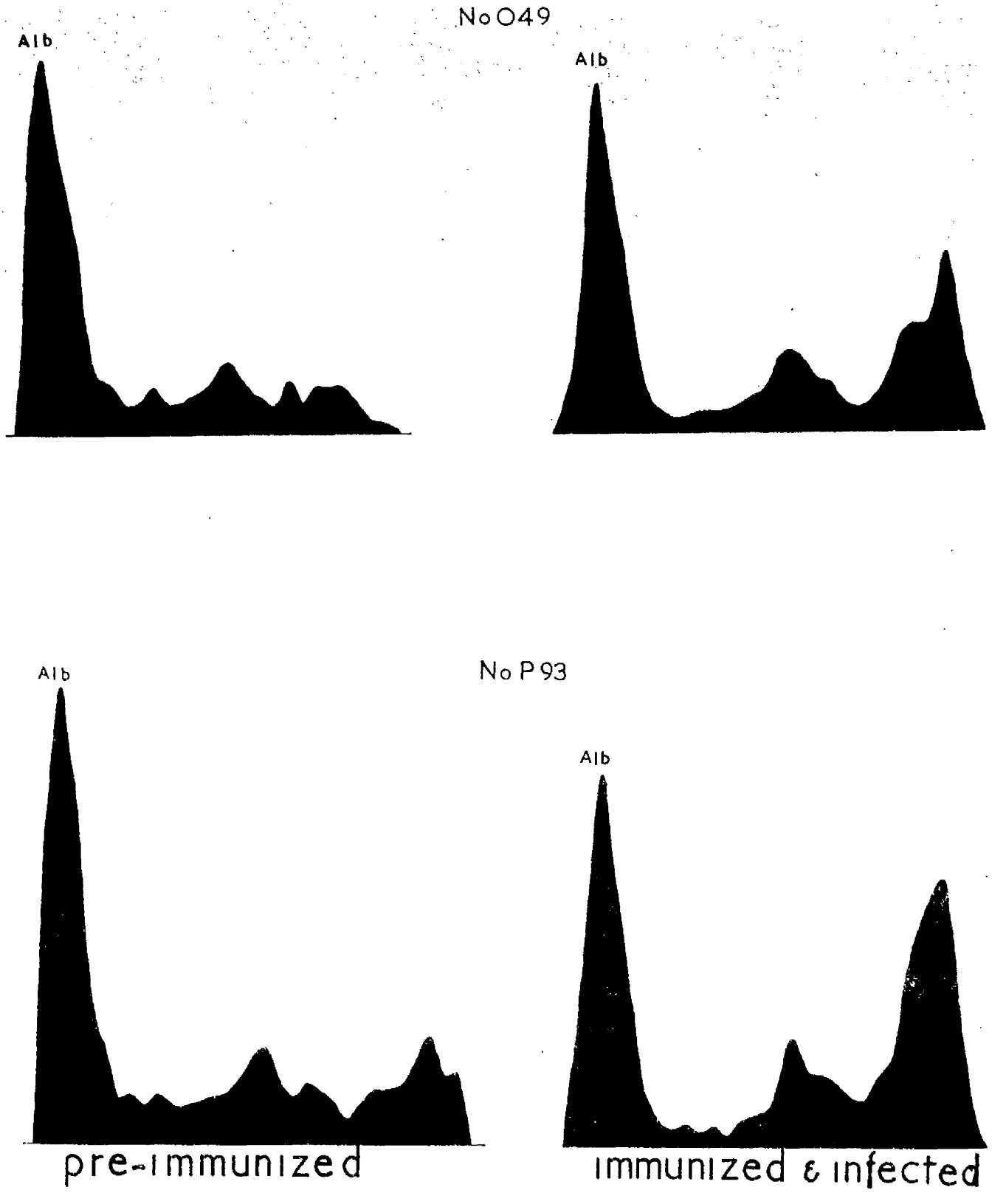


Fig. 6 Electrophoretic diagrams of the sera of rabbits before and after immunization and infection.

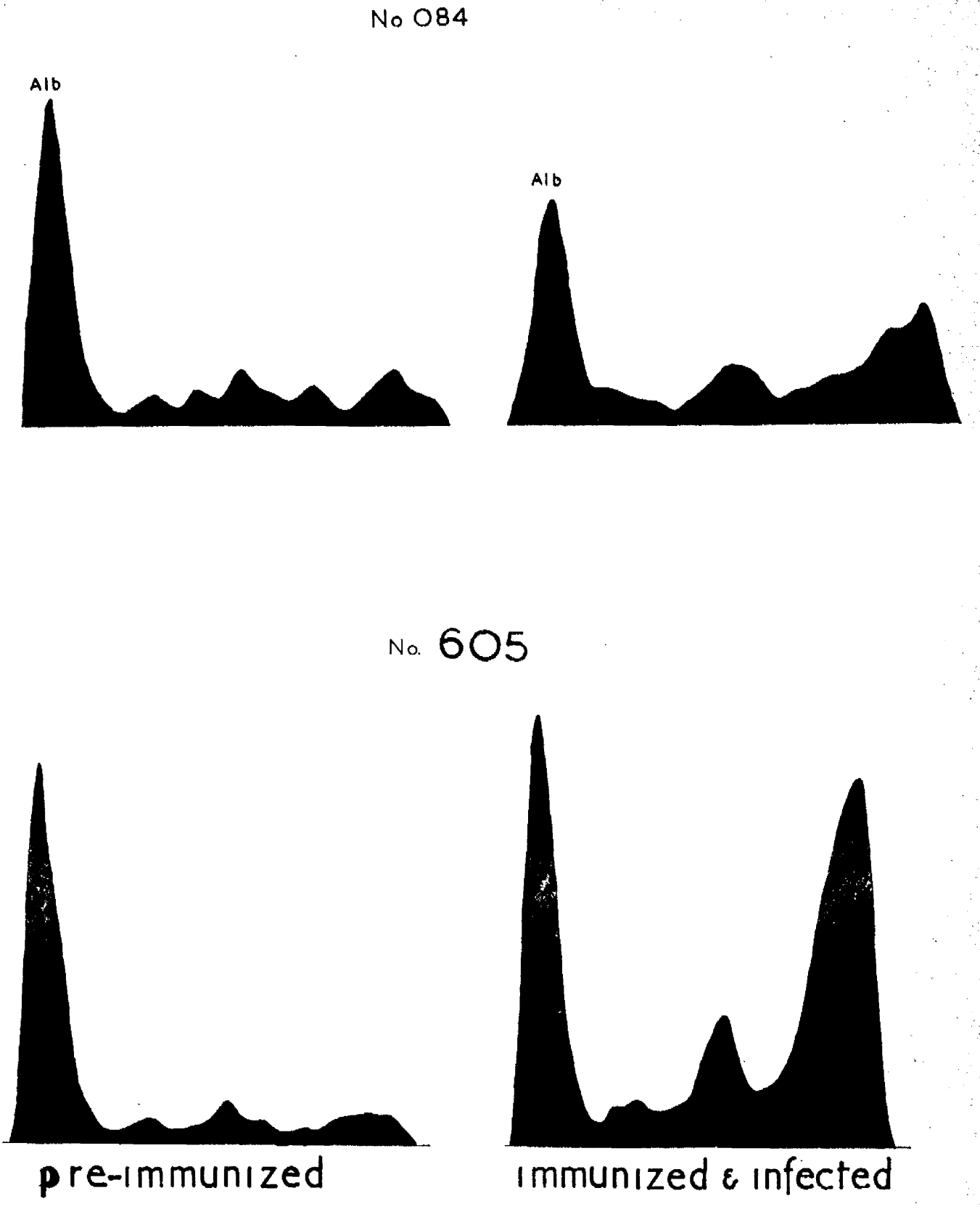


Fig. 7 Electrophoretic diagrams of the sera of rabbits before and after immunization and infection.

Autopsies on Immunized and Control Rabbits.

The experimental rabbits were killed 63 days after infection and the number of flukes recovered from each of the livers at autopsy is recorded in Table 3.

It is evident from the results shown in Table 3 that the average number of flukes in the rabbits in the immunized groups is not significantly lower than the average in the control group. However, the flukes recovered from the immunized groups were invariably smaller than those from the control group. This retarded development was particularly marked in the flukes obtained from rabbits V2, V1, 049 and 605. In order to obtain more definite information on this retardation of development, the flukes obtained from group B and from some of the control rabbits were analysed for total N. The flukes were removed from the liver, rinsed in saline, and analysed for total N. by the micro-Kjeldahl procedure. The results of these determinations are shown in Table 4. The figures obtained confirmed the observations on the inhibited development of the flukes in the immunized rabbits, i.e. the 125 flukes obtained from group B had an average total N. content of 0.41 mgN, whereas the 108 flukes from 5 control rabbits had an average total N content of 1.13 mg. Examination of the most retarded of these flukes showed that they were sexually immature.

The pathological examination was carried out by Dr. G.M. Urquhart and his report was as follows:

TABLE 3

Number of flukes found in immunized and control rabbits on autopsy.

	Rabbit No.	Number of flukes
Immunized rabbits Group A	V1	10
	V2	3
	M856	6
	M851	10
	0530	13
	0529	18
	0571	26
		Average 12.3 ± 7.1
Immunized rabbits Group B	P93	14
	073	14
	049	5
	084	31
	947	17
	076	20
	605	26
		Average 17.9 ± 9.1
Control rabbits	C2	6
	C4	18
	C8	20
	C9	25
	C14	25
	C7	6
	x	6
	9	25
	0	18
	3	34
	1	25
		Average 18.1 ± 9.4

TABLE 4

Comparative development of flukes expressed in terms of their average total nitrogen content in immunized and control rabbits.

	Rabbit No.	Number of flukes	Total fluke nitrogen (mg)	Nitrogen per fluke (mg)
Immunized rabbits Group B	P93	14	7.28	0.520
	073	14	5.96	0.426
	049	3	0.56	0.187
	947	17	7.54	0.444
	076	20	14.40	0.720
			3.18	0.122
				Average 0.41 ± 0.20
Control rabbits	X	6	4.22	0.704
	9	25	32.50	0.30
	0	18	24.20	1.35
	Z	34	38.80	1.14
	1	25	29.00	1.16
				Average 1.13 ± 0.25

"The livers of the immunized animals showed similar lesions to those of the control group, i.e. moderate fibrinous peritonitis, a degree of fibrosis approximately proportional to the number of flukes present, and a grossly distended bile duct which usually contained about half of the total number of flukes, the remainder being found in the bile ducts in the liver. In rabbits V2, V1, 605 and 049, however, from which the smallest flukes were obtained, there was very extensive fibrinous peritonitis, the livers were very enlarged with numerous large fibrous nodules and showed extensive areas of recent necrosis, caused by damage to blood vessels by migrating flukes". In V2 and 049 the common bile duct was normal in size and contained no flukes, while in V1 and 605 there was a slightly enlarged common bile duct containing a small number of flukes. The majority of the flukes from these 4 rabbits were found either migrating through liver tissue or in very fine bile ducts.

DISCUSSION.

From the present investigation, designed largely to study the antigenic properties of proteins from F. hepatica and the protective properties, if any, of the antibodies which they elicit, it was evident that the proteins of the liver fluke were highly immunogenic in that they stimulated the production of precipitins, and rabbits immunized with these antigens showed a large increase in the γ globulin fraction of the serum after challenge (see Figs. 6 and 7). However, prior immunization with these fluke proteins has no influence

on the number of F. hepatica developing from a challenging infection. The infection of normal rabbits with 50 7-day-old cercariae has been shown to yield an average "take" of 18.6 ± 8.8. This average is apparently not influenced by the sex of the rabbit nor by the use of cercariae from different snails (Urquhart, 1954). In the experiments described here the control rabbits showed a take of 18.1 ± 9.4, while the immunized groups showed takes of 12.3 ± 7.1 (group A) and 17.9 ± 9.1 (group B), respectively. The reduction in take in group A as compared to that of the control group is not statistically significant by the 't' test, (Chambers, 1952). Kerr and Petkovitch (1935) reported that it seemed possible to establish an active immunity in rabbits to F. hepatica by injections of dried fluke material. In their experiments 7 immunized rabbits, each infected with 13 cercariae, yielded 1, 2, 2, 2, 2, 3, and 3 flukes respectively, at autopsy. Several of these flukes were described as calcified, and repeated faecal examinations failed to demonstrate the presence of fluke eggs. Three control rabbits (also infected with 13 cercariae) had 11, 5 and 9 flukes at autopsy. None of these flukes was calcified and eggs were found in the faeces of all the rabbits. It is difficult to conclude from this experiment how significant is the apparent reduction in take of the immunized group as compared to controls because of the small number of rabbits (3) in the control group, and the variation in take which occurs in normal rabbits. If this reduction is real it might be due to the influence of antigens other than the proteins.

In the present experiment there is no correlation in either the immunized or control rabbits between the number of flukes found in a rabbit and the level of circulating precipitin to the protein antigen, as shown by the quantitative tests. It must be noted that in the precipitin tests the level of antibody to only one protein fraction of the parasite was measured and the results are expressed in terms of the total N. (antibody N. + antigen N.) precipitated.

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

It is interesting that the four livers in which the most retarded flukes were found also showed by far the most severe inflammatory reactions. This may have been due to the prolonged migratory period of the retarded flukes, but on the other hand, there were only three flukes each in two of these livers (Table 3). It is possible that a state of specific hypersensitivity had developed as a result of previous immunization and

was responsible for the exaggerated cellular reaction to the presence of the parasites. Similar phenomena have been observed in the study of natural acquired immunity to oesophagostomiasis in sheep (Fourie, 1936), to N. muris infections in rate (Sarles and Taliaferro, 1936; Taliaferro and Sarles, 1939), to anclystomiasis of mice and ascariasis of guinea pigs (Kerr, 1936; 1938b) and to Cooperia infections in sheep (Andrews, 1939).

Since the present work was completed (Urquhart, Mulligan and Jennings, 1954), other reports have been published. Healy (1955) reported that there was no evidence of resistance to infection when rabbits were immunized with extracts of tissue or regurgitated caecal contents of F. hepatica and challenged with 50 cercariae, although there was some evidence of retardation of development. Shibani, Tozawa, Takahashi and Isoda (1956) reported that in immunized rabbits 56 days after challenge with F. hepatica cercariae, the liver lesions, both macroscopic and microscopic, were much less severe in the rabbits which had been immunized intradermally or subcutaneously than in the control rabbits. The liver lesions of those given vaccine intraperitoneally or by mouth were also less marked than the controls but were more severe than the liver lesions of the intradermally and subcutaneously injected rabbits. However these observations were based on 3 rabbits given 0.2ml intradermally, 1 rabbit 1.0ml subcutaneously, 1 rabbit 1.0ml intraperitoneally of a 20% suspension of dried fluke material, and 1 rabbit given an oral dose of 1.0g of dried fluke material,

while 3 rabbits acted as controls for the experiment.

Ershov (1959) at the International Veterinary Congress at Madrid stated that workers in Moscow using an antigen of F. hepatica containing 60% polysaccharide and 30-40% 'albumin' found that immunization proved less effective than chemical prophylaxis because of the small percentage of the animals protected, the short duration of the immunity and the high cost of the antigen. In tests on 97 lambs only 10 - 25% were found to be immune and the rest showed a lower level of infection which was not sufficient for general application. The duration of immunity was short, as those sheep challenged 45 days after immunization showed no immunity.

SUMMARY.

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

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

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

B. The use of polysaccharide fractions of F. hepatica as immunizing antigen.

Introduction.

The possible role of polysaccharides in the host-parasite relationship was reviewed by Becker (1953), but little work has been done in attempting to stimulate an acquired immunity using parasitic polysaccharide fractions similar to the work reported with the pneumococcal polysaccharides. Campbell (1936a) reported on the protective action of a variety of chemical fractions including a polysaccharide from Taenia taeniaeformis and its larval form; using a purified polysaccharide antigen prepared from the larval form he was able to demonstrate the formation of precipitins and was able to show some immunity to the disease. Campbell (1936b) also isolated a polysaccharide from Ascaris lumbricoides capable of eliciting specific antibodies, but he did not test for the development of immunity.

Methods and Materials.

Polysaccharide antigen.

The polysaccharide antigen was prepared from "freeze-dried" whole flukes by a method similar to that described by Melcher and Campbell (1942) for the separation of a polysaccharide from Trichinella spiralis.

The flukes were removed from the bile ducts of infected sheep livers and freed from bile and other contaminating materials by washing in running tap water.

The superficial water was removed and the flukes placed in Petri dishes and refrigerated at -20°C. They were then dried in the frozen state in a vacuum desiccator with P_2O_5 as desiccant. The dried flukes were then finely ground in a mortar and stored at -20°C until required.

The finely ground material was suspended in 20 vols. of phosphate buffer pH8.0 placed in a boiling water bath and stirred vigorously with a mechanical stirrer. After 30 minutes the mixture was removed, cooled and centrifuged. The residue was washed once with the phosphate buffer and this washing was added to the original extract. The polysaccharide along with other soluble worm material was precipitated by the addition of 5 vols. of chilled 95% ethanol after the addition of sufficient NaCl to bring the concentration to 0.5%. After refrigeration at 0 - 4°C for 4 hours the resulting precipitate was centrifuged off and the supernatant discarded. The residue was resuspended in approximately 20 vols. of acetate buffer pH 4.6 and the insoluble residue centrifuged and discarded. The solution which contained mostly polysaccharide was adjusted to pH 8.0 by the addition of 1.0 N Na_2CO_3 and the polysaccharide precipitated by the addition of NaCl (0.5%) and 2 vols. of 95% ethanol.

Reprecipitation at pH 8.0 and redissolving at pH 4.6 was repeated until there was no insoluble residue obtained when the product was dissolved in the acetate buffer. The polysaccharide was finally precipitated and dried.

Immunization.

The five rabbits in Experiment 1 and the twelve rabbits in Experiment 2 received a total of seven intraperitoneal injections each of 200mg of polysaccharide in 5.0ml saline at weekly intervals.

Infection.

In Experiment 1 the five experimental and five control rabbits, and in Experiment 2 the twelve experimental and nine control rabbits were each infected using the technique described by Urquhart (1954) with 50 cercariae of F. hepatica one week after the last injection of immunized groups.

Results and Discussion.

The experimental and control rabbits were killed 63 days after infection, the livers removed and the flukes collected. The results are recorded in Table 5. In Experiment 2 total nitrogen determinations were carried out on the flukes recovered from both the immunized and control rabbits and the results of these are also shown in Table 5.

In the first experiment it was evident from the results that immunization had reduced the number of flukes which developed from the standard challenge, the immunized group had an average of 5.2 ± 4.3 as compared to 17.6 ± 9.8 flukes in the control rabbits. This was statistically significant by the 't' test ($P > 0.05$).

In the second experiment the immunized rabbits

TABLE 5

The Number and N. Content of Flukes Recovered from Immunized and Control Rabbits.

	Rabbit No.	Number of flukes	Total fluke nitrogen (mg)	Nitrogen per fluke (mg)
Immunized Group Experiment 1	40	3	---	---
	36	3	---	---
	30	1	---	---
	37	11	---	---
	34	9	---	---
	Mean	5.2 ± 4.3		
Control Group Experiment 1	42	33	---	---
	44	13	---	---
	27	15	---	---
	26	7	---	---
	2	20	---	---
	Mean	17.6 ± 9.8		
Immunized Group Experiment 2	42	9	5.8	0.64
	44	20	9.7	0.49
	45	32	26.1	0.82
	46	20	11.0	0.55
	47	6	5.2	0.87
	56	12	9.1	0.76
	57	34	14.8	0.44
	58	26	21.2	0.82
	59	15	14.8	0.99
	61	18	10.9	0.60
	62	21	---	---
	64	18	11.3	0.63
	Mean	19.3 ± 8.4		0.69 ± 0.17
Control Group Experiment 2	6	17	14.5	0.85
	9	20	15.7	0.79
	10	6	5.7	0.94
	16	32	25.4	0.79
	17	31	23.2	0.75
	18	30	30.5	1.02
	19	24	27.2	1.13
	51	27	20.5	0.76
	54	11	7.9	0.72
	Mean	22.0 ± 9.2		0.86 ± 0.14

had an average of 19.3 ± 8.4 as compared to 22.0 ± 9.2 flukes present in the controls which was not significant ($P < 0.9$). The only manifestation of immunity in this experiment was found in the development of the flukes, those in the control rabbits had an average of 0.86 ± 0.14mg nitrogen per fluke while the immunized group had an average of 0.69 ± 0.17mg nitrogen per fluke. This is a highly significant reduction ($P > 0.02$).

The results of these two experiments are difficult to explain but some of the factors which must be considered are (a) the polysaccharide antigens used in the two experiments may not have been absolutely identical although prepared by the same method, (b) the possibility that in the second experiment some degree of immunological paralysis might have been produced similar to that reported by Felton and Ottinger (1942); Felton, Kauffmann, Prescott and Ottinger (1955); Felton, Prescott, Kauffmann and Ottinger (1955). These workers found that relatively small amounts of polysaccharide antigens could produce a state of immunological paralysis or unresponsiveness which they attributed to the relative slowness with which such compounds are metabolised in the body.

Finally, one might be hesitant in arriving at too firm a conclusion in the first experiment where the number of animals is relatively small.

Conclusions.

In view of the conflicting results obtained in

the two experiments one cannot draw any definite conclusion about the use of a polysaccharide antigen from F. hepatica. The indication from experiment 1 is that it might be successfully used as an immunizing antigen, but further work is indicated using a standardized polysaccharide preparation and also reducing the quantities used to avoid the possibility of immunological paralysis.

Since this work was completed Ershov (1959) using an antigen which consisted of a 'polysaccharide-albumin' complex containing approximately 60% polysaccharide and 30-40% 'albumin' was able to successfully immunize 10-20% of a total of 97 sheep against F. hepatica. With similarly prepared antigens he was able to get 80% protection against ascaridosis in pigs, 28% protection against ascaridosis in chickens and 60% protection against monieziosis in lambs. In the case of immunization against F. hepatica the immunity engendered was of short duration, those sheep infected 45 days after immunization showing no immunity. In view of the results obtained by these Russian workers it is unlikely that immunization with polysaccharide fractions will be of practical application.

Summary.

1. Two experiments are described using a polysaccharide fraction prepared from the adult Fasciola hepatica.

2. The results in experiment 1 indicated that this treatment reduced the number of F. hepatica developing

from a challenging infection.

3. In experiment 2 immunization with polysaccharide resulted in a retardation of development as measured by protein N. of the flukes but there was no reduction in the number of F. hepatica developing from a challenge infection of 50 cercariae.

Section II.

Studies on the anaemia produced in rabbits by *Fasciola hepatica* infections.

INTRODUCTION.

Each of the three standard textbooks on Veterinary Helminthology makes reference to the fact that a degree of anaemia is constantly associated with chronic fascioliasis in sheep and cattle (Cameron, 1951; Monnig, 1950; Lapage, 1956). Regarding the cause of the anaemia Cameron, 1951, states that "the flukes feed on blood and bile but any loss from this source is generally replaced by the host.... the toxin secreted by the fluke causing a more or less profound anaemia". This reference to haemolytic toxin being responsible for the anaemia is repeated in all of these textbooks and although none of the authors cites any references in support of this statement, it is probably derived from the conclusions of Marcone (1940) and of Balian (1940). The former claimed that normal red cells were haemolysed by the sera from fluke infected sheep, while the latter, apparently by analogy with some human haemolytic anaemias concluded that a haemolytic factor was present.

The nutrition of F. hepatica has an important bearing on the etiology of the anaemia and it has been the subject of some controversy. It was first believed that flukes lived on bile; but Sommer (1880) concluded after a histological study that they lived on erythrocytes and bile duct epithelium. Railliet (1890), after injecting a plaster containing a blue dye into infected sheep, was able to recover both plaster and dye from the caeca of the flukes, but was unable to recover

the dye from the bile. From this he concluded that the parasite lived on blood. This work of Railliet's received criticism from Müller (1923) who pointed out that the dye might have been present in the bile as a colourless leucobase, whose colour re-appeared when the bile was taken into the fluke intestine. It is fairly reasonable to assume that the dye may have reached the fluke in this manner, but it is difficult to believe that the route of excretion of the plaster would also be via the bile. Müller concluded from his investigations that the parasites lived on the mucus content of the bile, the desquamated biliary epithelium and on leucocytes. Weinland and Von Brand (1926) demonstrated that flukes can feed on blood clots in vitro, and Hsü (1939) on histological examination found erythrocytes and leucocytes in the intestine of the flukes in practically the same proportion as in normal blood, also present was a small amount of bile-duct epithelium. The number of flukes examined by Hsü was only ten from a single host and therefore too much emphasis should not be placed on these findings. Stephenson (1947) as a result of a histological and spectroscopical examination of the intestinal contents of the flukes came to the conclusion that the adults lived mainly if not exclusively on the blood, and further experimental evidence that blood constitutes the main source of food for F. hepatica was given by Van Grembergen (1950). In a detailed examination of flukes and the fluke intestinal contents using histological, spectroscopical, enzymic and chemical techniques he came to the conclusion that

adult F. hepatica (a) possess a naturally occurring pigment similar to haemoglobin, (b) ingest blood from the host and (c) also ingest hepatic cells at the same time as the blood.

More recently Urquhart (1955) as a result of haematological observations on experimentally infected rabbits concluded that the anaemia was similar in all essential respects to that produced in rabbits by the repeated removal of blood. He was also able to consistently demonstrate the presence of blood in the contents of the bile ducts and in the caeca of the flukes.

It would appear that final proof of the haemorrhagic nature of the anaemia depends upon the demonstration that the amount of blood loss by the host, due to the flukes in the bile ducts is of sufficient magnitude to produce the degree of anaemia observed.

This section of the thesis describes attempts to measure quantitatively by the use of isotopically labelled blood the amount of blood lost by rabbits infected with F. hepatica. These experiments were carried out initially using ^{32}P-labelled red cells and ^{131}I-labelled serum albumin used separately. In later experiments labelled red cells and labelled serum albumin were used together. In addition preliminary experiments are reported on the use of ^{51}Cr-labelled red cells.

Methods and Materials

^{32}P-Labelled Red Cells.

These were prepared essentially as described by Hevesy, Koster, Sorenson, Warburg and Zerahn (1944). Freshly drawn heparinized rabbit blood (5 ml) was treated with 0.10 ml of radioactive phosphate solution in 0.9% NaCl containing about 100 μc ^{32}P and a minute amount of carrier phosphate. After incubation at 37°C for 2 hours with frequent mixing, the labelled cells were centrifuged and washed four times with 10 volumes each time of ice-cold isotonic saline. Injection of the labelled cells was carried out within a few minutes of the final washing.

^{131}I-Labelled Serum Albumin.

An 'albumin' solution was prepared from normal rabbit serum by the addition of anhydrous sodium sulphate to a final concentration of 18%. After centrifuging down the precipitated globulins the supernatant solution (mainly composed of albumin) was transferred to a dialysis sack and dialysed against repeated large volumes of 0.85% NaCl until no trace of sulphate could be detected in the dialysate with $BaCl_2$.

This albumin was trace labelled with ^{131}I essentially as described by Francis, Mulligan and Wormall (1951), i.e. the protein in ammoniacal solution was treated with a solution of I_2 in KI to which had been added Na ^{131}I. It is necessary in this reaction to keep the amount of KI in the iodinating mixture to a minimum and this is

done by saturating a solution of KI of appropriate concentration with elementary iodine and filtering off the excess iodine. A measured amount of this I_2 in KI is treated with the Na ^{131}I before use. The amounts of I_2 in KI and Na ^{131}I required to give a labelled protein of a certain iodine content and specific radioactivity can be calculated since in this iodination reaction about $^1/_3$ of the free I_2 and $^1/_6$ of the ^{131}I becomes attached to the protein. The method of labelling the albumin solution is outlined below:-

Na ^{131}I + I_2 in KI — Alb. in ammoniacal soln.
1.8mg free I_2/100mg protein — 0.1 ml 5N NH_4OH/ml prot. soln.

↓

Mix slowly with constant stirring

↓

Trace iodinated albumin (containing ^{131}I)
plus unbound ^{131}I as iodide.

↓

Neutralise with acetic acid (5N & 2N).

↓

Removal of unbound ^{131}I by dialysis.

↓

Trace iodinated albumin free
from unbound activity containing
approximately 0.6% iodine.

The labelling of proteins to contain about 0.6% iodine has been shown to cause little or no change in the physico-chemical properties of the proteins (Francis, Mulligan and Wormall, 1955).

Kjeldahl Analyses.

In some of the experiments Kjeldahl analyses on the flukes were carried out to correct for differences in the sizes of the parasites thus obtaining a more standard reference in calculating blood loss.

Radioactivity Determinations.

All determinations were made using the Veall (1948) type liquid counter. Each sample was counted for a sufficient time to give at least 2,500 counts. Two such determinations were carried out on each sample and where the duplicate counts were not in good agreement, a third determination was made.

Determination of ^{131}I and ^{32}P on the Same Sample*

In the experiment where ^{131}I-labelled serum albumin and ^{32}P-labelled red cells were injected simultaneously, ^{131}I and ^{32}P activities were determined as follows. The total radioactivity (^{131}I + ^{32}P) of each sample was first determined; the ^{131}I was then removed by sulphuric acid digestion and the ^{32}P activity in the digest measured. The ^{131}I activity was calculated by difference.

* The determination of ^{131}I and ^{32}P on the same sample is much more easily done by a combination of scintillation counting and Geiger-Muller counting. The scintillation counter gives mainly ^{131}I while the M6 liquid counter will give activity due to ^{32}P and ^{131}I. At the time these experiments were carried out no scintillation counting equipment was available.

As a check on the validity of this method the following two experiments were carried out:-

1. Efficiency of the removal of ^{131}I.

Samples (1 ml) of normal red cell suspensions were mixed with ^{131}I-labelled serum albumin solution (1, 2 and 3 ml) in micro-Kjeldahl incineration flasks. Concentrated H_2SO_4 (4 ml) was added followed by a few milligrams of catalyst and about 0.2 gm NaI to act as carrier. The flasks were then boiled vigorously until the organic matter had disappeared and the iodine had sublimed off. The neck of each flask was heated to ensure that all the iodine was expelled. The flasks were cooled, the contents diluted with a few millilitres of water, more carrier iodide added and digestion continued as before until all the iodine had sublimed off. The flasks were again cooled and this process repeated. The contents of each flask were then transferred quantitatively to a volumetric flask, made up to 10 ml with distilled water and counted in the M6 liquid counter. The results of this experiment are in Table 6. It is evident from the results that almost complete removal of the ^{131}I can be effected by this method.

TABLE 6

Removal of ^{131}I by Digestion.

^{131}I labelled serum albumin added (c/min)	^{131}I remaining after digestion (c/min)
1,758	3.4
1,758	0
1,758	0.2
3,516	0
3,516	0
3,516	0
5,274	1.9
5,274	0
5,274	2.9

2. Recovery of ^{32}P.

To determine if ^{32}P could be recovered completely after the above digestion, varying amounts of labelled red cells of known radioactivity were added to samples of non-radioactive iodine-labelled serum albumin and the mixture incinerated as for the removal of ^{131}I. The digests were diluted to 10 ml and radioactivity determinations carried out as before. The results are shown in Table 7. The recovery of ^{32}P in all cases was within the limits of experimental error.

TABLE 7

Recovery of ^{32}P

^{32}P Activity present (c/min)	^{32}P Activity found after digestion (c/min)	Recovery
1402	1360	97.0
1655	1652	99.9
2152	2134	99.2
3271	3324	101.6
6429	6411	99.7
8409	8438	100.3

3. Self-Absorption of the Radiations from ^{131}I.

In ^{131}I determinations on homogenates the possibility of self-absorption of the relatively soft β-radiations must be considered. An experiment was therefore carried out in which a standard amount of ^{131}I-labelled serum albumin was added to 10 ml samples of fluke homogenates of increasing concentration as determined by Kjeldahl analysis. The radioactivities of these samples are detailed in Table 8. In most of our experiments the concentrations of the fluke homogenates used were within the range shown in Table 8 and therefore corrections were unnecessary.

TABLE 8

Radioactivity of a Standard Amount of ^{131}I-labelled Serum Albumin Added to Increasing Concentration of Fluke Homogenate.

Fluke homogenate (mg N)	Radioactivity (c/min)
0	1758
3.8	1776
7.6	1743
11.4	1744
19.0	1771

Infection of Rabbits.

Each of the experimental rabbits was infected with 50 7-day-old cercariae encysted on Cellophane and fed in cabbage as described by Urquhart (1954). The isotope experiments were carried out at least 9 weeks after infection.

Results.

Experiments with ^{32}P-Labelled Red Cells.

Red cells from fluke infected and normal rabbits were labelled with ^{32}P as described. The labelled cells were then suspended in an equal volume of 0.9% NaCl and injected into the marginal vein of the left ear. Each rabbit was injected with its own red cells. Blood samples (5ml) from the marginal vein of the right ear were collected in heparinized bottles, 10 minutes and

1 hour after injection. Duplicate 0.20 ml samples of each bleeding were made up to 10 ml for counting. Each rabbit was killed immediately after the withdrawal of the second blood sample, the abdomen was opened, the common bile duct ligated at its junction with the duodenum, and the liver removed and washed free from superficial blood. The surfaces of the common bile duct and gall bladder were seared with a hot spatula to prevent haemorrhage, a Pasteur pipette was inserted through the seared area and the bile withdrawn and transferred to a 10 ml graduated cylinder and made up to volume for counting.

The large extrahepatic bile ducts which usually contained most of the parasites were carefully opened, the flukes removed, rinsed well in 0.9% NaCl and transferred to a Kjeldahl incineration flask. The lobes of the liver were carefully dissected and any flukes in the intrahepatic bile ducts were collected, rinsed and added to the incineration flask. After digestion was completed the digest was quantitatively transferred to a volumetric flask and made up to 10 ml for counting. In some of the experiments aliquots of this digest, after the radioactivity determinations had been carried out, were analyzed for nitrogen by the micro-Kjeldahl procedure.

Radioactivity determinations were carried out on the blood samples, bile and fluke digests and the results of these determinations are shown in Table 9. In all cases the radioactivity of the flukes greatly exceeded

TABLE 9

Radioactivity of Flukes, Blood and Bile from Infected Rabbits, and Blood and Bile from Control Rabbits.

	Rabbit No.	Method	60 min blood (0.2ml)	Activity c/min Bile[a] (total)	Activity c/min Fluke[b] (total)
INFECTED	1	^{32}P cells	3388	78 (1.2)	474 (6)
	2	"	2448	50 (0.8)	2311 (25)
	3	"	2425	49 (1.1)	1293 (18)
	4	"	1985	56 (0.8)	1994 (25)
	5	"	2509	157 (1.15)	3985 (34)
	15	"	1559	56 (1.15)	1698 (13)
	16	"	2054	38 (3.0)	732 (20)
CONTROL	Y	"	3240	15 (0.5)	
	071	"	1064	67 (0.7)	
	G3	"	1893	8 (0.7)	
	24	"	4870	9 (0.3)	
	26	"	2899	17 (0.4)	
	27	"	4069	23 (0.5)	
	33	"	5577	10 (0.2)	
	37	"	5526	6 (0.1)	

[a] Values in parenthesis are volumes of bile (ml).

[b] Values in parenthesis are numbers of flukes.

that of the corresponding bile. The quantity of 'blood' in the flukes from each rabbit was calculated as follows:-

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

This figure for the amount of blood in the flukes was multiplied by 24 to obtain an approximate measure of the blood loss per day. The results of this experiment are shown in Table 10.

The radioactivities of the 10 minute blood samples when compared with those of the 60 minute blood samples showed that no abnormal loss of the label from the red cells occurred during the experimental period.

Experiments with ^{131}I Trace-Labelled Serum Albumin.

^{131}I-labelled serum albumin (1 ml) was injected into the marginal vein of the left ear of each of a number of normal and infected rabbits. Blood samples from the opposite ear were taken at 10 minutes and again immediately before the rabbits were sacrificed at 1 hour. Bile was collected and counted as before. The flukes were macerated in a Griffith's tube and the homogenate transferred to a 10-ml graduated cylinder and made up to volume for counting. The blood samples were diluted 0.20 ml to 10 ml for counting, and the amount of 'blood' in the flukes calculated from the

TABLE 10

Calculated Blood Loss of Infected Rabbits.

Method	Rabbit No.	No. of flukes	Total blood loss in 24 hr. calculated from ^{32}P (ml)	Total blood loss in 24 hr. calculated from ^{131}I (ml)	Blood loss per mg. Fluke N in 24 hours calculated from ^{32}P (ml)	Blood loss per mg. Fluke N in 24 hours calculated from ^{131}I (ml)
^{32}P labelled red cells	1	6	0.67	-	0.16	-
	2	25	4.80	-	0.15	-
	3	18	2.60	-	0.11	-
	4	25	4.82	-	0.17	-
	5	34	7.60	-	0.20	-
	15	13	7.70	-	-	-
	16	20	4.20	-	-	-
	17	7	3.30	-	-	-
^{131}I labelled serum albumin	18	32	-	3.86	-	-
	19	20	-	2.10	-	-
	20	20	-	2.13	-	-
^{32}P labelled red cells + ^{131}I labelled serum albumin	6	30	5.70	3.08	0.18	0.10
	7	27	2.40	1.29	0.12	0.06
	8	31	3.01	1.65	0.13	0.07
	9	17	3.41	1.77	0.24	0.12
	10	11	1.80	1.19	0.23	0.15
	11	6	0.87	0.49	0.15	0.09
	12	24	6.32	4.54	0.23	0.17
	13	20	2.27	1.18	0.14	0.08
	14	32	3.61	1.97	0.14	0.08

radioactivities of the fluke homogenates and the corresponding 60 minute blood samples. The results are also shown in Table 10.

From Table 10 there appears to be a difference in the daily blood loss as determined by the two different isotopic methods, the ^{32}P method giving consistently higher results. This is most apparent where both methods have been used simultaneously.

There is a correlation ($P = 0.05$, Chambers, 1952) between the estimated blood loss in 24 hours determined by ^{32}P measurements and the number of flukes present. A much closer correlation ($P < 0.001$) is obtained if estimated daily blood loss is correlated with the total amount of fluke nitrogen present in each rabbit, which takes into account differences in size of the parasites.

Experiments with ^{32}P-Labelled Red Cells and ^{131}I-Labelled Serum Albumin Injected Simultaneously.

In some experiments ^{32}P-labelled cells and ^{131}I-labelled albumin were injected simultaneously into each rabbit. It was therefore possible to calculate blood loss by the two methods (see Table 10) and also to compare the $^{32}P/^{131}I$ ratios of the flukes with that of the corresponding blood. If the flukes are ingesting whole blood then the $^{32}P/^{131}I$ ratios of the flukes should theoretically be the same as that found in the blood.

In these experiments the activity of one of the isotopes was determined by difference between total

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

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

A sample (2.0 ml) of labelled cell suspension from each rabbit and 2.0 ml ^{131}I-labelled albumin solution were mixed thoroughly in a small beaker. 2.0 ml of the mixture was injected into the marginal vein of the left ear. Blood samples (6.0 ml) were collected in heparinized bottles from the right ear at 10 minutes and immediately before killing the animal at 60 minutes. Flukes and bile were removed as before. The flukes from each animal were homogenized in a Griffith's tube and made up to 10 ml. The total bile and duplicate blood samples (1.0 ml) from the 10 minute and 60 minute bleedings were also diluted to 10 ml. Total radioactivity (^{32}P + ^{131}I) was determined on the fluke homogenate, bile and blood samples.

Triplicate blood samples (1.0 ml) from each bleeding, duplicate aliquots (4.0 ml) of the fluke homogenate, and the total bile were freed from ^{131}I by digestion as previously described. The digests were each made up to 10 ml for counting and the ^{32}P activity determined. ^{131}I activity was calculated by difference. It was thus possible to compare the $^{32}P/^{131}I$ ratio in the blood with that in the flukes (Table 11). Some difficulty was encountered in the digestion of the bile samples (losses occurred due to foaming and spurting) and the results are therefore not included in Table 11.

It can be seen from Table 11 that the $^{32}P/^{131}I$ ratios in the flukes are in all cases greater than those in the 60 minute blood sample. If these ratios had been equal it might have been interpreted as due to the ingestion by the flukes of red cells and plasma in the same proportions as found in the whole blood. The $^{32}P/^{131}I$ ratios in the blood at 10 minutes and 60 minutes show that no marked variation in this ratio took place during the experimental period. When the $^{32}P/^{131}I$ ratios in the flukes are divided by the corresponding figure for the 60 minute blood sample a fairly constant value is obtained (1.77 ± 0.17).

TABLE 11

$^{32}P/^{131}I$ Ratios in Flukes and Blood.

Rabbit No.	$^{32}P/^{131}I$ Ratio			$(^{32}P/^{131}I)$ Flukes / $(^{32}P/^{131}I)$ 60 min blood
	10 min blood	60 min blood	Flukes	
6	0.95	0.91	1.68	1.85
7	0.86	0.82	1.52	1.87
8	1.47	1.61	2.95	1.83
9	0.93	0.96	1.84	1.92
10	1.00	1.04	1.58	1.52
11	0.93	0.92	1.62	1.76
12	1.54	1.56	2.17	1.39
13	1.83	1.81	3.47	1.92
14	1.65	1.85	3.39	1.83
				Mean = 1.77 ± 0.17

Discussion.

In the experiments with ^{32}P-labelled red cells, it has been calculated that the blood loss is approximately 0.2 ml per day per fluke and in the rabbits where ^{131}I-labelled serum albumin was used, about half this amount. Using the mean ^{32}P figure for blood loss, a rabbit having 30 flukes in its bile ducts would suffer a blood loss of about 6.0 ml per day. That this amount of daily blood loss is sufficient to produce a marked anaemia is substantiated by the work of Oberg, (1949)

and of Steele(1933) who removed from rabbits a quantity of blood equivalent to 3-6 ml per day over a period of several months, with the production of a degree of anaemia of similar order to that found in fluke-infected rabbits (Urquhart, 1955).

An interesting result was obtained in the experiments where ^{32}P-labelled cells and ^{131}I-labelled albumin were used simultaneously. The $^{32}P/^{131}I$ ratio in the flukes was consistently higher than the corresponding ratio in the blood (see Table 11). This might indicate a preferential uptake and/or retention of red cells relative to plasma. A finding which would support either hypothesis is that in the three rabbits, 6, 8, and 11, where it was possible to obtain analyses for both isotopes in the bile, the $^{32}P/^{131}I$ ratios in the bile were 0.39, 0.40 and 0.14 respectively, i.e. much smaller than the corresponding bloods and flukes. Stephenson (1947) found flukes placed on clotted sheep's blood contained in saline, the blood was removed from the clot and concentrated in the fluke by removal of fluid.

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

products. Normal bile contains phosphate and it is also recognized that the bile is one of the pathways for the excretion of iodine. It is to be expected therefore that after the injection of ^{32}P labelled cells or ^{131}I labelled serum albumin, some radioactivity will appear in the bile. It might be suggested therefore that the radioactivity of the flukes in these experiments was due to the uptake by the parasites not of blood but of labelled phosphorous and iodine compounds present in the bile. It is not thought that this is the case because of the large quantitative differences between the radioactivity of the flukes and that of the corresponding bile (Table 9). It is felt however, that further experiments using different labelling techniques for red cells and plasma proteins are indicated. Since some loss of label from the cells and a similar loss due to catabolism of the plasma proteins are inevitable, it would be of great value to work with red cells containing the label in different types of cellular constituents (^{32}P in organic P compounds, radio-iron in the haemoglobin); and likewise plasma proteins labelled at different points in the molecule (^{131}I attached to tyrosine residues, ^{35}S in sulphur containing amino acids).

It is considered that the isotopic approach to this type of problem is a valuable one and might be applied in the assessment of blood consumption by hookworms and similar blood-sucking parasites.

Summary.

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

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

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

Preliminary Experiments using ^{51}Cr-labelled red cells.

As an extension of the previous experiments, the use of ^{51}Cr-labelled red cells was considered, as this label is much more stable than either ^{131}I-labelled albumin or ^{32}P-labelled cells. Chromium-51 has been used both in blood volume and red cell survival studies (e.g. Sterling and Gray, 1950; Necheles, Weinstein and Le Roy, 1953; Weinstein and Le Roy, 1953; Small and Verloop, 1956). Chromium-51 labelled cells have also been used in the detection of gastro-intestinal haemorrhages (Owen, Bollman and Grindlay, 1954; Matsumoto and Grossman, 1959). This background information indicated that it might be possible to estimate the blood loss in a fluke infected animal by measurement of ^{51}Cr excreted via the faeces over a long period and enable one to obtain a more accurate figure for the daily blood loss.

As a preliminary it was necessary to find out if there was any appreciable absorption of ^{51}Cr from the alimentary canal. Chromium oxide has been used extensively as a marker in digestibility trials, but in the experiment envisaged the chemical state of the chromium, after combination with the red cells, and having passed through the digestive tract of the liver fluke and finally passing out with the bile, is unknown. In an attempt to elucidate the situation which might exist in the fluke infected animal the following experiments were carried out:-

(1) Oral administration of ^{51}Cr-labelled red cells.
(2) Oral administration of sodium $^{51}Chromate$.
(3) Intravenous injection of haemolysed ^{51}Cr-labelled red cells.

Experiment 3 was performed in order to find out the pathway of excretion of the ^{51}Cr released by the normal metabolic breakdown of the labelled red cells and/or the influence of a haemolytic toxin.

Methods and Materials.

Approximately 30 ml heparinized whole blood was incubated at room temperature with 1800 µc. ^{51}Cr for 2 hours.

The labelled blood was then washed three times with 0.85 NaCl and finally made up to approximately 30 ml with normal saline.

The rabbits were normal adults, and were placed in metabolism cages two days before the experiments were started.

Experiment 1. Two rabbits V312 and V243 were given by stomach tube 5.0 ml of the labelled cell suspension. A standard was prepared by diluting 2.0 ml of the preparation to 1 litre.

Experiment 2. Two rabbits V298 and V292 were given by stomach 5 ml of a solution of normal saline containing approximately 225 µc. sodium ^{51}Chromate. A standard was prepared by diluting 2 ml of this solution to 1 litre as before.

Experiment 3. The remaining ^{51}Cr-labelled red cells from Experiment 1 were haemolysed completely by freezing and thawing and then 5 ml of the haemolysed solution was injected intravenously into the marginal ear vein of two rabbits V297 and V306. A standard was prepared using

2 ml of haemolysed cells to 1 litre.

In each case faeces and urine were collected at least twice daily and bulked into 24 hour samples. The volumes of urine were noted and 10 ml samples taken for radioactivity determinations in a scintillation counter.

The faeces for each day were homogenised in a macerator adding sufficient water to make the homogenate a fluid consistency. The volumes were noted and 10 ml aliquots taken for counting.

The results of these experiments are shown in Table 12.

TABLE 12

Rabbit	Treatment	%age of administered dose recovered		
		urine	faeces	
V312	^{51}Cr labelled red cells by stomach tube.	0.4%	98.4%	106 hrs.
V243		0.2%	98.5%	
V298	$Na^{51}CrO_4$ soln. by a stomach tube.	0.1%	97.7%	106 hrs.
V292		0.1%	92.5%	
V297	haemolysed ^{51}Cr labelled red cells I.V.	61.1%	6.2%	240 hrs.
V306		48.3%	9.6%	

It is clear from these results that ^{51}Cr either in the form of chromate or bound to red cells will not be absorbed from the alimentary canal in appreciable

quantities. The small amount recorded in the urine may be due to a large extent on faeces contamination. Conversely the activities recorded in the faeces of the two rabbits V297 and V306 which received the haemolysed labelled cells may be due to urine contamination.

From these preliminary experiments one can postulate that if intravascular haemolysis occurs then the majority of the activity will appear in the urine although the possibility of part of this released chromium-51 recombining with other red cells cannot be completely ruled out. If, however, the anaemia is haemorrhagic and the flukes consume blood then the chromium-51 should be excreted in the faeces.

The Use of ^{51}Cr-labelled red cells in the estimation of blood loss by F. hepatica (Pilot Experiment).

Methods and Materials.

Three adult rabbits T503, V238 and V283 were placed in metabolism cages. Rabbit T503 had been infected with 50 cercariae of F. hepatica approximately 12 months previously, while the other two rabbits were normal and acted as controls for the experiment.

Approximately 10 ml of blood from each rabbit was collected into bottles containing the requisite quantity of heparin. After the addition of approximately 250 μc ^{51}Cr as sodium chromate to each sample, the bloods were allowed to stand at room temperature, with frequent mixing, for two hours. The labelled red cells were then

washed three times with approximately 10 ml saline each time, and finally made up to a volume of approximately 10 ml with saline for injection. 5.0 ml of homologous labelled red cells were injected into the marginal ear vein of each of the three rabbits, and in each case a standard was prepared by diluting 2 ml of the labelled cells to 1 litre.

Heparinised blood samples (2.0 ml) were withdrawn from each rabbit at 10 minutes, and at intervals after injection over a period of 16 days. The radioactivities per ml of whole blood were determined on the scintillation counter and these activities expressed as a percentage of the 10 minutes sample and plotted on semi-log paper (Fig.8). From this graph it was possible to calculate the half life of the cells.

Faeces and urine were collected at least twice a day, and bulked into 24 hour samples. The urines were made up to a suitable volume and 10 ml aliquots were used for radioactivity determinations. The faeces were homogenised in a macerator with the addition of sufficient water to obtain fluid consistency, after making up to suitable volume, duplicate 10 ml aliquots were taken for radioactivity determinations and where these determinations failed to agree at least one more sample was taken and radioactivity determined.

At the end of the experiment each rabbit was autopsied, the alimentary canal removed, and radioactivity measurements made on the contents. In the case of the fluke infected rabbit, the liver was removed, after ligation of the common bile duct, the flukes were removed, rinsed in

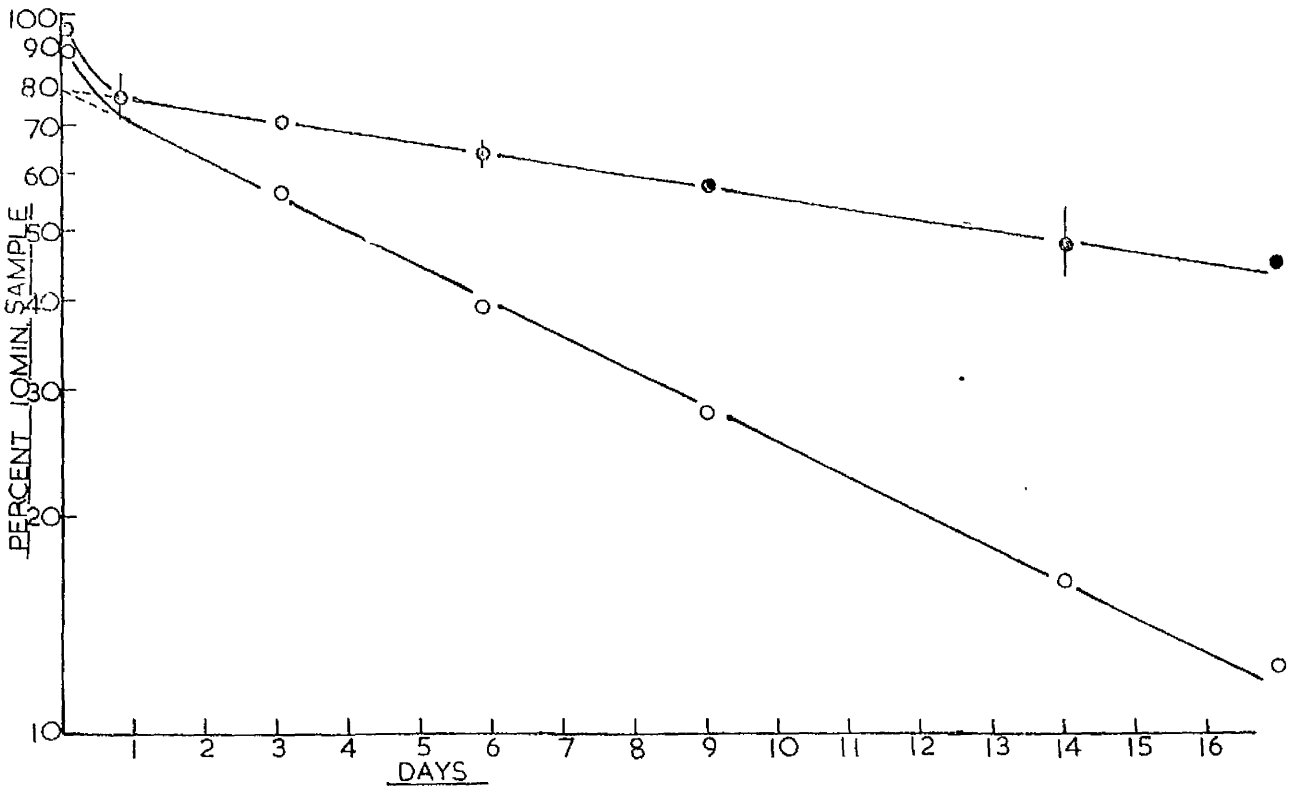


Fig. 8 Levels of chromium-51 activity expressed as a percentage of the 10 min. sample in fluke infected and normal rabbits. (Blood ^{51}Cr).
●——● control rabbits
o——o infected rabbit.

saline and homogenised and made up to 10 ml for radioactivity determinations.

The results in Table 13 show the percentage of the injected radioactivity which was recovered in the faeces of the fluke infected rabbit (62%) and that in normal rabbits (3.5% and 3.2%) during the period of the experiment. The 20 flukes recovered from the infected rabbit contained, if calculated from the activity of the circulating blood at the time of killing, the equivalent of 1.15 ml of blood, i.e. 0.06 ml/fluke. This is much higher than the amount present in the flukes in the previous experiments using ^{32}P and sacrificing the rabbit after 1 hour (approximately 0.01 ml/fluke). Some problems arise in converting the activity found in the faeces to volumes of blood because of the difficulty of correlating the activity of the blood with that in the faeces, and it is complicated by the rabbit's natural practice of eating its "night faeces" and therefore some of the activity may have traversed the alimentary canal more than once. Owen, Bollman and Grindlay (1954) in their investigations on the use of the method in the detection of intestinal haemorrhage, using the dog as an experimental animal, removed a volume of labelled blood from the circulation and administered it by stomach tube. The amount of radioactivity recovered in the faeces was converted to ml blood by taking the average blood level of activity during the experiment. In their case, however, the loss of activity from the blood was relatively small (corresponded to that found in the normal rabbit) and therefore the possible error was reduced. In the infected rabbit the blood activity fell during the experimental

TABLE 13

Chromium-51 activities recovered in the faeces and urine of the fluke infected and two normal rabbits after the intravenous administration of homologous labelled red cells.

Day	Rabbit T503 (Infected)		Rabbit V238 (Normal)		Rabbit V283 (Normal)	
	Urine c x 10^3 per min.	Faeces c x 10^3 per min.	Urine c x 10^3 per min.	Faeces c x 10^3 per min.	Urine c x 10^3 per min.	Faeces c x 10^3 per min.
1	81.6	17.7	46.7	0.8	51.9	3.1
2	45.8	97.6	71.6	3.7	50.6	1.5
3	32.5	136.3	36.6	1.9	45.6	4.7
4	42.6	155.3	66.4	5.6	62.7	6.3
5	21.3	99.2	39.9	4.4	36.4	3.7
6	26.4	81.9	21.9	2.4	15.4	4.4
7	23.0	99.6	44.1	3.9	28.3	3.8
8	22.3	83.8	31.7	8.6	29.4	3.0
9	15.2	101.5	29.1	1.2	31.3	3.7
10	16.0	80.5	39.0	0.3	41.1	4.8
11	13.5	61.9	45.6	12.8	44.3	4.5
12	15.2	70.1	40.5	8.9	10.5	2.5
13	12.3	62.6	12.8	2.3	13.2	0.6
14	12.0	64.2	28.0	4.4	31.7	2.5
15	9.7	47.6	16.6	2.5	25.5	2.2
16	10.3	49.0	33.1	5.8	27.3	2.2
17	6.5	37.9	28.6	5.4	24.8	2.6
18	9.9	26.3	31.0	7.2	30.1	4.7
Alimentary Canal		41.1		11.0		14.3
Total	416.1	1,414.1	663.2	93.1	600.1	74.8
	18.3%	62.0%	24.2%	3.5%	25.7%	3.2%
	80.3%		27.7%		28.9%	

period to below 12% of the value at the beginning, and calculation of blood loss based on the average blood activity during the whole experiment gave a total loss of 298 ml i.e. 15.7 ml/day which is equivalent to 11.5% of its total blood volume per day. Using the same basis for the calculation the normal rabbits V238 and V283 lost a total of 6.9 and 5.1 ml respectively during the experimental period. If this is subtracted from the amount lost by the infected rabbit to find the blood loss due to the flukes, the total volume is still 292 ml or 15.4 ml per day which is still high when compared to our estimate based on ^{32}P and ^{131}I experiments.

Considering the rate of removal of the labelled red cells from the circulation (see Fig.8) and making two assumptions (1) that the blood volume of the rabbit remains constant during the experiment and (2) that the rate of blood loss is constant then one can calculate the volume of blood lost from the half life of the labelled cells using the following equation:-

$$(1 - \frac{x}{k})^n = \frac{1}{2}$$

Where x = ml of blood loss per unit time (hours)
k = blood volume of the rabbit
n = number of units time (hours) in which half the activity has disappeared from the circulation, i.e. half life value.

k was calculated from the total c/min injected and the c/min/ml of the 10 min. sample and in the case of the infected rabbit was 137 ml:n was calculated from Fig.8. In the case of the infected rabbit n = 145 hrs.

and the normal rabbits n = 425 hrs. Substitution of these values gives in the infected rabbit a blood loss of 0.67 ml/hr. and in the normal rabbits a loss of 0.22 ml/hr. or a loss due to the flukes of 0.45 ml/hr., i.e. 10.8 ml/day or 0.54 ml/fluke/day. This figure is of the same general order as the previous one based on average blood activity.

An examination of the amount of radioactivity excreted in the urine indicated that the infected rabbit was similar to the two controls, 18.3% as compared to 24.2% and 25.7% and this is more heavily weighted in favour of the infected rabbit if one considers that in this case the urine is much more likely to have been significantly contaminated by radioactive faeces due to the method of collection. Qualitatively the results of this experiment are in agreement with the theory that the anaemia is not due to intravascular haemolysis, but to a loss of blood via the bile to the gut.

Although it would be imprudent to place too much emphasis on the results obtained from one fluke infected rabbit, it does seem that a measurement of blood loss based on ^{51}Cr-labelled red cells is higher than the estimate obtained when ^{32}P-labelled cells and ^{131}I-labelled albumin are used. This may be due to fundamental differences between the two methods used. In the ^{32}P and ^{131}I experiments the rabbits were killed one hour after administration of the labelled materials. This timing is purely arbitrary and an assessment of blood loss assumes that the flukes consume the blood at a constant rate. Also a direct estimation of the

radioactivity in the flukes is made which will not include any concomitant haemorrhage directly into the bile while the flukes are feeding nor any bleeding caused by the spines on the dorsal side of the mature parasite. In addition this direct estimation does not allow for any excretion by the flukes during the experimental period which certainly is indicated in the combined ^{32}P, ^{131}I experiment. The experiment using chromium-51 allows one to make an estimate of the total blood loss by the host over a long period due to both the blood-sucking of the parasites and any concurrent haemorrhage. In view of these factors it is not unreasonable to assume that any estimate of blood loss based on chromium-51 might be in excess of that based on short term direct estimates of ^{32}P or ^{131}I in the flukes.

It is intended to extend these experiments using chromium-51 labelled red cells as soon as infected rabbits are available.

Summary.

1. An attempt was made to estimate quantitatively the blood loss in a rabbit infected with F. hepatica using ^{51}Cr.

2. The blood loss was calculated from the activity in the faeces and also from the half life value of the circulating red cells.

3. The estimated blood loss based on ^{51}Cr-labelled red cells was higher than that obtained when either ^{32}P-labelled cells or ^{131}I-labelled serum albumin was used.

Section III

Studies on *Dictyocaulus viviparus* infection in the bovine.

A. The immunity resulting from natural infection.

B. The immunity resulting from passive immunization.

C. The immunity resulting from artificial immunization with whole worm antigen.

General Introduction.

Parasitic bronchitis, caused by the parasite Dictyocaulus viviparus, is of considerable economic importance in Great Britain and in many other countries. Although many drugs have been tested (e.g. Florent, 1951; Sellars and Taylor, 1951; Thomsen, 1953) before the work reported in this thesis was done, none had appeared to be of any great value either as a therapeutic or a prophylactic agent.

The life cycle of the parasite is relatively simple, the adult worms being found in the trachea and bronchi of the lungs in cattle. The female worm which is larger and more opaque than the male, lays larvated eggs, which are normally coughed up and swallowed. These eggs hatch as they pass through the digestive tract and pass out in the faeces as first stage larvae. At this stage the intestinal cells and body cavity of the larvae are filled with brownish granules and apparently the free living stages of the parasite do not feed but exist on these granules as they gradually disappear as the larvae become older, (Daubney, 1920; Monnig, 1950). After one or two days the larvae undergo a moult, the cuticle being retained as a protective sheath and these are known as the second stage larvae. A second moult one or two days later produces third stage larvae and for a short time the larvae are surrounded by two sheaths. The larvae at this stage in their development have reached the infective stage, which under

suitable conditions should be reached 5 - 7 days after being voided in the faeces. Further development of the infective, third stage larvae is only possible if they are ingested by a susceptible bovine, where on reaching the intestine they throw off their protective sheaths and penetrate the intestinal mucosa. The route of migration followed by the parasite from this point until it reaches the bronchi is not completely known, but from the work of Soliman (1953) and by analogy with the life-cycle of the related lungworm of sheep, Dictyocaulus filaria, which has been more thoroughly investigated (Romanovich and Slavine, 1914; Guberlet, 1919; Daubney, 1920; Hobmaier and Hobmaier, 1933; Kauzal, 1933, 1934), it is probable that the route is as described below.

The larvae, after penetration of the intestinal mucosa, enter the lymphatic vessels and reach the mesenteric lymph nodes where a further moult takes place about four days after infection. The young worms then pass via the lymph and blood vessels to the heart and are carried to the lungs via the pulmonary artery. The young worms break out through the walls of the pulmonary capillaries into the alveolar areas of the lungs and migrate into the bronchi where the final moult takes place and the fully developed adult stage is reached.

The prepatent period, i.e. the time interval between a susceptible bovine ingesting the infective larvae, and the appearance of larvae in the faeces is about twenty-three days.

In the field there appears to be a strong resistance to reinfection (Wetzel, 1948; Taylor, 1951; Jarrett, McIntyre and Urquhart, 1954). This gave some indication that it might be possible to protect animals artificially against the disease and this section of the thesis deals with work mainly along these lines.

A. The Immunity Resulting from Natural Infection.

Introduction.

At the commencement of these studies the only experimental work done on this disease was by Porter and Cauthen (1942) and by workers at Weybridge Laboratories (e.g. Taylor and Michel, 1952). Porter and Cauthen artificially reinfected three calves which had recovered from a primary infection and found that one of them completely resisted this challenge infection. The other two calves became lightly infected, indicating a reasonable degree of resistance. Taylor and Michel (1952) reported that the larvae of *D. viviparus* invading the lungs of a partially resistant host were retarded in their development.

The following experiments were set up to study the resistance acquired by repeated infections and the pattern of antibody response.

Materials and Methods.

Serological Methods.

The use of a precipitin test was investigated, but no precipitating antibodies could be detected in any of the sera tested. The complement fixation method

was then investigated and after testing a saline extract of adult lungworms, the antigen finally adopted was a boiled extract similar to that described by Stewart (1950a) for Haemonchus contortus. The complement fixation method adopted was a modification of a quantitative technique described by Maltaner and his colleagues (for references see Kabat and Mayer, 1948). The experimental details are outlined below.

Glassware: All glassware used for the test was thoroughly steeped in chromic acid overnight, rinsed in tap water, finally in distilled water, and dried in a hot air oven.

Test Sera: Blood samples were collected at approximately weekly intervals, from the jugular vein of the experimental animals, allowed to clot at room temperature, and the sera separated by centrifugation. All sera were stored at -20°C.

Complement: Bulked guinea pig serum in quantities up to 200 ml was collected and preserved by freeze-drying in 2 ml ampoules which were stored at 0 - 4°C. This material was found to maintain its titre for periods up to one year. A titration was carried out on each batch and for each day's testing a suitable dilution was prepared so that 0.025 ml corresponded to approximately one 50% haemolytic unit. This particular volume was chosen so that the 1, 2, 4 and 8 units could then be measured with E-Mil auto-zero micropipettes (which are accurate and comparatively fast to manipulate).

Antigen: The adult lungworms were collected from the bronchi of experimentally infected animals as soon as possible at autopsy. These worms were rinsed in saline and placed in a Petri dish and the superficial moisture removed with filter paper. The dish containing the worms was put into the deep freeze, and as soon as it was completely frozen, it was removed and placed either in a vacuum desiccator over P_2O_5, or in the freeze drier. In this way it was possible to dry the worms without thawing taking place. When completely dry the worms were pulverized in a mortar, transferred to 10 ml ampoules which were sealed under vacuum and stored at 0 - 4°C until required.

The test antigen was prepared by taking 0.3g of the dried lungworms suspended in 25 ml double distilled water in a boiling tube. The tube and contents were placed in a boiling bath of 20% NaCl and kept at 100°C for exactly 10 minutes. The tube was then cooled, the contents transferred quantitatively to a graduated cylinder and the volume made up to 25 ml. This was made isotonic by the addition of 0.22 g NaCl. The antigen preparation at this stage could be stored overnight at 0 - 4°C or used immediately. Just before use the antigen was centrifuged for 10 minutes at 1,500 r.p.m. and the supernatant was diluted 10 times with 0.85% NaCl. The optimum level of antigen in the system was taken as 1 ml of 1:10 dilution. As shown in Table 14 there was a wide range of antigen concentration suitable, there being little difference between dilutions of antigen 1:5, 1:10, and 1:20.

TABLE 14

The effect of antigen concentration on the Index Ratio* of immune serum.

Antigen Concentration		Index Ratios (corrected for dilutions 2 & 3). 1 : 5	1 : 10	1 : 20	1 : 40
	dil.1	16	15	13	9
Serum	dil.2	20	24	19	11
1	dil.3	28	27	25	20
Mean Index Ratio		21	22	19	13
	dil.1	17	17	13	8
Serum	dil.2	26	25	20	10
2	dil.3	24	25	19	---
Mean Index Ratio		22	22	17	9

* As explained later in the text the Index Ratio gives a measure of the antibody content of the serum (See pages 67-68).

The antigen was always used as soon as possible, as it was found that the titre of the standard serum tended to vary if the antigen used in the test was prepared more than 24 hours in advance.

Sheep Red Cells: Sheep blood was collected using heparin as an anti-coagulant; the cells were washed three to four times with cold saline before use. When the cells were not used on the day of collection, they were suspended in an equal volume of Alsever's solution (see Kabat and Mayer, 1948, p.107) and stored at 0 - 4°C. The washed cell suspension was adjusted so that 0.25 ml lysed and diluted with water to a volume of 3.75 ml in a 4 ml colorimeter tube gave a reading in the E.E.L. colorimeter of approximately 50 using filter no.625. This concentration should approximately be equivalent to the point of 100% haemolysis in the actual test and ensured that the range of haemolysis falls into a suitable scale, and also it ensured that the concentration of red cells added to the system on different days was relatively standard.

Haemolytic System: Haemolysin (Burroughs Wellcome and Co.) of haemolytic titre between 1 in 1000 and 1 in 2000 was diluted 1 to 200 with 0.85% NaCl solution. An equal volume of haemolysin solution was added slowly with constant stirring to the adjusted sheep cell suspension and incubated at room temperature for at least 30 minutes before use.

Standard Serum: A large bulked sample of relatively high titre was obtained from an animal which had been

reinfected after recovery from a primary infection. This serum was divided into 3 ml aliquots and stored at -20°C. Each day on which testing was carried out, one of these samples was taken, dilutions prepared and the titre determined. The use of such a serum has been recommended by Rice (1942) in order to correct for variations in the 'fixability' of complement. It enables one to correct for day to day variations in the test, and also to correlate results obtained with different batches of complement. The variations in the Index Ratios* from day to day and with different batches of complement are shown in Table 15. This also shows the stability of the test serum as there was a time interval of three years between first and last results.

TABLE 15

Variations in the Index Ratio* of a standard serum from day to day and with different batches of complement.

Complement A	Complement C
Index Ratio 21,27,22,25,27.	Index Ratio 10,11,11,12.
Complement B	Complement D
Index Ratio 18,17,20,13,16.	Index Ratio 18,19,24,18.

* Index Ratio. See pages 67-68.

Pre-colostral serum: Pre-colostral serum (P.C.) was collected from a newborn calf prior to the ingestion of colostrum and contained no detectable antibodies when tested with the antigen. It was found most useful as a control serum to check on any anticomplementary effects of the antigen and also as a diluent for the test sera. It was important that the calf should not have been allowed access to colostrum before collection of the serum as antibodies which react with the antigen are transferred by it; this is illustrated in Fig. 9, which shows the level of complement fixing antibodies to the lungworm antigen before and after the ingestion of colostrum.

Setting up of test: Doubling dilutions of the sera to be tested were carried out in centrifuge tubes using pre-colostral serum as the diluent in order to maintain the protein concentration; the number of dilutions prepared from each serum depending upon its probable titre. After inactivation at 56°C. for 30 min. a further dilution (1 - 5) with 0.85% NaCl was carried out, followed by mixing and centrifugation at 0°C. for 20 min. The sera were then ready for testing. The test was carried out in E.E.L. colorimeter tubes (4 ml) and was set up as shown in Table 16.

The tubes containing the reagents described in Table 16 were agitated and incubated at 37°C. for 1 hour; 0.5 ml haemolytic system was then added to each tube. The tubes were thoroughly shaken and reincubated at 37°C. for a further hour. After refrigeration at 4°C. overnight the tubes were prepared for reading by the addition of a suitable volume (2.0 ml) of 0.85% NaCl to each, mixing and centrifugation.

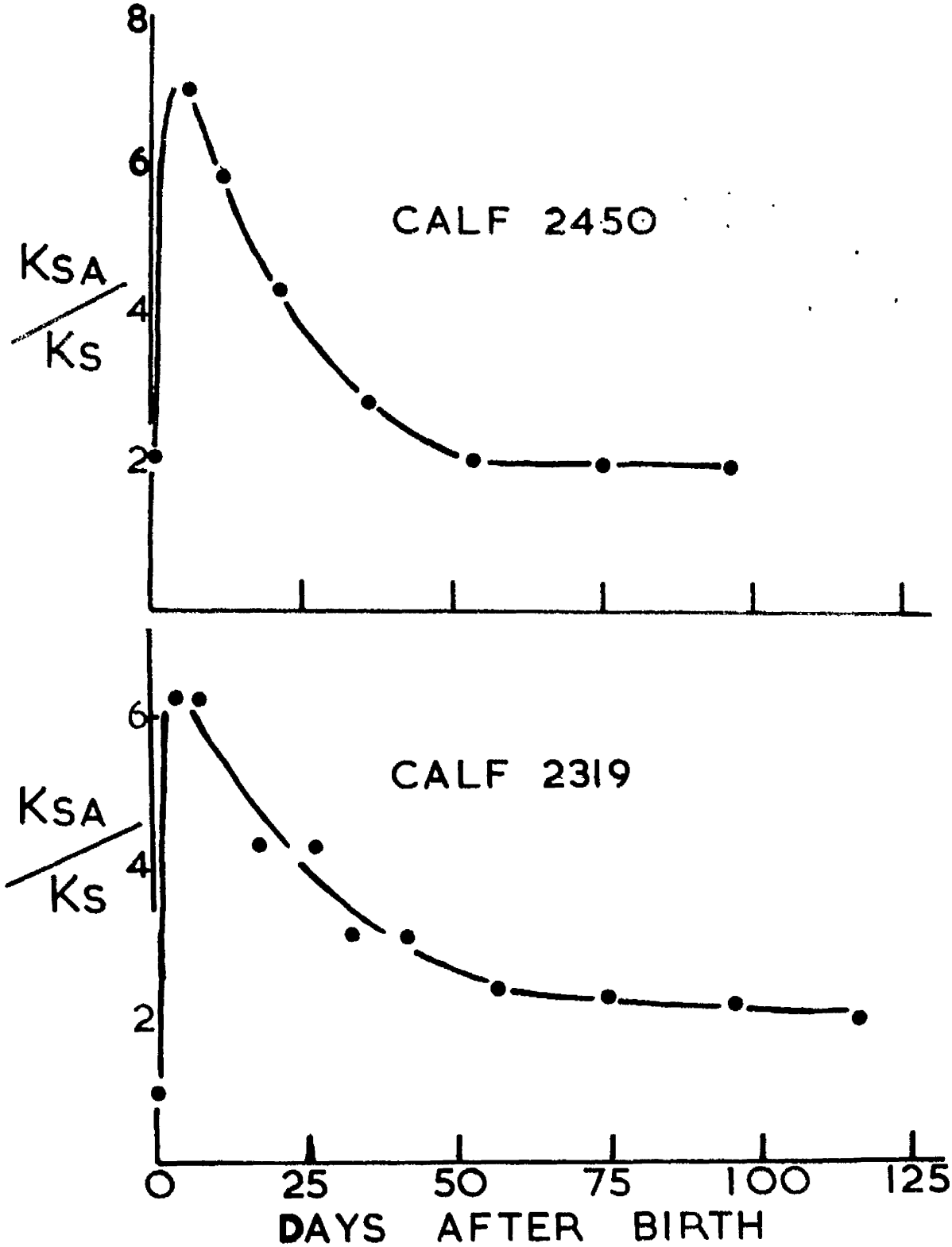


Fig. 9 The effect of ingestion of colostrum on the index ratios in the serum of two calves tested with D. viviparus antigen.

TABLE 16

Setting up the Test*

			Tube No.	Complement arbitrary 50% units	Serum (ml)	Antigen (ml)	0.85%NaCl (ml)
Series A	100% Haemolysis		1	0	0	0	1.25
			2	0	0	0	1.25
			3	8	0	0	1.25
			4	8	0	0	1.25
Series B	P.C. Control		5	0	0.25	0	1.0
			6	1	0.25	0	1.0
			7	2	0.25	0	1.0
			8	1	0.25	1.0	0
			9	2	0.25	1.0	0
Series C	Test Serum	dil. 1	10	0	0.25	0	1.0
			11	1	0.25	0	1.0
			12	2	0.25	0	1.0
			13	2	0.25	1.0	0
			14	4	0.25	1.0	0
			15	8	0.25	1.0	0
		dil. 2	16	2	0.25	1.0	0
			17	4	0.25	1.0	0
			18	8	0.25	1.0	0
		dil. 4	19	2	0.25	1.0	0
			20	4	0.25	1.0	0
			21	8	0.25	1.0	0
		dil. 8 etc.					

* Serum samples and complement were measured with E-Mil auto-zero micropipettes (H.J. Elliot Ltd.) and antigen and saline with the Hawkins Automatic Syringe Pipette (A.L. Hawkins & Co. Ltd.).

Reading the test: The tubes were read in the E.E.L. colorimeter using filter 625 as follows:-

Series A - Tubes 1 and 2 which contain only haemolytic system and saline were used to zero-set the instrument for the reading of tubes 3 and 4 which contained haemolytic system and excess complement. From these measurements one obtained a reading for 100% haemolysis.

Series C - Tube No. 10 which contained only test serum and haemolytic system was used to zero-set the instrument for the rest of the series. This corrected for any background colour due to the serum itself. Tubes 11 and 12 gave a measure of the haemolysis occurring with serum, complement and no antigen; 13, 14 and 15 that with serum, complement and antigen. Tubes 16 to 18, 19 to 21, etc. were the counterparts of 13 to 15, with higher dilutions of serum.

Series B - These tubes covered a complete test with a known negative serum. It was therefore only necessary to test at one dilution of this serum.

Calculation: By comparing the colorimeter readings of the tubes of series B and C with those of series A the % haemolysis in each tube was calculated. From the % haemolysis and the number of arbitrary 50% units present in any one tube, the number of units of complement required to produce exactly 50% haemolysis was calculated by reference to a set of tables based on the von Krogh equation (see Kabat and Mayer, 1948, p.102). Only tubes where the % haemolysis lay between 20% and 80% were used for the calculation as this range corresponds to the

sensitive part of the haemolysis curve. The potency of a serum was expressed in terms of its 'Index Ratio' (K_{sa}/K_s) as recommended by Rice (1942) where K_{sa} is the amount of complement required to give 50% haemolysis in the presence of immune serum and antigen, and K_s the amount required to give 50% haemolysis in the presence of immune serum alone. This method of expressing the result automatically corrected for any anti-complementary effect due to the serum. In testing a serum the dilution where suitable partial haemolysis occurred depended on the potency of that particular serum. All index ratios obtained at dilutions 2, 4, 8 etc. had to be suitably corrected. In making this correction, it had to be borne in mind that the index ratio of a serum could not be less than 1.0 as one unit of complement was necessary to produce 50% haemolysis in the absence of antibody. Thus if X is the index ratio obtained for a serum at dilution Y then $((X - 1)Y) + 1$ gives the index ratio at dilution Y. In the case of a serum where it was possible to calculate index ratios from more than one dilution good agreement was usually obtained.

The test with pre-colostral serum (Series B) enabled one to detect possible anti-complementary effects of the antigen.

Experimental Animals.

Ayrshire bull calves were purchased when 3 - 7 days old and reared in individual metal houses.

Parasitology.

Culture of D. viviparus larvae: Faeces containing lungworm larvae were obtained from a series of experimentally infected culture calves. The faeces were placed in culture dishes to a depth of 1 - 2 inches and stored in the dark at maximum humidity. After 7 - 10 days the infective larvae were removed from the culture by washing the surface of the faeces and the sides of the vessel with a little water; in this way a suspension containing large numbers of infective larvae was obtained (Jarrett et al., 1954).

Preparation and administration of doses: A suitable number of representative samples (0.05 ml) of the larval suspension were examined microscopically and the number of larvae counted. In this way the total number of larvae in the suspension was estimated. The number of larvae required for administration to each calf was then transferred to a dosing bottle and diluted to 50 ml with tap water. The calves were dosed orally with this suspension, care being taken to ensure that the full dose was administered.

Faecal examination: Faecal examinations were carried out throughout the course of the experiments using the McMaster and Baerman techniques (Craig and Faust, 1951).

Post-mortem Examination.

Each calf was killed and the lungs removed. The trachea and bronchi were cut open and all visible worms removed, then the lungs were thoroughly washed in warm

saline. The washing from each pair of lungs was allowed to stand overnight and the sediment examined. As a further check for the presence of worms, approximately 50% of each pair of lungs was minced and examined by the Baerman technique.

Results.

Experiment 1.

Infection with three consecutive single doses of D. viviparus larvae.

In this experiment ten 2-month-old calves were each infected with 2,500 infective D. viviparus larvae; 160 days later each of the calves was dosed with 5,000 larvae and after a lapse of a further 150 days, 13,000 larvae were administered to each calf. All the calves were killed and autopsied 30 days after the last infection. The parasitological and serological findings are described below.

Parasitology.

First infection: McMaster examination showed that the faeces of all the calves contained appreciable numbers of larvae from 24 days after infection. The mean daily larval output per gramme of faeces as determined by the McMaster technique is shown in Fig. 10. while the maximal number of larvae/g of faeces recorded for each calf is shown in Table 17.

Second infection: The faeces of all the calves were negative to McMaster examination throughout and in spite

of repeated Baerman examination larvae were not found in the faeces of 6 of the calves at any point after the second infection. The faeces of the remaining 4 calves contained larvae on several occasions between the 35th and 71st day after the second infection (see Table 17).

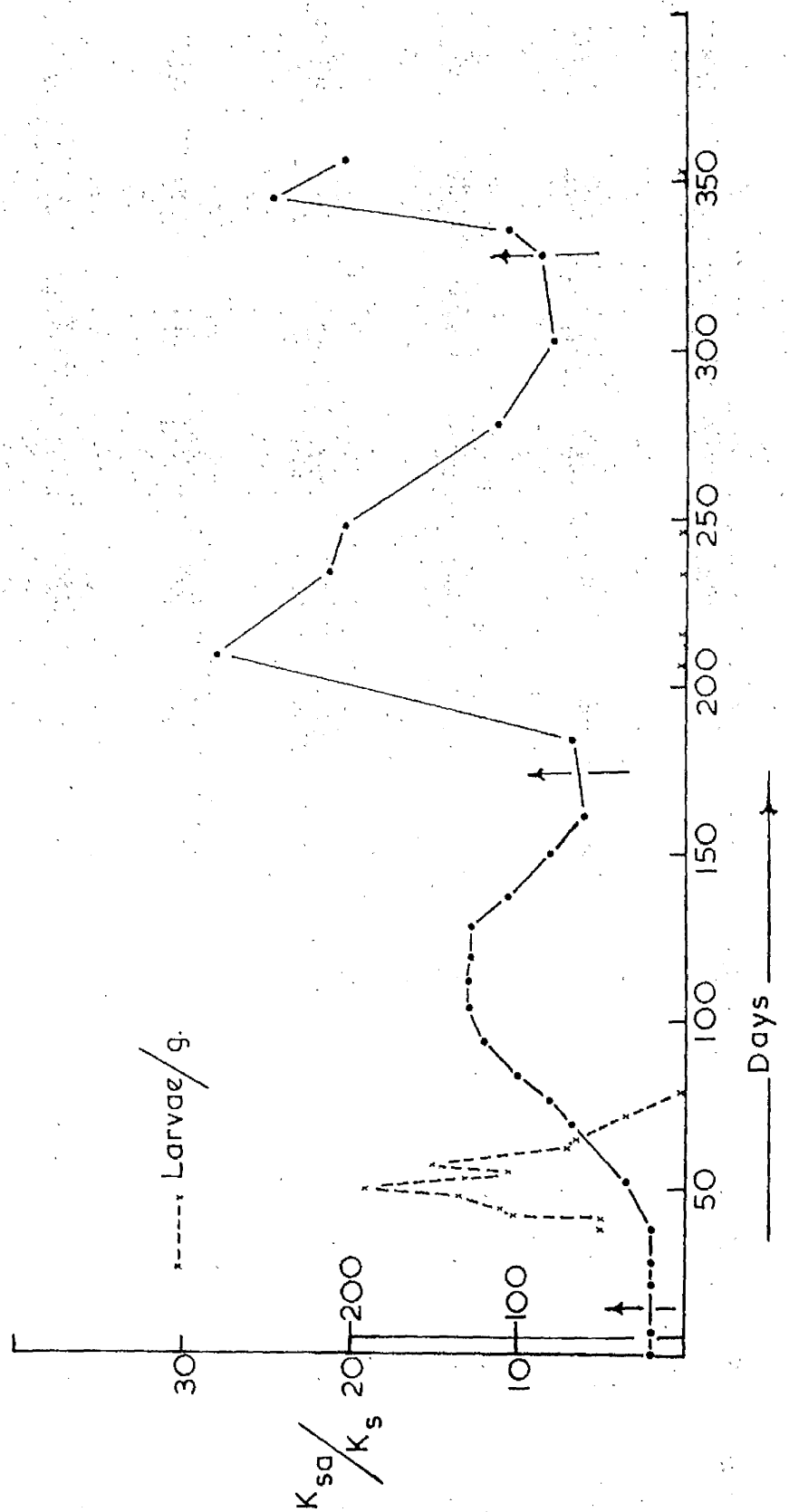
TABLE 17

Faecal Larval Counts during Three Consecutive Infections with D. viviparus.

Calf No.	Infection: First Max. McMaster Larvae/g	Second Baerman	Third Examination	No. of worms in lungs at autopsy.
23	450	-	-	0
25	200	-	-	0
26	100	+	-	0
27	150	+	+	165*
28	225	+	-	6*
29	250	-	-	0
30	625	-	-	0
31	150	-	-	0
32	200	-	-	0
34	350	+	+	12*

* Small adults or immature worms.

Fig. 10 Mean faecal larval output and serum antibody level during three consecutive infections with D. viviparus larvae.



Third infection: Larvae were not found in faeces of the 6 calves whose faecal larval counts had been negative during the course of the second infection, nor in the faeces of two of the calves which had shown positive faecal larval counts at that time. The faeces of the remaining two calves were positive on Baerman examination on the 25th and 28th day after infection respectively (see Table 17).

Serology.

The mean level of complement fixing antibodies for the 10 calves throughout the course of the experiment is shown in Fig. 10.

It can be seen that complement fixing antibodies do not appear in the serum until 30 - 35 days after the initial infection, while the maximal antibody level is not attained until about 80 days after infection, i.e. when the bulk of the infection has been thrown off (Jarrett et al., 1954). The increase in circulating antibody following the second and third infections is characteristic of a secondary response.

Pathology.*

At autopsy the lungs were almost completely normal. In each case there were a few areas of consolidation, involving only three or four lobules. Results of the detailed microscopic examination of the areas of consolidation have been reported elsewhere by Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart (1959).

* Carried out by W.F.H. Jarrett.

Experiment 2.

Infection of calves previously exposed to small repeated doses of D. viviparus larvae.

In this experiment each of 5 calves was infected with 25 doses of 300 infective larvae. The doses were separated by intervals of 2 or 3 days and extended over a period of 62 days (see Fig.11). One hundred and fifty-five days after the last dose of the infection schedule, 4 of the calves were challenged with 14,000 infective larvae each, while the fifth calf received 90,000 larvae. The calves were killed and autopsied 30 days after this challenge.

Parasitology.

First infection: The course of the mean daily larval output of the 5 calves is shown in Fig.11, and the peak numbers of larvae/g faeces recorded from each calf are shown in Table 18.

Although a total of 7,500 larvae was administered in the divided doses to each calf it can be seen that the highest faecal count recorded in Fig.11 was only 120 larvae/g and that larval output terminated 62 days after the first dose of the infection schedule (cf.Fig.10).

Second infection: Faecal examination up to the date of autopsy failed to reveal any larvae. The number of worms recovered from the lungs at autopsy is recorded at Table 18.

TABLE 18.

Faecal Larvae Counts During a Serial Infection Followed by a Single Infection with D. viviparus.

Calf No.	Serial Infection Maximal McMaster count/g.	Challenge Infection Size of Dose	Baerman Results	No. of worms in Lungs at Autopsy.
1	50	90,000	-	32*
2	250	15,000	-	46*
3	150	15,000	-	7*
4	150	15,000	-	14*
5	100	15,000	-	20*

* Immature Worms.

Serology.

The mean level of complement fixing antibodies in the sera of the 5 calves throughout the course of the experiment is shown in Fig.11. As in Experiment 1 the antibodies first appeared in the serum 35 days after the first dose of larvae, the maximal antibody level was attained about 70 days later, and the challenging infection produced a marked secondary response.

The lungs at autopsy were almost completely normal with a few small areas of consolidation; the histopathology has been reported (Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart, 1959).

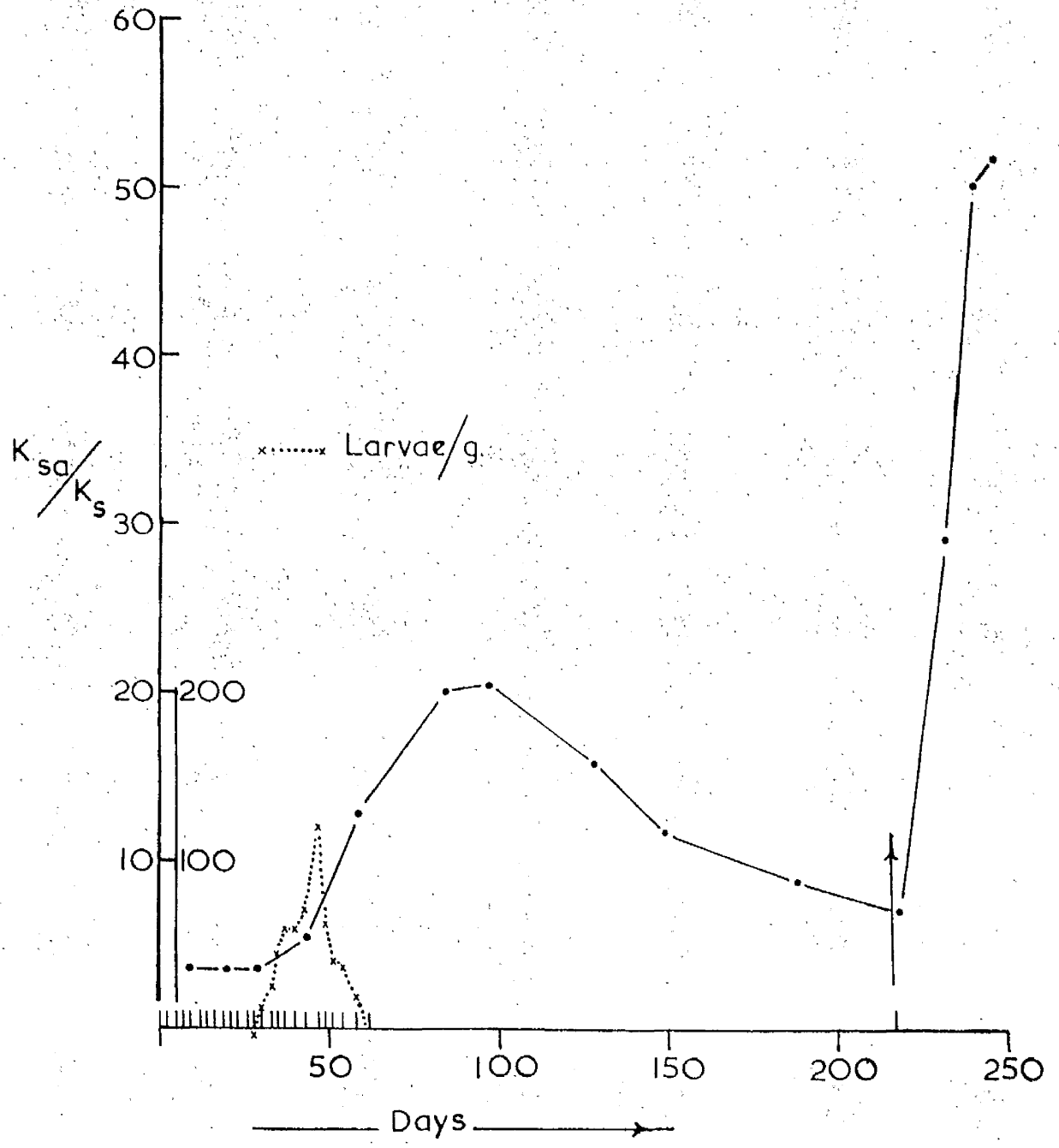


Fig. 11 Mean faecal larval output and serum antibody level resulting from successive small doses of D. viviparus larvae followed later by a single large dose.

Discussion.

From the results of Experiments 1 and 2 above it is clear that an infection with D. viviparus confers a high degree of resistance to a subsequent reinfection, and that this acquired immunity can result from a single infection with a sub-lethal dose of larvae or from a series of repeated doses of small numbers of larvae.

In Experiment 1 the acquired immunity is manifested strikingly by the differences in the faecal larval output corresponding to the different infections. At the first infection larvae appear in the faeces in significant numbers from 24 days after administration of the infecting dose, the mean faecal larval output for the group rising sharply as shown in Fig.10 to a maximum of about 200 larvae/g. Throughout the second and third infections although larvae did appear in the faeces of some of the calves the numbers were in every case too small to be measured by the McMaster technique. It is interesting to note the relationship in this experiment between the presence of larvae in the faeces and the level of complement-fixing antibodies in the serum. At the first infection when larval output was maximal the level of circulating antibodies was just beginning to rise and the peak titre was reached some 50-70 days after the peak larval output, i.e. when faeces samples were completely negative. Because of this relationship serological tests on field cases of the disease can sometimes give puzzling results, e.g. animals which are severely affected and putting out large numbers of larvae in the faeces may show low and sometimes

negative titres and others which are apparently normal on parasitological examination can show very high levels of circulating antibody. At the second and third infections due to the secondary response phenomenon the antibody level rose much more quickly after larval administration so that where larvae did appear in the faeces their presence coincided with elevated levels of circulating antibodies in the infected animals. The serological and parasitological picture at the second and third infections in Experiment 1 is very similar to that which we have found in the experimental reinfection of a number of recovered field cases of the disease.

The strong acquired immunity developing in Experiment 1 was shown not only by the faecal larval output of the experimental calves but also by the number of worms present in the lungs of these animals at autopsy (Table 17), seven of the calves having no worms in the lungs whatsoever. It should be pointed out that the number of larvae (13,000) administered to each animal at the third infection would prove fatal in a high proportion of non-immune animals, and those surviving until 30 days after challenge would have several thousand worms in their lungs.

The serological and parasitological findings in Experiment 2 are not very different from those obtained up to the second infection in Experiment 1, i.e. the same sort of time relationships were shown between larval output and antibody response and a strong acquired immunity was evident. The immunizing dose

of larvae in Experiment 2 consisted of 25 doses of 300 larvae each administered over a period of 62 days, i.e. a total of 7,500 larvae. It is interesting to note that mean faecal larval output in this experiment rose to a maximum of about 140/g as compared to 200/g in Experiment 1, when only 2,500 larvae were given at the first infection. It seems likely that some degree of immunity was developing in the calves of Experiment 2 during the period of larval administration so that there was a reduced "take" in the later doses in the schedule.

In both experiments the worms found in the lungs at autopsy were significantly smaller than those obtained from a thirty day normal infection and it is possible that in Experiment 1 some of the worms found at autopsy may have resulted from the second infection, (cf. Taylor and Michel, 1952).

Since these results were obtained and published (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1957) and (Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart, 1959), further studies by other workers have been reported. Weber (1958) in his studies on the immune response of calves to D. viviparus used an adult whole worm antigen prepared by a low temperature method and a complement fixation test of the serum dilution type, employing the end-point of complete inhibition of haemolysis with 2 100% units of complement. His results indicated that the immune response to a primary dose of infective larvae occurred at 12 days reaching a peak at 16 days, and this remained

fairly constant until 63 days. This pattern of response was virtually unchanged by subsequent exposure to infective larvae. These results of Weber do not agree with the serological response described here, although a different type of antigen was employed, which might contain a number of different antigenic substances as compared to the boiled antigen used in our studies. However, Cornwell and Michel (1960) have confirmed our results using a similar antigen to the one used in these studies. They also state that they have compared the two types of antigen and found no difference in the time of onset of antibody titre in the same series of serum samples.

The timing of the initial antibody response described by Weber is much more consistent with a secondary response and it may be that his calves had some previous contact with the disease before the experiment.

Summary.

1. It has been shown experimentally that infection of calves with D. viviparus confers a high degree of resistance to a subsequent reinfection.

2. This acquired immunity can result from a single infection with a sub-lethal dose of larvae or from a series of repeated doses of small numbers of larvae.

3. Animals immunized by a previous infection exhibit on challenge a rapid antibody response and a striking reduction (in some cases to zero) in the numbers of worms present in the lungs, and in the numbers of larvae appearing in the faeces.

B. The immunity resulting from passive immunization.

Introduction.

Attempts at passive immunization in helminth diseases have produced variable results, and while there was no published information on the effects of passive immunization in the case of *D. viviparus* it was shown that a strong acquired resistance to the disease could be produced experimentally (see previous section) and that this immunity was accompanied by a rise in the complement fixing antibodies in the serum. Although there was no evidence to show that the actual titre which was determined in these experiments was a measure of protective antibodies it was decided to investigate the protective action of a high titre serum by injecting it into young susceptible calves and subsequently to challenge them by infection.

Methods and Materials.

Immune Serum.

Immune serum was obtained from six recovered field cases of the disease whose antibody titre had been boosted by experimental reinfection with doses ranging from 50,000 to 200,000 infective larvae of *D. viviparus*. All these animals showed a typical "secondary response" as measured by the technique described previously, the complement fixing antibodies reaching a maximum approximately 2 weeks after the experimental infection. These six animals showed almost complete resistance to reinfection viz. in only one case did respiratory

symptoms occur, and larvae did not appear in the faeces of any of the animals up to five months after reinfection. When the level of complement fixing antibodies was approximately at its maximum, the animals were each bled twice, with a three day interval between the bleedings. The blood was allowed to clot, the serum separated, centrifuged and pooled. A total of 23 litres of serum was obtained.

In order to reduce the volume and the amount of protein used in the passive immunization, a globulin preparation was obtained by adding anhydrous sodium sulphate to a final concentration of 18%. No attempt was made to isolate further the antibody-containing globulin fractions in view of the lack of precise information on the distribution and location of helminth antibodies in bovine serum. The mixed globulin precipitate was freed from sulphate by dialysis overnight against running tap water and then for four days against a large volume of isotonic NaCl with frequent changes. The final volume was 7.5 litres with a protein concentration of 10.8g per 100ml.

Experimental calves.

Ten Ayrshire bull calves were purchased when 3 - 7 days old and reared in isolation in individual metal pens.

Immunization and Infection.

When 10 weeks old, each of five calves was injected intraperitoneally with 500 ml of the immune globulin

preparation daily on three consecutive days. Two days after the last injection these calves together with 5 control calves were each infected with 4,000 D. viviparus larvae, cultured and prepared as described previously.

Blood samples were collected prior to the injection of immune serum and at intervals during the experiment. The calves were killed and autopsied 30 days after infection, the lungs removed and the lungworms removed and counted. The worms from each calf were dried in a separate Petri-dish over P_2O_5 in a vacuum desiccator and weighed.

Results.

Clinical examination* of the 10 calves was carried out 14 days after infection and repeated every third day until the experiment termination. A difference between the two groups became apparent on the 17th day and became progressively more marked with time. The individual respiratory rates are shown in Table 19, and the mean respiratory rates for both groups are shown in Fig. 12.

* Carried out by W.I.M. McIntyre.

TABLE 19.

Respiratory rates of immunized and control calves.

Days after infection		14	17	20	23	25	28
Respiratory rates of immunized group.	No.1	45	50	40	70	70	70
	No.2	30	30	35	50	35	30
	No.3	30	50	50	50	45	45
	No.4	35	35	35	40	45	45
	No.5	35	35	50	65	60	60
	Mean	35	40	42	55	51	50
Respiratory rates of control group.	No.6	40	60	70	80	80	80
	No.7	35	60	70	80	80	70
	No.8	40	60	70	80	100	110
	No.9	60	90	90	90	100	100
	No.10	50	60	60	60	65	65
	Mean	45	66	72	78	85	85

With the exception of calf 1 the immunized calves did not develop severe clinical pneumonia at any time during the experiment and it is interesting to note that this calf had no worms in its lungs at autopsy and therefore the rise in respiratory rate may have been due to some intercurrent infection. All the control calves, except No. 10 were very severely affected yet this calf had the highest worm burden at autopsy.

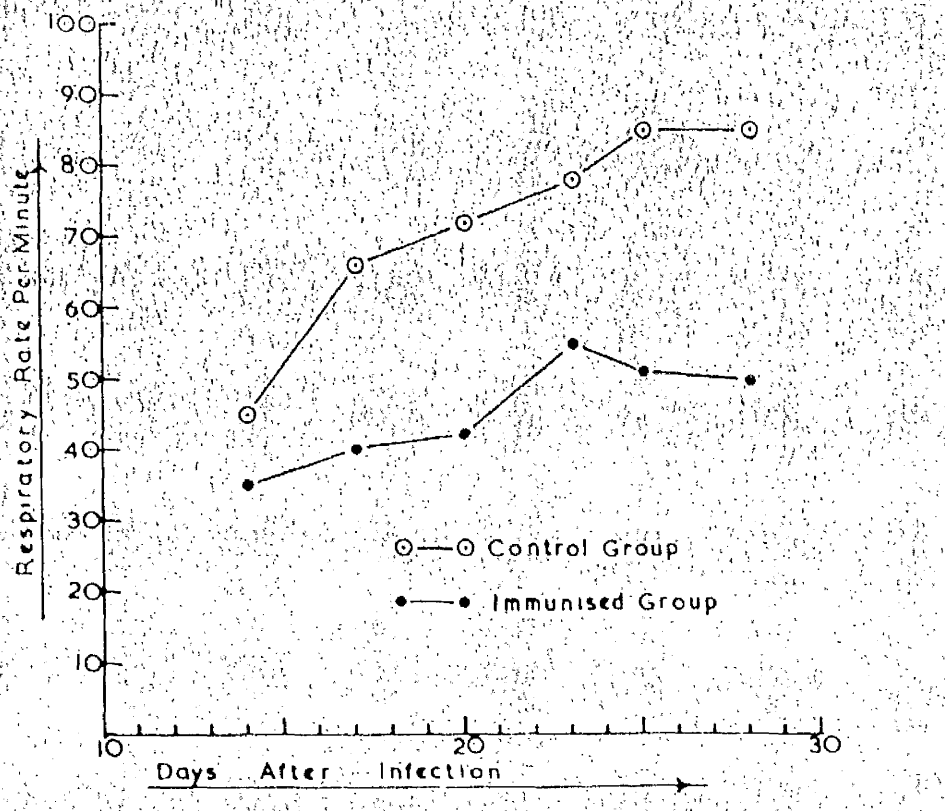


Fig. 12 Mean respiratory rates of control and immunized groups.

Larval counts on the faeces of the immunized and control animals were carried out on the 25th and 29th day after infection. The results are shown in Table 20.

TABLE 20.

Faecal larval counts of immunized and control calves.

		25th day		29th day	
	Calf No.	McMaster (larvae/g)	Baerman	McMaster (larvae/g)	Baerman
Immunized group	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	+
	4	0	0	0	+
	5	0	0	0	0
Control group	6	200	-	650	-
	7	150	-	100	-
	8	300	-	250	-
	9	50	-	150	-
	10	500	-	900	-

The numbers of worms recovered from each pair of lungs are shown in Table 21, and illustrated in Fig.13, the total weight of dried worms and the average dry matter per worm are also shown in Table 21.

The average number of worms in the control group was 786 as compared with 37.4 in the immunized group. There was also a reduction in the average weight per worm measured as dry matter in the immunized group,

TABLE 21

Numbers and weights of lungworms recovered from immunized and control calves.

	Calf No.	No. of worms	Total weight of dried worms (mg.)	Average D.M. per worm (mg.)
	A	60	-	-
	B	0	-	-
	1	0	0	0
	2	0	0	0
Immunized	3	142	89.6	0.631
group	4	49	16.2	0.331
	5	11	2.4	0.218
	Total	262	108.2	1.180
	Mean	37.4		0.536
	C	311	-	-
	D	712	-	-
	6	1224	906.6	0.741
Control	7	83	28.0	0.337
group	8	1131	890.6	0.787
	9	778	558.1	0.717
	10	1262	1362.8	1.080
	Total	5501	3746.1	3.662
	Mean	786		0.837

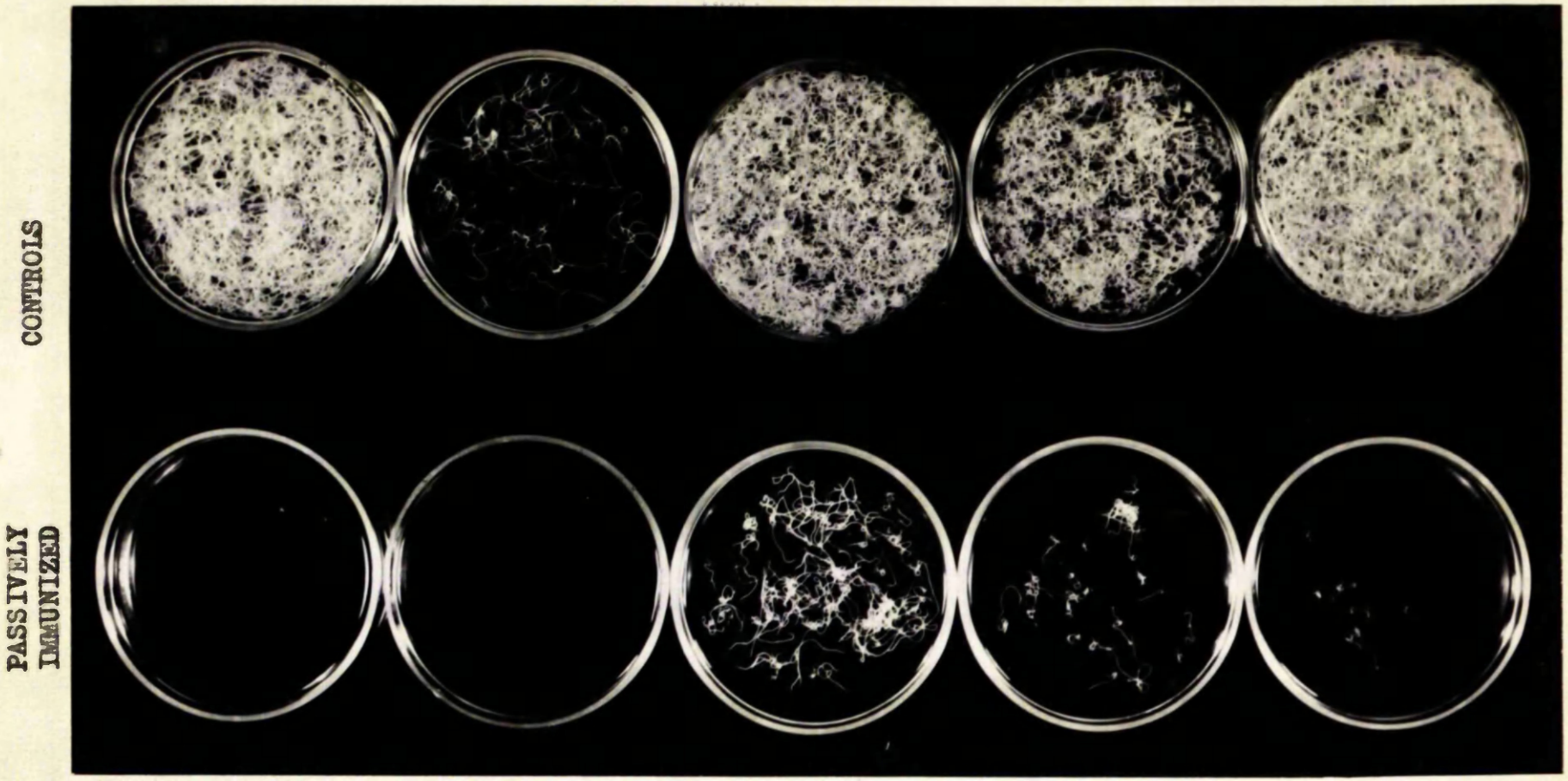


Fig. 13 D. viviparus recovered from the lungs of passively immunized and control calves

but this is probably not significant as it may be a function of the number of worms present in the lungs, there being a statistically significant correlation between number of worms and the average weight per worm in each lung. Examination for immature worms in bronchial mucus and in Baerman sediments of minced lung were negative in all cases.

The results of complement fixation test on the sera of all the experimental animals are shown in Fig. 14. This indicated that the absorption of intraperitoneally injected immune globulin was rapid and resulted in a high circulating titre at the time of infection and that this titre fell normally during the course of the experiment and was still positive at its termination. The small increase in titre of the sera of the control group indicated some active production of antibody.

The numbers of worms present in the bronchi of the calves were found to parallel closely the degree of consolidation of the diaphragmatic lobes seen at autopsy (Table 22). The superficial lesions in the lungs of the control group were more extensive than those in the lungs of the immunized group as shown in Fig. 15. The lungs of the control group also appear larger than the immunized group and this was due to fairly well marked interstitial emphysema.

TABLE 22.

Correlation between numbers of worms and gross lesions* in the lungs of immunized and control calves.

	Calf	Number of worms	Diaphragmatic consolidation.	Emphysema.
	1	0	-	-
	2	0	-	-
Immunized	3	142	++	-
group	4	49	+	-
	5	11	trace	-
	6	1224	++++	+
	7	83	++++	+
Control	8	1131	+++	+
group	9	778	+++	+
	10	1262	++++	+

* Examination carried out by W.F.H. Jarrett.

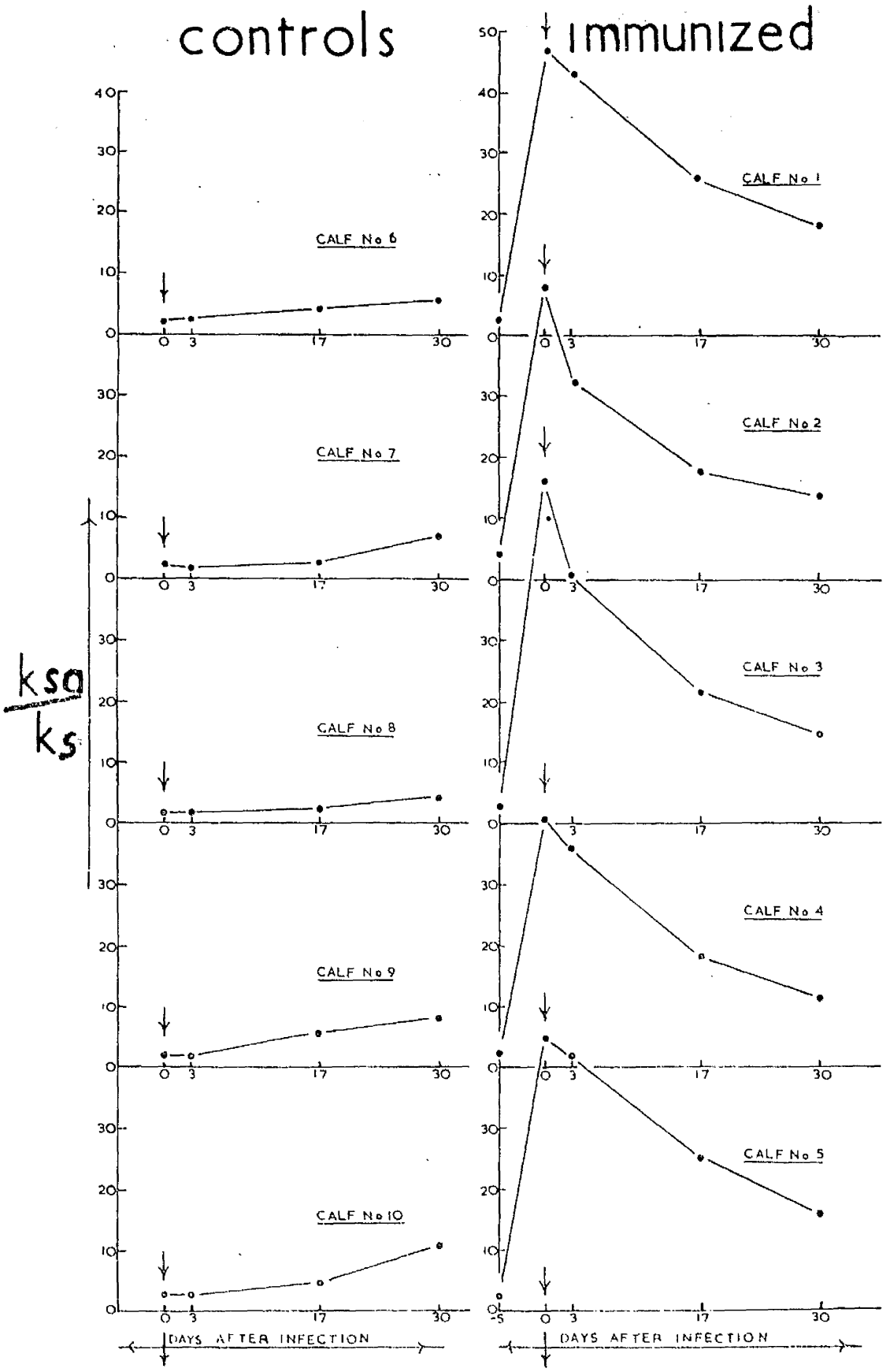


Fig. 14 The levels of complement-fixing antibodies in the sera of immunized and control calves.

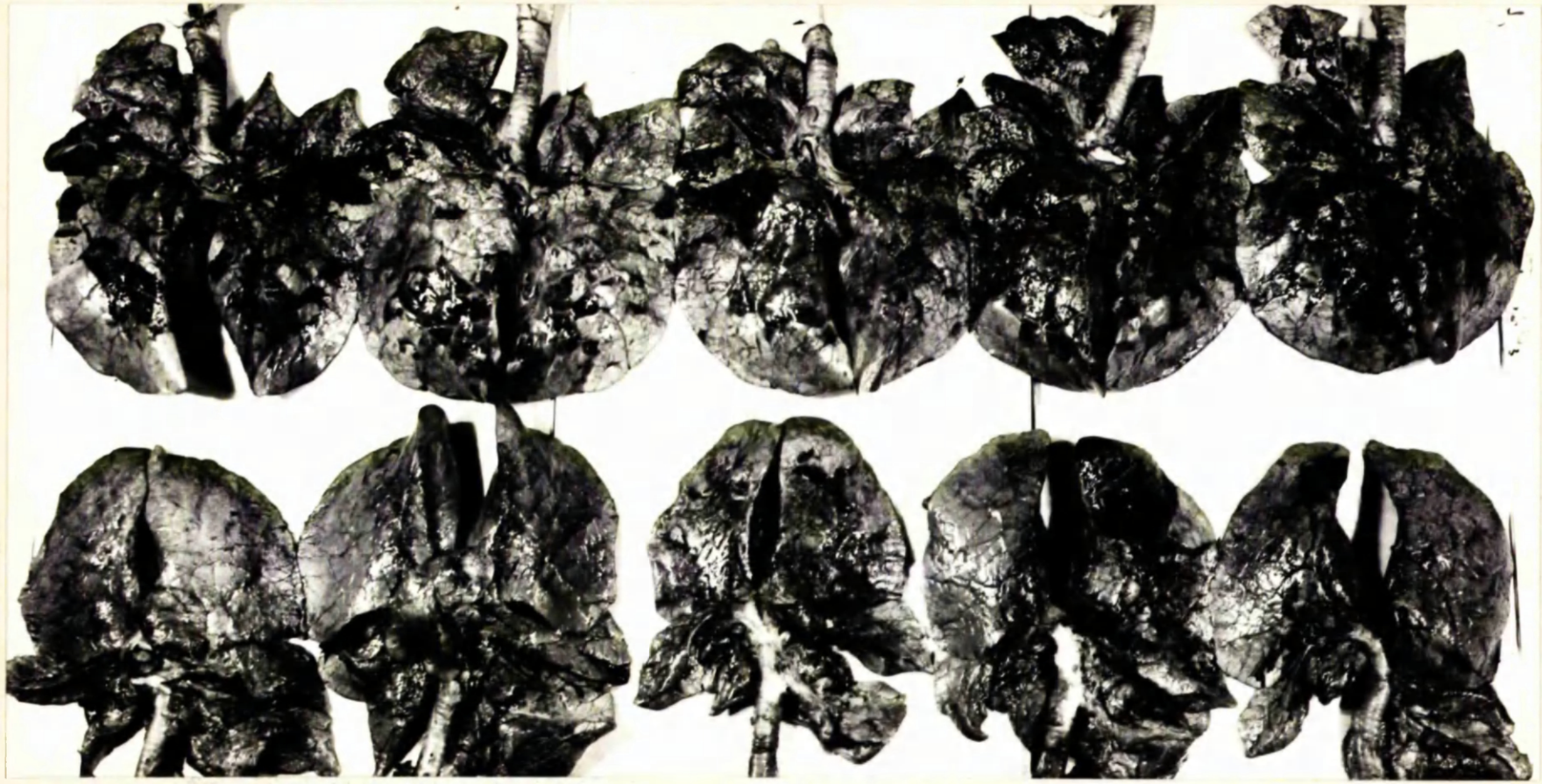


Fig. 15 Lungs of passively immunized and control calves

Discussion.

There is no doubt from the results obtained in this experiment that passive immunization with serum of the titre and the amounts used conferred a high degree of protection against a challenge infection of 4,000 normal infective D. viviparus larvae. This was manifested in three ways, each of which is of great importance in this disease, and are the marked reduction in clinical symptoms; the marked reduction in the extent of lung damage and the marked reduction in the numbers of worms developing from the challenge infection. The respiratory rates of the control calves were significantly higher than the immunized animals, and the former all showed fairly well marked interstitial emphysema. The number of worms recovered from the control group was 5,501 compared to 262 in the immunized group corresponding to mean worm burdens of 786 and 37.4 respectively. In the immunized group the lungs of three of the calves contained no worms.

These findings offer some hope that a practical method of passive immunization might be devised. There is however, the difficulty that in this experiment a large quantity of serum was used (23 litres) to protect only five calves, and further experiments would have to be carried out in an attempt to obtain a method of producing very high titre serum in the donor animals, and therefore reduce the amount of immune serum necessary for protection.

There is also the problem that any immunity conferred passively would be of a transient nature and experiments would also have to be carried out correlating the titre of the serum and the duration of immunity, as the time of infection after immunization would be very variable.

Since this work was completed, Rubin and Weber (1955) reported that the intravenous administration of 5.0 ml per lb body weight of immune serum enabled two calves to survive a known lethal dose of 50,000 lungworm larvae. Although these results by themselves could not be regarded as significant because of the small number of animals, when considered in conjunction with the result achieved in the passive immunization experiment reported here, it does suggest that smaller amounts of immune serum might exert a protective influence.

Summary.

(1) Hyperimmune serum was obtained by experimental infection of recovered field cases of parasitic bronchitis.

(2) A globulin preparation from the high titre serum was prepared by sodium sulphate fractionation and this preparation when injected intraperitoneally into susceptible calves conferred a high degree of immunity to experimental infection.

C. The immunity resulting from artificial immunization with whole worm antigen.

Introduction.

It has been shown that the infection of bovines with Dictyocaulus viviparus confers a high degree of resistance to reinfection and that this acquired resistance can be passively transferred. These findings suggested that it might be possible to protect animals against the disease by some method of active immunization and this section of the thesis describes experiments carried out along these lines.

Materials and Methods.

Immunizing antigen.

The freeze-dried worm material was prepared as described in the previous section. The immunizing antigen preparation was a water-in-oil emulsion as described by Freund, Thomson, Hough, Sommer and Pisani (1948). The emulsion was prepared in a M.S.E. homogeniser, each 100 ml containing 1g dried lungworm, 0.625g heat-killed Mycobacterium phlei, 50 ml 0.85% NaCl, 50 ml low viscosity liquid paraffin, and 2g "Emocithine". *

Serology.

Sera were collected at approximately weekly intervals and complement fixing antibodies were estimated by the method described in Section IIIA of this thesis.

* (W.A. Scholten's Chemische Fabrieken, N.V.Foxhol (Gr.), Netherlands). The "Emocithine" was used as the stabilizer for the emulsion.

Experimental animals.

Ayrshire bull calves were purchased when 3 - 7 days old. In the first experiment the 24 calves involved, and in the third experiment the 15 calves involved were kept in metal isolation huts. In the second experiment (carried out at the Agricultural Research Council's Field Station, Compton, Berkshire), the 33 calves were reared in groups of 11 in large loose-boxes. In all cases the calves were 2 - 3 months old when the experiments commenced.

Injection procedure.

10 ml of the immunizing antigen preparation prepared as described above was given at each injection. The dose was administered intramuscularly in the hind leg. The exception to this procedure was in experiment 3 where the injections were intraperitoneal and in one group the antigen was a saline homogenate of fresh worm material.

Infection procedure.

The larvae were cultured and the calves were infected as described previously.

Results.

Experiment 1:

Whole worm vaccine with single challenge.

The 24 calves in this experiment were divided into 4 groups as shown in Table 23. In group A each calf received vaccine at 2 months and at 1 month before challenge; in group B one injection was given to each calf 2 months before challenge; in group C one injection

TABLE 23.

Numbers of worms found at autopsy in immunized and control calves.

	Calf No.	Injection dates		Challenge dates	No. of worms found at autopsy
Group A	1	22/6	20/7	20/8	91
	2				768
	3				676
	4				685
	5				599
	6				826
					Mean 608 ± 242
Group B	7	22/6	-	20/8	422
	8				34
	9				694
	10				590
	11				245
	12				377
					Mean 394 ± 203
Group C	13*	-	20/7	20/8	
	14				913
	15				688
	16				174
	18				290
	19				374
					Mean 569 ± 308
Group D	17	-	-	20/8	1049
	20				388
	21				1357
	22				411
	23				443
	24				481
					Mean 688 ± 375

* Calf 13 died shortly after infection and is not included in this table.

was given 1 month before challenge. The calves in group D acted as control animals.

Each injection was given intramuscularly and consisted of 10 ml (100 mg dried lungworm) antigen preparation in water-in-oil emulsion as described above. The resulting immunity was tested by a challenge of 4,000 infective D. viviparus larvae administered orally to each calf. Autopsies were carried out 30 days after challenge.

The faeces of all the calves were examined 24, 25, 26 and 27 days after challenge. On the earliest date larvae were present in the faeces of the majority of the immunized and control calves, and subsequent faecal counts of the various groups showed no significant differences. The numbers of worms recovered from the lungs at autopsy are shown in Table 23. Sex ratios were determined on representative samples of worms from each calf and all the worms recovered from each pair of lungs were dried to constant weight over P_2O_5 in a vacuum desiccator. There was no significant difference in size as measured by the amount of dry matter per worm between the parasites obtained from the immunized and control calves, nor was any variation in sex ratio apparent.

Only in the calves of group B, where the course of immunization consisted of 1 injection 2 months before challenge, was there an apparent reduction in worm burden as compared to the control calves, but this just failed on statistical analysis to be significant.

Fig. 16 Individual levels of complement-fixing antibodies after immunization with adjuvanted whole worm vaccine and challenge with 4000 D. viviparus larvae.

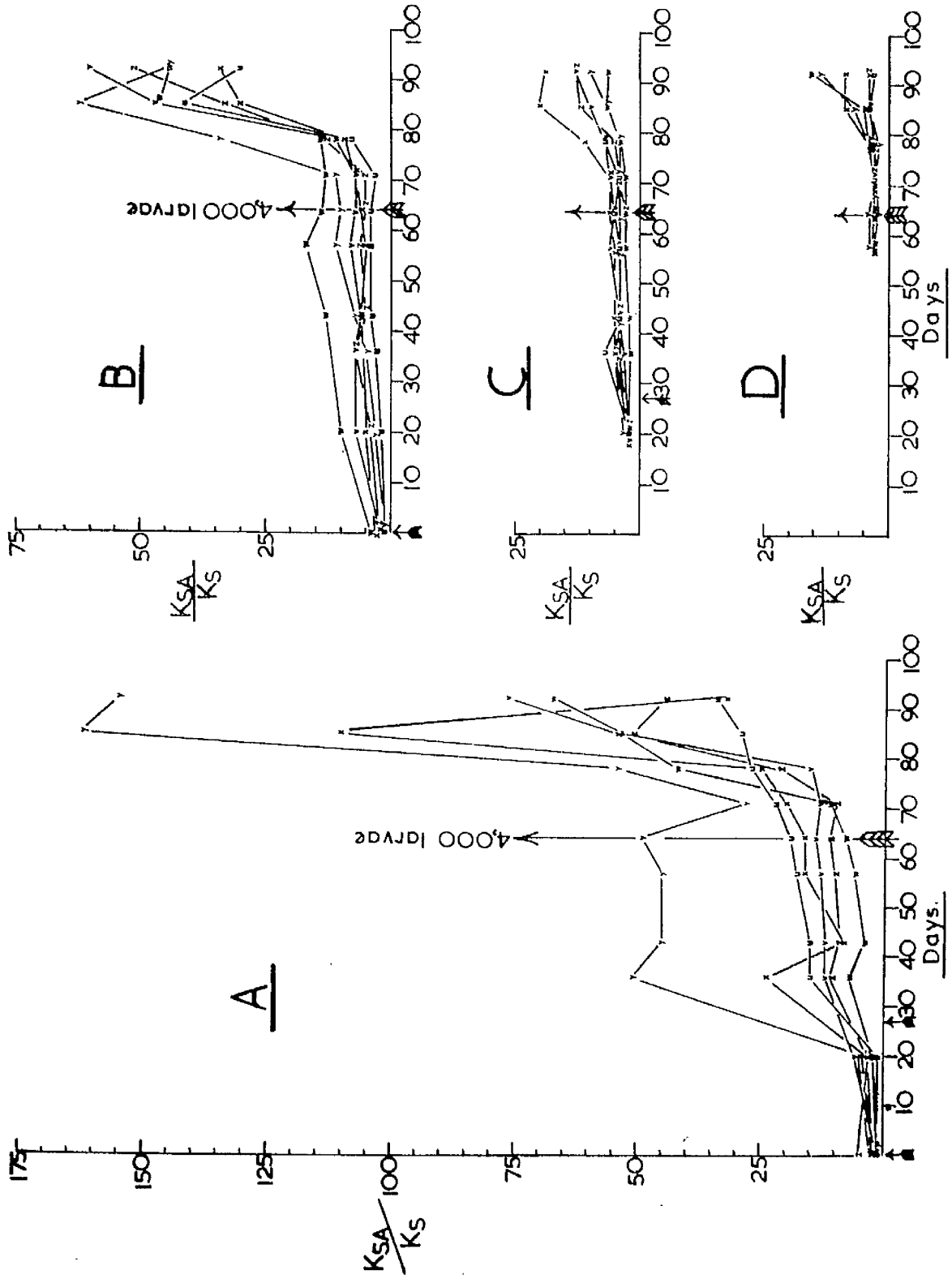
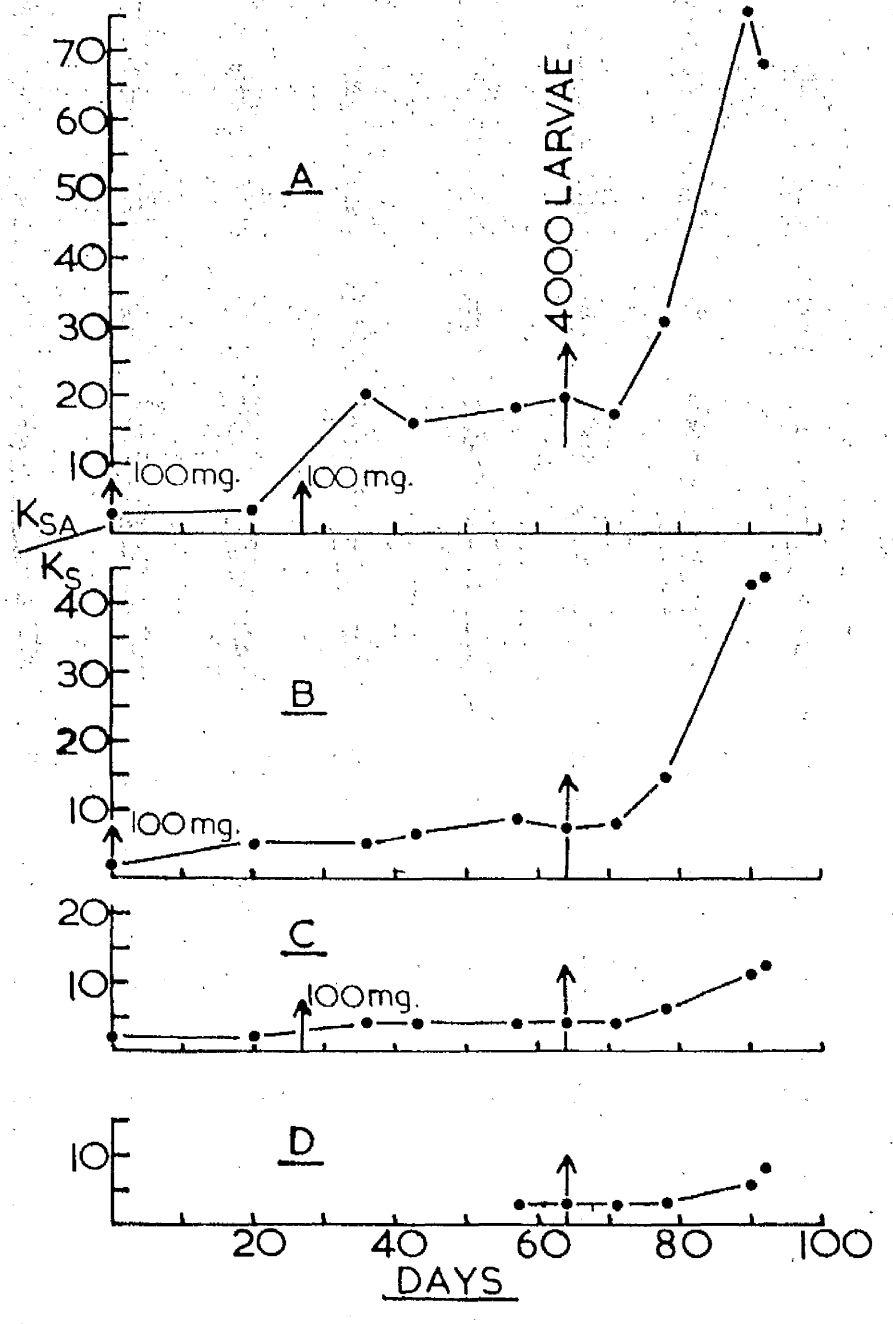


Fig. 17 Mean level of complement-fixing antibodies after immunization with adjuvanted whole worm vaccine and challenge with 4000 *D. viviparus* larvae.



The animals of group A gave a good antibody response to the second injection, and a very marked response to challenge. In groups B and C where only one injection was given, the antibody response at challenge was better in group B (i.e. where the time interval between injection and challenge was longer). There was no apparent correlation between antibody response resulting from challenge and the number of worms found at autopsy. The levels of complement fixing antibody for each calf are shown in Fig. 16, and the mean level for each group is shown in Fig. 17.

The lungs of all the calves in each group showed some degree of consolidation and details of the histopathology of the lesions have been reported (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1960).

Experiment 2. Whole worm adjuvanted vaccine with a range of challenge.

Although the results of Experiment 1 were disappointing in that the course of vaccination failed to confer any marked degree of protection against the challenge dose, it was felt that further experiments were desirable to determine the efficacy of this method of vaccination against (a) smaller challenge doses and (b) a challenge in the form of small repeated doses of infective larvae.

The experimental calves were divided into 4 main groups as shown in Table 24. Half of the animals in

TABLE 24

Number of Worms found at Autopsy in Immunized and Control Calves challenged with 'Single' and 'Divided' doses of *D.viviparus* larvae.

Challenge	Vaccinated			Control		
	Calf No.	No. of worms	Lesion score	Calf No.	No. of worms	Lesion score
Group A 2000 larvae single dose	596	60	6	589	-	-
	592	-	-	606	137	8
	600	-	-	594	330	4
	554	325	4	549	120	6
	555	90	4	545	450	6
	570	-	-	573	590	6
	546	40	4	588	550	2
	580	-	-	632	380	2
	526	147	4	527	-	-
	623	100	6	572	226	4
	556	200	4	609	180	2
Mean		123±92	4.5		329±162	4.5
Group B 2000 larvae administered as 200/day on alternate days	528	238	4	539	480	4
	622	123	2	522	203	4
	576	185	4	518	240	-
	544	73	4	599	277	6
	593	140	4	617	195	1
	540	21	4	575	212	4
	621	128	2	634	164	4
	532	140	4	605	333	6
	602	73	4	558	224	6
	553	101	4	562	105	1
	531	0	2	587	312	4
Mean		111±65	3.5		250±95	3.8

TABLE 24 (continued)

Challenge	Calf No.	No. of worms	Lesion score	Calf No.	No. of worms	Lesion score
Group C 500 larvae single dose	586	40	6	578	96	6
	534	4	2	568	221	4
	581	42	4	533	105	2
	519	63	4	559	88	6
	585	71	4	571	94	4
	629	120	4	550	53	4
	574	69	4	628	80	4
	616	36	2	567	115	6
	547	0	1	583	70	4
	612	93	4	633	2	2
	565	28	-	563	50	4
Mean		51±35	3.5		89±52	4
Group D 500 larvae administered on alternate days as 50/day.	552	8	2	607	69	2
	569	135	2	543	90	2
	604	7	2	566	56	2
	564	24	2	584	83	4
	541	23	2	536	111	4
	590	66	2	561	63	4
	582	39	2	625	6	2
	608	22	2	517	65	2
	529	61	2	597	80	4
	598	1	2	635	56	4
	551	49	1	618	73	2
Mean		39±37	2		68±27	3

each group received a course of 2 intramuscular injections of adjuvanted vaccine with an interval of 3 weeks between injections. All the calves were challenged with infective larvae according to the schedule shown in Table 24. The total dose in the case of the single challenge (groups A and C) and the first dose of the series in the case of the multiple dose challenge (groups B and D) were administered 6 weeks after the second injection. After a lapse of a further 35 days all the calves were autopsied. The numbers of worms recovered from the lungs are shown in Table 24. At autopsy a lesion score* ranging from 1 to 10 was allocated to each pair of lungs. This was compiled on a basis of 10 points to lungs, corresponding in degree of consolidation to that of a susceptible calf given 4,000 larvae (i.e. about 60% consolidation) and a score of 1 corresponding to a few scattered lobules of collapse. The other intervals used were 2, 4, 6 and 8. The lesions score for each pair of lungs are recorded in Table 24.

In each group there was an apparent reduction in "take" between vaccinated animals and controls, in groups A and B this was just significant ($P = .05$) while in groups C and D this just failed to be significant. There was no apparent difference between the two methods of administration of a standard number of larvae. The amount of lung damage as measured by the lesion score was relatively constant throughout the groups as a whole, tending to decrease as the number of larvae administered was reduced, and the amount of damage between vaccinates

* Pathological examination carried out by W.F.H. Jarrett.

and controls in each group was also constant despite the reduced numbers of worms reaching the lungs in the vaccinated animals. These lesions were different on microscopic examination (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1960) from those produced by an uncomplicated primary infection and can be regarded as an exaggerated response on the part of the host to the parasite. This general type of reaction has been recorded by Taliaferro and Sarles (1939) in the skin and lungs of rats actively immunized by repeated infections, and passively immunized by hyperimmune serum against Nippostronglus muris.

Experiment 3. Intraperitoneal Vaccination.

It is possible that one of the "immunological barriers" which is operative in the natural infection is the mesenteric lymph nodes through which the larvae pass in their migration to the lungs, and in an attempt to bring the antigenic stimulus into as intimate contact with these nodes as possible it was decided to attempt active immunization by intraperitoneal injection.

The calves involved in this experiment were divided into three groups as indicated in Table 25. Group A received an intraperitoneal injection of adjuvanted vaccine (10 ml) as described previously, group B received an intraperitoneal injection of fresh lungworms homogenised in saline (equivalent to approx. 100 mg dried worm material). Approximately six weeks after this injection each calf in group A and B, and

TABLE 25.

Number of worms recovered in immunized and control animals.

	Calf No.	No. of worms	Lesion Score
Group A	38	290	8
i/p whole	39	350	8
worm in	40	400	6
saline.	41	300	8
		Mean 335 ± 51	7.5
Group B	49	0	7
i/p adjuvanted	50	310	10
dried worm	53	250	10
material.	54	96	7
	56	145	7
		Mean 160 ± 123	8.2
Group C	57	490	10
Control.	58	22	7
	60	340	10
	61	600	-
	62	650	-
		Mean 420 ± 252	9.0

each calf of the control group C received 4,000 normal infective D. viviparus larvae administered orally. The calves were killed and autopsied 30 days later. The numbers of worms recovered from each pair of lungs together with an assessment of the amount of lung damage (lesion score) are recorded in Table 25.

There was an apparent reduction in the worm burden in Group B as compared to control calves, this just fails to be significant ($P > .05$). There was no reduction in the amount of lung damage, and even in the case of the calf in which no worms were found at autopsy (calf 49) the lung damage was extensive.

Discussion.

These experiments on active immunization against D. viviparus infections using whole worm antigens were to a large extent unsuccessful, but several interesting points were raised. In Experiment 1 no significant reduction in worm burden to a challenge infection of 4000 normal infective larvae could be demonstrated, although there was a marked serological response to the test antigen. In Experiment 2 using a smaller challenging dose of infective larvae Groups A and B showed a significant ($P = .05$) reduction. This beneficial effect is cancelled out on examination of the amount of lung damage in these groups and the fact that in Group A 4 control animals and 2 experimental animals died. Groups C and D of this experiment just failed to be significant. In Experiment 3 the only group where there was an apparent reduction in "take" was group B

but this again failed to be significant and once again the amount of lung damage was of the same as in the control animals.

This exaggerated response in the lungs of vaccinated animals to the smaller number of worms present, illustrates a fundamental principle in vaccinating against a disease of this type and that is, that the immunity produced must not only be strong enough to reduce the number of worms developing from a challenging infection, but the amount of lung damage must be reduced to such a level that the clinical response (reflecting pathological changes) is insignificant.

The results of these experiments show that it is possible to stimulate a 'degree of immunity' against D. viviparus by injection of dead whole worm material, but this immunity is not of such a high degree as would justify its use under practical conditions, and in addition the manifestation of the exaggerated response in the lungs resulting in equally severe lung damage as in normal infection would make it unacceptable as a method of controlling the disease.

The use of a whole worm vaccine in the field would also be obviously limited since lungworms for the production of such a vaccine would have to be obtained from the lungs of culture animals infected with the disease, and the cost would prohibitive.

Summary.

1. Experiments on the vaccination of calves against D. viviparus infection using whole worm antigens

are described.

2. A marked serological response occurred when the immunized calves were subjected to a challenge infection, but this was not accompanied by a significant reduction in the number of parasites.

3. In all the immunized animals the amount of lung damage was comparable to that found in the controls.

Section IV

Studies on *Dictyocaulus viviparus* infection in the bovine.

The immunity resulting from the administration of x-irradiated larvae.

The immunity resulting from the administration of x-irradiated larvae.

Introduction.

It has been established that in experimental infections calves which have recovered from an initial infection with *D. viviparus* are strongly resistant to a subsequent reinfection with this parasite, and that this immunity can be transferred passively.

The results of subsequent attempts at active immunization with freeze-dried adult lungworm preparations were, however, relatively disappointing, and it may be that the stimulation of protective antibodies may be largely dependent upon the presence of antigens associated with the actively metabolising parasites (cf. Thorson, 1953). Another factor which may determine the efficacy of the natural infection in conferring an immunity is the route of stimulation. If these considerations are valid the ideal immunogenic agent would contribute all the antigens of the actively metabolising larvae and would bring these to bear on all the antibody producing sites in the body which would be stimulated during the course of a natural infection. It was considered that these conditions might be simulated by the use of larvae altered by some treatment so that, although stimulating immunity, they would not become adult and give rise to the clinical disease.

It has been known since 1916 (Tyzzer and Honeij, 1916) that x-rays have a deleterious effect on *Trichinella spiralis* larvae. They found that if encysted larvae were

subjected to the radiations of radium, it rendered them non-infective to mice. Schwartz (1921) fed rats on pork infected with T. spiralis which had been subjected to x-rays, and found that "relatively large doses" of x-rays prevent the parasites becoming adult, while smaller doses allowed them to reach structural but not sexual maturity. Semrad (1937) obtained more or less the same result, but states that 1200r. or more allows the parasites to become adult but the females are sterile. Evans, Levin and Sulkin (1941) also studied the effect of x-rays on development to sexual maturity, and found that a dose of 5000r. inhibited the development of the larvae while a dose of 2000-2500r. prevented the females becoming sexually mature. Alicata (1951) found that much higher doses were necessary to prevent sexual maturity, 9000-10,000r. Since 1953 a series of papers by American workers mainly on the effect of radiation, both with x-rays and with γ-rays from cobalt-60, on the development of T. spiralis and the possibility of irradiation of infected pork to make it safe for human consumption, have been published, (Gomberg and Gould, 1953; Gould, Von Dyke and Gomberg, 1953; Gould, Gomberg and Bethell, 1954; Gomberg, Gould, Nehemias and Brownell, 1954; Gould, Gomberg, Bethell, Villella and Hertz, 1955; Gould, Gomberg, Villella and Hertz, 1957).

In only two of these papers was there any indication that the feeding of x-irradiated larvae of T. spiralis resulted in an increased resistance to reinfection with the parasite (Levin and Evans, 1942; Gould et al. 1955, (Part IV)).

In view of the findings of these two groups, and although the life cycle of the two parasites are dissimilar, it was decided to attempt attenuation of D. viviparus infective larvae with x-rays, and the following part of this thesis describes experiments using these x-irradiated larvae as immunizing agent.

Methods and Materials.

Infective Larvae.

The larvae were cultured as described previously.

X-irradiation of infective larvae.

An aqueous suspension of D. viviparus infective larvae were placed in a Petri dish to a depth of 1cm. This dish was placed in the beam of an x-ray machine running at 140 Kv, 5 mA with external filtration of .25 mm. Cu and 1.0 mm. Al. until the requisite dose (measured at the surface of the suspension) had been delivered. The calibration* of the x-ray machine was carried out using a Baldwin-Farmer Sub-standard dose meter.

Experimental animals.

Ayrshire bull calves were purchased when 2 - 7 days old and reared in individual metal houses. The calves were 8 - 10 weeks old at the commencement of the experiment.

* Calibration was carried out by the staff of the Western Regional Physics Department.

Infection of calves.

The infection of the calves with the x-irradiated and normal D. viviparus larvae was carried out as described previously.

Serology.

Blood samples were removed from each of the experimental animals at approximately weekly intervals, the serum removed and deep frozen until the experiment was completed. The serum samples were then tested for complement fixing antibodies as described previously.

Results.

Experiment 1.

An initial pilot experiment was carried out on nine calves divided into 3 groups and 4000 x-irradiated D. viviparus larvae were administered by mouth. The three levels of irradiation in the first experiment were 5000r, 10,000r and 20,000r. These calves were challenged 50 days after vaccination with 4000 normal infective D. viviparus larvae and autopsied 30 days after this challenge dose. During the immunizing phase all the calves showed clinical symptoms which in general were graduated in severity, 5000> 10,000> 20,000 and in all the calves the disease became patent. On autopsy 30 days after the challenge dose, the numbers of worms recovered indicated a high level of immunity, but as no control animals were available, and the fact that the disease had become patent during the immunizing phase, no conclusions about the efficacy of the

immunizing dose could be drawn from this pilot experiment. However, the graduation in clinical symptoms during the immunization phase gave an indication that the degree of attenuation of the larvae was insufficient to prevent patency during this phase and that further experiments were desirable using higher levels of irradiation.

Experiment 2.

The 45 calves in this experiment were divided into 9 groups of 5 calves each as indicated in Table 26. Groups A_1, A_2 received 4000 D. viviparus larvae subjected to 20,000r., groups B_1, B_2 received 4000 larvae at 40,000r. level and C_1, C_2 received 4000 larvae at 60,000r. level. D_1, D_2 received 4000 normal infective larvae and group E, which acted as controls for the challenge infection, did not receive larvae until groups A_2, B_2, C_2, D_2 received their challenging dose of 4000 normal infective larvae.

Groups A_1, B_1, C_1, D_1 were autopsied 35 days after the initial dose of larvae, the worms removed from the bronchi and counted. The number of worms recovered from the three irradiated groups, compared with those which developed when normal infective larvae were administered, gave a measure of the degree of inactivation produced by the three levels of irradiation (Table 26).

The remaining calves (groups A_2, B_2, C_2, D_2) were challenged with 4000 normal infective D. viviparus larvae 50 days after the initial immunizing dose, along with group E, which acted as controls. These calves were then autopsied 35 days after the administration of this

TABLE 26

Immunization of calves with *D viviparus* larvae irradiated at different levels.

Group	Calf No.	Immunizing phase: X-ray dose (roentgens)	Immunizing phase: No. of larvae day 0	Immunizing phase: No. of worms day 35	Immunizing phase: Lesion score day 35		Challenge phase: No. of worms day 85	Challenge phase: Lesion score day 85
A1	1			7	1			
	2	20,000		6	1			
	3		4000	0	2			
	4			8	4			
	5			0	1			
				4.2 ±3.5	1.8			
A2	6					Challenged 4,000 normal larvae Day 50	48	8
	7						4	4
	8	20,000	4000				14	4
	9						2	2
	10						10	2
							15.6 ±16.7	4.0
B1	11			2	1			
	12			0	0			
	13	40,000	4000	2	4			
	14			5	2			
	15			1	0			
				2.0 ±1.7	1.4			
B2	16						11	2
	17						4	2
	18	40,000	4000				2	0
	19						49	2
	20						2	2
							13.6 ±18	1.6

TABLE 26 (continued).

Group	Calf No.	Immunizing phase: X-ray dose (roentgens)	Immunizing phase: No. of larvae day 0	Immunizing phase: No. of worms day 35	Immunizing phase: Lesion score day 35	Challenge phase: No. of worms day 85	Challenge phase: Lesion score day 85
C1	21			0	0		
	22			0	0		
	23	60,000	4000	0	0		
	24			0	0		
	25			0	0		
				0	0		
C2	26					1650	10
	27					314	4
	28	60,000	4000			153	4
	29					909	6
	30					1573	6
						919.8 ±619	6.0
D1	31			180	2		
	32			1081	8		
	33	0	4000	1100	8		
	34			950	4		
	35			1120	10		
				906.2 ±373	6.4		
D2	36					27	10
	37					4	4
	38	0	4000			15	4
	39					2	4
	40					9	2
						11.4 ± 9	4.8
E	41					825	8
	42					865	8
	43	0	0			1452	10
	44					1788	10
	45					992	8
						1188.4 ±375	8.8

Challenged 4,000 normal larvae Day 50

challenging dose, the worms removed from the lungs and counted. These results are also shown in Table 26.

The results indicated that only small numbers of worms developed when larvae irradiated at 20,000 and 40,000r. were administered, while the efficacy of this immunizing dose was also clearly seen, the immunity produced was equivalent to that of animals which had been through a full scale infection and were reinfected (group D_2). In the case of larvae irradiated with 60,000r. no worms developed (group C_1). On challenge these animals reacted (with the exception of the serological responses which will be discussed later) as if they had never been in contact with the disease, as similar numbers of worms were recovered from the lungs of this group and the controls (group E).

A distinct increase in respiratory rate in the experimental calves indicated a clinical reaction during the immunizing phase, the exception being the 60,000r. group, which showed no reaction. This group however, reacted similarly to the control group E on challenge.

The mean serological responses of the different groups are shown in Fig. 18. Until challenge at 50 days, the individual lines represent the mean of the 10 calves in each group ($A_1 + A_2$, $B_1 + B_2$ etc.) while after challenge they represent the mean of 5 calves (groups A_2, B_2, etc.). The serological response in all groups during the immunizing phase was negligible. After receiving a challenge of 4000 normal larvae, all the animals showed a marked secondary response, with the exception of those receiving larvae irradiated at

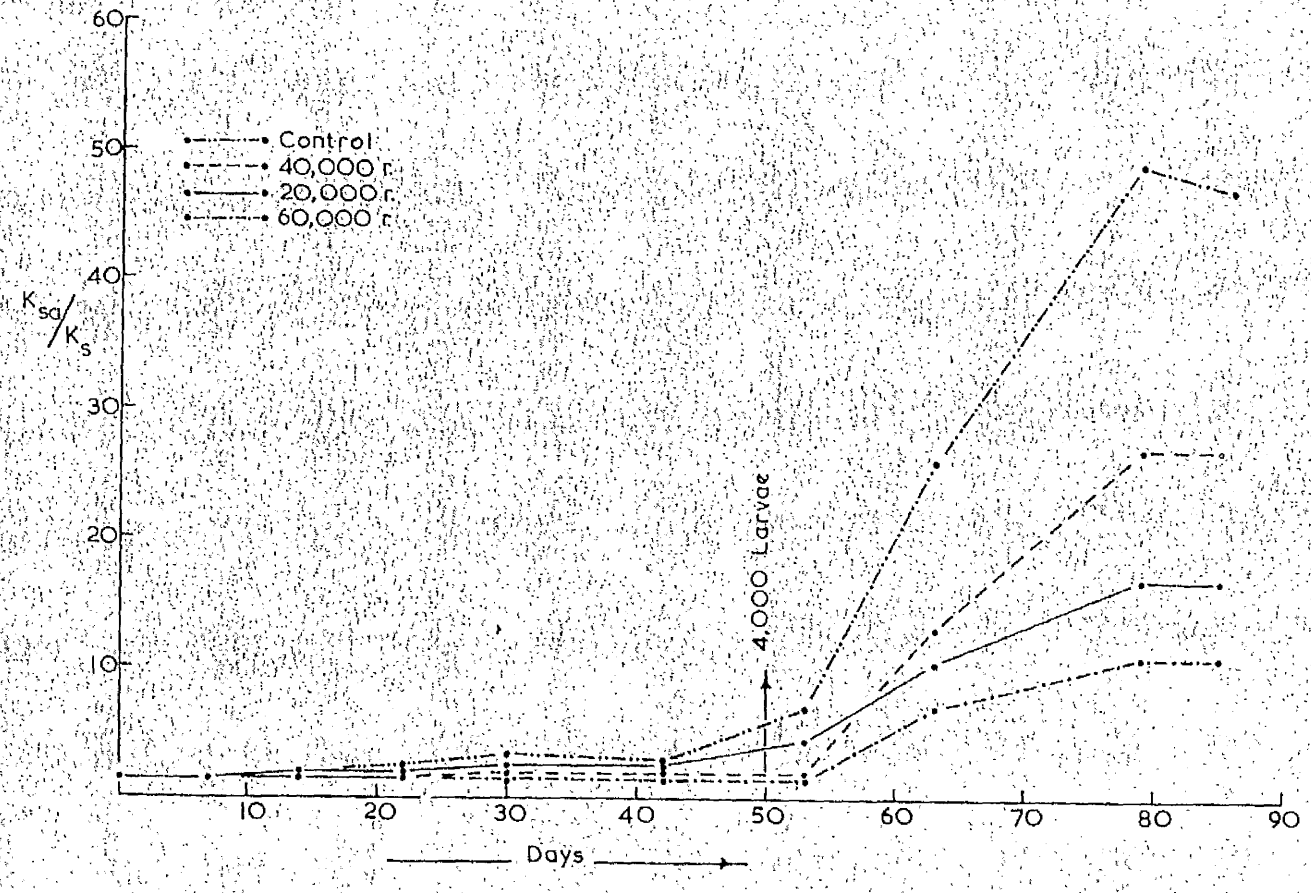


Fig. 18 Mean serum antibody levels in groups of calves immunized at day 0 with irradiated larvae and later challenged with 4000 normal larvae per calf. Control group received only 4000 normal larvae per calf at day 50.

60,000 roentgens. In this group, the response was very much smaller and was due to only two of five animals. Also on examination of clinical symptoms and numbers of worms recovered on autopsy (Table 26) there was no evidence of immunity in this group although some of the animals reacted serologically as if they had been in previous contact with the disease. The conclusions drawn from these results was that irradiation with 60,000r was outside the upper limit for the production of immunity, but was not sufficient in every case in preventing larvae from penetrating the gut wall and sensitizing the antibody producing sites in the body.

The general picture emerging from the results of this experiment was that 4000 D. viviparus larvae exposed to at least 40,000 and certainly less than 60,000r of x-irradiation will give rise to an immunity which will withstand a challenge of at least 4000 normal infective larvae, i.e. a dose of larvae which would produce in the non-immune animal a severe case of clinical disease. However, as there was a clinical response in the calves during vaccination, it was fairly clear that attempts would have to be made to reduce the number of irradiated larvae. In addition if complete failure of the attenuation of the larvae should occur, 4000 larvae would constitute a sub-lethal dose and severe clinical husk would result. In view of these considerations a further experiment was set up to test the degree of immunity produced by administration of smaller numbers of x-irradiated larvae.

Experiment 3.

The 25 calves were divided into 5 groups as shown in Table 27. Each of the calves in group A received 1000 larvae and those in group B received 2000 larvae, all of which had received 40,000r. of x-rays. In view of the findings in the previous experiment, that larvae irradiated with 60,000r produced no immunity, and that there was evidence in two of the calves that some of the larvae were reaching the antibody producing sites, it was decided to investigate the effects of a dose of radiation intermediate between 40,000 and 60,000r. Therefore, the calves of group C each received as an immunizing dose 4000 larvae irradiated with 50,000r.

There was also the possibility that the irradiation procedure killed a large proportion of the larvae, and the immunization effect observed was due to the few survivors. Therefore group D received 100 normal larvae as an immunizing dose.

Group E acted as controls for the experiment and received no treatment until the immunized animals were challenged with 4000 normal larvae.

The numbers of worms recovered at autopsy 30 days after the challenge dose of 4000 normal infective larvae are recorded in Table 27, together with the assessment of the pathological lesions * based on the severe case of husk in the normal infection having a lesion score of 10.

* Carried out by W.F.H. Jarrett.

TABLE 27

Number of worms recovered from the lungs of calves receiving irradiated larvae and challenged with 4000 normal larvae.

Group	Calf No.	Immunizing dose day 0		No. of worms day 80	Lesion Score
A	11		Challenged 4,000 normal larvae Day 50	0	2
	12	1000 larvae		0	1
	13			43	3
	14	40,000r.		68	3
	15			0	1
				22.5	1
B	17			1	0
	18	2000 larvae		3	1
	19			0	1
	20	40,000r.		2	2
	21			6	4
				2.4	1.6
C	24			0	0
	25	4000 larvae		8	4
	26			0	2
	27	50,000r.		0	1
	28			0	3
				1.6	2.0
D	29			4	4
	30	100		1	4
	32	normal		188	4
	33	larvae		285	8
	34			1	6
				95.8	5.2
E	57			490	10
	58			22	7
	60	0		340	10
	61			600	-
	62			650	-
				420	9.0

In the calves of group A which received 1000 larvae irradiated with 40,000r, an average of 22.5 worms was found in the lungs at autopsy and 3 out of the 5 calves were completely immune to this challenge dose. The other two calves had 43 and 68 worms respectively.

The calves receiving 2000 larvae irradiated with 40,000r (group B) showed a higher degree of immunity, having an average of only 2.4 worms at autopsy. Similarly those calves which received 4000 larvae irradiated with 50,000r (group C) also were immune to challenge and had a mean of 1.6 worms in the lungs at autopsy.

The calves which received 100 normal larvae as an immunizing dose (group D) also showed a degree of immunity as the mean worm burden of 95.8 was largely due to 2 out of the 5 calves in the group. However, on examination of the gross lesions in the lungs, this group was very much more severely affected than those groups receiving the irradiated larvae.

These results indicated that even a dose of 1000 larvae irradiated with 40,000r gave a fairly good degree of immunity when the calf was challenged with 4000 normal infective larvae, and that the amount of lung damage was very small. However, the control animals which had received 4000 normal larvae had a mean worm burden of only 420 $\pm$ 252 instead of the more usual one of approximately 1000 worms from this size of larval dose. This may have been due to a lower infectivity of the larvae, and as a consequence the challenge may only have been in the order of 1600 viable larvae.

The conclusions drawn from this experiment were

therefore qualified by this supposedly low challenge and were that 1000 larvae irradiated at 40,000r would give a reasonable degree of immunity to a relatively low challenge dose of larvae and that the clinical symptoms both during the immunizing and challenge phase (of lesion score) were of such a low order that this method of immunization could safely be used under field conditions.

Summary.

1. Experiments are described on the immunity produced by third stage D. viviparus larvae which had been treated with various levels of x-irradiation.
2. A high degree of immunity was produced by the use of 4000 D. viviparus larvae treated with 40,000 roentgens. The animals withstanding a challenge dose of 4000 normal infective larvae.
3. A still satisfactory, but lower immunity, was obtained with 1000 larvae treated with 40,000r.

GENERAL DISCUSSION.

There is no doubt that an immunity to many helminth diseases can be acquired by the host animal. The ease with which this resistance is acquired and the strength and duration of the immunity produced vary widely with different host-parasite systems. It has also been demonstrated that the presence of living larvae in the host animal is necessary in order to obtain a high degree of immunity. The injection of dead worm materials, although highly immunogenic, fails to elicit to any extent the production of functional protective antibodies.

If living larvae are used for immunization and are administered to the host animal by the normal route of infection, a degree of parasitism results with consequential spread of the disease. Attempts to overcome this objection by administration of the infective larvae by an abnormal route have met with some success in Ascaris lumbricoides infections in guinea pigs (Oliver-Gonzalez, 1956; Soulsby, 1957). In the case of Haemonchus contortus infections of sheep, although Stoll (1958) reported that he could obtain a good degree of immunity by either subcutaneous or intraperitoneal injections of exsheathed third stage larvae, neither Stewart (1959) nor Silverman and Patterson (1960) could confirm his results. There is also the danger that even when infective larvae are administered by an abnormal route some may eventually reach their usual loci and develop normally with dissemination of the disease.

Davtyan (1955) states that in the case of active immunization against Dictyocaulus filaria, the sheep lungworm, the parenteral injection of live larvae cannot be recommended as some may develop normally.

If one accepts that live larvae and/or antigens produced by living larvae are necessary for the development of a good immunity, then there are a number of possible approaches to the immunization problem.

Firstly, one can attempt to maintain and grow larvae in axenic culture and collect the antigens released into the media at the various stages in the worms' development. These antigens can then be administered parenterally to the host animal to stimulate immunity. Work along these lines is being done in a number of laboratories but as yet no results either of success or failure have been published.

Secondly, one may administer larvae by the normal route of infection and then after a suitable interval attempt to eliminate this infection. This method is dependent on an exact knowledge of 'development - time' relationships of the parasites, of which particular stage or stages give rise to immunity, and finally on having a really effective larvaecidal drug which will prevent all the parasites reaching maturity.

Thirdly, there is the approach carried out in this thesis, i.e. using larvae inactivated by treating with ionizing radiation. In considering vaccination by this or any other method two important factors must be considered. The immunization procedure should produce

as mild a clinical reaction as possible and this should be of short duration, i.e. the pathogenicity of the vaccine should be of a low order. Furthermore, this vaccination procedure should not give rise to an adult worm population capable of producing fertile eggs and/or larvae with the danger of spread of the parasitic disease. In the case of Dictyocaulus viviparus these two conditions have been satisfied by using a suitable dose of x-rays for the irradiation of the infective larvae. These irradiated larvae when administered to the host animal complete only part of their life cycle but are still capable of eliciting a good immunity. The dose of x-rays required for sterilization of the parasite is considerably less than that required to stop the majority completing their normal life cycle, and therefore the few worms which occasionally reach the adult stage in the lungs are sterile and the danger of spread of infection by the immunization procedure is negligible.

An additional advantage of the use of irradiated larvae for immunization is that they can be administered via the normal route of infection thus stimulating all the sites involved in the normal infection. The importance of this factor is difficult to assess in the light of present knowledge or lack of knowledge on the significance of local immunity in helminth infections.

Apart from their use in active immunization irradiated larvae may have important applications as an experimental tool in fundamental studies on helminth immunity. It may be possible by the use of a range of

x-ray doses to stop larval development at various stages, thus enabling one to determine the relative importance of different parasitic stages in the production of immunity in the host animal.

In the case of gastro-intestinal parasites such as Haemonchus contortus, the inability to distinguish between the worm populations which develop from a primary and secondary infection has been a problem. One solution is to administer larvae subjected to an appropriate dose of x-irradiation as the secondary infection. This population being composed of sterile females can be easily distinguished from the normal worm burden of the primary infection.

The most important applications for x-irradiated larvae will probably be in the field of vaccination against parasitic infections. As reported in this thesis, when it was established that 4000 D. viviparus larvae irradiated with 40,000r could protect animals against a sub-lethal dose of 4000 normal larvae, the possibility of applying this as a field vaccine was envisaged. It was felt necessary, because of the number of unknown factors in the field, to lower the immunizing dose of irradiated larvae, and therefore it was reduced to 1000. This allowed a greater safety margin as even 1000 normal larvae should not prove fatal to calves.

This work has since been extended and a single dose of 1000 larvae irradiated with 40,000r. has been used with success both under normal farm conditions and in an experimental field trial (Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp and Urquhart, 1958).

At the same time experiments were undertaken to determine the efficacy of two doses of irradiated larvae (Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart, 1959). Two doses of irradiated larvae were found to give superior immunization and this schedule has been used in an experimental field trial (Jarrett, Jennings, McIntyre, Mulligan and Sharp, 1961). A vaccine against parasitic bronchitis based on this work is now being widely used on farms.

Subsequent to the success achieved in the initial irradiation experiments with D. viviparus, this method has been applied with considerable success to other host-parasite systems, e.g. in Haemonchus contortus infections in sheep (Jarrett, Jennings, McIntyre, Mulligan and Sharp, 1959), in H. placei infections in calves (Ross, Armour, Hart and Lee, 1959), in Trichostronglus colubriformis infections in sheep (Jarrett, Jennings, McIntyre and Sharp, 1960), in T. colubriformis infections in guinea pigs (Gordon, Mulligan and Reinecke, 1960), and in Uncinaria stenocephala infections in dogs (Dow, Jarrett, Jennings, McIntyre and Mulligan, 1959, 1961).

GENERAL SUMMARY

Section I

1. Prior immunization of rabbits with the proteins of Fasciola hepatica resulted in a retardation in development of the parasites without a significant reduction in the numbers which developed from a challenge with 50 cercariae. The F. hepatica proteins were highly immunogenic as they elicited a marked antibody response in the sera of the immunized rabbits.

2. Prior immunization of rabbits with a polysaccharide fraction of F. hepatica resulted in one experiment in a significant reduction in the numbers of flukes but in a second experiment only in a retardation of development of the flukes. In both experiments the rabbits were challenged with 50 cercariae.

Section II

3. A measurement of the amount of blood lost by fluke-infected rabbits was made using ^{32}P-labelled red cells and ^{131}I-labelled plasma albumin. The amount of blood present in the flukes was calculated by a comparison of their radioactivity with that of the circulating blood at the time of autopsy. The calculated blood loss per day was consistent with the observed degree of anaemia.

4. The simultaneous use of ^{32}P-labelled red cells and ^{131}I-labelled plasma albumin suggested that the flukes preferentially absorbed and/or retained ^{32}P (labelled red cells) relative to ^{131}I (labelled plasma albumin).

5. A preliminary experiment on the use of ^{51}Cr-labelled red cells in estimating blood loss gave a higher value for blood loss per fluke than either ^{32}P-labelled red cells or ^{131}I-labelled plasma albumin.

Section III

6. A study was made of the serological response and the degree of immunity in calves resulting from primary and subsequent infections with the bovine lungworm, Dictyocaulus viviparus. During the primary infection the level of complement fixing antibodies in the serum rose slowly and reached a peak after the bulk of the infection had been thrown off. At the second and third infection a typical secondary response was observed. Administration of the primary infection as a series of divided doses did not materially alter the serological response. The resulting immunity of both single and divided primary infections was very good.

7. Passive immunization of calves with serum from 'hyperimmune' donor animals resulted in a high degree of immunity when the calves were challenged with 4000 normal infective D. viviparus larvae.

8. Prior immunization of calves with whole worm antigens of D. viviparus did not produce a significant reduction in the numbers of worms which developed from a challenge infection with normal infective larvae.

Section IV

9. Prior immunization of calves with D. viviparus larvae treated with a suitable dose of x-rays resulted in a very high degree of immunity. An immunization dose of 4000 larvae treated with 40,000r enabled calves to withstand a challenge of 4000 normal larvae.

10. Prior immunization of calves with 1000 D.viviparus larvae irradiated with 40,000r resulted in an acceptable degree of immunity without any marked clinical reaction during immunization.

11. The potential value of the irradiation method both in immunization against worm diseases and as an experimental tool in the study of helminth immunity is discussed.

12. An extension of this work has led to the first field vaccine against a parasitic worm.

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