Immunology, 1959, **2**, 252.

Immunological Studies on *Dictyocaulus viviparus* Infection

THE IMMUNITY RESULTING FROM EXPERIMENTAL INFECTION

W. F. H. JARRETT, F. W. JENNINGS, W. I. M. MCINTYRE,
W. MULLIGAN, BEATRICE A. C. THOMAS AND G. M. URQUHART*

Glasgow University Veterinary School

* Present address: East African Veterinary Research Organisation, Muguga, Kikuyu, Kenya.

Summary. It has been shown experimentally that infection of calves with *D. viviparus* confers a high degree of resistance to a subsequent reinfection. 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. 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 reaching the lungs, and in the numbers of larvae appearing in the faeces.

INTRODUCTION

PARASITIC bronchitis is an important disease of cattle and is caused by the helminth *Dictyocaulus viviparus*. It is well established that a strong immunity to this infection can be acquired under field conditions (Wetzel, 1948; Taylor, 1951; Jarrett, McIntyre and Urquhart, 1954). Until recently, little work had been done on experimental animals to demonstrate the nature of this acquired resistance and to explore its quantitative aspects both from the serological and protective points of view.

Porter and Cauthen (1942) and Rubin and Lucker (1956) have shown the development of immunity to reinfections in experimental calves. Taylor and Michel (1952) reported that larvae of *D. viviparus* invading the lungs of a partially resistant host were retarded in their development. The experiments described in the present paper were designed to study the resistance acquired by repeated infections and the pattern of antibody response. These experiments were the first in a series in which immunity was demonstrated by reinfection, by hyperimmune serum (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1955), by whole worm adjuvanted vaccine and by larvae treated by X or by gamma rays. Preliminary accounts of some of this work have been reported elsewhere (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1957).

MATERIALS AND METHODS

SEROLOGICAL METHODS

Sera from the experimental animals were tested for complement fixing antibodies by a modification of the quantitative procedure described by Maltaner and his colleagues (for references see Kabat and Mayer, 1948, p. 97). The experimental details are outlined below.

Test Sera

Blood samples were collected from the jugular vein of the experimental animals, allowed to clot at room temperature and the sera separated. 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 of up to one year. A titration was carried out on each batch and for each day's testing a dilution was prepared so that 0·025 ml. corresponded approximately to one 50 per cent haemolytic unit (the 'arbitrary' 50 per cent unit).

Antigen

The test antigen was prepared by a method similar to that employed by Stewart (1950) for *H. contortus* antigen: 0·3 g. of freeze-dried adult worm material was used per 25 ml. After centrifugation the supernatant was diluted ten times with 0·85 per cent NaCl for use in the test. The antigen was always used within 24 hours of preparation, as it was found that the titre of a standard serum varied slightly with the age of the antigen.

Sheep Red Cells

Sheep blood was collected using heparin as anti-coagulant and the cells were washed at least four times with ice-cold saline before use; when cells were not used on the day of collection they were stored at 0–4° in an equal volume of modified Alsever's solution (see Kabat and Mayer, 1948, p. 107). The concentration of 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. tube gave a galvanometer reading of approximately 50 in the E.E.L. colorimeter with filter No. 625.

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 per cent NaCl. Equal volumes of adjusted sheep red cell suspension and haemolysin solution were mixed and kept at room temperature for at least 30 minutes before use.

Standard Serum

A large sample of relatively high titre serum was divided into 3 ml. lots and stored at −20°. For each day's testing, one of these samples was removed, dilutions prepared and the titre determined. The use of such a standard serum has been recommended (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 as well as to correlate results obtained with different batches of complement.

Pre-colostral Serum

Pre-colostral serum (P.C.) is that obtained from a calf prior to the ingestion of colostrum and contains no antibodies. It was found most useful as a control serum and also as a diluent for test sera.

Setting up of Test

Doubling dilutions of the sera to be tested are 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 inacti-

vation at 56° C. for 30 min. a further dilution (1–5) with 0·85 per cent NaCl is carried out, followed by mixing and centrifugation at 0° C. for 20 min. The sera are then ready for testing. The test is carried out in E.E.L. colorimeter tubes (4 ml.) and is set up as shown in Table 1.

TABLE 1

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
	Dil. 8	21	8	0·25	1·0	0

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

† The volumes contributed by the complement are in all cases very small (e.g. 8 units equals 0·2 ml.) and it has been found convenient to ignore these, since the final volume in the tubes at the time of reading is 3·75 ml.

The tubes containing the reagents described in Table 1 are agitated and incubated for one hour at 37° C.; 0·5 ml. haemolytic system is then added to each tube. The tubes are thoroughly shaken and reincubated at 37° C. for a further hour. After refrigeration overnight the tubes are prepared for reading by the addition of a suitable volume (2·0 ml.) of 0·85 per cent NaCl to each, mixing and centrifugation.

Reading the Test

The tubes are 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 are used to zero set the instrument for the reading of tubes 3 and 4 which contain haemolytic system and excess complement. From these measurements one obtains a reading for 100 per cent haemolysis.

Series C. Tube No. 10 which contains only test serum and haemolytic system is used to zero set the instrument for the rest of the series. This corrects for any background colour due to the serum itself. Tubes 11 and 12 give a measure of the haemolysis occurring with serum, complement and no antigen; 13, 14 and 15 that with serum, complement and

antigen. Tubes 16–18, 19–21, etc., are the counterparts of 13–15, with the further dilutions of serum.

Series B. These tubes cover a complete test with a known negative serum. It is 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 one can calculate the percentage haemolysis in each tube. From the percentage haemolysis and the number of arbitrary 50 per cent units present in any one tube the number of units of complement required to produce exactly 50 per cent haemolysis is 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 percentage haemolysis lies between 20 and 80 per cent are used for the calculation as this range corresponds to the sensitive part of the haemolysis curve. The potency of a serum is 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 per cent haemolysis in the presence of immune serum and antigen, and K_s the amount required to give 50 per cent haemolysis in the presence of immune serum alone. This method of expressing the result automatically corrects for any anti-complementary effect due to the serum. In testing a serum the dilution where suitable partial haemolysis occurs will depend on the potency of that particular serum. All index ratios obtained at dilution 2, 4, 8, etc., have to be suitably corrected. In making this correction it must be borne in mind that the index ratio of a serum cannot be less than 1·0, as one unit of complement is necessary to produce 50 per cent 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 1. In the case of a serum where it is possible to calculate index ratios from more than one dilution good agreement is usually obtained.

The test with pre-colostral serum (Series B) enables 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 a dark damp atmosphere. After 7–10 days the infective larvae were extracted 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 was examined microscopically and the numbers 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 washings from each pair of lungs were allowed to stand overnight and the sediment examined. As a further check for the presence of worms, approximately 50 per cent of each pair of lungs was minced and examined by the Baerman technique.

Before washing, a number of blocks of tissue were removed from the lungs for histological examination. Fixation was by formol-sublimate, the blocks being dehydrated and cleared in an alcohol-amyl acetate-benzene series and finally embedded in paraffin under reduced air pressure. Routine staining was by Haemalum and Eosin.

RESULTS

EXPERIMENT I

Infection with Three Consecutive Single Doses of D. viviparus *Larvae*

In this experiment ten two-month-old calves were each infected with 2500 infective *D. viviparus* larvae; 160 days later each of the calves was dosed with 4500 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 32 days after the last infection. The parasitological and serological findings are described below.

TABLE 2

FAECAL LARVAL COUNTS DURING THREE CONSECUTIVE INFECTIONS WITH *D. viviparus*

Calf No.	*First infection Maximal McMaster count/g.*	*Results of Baerman Examination*		*Number of worms in lungs at autopsy*
		Second infection	*Third infection*	
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.

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 g. of faeces as determined by the McMaster technique is shown in Fig. 1, while the maximal number of larvae per g. of faeces recorded for each calf is shown in Table 2.

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 six of the calves at any point during the course of the second infection. The faeces of the remaining four calves contained larvae on several occasions between the thirty-fifth and seventy-first day after the second infection (see Table 2).

Third Infection. Larvae were not found in faeces of the six calves whose faecal larval output had been negative during the course of the second infection, nor in the faeces of two of the calves which had been positive on Baerman examination at that time. The

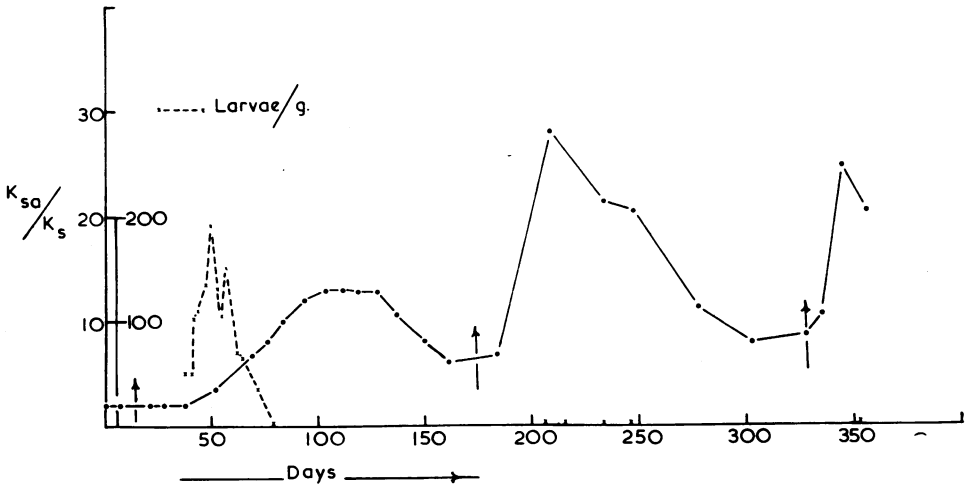

FIG. 1. Mean faecal larval output (-x---x-) and serum antibody level (—·—·) during three consecutive infections with *D. viviparus larvae.* Arrows indicate the administration of larvae.

faeces of the remaining two calves were positive on Baerman examination on the twenty-fifth and twenty-eighth day after infection respectively (see Table 2).

Serology

The mean level of complement fixing antibodies for the ten calves throughout the course of the experiment is shown in Fig. 1.

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

Morbid Anatomy and Histology

At autopsy the lungs appeared almost completely normal. In each case there were a few lobular areas of consolidation, and histologically these were typical of an infection

of over 120 days' duration (Jarrett, McIntyre and Urquhart, 1957). In six of the ten calves there were present a few lesions of the type frequently found in immune animals and consisting of a cellular reaction to dead parasites: these changes will be described in detail elsewhere.

EXPERIMENT 2: INFECTION OF CALVES PREVIOUSLY EXPOSED TO SMALL REPEATED DOSES OF *D. viviparus* LARVAE

In this experiment each of five 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

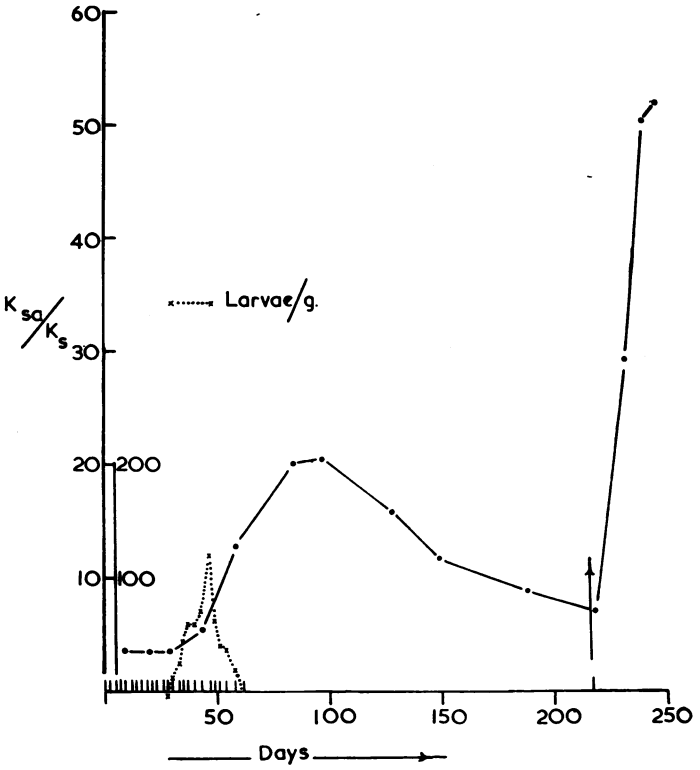

FIG. 2. Mean faecal larval output (-x—x-) and serum antibody level (——·) resulting from successive small doses of *D. viviparus* larvae followed later by a single large dose. Arrows indicate administration of larvae.

days (see Fig. 2). One hundred and fifty-five days after the last dose of the infection schedule, four of the calves were challenged with 15,000 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 five calves is shown in Fig. 2, and the peak numbers of larvae per g. of faeces recorded from each calf are shown in Table 3.

Although a total of 7500 larvae was administered in the divided doses to each calf it can be seen that the highest faecal count recorded in Fig. 2 was only 120 per g. and that larval output terminated 62 days after the first dose of the infection schedule (*cf.* Fig. 1).

Second Infection. Faecal examination up to the date of autopsy failed to reveal any larvae. The numbers of worms recovered from the lungs at autopsy are recorded at Table 3.

TABLE 3

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*		*Number of worms in lungs at autopsy*
		Size of dose	*Baerman results*	
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 five calves throughout the course of the experiment is shown in Fig. 2. 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.

Histology

In four calves which were challenged with 15,000 larvae, the lungs were almost completely normal; one calf (2) showed a few lobules of active parasitic pneumonia and in all there were a few bronchi with an excess of plasma cells in the lamina propria. The broncho-mediastinal lymph nodes were grossly hyperplastic.

The calf (1) which was challenged with 90,000 larvae showed a marked pulmonary cellular response to dead larvae and adult worms. The detailed lesions will be described elsewhere; they represent a foreign body response to worms which have reached the lungs before being killed by the immune reaction.

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. 1 to a maximum of about 200 larvae per 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 faecal 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, owing 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 is 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 2), 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 (Jarrett, McIntyre and Urquhart, 1957).

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 twenty-five doses of 300 larvae each administered over a period of 62 days, i.e. a total of 7500 larvae. It is interesting to note that mean faecal larval output in this experiment rose to a maximum of about 120/g. as compared to 200/g. in Experiment 1, when only 2500 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 30-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). Likewise it is not possible to be categorical concerning the origin in time of the lesions found in the lungs at autopsy. In Experiment 1 the consolidated areas were of a type that suggested that they resulted from a primary infection, although the first re-infection cannot be excluded. The lymphoid and giant cell reaction to the fourth stage larvae and adults, however, probably occurred during the second reinfection and may represent an exaggerated reaction to the few nematodes dying on reaching the lung. We have found this lesion causing widespread pulmonary damage in adult bovines dying from natural exposure to *D. viviparus*, and it seems likely that this arises when a partially immune animal is subjected to a massive challenge. The pathogenesis and significance of this lesion will be discussed in a later paper.

ACKNOWLEDGMENTS

This work was supported by a grant from the Agricultural Research Council.

REFERENCES

CRAIG, C. F. and FAUST, E. C. (1951). *Clinical Parasitology*, 5th ed. Henry Kimpton, London.

JARRETT, W. F. H., MCINTYRE, W. I. M. and URQUHART, G. M. (1954). 'Husk in cattle. A review of a year's work.' *Vet. Rec.*, **66**, 665.

JARRETT, W. F. H., JENNINGS, F. W., MCINTYRE, W. I. M., MULLIGAN, W. and URQUHART, G. M. (1955). 'Immunological studies on *Dictyocaulus viviparus* infection. Passive immunization.' *Vet. Rec.*, **67**, 291.

JARRETT, W. F. H., MCINTYRE, W. I. M. and URQUHART, G. M. (1957). 'The pathology of experimental bovine parasitic bronchitis.' *J. Path. Bact.*, **73**, 183/93.

JARRETT, W. F. H., JENNINGS, F. W., MCINTYRE, W. I. M., MULLIGAN, W. and URQUHART, G. M. (1957). 'The natural history of parastic bronchitis with notes on prophylaxis and treatment.' *Vet. Rec.*, **69**, 1329.

KABAT, E. A. and MAYER, M. M. (1948). 'Experimental Immunochemistry.' 1st ed. Charles C Thomas, Springfield, Illinois.

PORTER, D. A. and CAUTHEN, G. E. (1942). 'Experiments on the life history of the cattle lungworm *Dictyocaulus viviparus*.' *Am. J. vet. Res.*, **3**, 395.

RICE, CHRISTINE E. (1942). 'Studies of antipneumococcal serum. II. Complement-fixing activity of antipneumococcal rabbit-serum with homologous type-specific carbohydrate. Technic of Test. General quantitative relationships among reagents'. *J. Immunol.*, **43**, 129–48.

RUBIN, R. and LUCKER, J. T. (1956). 'Acquired resistance to *Dictyocaulus viviparus*, the lungworm of cattle.' *Cornell Vet.*, **46**, 88.

STEWART, D. F. (1950). 'Studies on resistance of sheep to infestation with *haemonchus contortus* and *trichostrongylus* species and on the immunological reactions of sheep exposed to infestation. 1. The preparation of antigens for the complement fixation test and the reactivity of the biochemical fractions of *haemonchus contortus*.' *Aust. J. Agric. Res.*, **1**, 285.

TAYLOR, E. L. (1951). 'Parasitic bronchitis in cattle.' *Vet. Rec.*, **63**, 859.

TAYLOR, E. L. and MICHEL, J. F. (1952). 'Inhibited development of Dictyocaulus larvae in the lungs of cattle and sheep.' *Nature, Lond.*, **169**, 753.

WETZEL, R. (1948). 'Zur Epidemiologie des Lungenwurmbefalls bei Rindern.' *Mh. Vet. Med.*, **3**, 141-8.