HOST-PARASITE RELATIONSHIPS OF *PARAMPHISTOMUM MICROBOTHRIUM* FISCHOEDER, 1901, IN EXPERIMENTALLY INFESTED RUMINANTS, WITH PARTICULAR REFERENCE TO SHEEP*

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CONTENTS

INTRODUCTION

Paramphistomiasis of sheep and cattle is a disease which results in serious economic loss to the wool, meat and milk industries. In the past decade hundreds of sheep have perished, while in 1965 alone, at least 64 deaths occurred in cattle in Southern Africa.

The conical fluke *Paramphistomum microbothrium* Fischoeder, 1901, is widely distributed in Africa, the adjacent territories of the Mediterranean and the Near East (Lengy, 1960; Arfaa, 1962; Eisa, 1963; Dinnik, 1964), and has been incriminated in all cases of clinical paramphistomiasis in Africa (Dinnik, 1964).

Boray (1959) and Dinnik (1964) summarized the publications on paramphistomiasis. A study of the literature indicates that little attention has been paid to the host-parasite relationships.

In the present study, therefore, emphasis has been placed on the host-parasite relationships in experimentally infested sheep, goats and cattle.

Throughout these investigations certain standard procedures were followed which will now be described.

MATERIALS AND METHODS

Metacercariae

Methods of collection, counting, storage and infestation have been described (Durie, 1955; Swart & Reinecke, 1962; Horak, 1962a, 1964).

Experimental animals

Animals reared and housed paramphistome-free were used throughout. The abbreviations S, G or B preceding the experimental number of an animal indicate whether that animal was a sheep, goat or bovine. The animals used in a particular experiment were not necessarily infested at the same time because of a shortage of metacercariae. Certain animals were used in more than one experiment, e.g. controls in an immunity experiment may also have been included in a life cycle study.

Faecal worm egg counts

The worm egg count technique was similar to that described previously (Anon., 1962). The modifications were: faeces were collected at midday; one gram of sheep and goat and two grams of cattle faeces were used; sedimentation time was reduced from 10 to six minutes; instead of decanting, the clear supernatant fluid was siphoned off.

Autopsy procedures

These have been described (Horak, 1962b, 1964, 1965b). In addition the first nine metres of the small intestine were divided by double ligatures into three equal portions of three metres each, and the remainder to the ileo-caecal valve regarded as a separate unit.

All ingesta was washed on a sieve with a strong stream of water and the material trapped on the surface examined for worms. A sieve with apertures of 53 microns (300 mesh to the linear inch) was used for infestations less than nine days of age,

while infestations containing worms between nine and fourteen days old were washed on 200 mesh sieves (aperture 76 microns). All older infestations were washed on 100 mesh sieves (aperture 152 microns). The gall-bladder was also examined for worms.

Occasionally the omasum, caecum and colon were not examined as these organs were generally found to harbour few worms.

Percentage take

Throughout these experiments this term will indicate the total number of paramphistomes recovered at autopsy expressed as a percentage of the total number of metacercariae dosed.

Fixation and examination of worms

A representative sample of the worms recovered was transferred to a bottle containing 100 ml of normal saline. This bottle was thoroughly shaken for two to three minutes and formalin added to a dilution of approximately four per cent. Shaking was continued for a further half minute and the paramphistomes stored until required. Thirty worms from each of a large number of animals were measured. A method of killing the worms at 70°C and then preserving with formalin was discarded as the measurements were too variable.

The measurements recorded are illustrated in Fig. 1 and were: length A to B; breadth C to D; depth E to F; acetabulum G to H.

EXPERIMENTAL OBSERVATIONS

The data are summarized in tables in the appendix and presented graphically in the text.
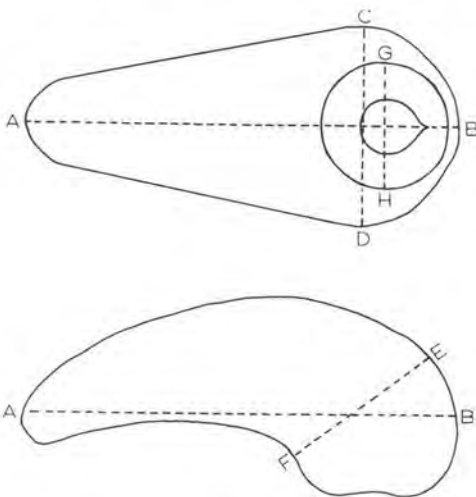
FIG. 1.—Measurements carried out on paramphistomes

Length:	A to B
Breadth:	C to D
Depth:	E to F
Acetabulum:	G to H

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PART I

PARAMPHISTOMUM MICROBOTHRIUM

1. *Metacercariae*

Because of the importance of using viable metacercariae an *in vitro* method of testing the viability of *P. microbothrium* metacercariae was developed by Horak (1962a). The reliability of this *in vitro* test was confirmed *in vivo*.

Srivastava (1938) found that a small percentage of the metacercariae of *Cotylophoron cotylophorum* (Fischoeder, 1901) Stiles & Goldberger, 1910, kept on moist leaves at room temperature, remained viable for a maximum period of four months.

Horak (1962a) showed that the viability of metacercariae of *P. microbothrium* was not affected for at least 29 days if stored at room temperature or at 4°C. He also showed that fungal contamination severely reduced metacercarial viability. This contamination became a problem particularly when metacercariae were stored at room temperature and for this reason all metacercariae were stored at 4°C.

Because of the large numbers of metacercariae required for some of the experiments to be described later, it was necessary to accumulate and store metacercariae for prolonged periods at 4°C. As the survival of these metacercariae was an unknown factor an experiment was carried out to ascertain their viability *in vivo*.

Experimental observations

Metacercariae were collected, counted and stored at 4°C. Ten sheep were divided into pairs; one member of each pair received metacercariae varying in age from 47 to 539 days and the other, metacercariae four to 18 days of age. Each pair was slaughtered seven to 26 days after infestation, the paramphistomes recovered and counted. The results are summarized in Table 1.

A comparison of the percentage take between members of each pair showed that metacercarial viability decreased slightly at 47 to 63 days of age and thereafter rapidly with increasing age.

Discussion

In the light of these results, wherever possible, metacercariae stored at 4°C and between the ages of four and 40 days were used. Occasionally older metacercariae were included, but only to supplement numbers when there was a shortage of metacercariae. Therefore, any reduction in the number of worms recovered, would be due to factors other than metacercarial viability.

Having solved the problem of metacercarial storage, life cycle studies could commence.

2. *Life cycle in the definitive hosts*

A. *Sheep, goats and cattle*

Dinnik & Dinnik (1954) found that after experimental infestations with metacercariae of *P. microbothrium*, the percentage take and size of worms were larger in cattle than in goats. Deiana, Lei & Arru (1962) noted that *Paramphistomum cervi* Schrank, 1790, recovered from goats were larger than those recovered from sheep.

The first eggs of *P. microbothrium* were passed in the faeces of sheep, 69 days after infestation (Arfaa, 1962). The first eggs passed in the faeces of calves were noted 87 to 107 days after experimental infestation, and faecal egg counts were maintained at a high level in cattle for many years (Dinnik & Dinnik, 1962).

The following experiments involved a comparison of the life cycle of *P. microbothrium* in three definitive hosts.

Experimental observations

(i) *A comparison of the life cycle in sheep, goats and cattle*

Twenty-one animals were divided into seven equal groups; each group consisting of a single sheep, goat and bovine. The animals in each group were infested with an equal number of 11-day old metacercariae and slaughtered four to 487 days later. Two similar additional groups were infested with either 5,000 or 10,000 metacercariae each. The cattle in these groups were not killed but the sheep and goats in both groups were slaughtered 391 days after infestation.
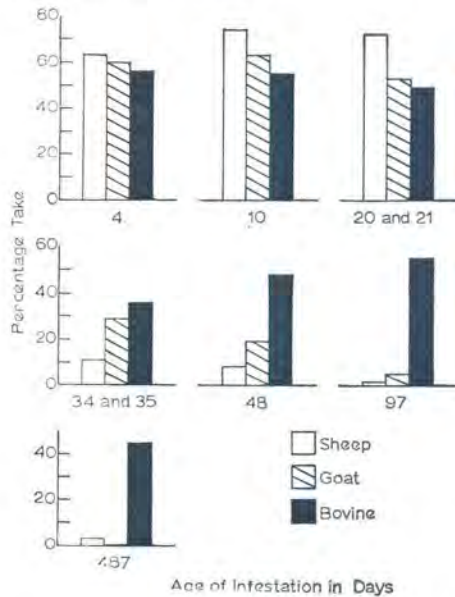
FIG. 2.—The percentage take in sheep, goats and cattle at various stages after infestation

Percentage take and rate of migration: These results are summarized in Table 2; Fig. 2 illustrates the percentage take.

A greater percentage take was recorded in sheep and goats than in cattle, four to 21 days after infestation. Thereafter as the age of infestation increased the percentage take in sheep and goats usually decreased, whereas in cattle it remained constant between 36·0 and 55·5 per cent. Forward migration in cattle was more rapid than in sheep, in which it was more rapid than in goats.

Mature worms in all hosts were found attached at two or three sites in the rumen. The majority of paramphistomes was present on the dorsal aspect of the anterior pillar, with lesser numbers attached to both the dorsal and ventral surfaces of the posterior pillar.

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Worm size: The results, summarized in Table 3, include the size of worms of similar ages recovered from the various hosts, as well as worms recovered three hours after artificial excystation. Fig. 3 to 8 illustrate the frequency distributions of the measurements of the worms four to 487 days old.

The worms grew rapidly and at four days of age were twice as large as those three hours old. They doubled in size between four and 10 days and again during the next 24 days. Finally the 34-day size was nearly trebled 487 days after infestation.

With the exception of four and 10-day old worms the paramphistomes recovered from cattle were always larger than those from goats, which were usually larger than those in sheep.

Comment: The total length measurement showed the greatest range of variation; the breadth, depth and acetabular measurements exhibited considerably smaller ranges of variation and appeared to be a more accurate indication of size than the total length. The variations in total length can be ascribed to the varying degrees of curvature of the worms after fixation (Fig. 1).

The reliability of the sample of 30 worms measured as an indication of size was tested when the breadths and acetabula of a further 100, 21-day old worms from a bovine (B3) were measured. The average measurements obtained were: breadth 1·04 mm; acetabulum 0·68 mm; while these measurements obtained from 30 worms were 1·03 mm and 0·66 mm respectively. Therefore, no useful purpose would be served by measuring more than 30 worms to obtain their average sizes.
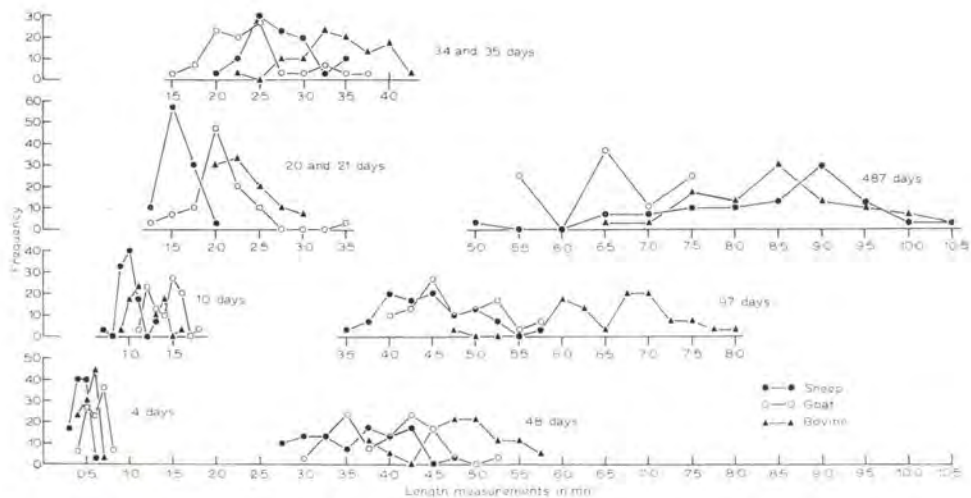
FIG. 3.—Frequency distributions of the length (A to B) of paramphistomes in sheep, goats and cattle at various stages after infestation
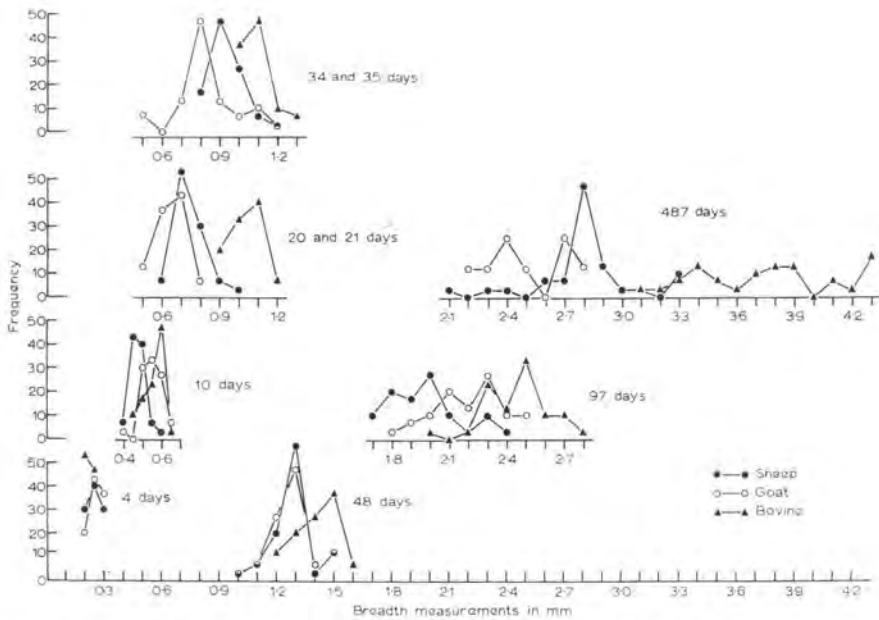
FIG. 4.—Frequency distributions of the breadth (C to D) of paramphistomes in sheep, goats and cattle at varous stages after infestation
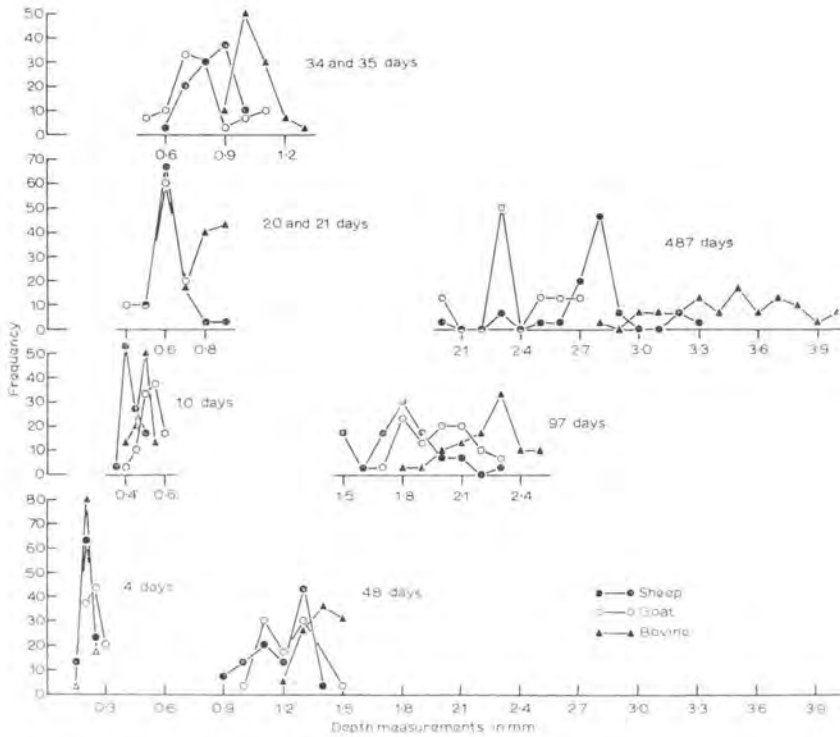
FIG. 5.—Frequency distributions of the depth (E to F) of paramphistomes in sheep, goats and cattle at various stages after infestation
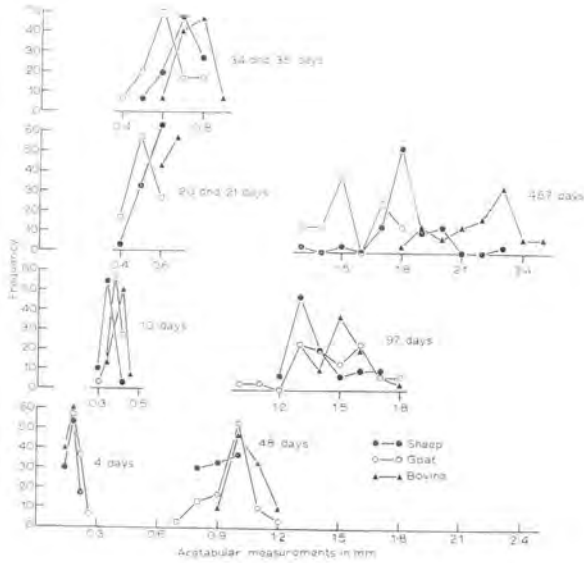
FIG. 6.—Frequency distributions of the acetabular size (G to H) of paramphistomes in sheep, goats and cattle at various stages after infestation

FIG. 7.—Frequency distributions of the length, breadth, depth and acetabular measurements of paramphistomes from a sheep and goat infested with 5,000 metacercariae and slaughtered 391 days later
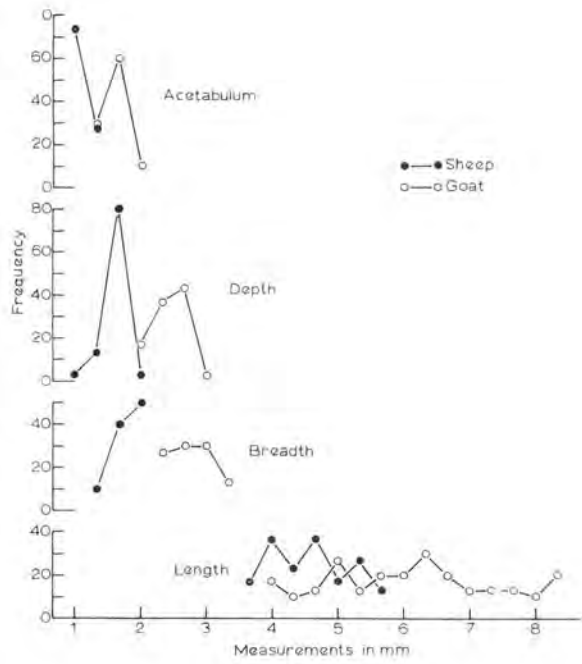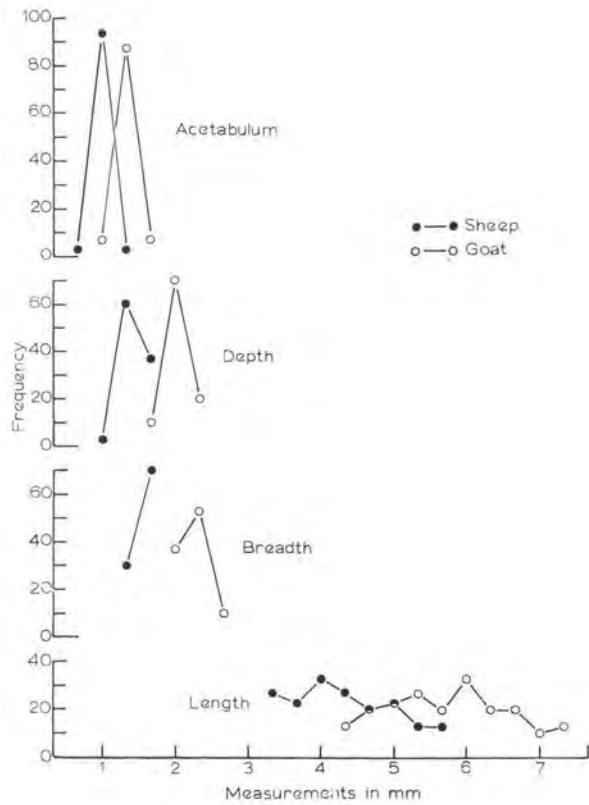
FIG. 8.—Frequency distributions of the length, breadth, depth and acetabular measurements of paramphistomes from a sheep and goat infested with 10,000 metacercariae and slaughtered 391 days later

The effect of worm size on migration: Until the majority of paramphistomes reach a certain size they are unable to migrate from the small intestine to the rumen. Using the average acetabular measurement as a criterion of size it can be seen that until this measurement reached 0·56 mm no significant migration from the small intestine took place (Tables 2 and 3).

In a sheep and a bovine 20 and 21 days after infestation the acetabulum had reached 0·56 and 0·66 mm respectively and migration from the intestine had commenced, whereas in a goat the acetabulum was 0·52 mm and nearly all the worms were still in the intestine. At 34 and 35 days after infestation the acetabular measurements in a sheep and a bovine were 0·70 and 0·77 mm respectively and were made from worms in the rumen as migration was practically complete. In a goat the acetabulum was 0·63 mm and migration had just commenced (Tables 2 and 3).

Faecal worm egg counts: In those infestations that reached patency, the day that the first egg appeared in the faeces was noted. The average number of eggs per gram (e.p.g.) of faeces during each 30-day period after infestation was recorded. This average was based on one to eight egg counts per 30-day period.

The average worm egg counts in sheep, goats and cattle for each 30-day period are shown in Fig. 9, each group of three animals having received a different number of metacercariae. Fig. 9 also indicates the day on which the first paramphistome egg was present in the faeces.

The first egg appeared in the faeces of cattle 56 days, of goats 69 days, and of sheep 93 days after infestation. Four sheep were subsequently infested with 10,000 metacercariae each. Three of these sheep passed paramphistome eggs in their faeces 71 days later.

The prepatent period in cattle is considerably shorter than that given by Dinnik & Dinnik (1962), while the prepatent periods in the sheep and goat confirm the results of Arfaa (1962) in an experimentally infested sheep.

In both sheep and goats infestation with 5,000 metacercariae resulted in higher faecal egg counts than infestations with 2,000 or 10,000 metacercariae; while in cattle the highest egg count followed infestation with 10,000 metacercariae.

Faecal egg counts were higher in a goat (G7) infested with 5,000 metacercariae than in cattle (B7 and B8) infested with 5,000 or 10,000 metacercariae respectively, or in a bovine (B9) which had approximately the same worm burden (Table 2). No direct comparison is possible as the faecal output of cattle exceeds that of goats and the greater mass of faeces in cattle would dilute the concentration of eggs.

Faecal egg counts in cattle reached a peak seven to 13 months after infestation and were maintained at this level for the duration of the experiment. In goats peak egg counts were reached in the fourth to eighth month but declined thereafter. In sheep egg counts remained at a low level.

(ii) *Worm distribution*

A goat, a sheep and a bovine were each infested with a single large dose of metacercariae; they died or were slaughtered 23 to 33 days later. At autopsy the first three-metre portion of the small intestine was divided by double ligatures into nine equal portions, each 33 cm long. The total number of worms in the first three metres of small intestine and their distribution in the nine equal sub-divisions of this intestine are summarized in Table 4.

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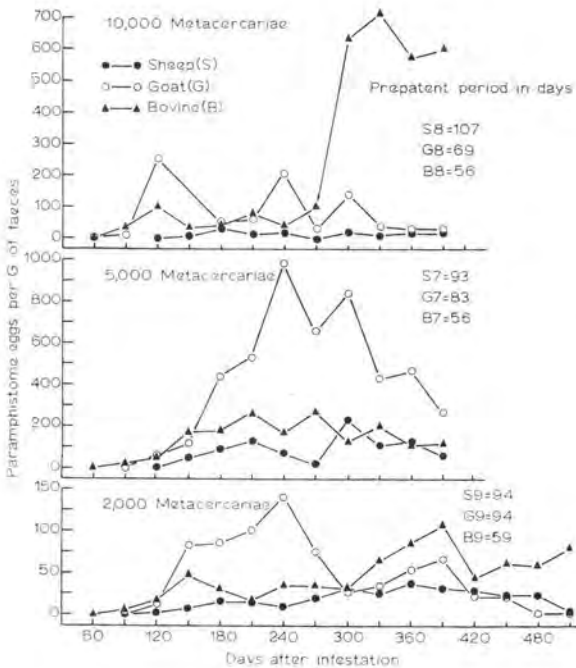
FIG. 9.—The average paramphistome egg counts in sheep, goats and cattle for each 30-day period after infestation

Worm distribution in the small intestine in infestations 23 to 33 days of age varied with the host species. In cattle worms were most numerous within 33 cm of the pylorus and fairly evenly distributed two to three metres from the pylorus. In sheep and goats worms were most numerous 66 to 100 cm from the pylorus, tending to decrease posteriorly.

In all species fewer worms were recovered 33 to 66 cm from the pylorus than elsewhere in the first metre of intestine. This is probably due to the fact that the bile duct opens in this portion or just anterior to it and the high concentration of bile creates an unfavourable environment.

(iii) *Fluctuations in faecal worm egg counts*

Faeces, for worm egg counts, were collected at various times throughout the day from infested housed sheep, goats and cattle, which were fed at 8.00 a.m. and again at 2.00 p.m. Examples of the fluctuations in egg counts are given in Table 5.

Faeces collected in the morning contained the least number of eggs. The egg counts rose sharply towards the middle of the day and then fell gradually during the afternoon.

The increase in worm egg counts during the middle of the day may be due to fluctuations in egg-laying by the worms, or to the physiological processes of the host such as more complete emptying of the reticulum during feeding. These findings are similar to those obtained by Dorsman (1956) in housed and grazing cattle infested with *Fasciola hepatica* Linnaeus, 1758.

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Faecal worm egg counts and moisture content: Faeces were collected at hourly to two hourly intervals from an infested goat, and the worm egg count and moisture content of each faecal sample determined.

In the goat (G7) used in this experiment worm egg counts rose from 42 e.p.g. at 6.00 a.m. to 304 e.p.g. at 12.00 noon and then showed an erratic decline to 240 e.p.g. at 6.00 p.m. (Table 5). The moisture content of the faeces fluctuated between 36·38 and 42·34 per cent throughout the day. These fluctuations would not account for the rise in egg counts at midday.

The average daily egg production of a single paramphistome: The total daily faecal output of a sheep with an adult patent infestation of *P. microbothrium* was collected for six days prior to slaughter. Faecal egg counts were done daily on two separate, one-gram representative samples and total daily output of eggs calculated. At autopsy the number, sites of attachment and percentage of paramphistomes containing eggs were noted, and the average daily egg production of a single paramphistome estimated. The results are presented in Table 6.

The similarity between egg counts of the two different faecal samples confirmed the validity of the method of assay.

Only when the faecal output decreased, i.e. days 3 and 4, did the average worm egg counts rise. Otherwise they were remarkably similar.

The infestation, comprising 837 worms, was 548 days old (S51, Table 7), but only 85·3 per cent of the worms contained eggs. The remainder may have been in a state of degeneration similar to that described in natural infestations in cattle by Dinnik & Dinnik (1962). The estimated average daily egg output of one of these fertile worms was 75·1.

Approximately equal numbers of paramphistomes were attached to the dorsal aspect of the anterior ruminal pillar and to the ventral aspect of the posterior pillar, a lesser number were attached to the dorsal surface of the posterior pillar (Table 6).

Discussion

The life cycle in domestic ruminants: Horak (1962a) has shown that after artificial infestation 38·0 to 90·0 per cent of metacercariae excyst in the alimentary tract and the remainder are excreted unexcysted with the faeces.

While most of these young worms attach themselves to the walls of the anterior six metres of small intestine, a few are situated posteriorly and a few are also found in the abomasum and growth commences immediately. Four to 10 days after infestation worms in the posterior intestine migrate to the anterior three metres of small intestine. Simultaneously worms are recovered in slightly larger numbers from the abomasum, indicating further forward migration. Migration to the rumen proceeds gradually as the worms have to grow to the size necessary for migration and the first few worms are recovered from the rumen 20 days after infestation. As the worms increase in size massive forward migration takes place which is virtually complete at 34 days in cattle and sheep but not in goats. After reaching the rumen worms migrate to their sites of attachment on the anterior and posterior ruminal pillars.

After 48 days some worms may contain eggs. The first paramphistome eggs are present in the faeces of the definitive hosts 56 days after infestation in cattle, 69 days in goats and 71 days in sheep. Faecal worm egg counts reach a peak seven

to 13 months after infestation and are maintained at a high level for the duration of the infestation in cattle, while they decline after eight months in goats. There is no obvious pattern in sheep and egg counts are low (less than 240 e.p.g.).

During their sojourn in the small intestine the young paramphistomes not only grow but undergo a colour change. Four day old worms are dark brown, nearly black, similar to metacercariae. This colour is due to stellate congregations of pigment mainly on the dorsal surface of the worms. As these dendritic patches do not grow as rapidly as the worms do, they become dispersed, lighter in colour and at 10 days the worms have a brown spotted appearance. At 20 days the worms are a uniform pink colour and the dendritic patches are not readily discernible unless the worms are stunted.

Although migration is practically complete at 34 days, growth continues, but is more gradual as the parasite becomes older.

The normal host: Cattle appear to be the most suitable hosts used in these experiments. The percentage take was more consistent; migration to the rumen more rapid; worms larger; prepatent period shorter; egg production maintained at a higher level, and adult worms lived longer in cattle than in either goats or sheep. If cattle are not the normal hosts at least they are better " adapted " hosts than either of the other domestic ruminants. Other evidence supports this contention.

Dixon (1964) determined the relative suitability of sheep and cattle as hosts for *F. hepatica.* He found that flukes in sheep grew faster, more uniformly and to a greater size than those in cattle. The worms in cattle, however, produced twice as many eggs a day as those in sheep.

Dixon states " considered in the light of the first alternative, sheep would appear to be the more suitable host, whereas, if the decision is based on the second alternative, cattle would appear to be more favoured. The latter conclusion could be open to revision were the relative lengths of the patent periods and the relative fertility of the eggs also considered ".

With the exception of the relative fertility of eggs, which was not investigated, all these requirements were fulfilled for *P. microbothrium* by a single host, viz. cattle.

An apparent contradiction exists when considering the relative suitability of sheep or goats as definitive hosts. Although egg counts are lower and paramphistomes smaller, the percentage take of *adult worms* is usually higher in sheep than in goats.

If the percentage take of adult worms is used as a criterion, sheep are better hosts than goats, whereas the reverse is true if worm size and egg production are used as criteria.

In infestations up to 21 days of age the percentage take in cattle is lower than that in sheep or goats. Horak & Clark (1963) found that *P. microbothrium* was most pathogenic just prior to and during migration. The lower percentage take in cattle infested with 10,000 metacercariae during this phase of the life cycle, seems to indicate some degree of natural resistance to the worms during the most pathogenic stage of the life cycle. Thereafter, the percentage take in cattle remains constant irrespective of the age of infestation, suggesting an association which assures the survival of the parasite.

The life cycle differs in sheep and goats. The percentage take in the first three weeks is high and pathogenic effects enhanced. Conversely the violent reaction of these hosts during migration leads to the loss of large numbers of worms. Further reduction in numbers generally occurs during maturation and after patency, thus seriously affecting the longevity of this species in sheep and goats. The combination of pathogenic effects followed by poor survival of the parasites indicates that these animals are not suitable hosts.

Dineen (1963) states: " So long as infection with the parasite is potentially able to produce significant pathogenic effects then survival of the relationship will be favoured by some degree of relevant antigenic disparity. The effect of such disparity will be to confine pathogenicity within tolerable limits by control of the size of parasitic burdens. It is concluded, therefore, that the rôle of the immunological response in the ' adapted ' host-parasite relationship is to control the parasitic burden rather than to cause complete elimination of the infection ".

This statement supports the contention that cattle are the normal or " adapted " hosts of *P. microbothrium*. During that stage of the life cycle when significant pathogenic effects may occur the worm burden allowed is smaller than that in sheep or goats. The worm burden is not completely eliminated and adult worms persist in a long-term well tolerated association with the host. In sheep and goats this tolerance does not develop and the adult worm burdens gradually diminish.

Paramphistome populations: Overcrowding caused stunted growth. Evidence of this stunting is noted when sheep and goats dosed 5,000 and 10,000 metacercariae are compared (Compare S7 and G7 with S8 and G8, Table 3, Fig. 7 and 8).

Further investigations were necessary to determine the effect, if any, of overpopulation on the life cycle of *P. microbothrium*. This is of great veterinary importance as pathogenesis is directly related to the number of worms present.

B. *The effect of massive infestation on the life cycle in sheep*

Materials and methods

Forty-four sheep were each infested with a single dose of metacercariae varying from 5,000 to 210,000.

These sheep were slaughtered or died at different intervals after infestation. At autopsy the number, percentage take, distribution and size of the paramphistomes recovered were recorded. In seven autopsies worms recovered from more than one site of attachment were measured.

Results

The percentage take and the migratory pattern of *P. microbothrium* in 33 sheep are given in Table 7. Where possible sheep with moderate or severe infestations of approximately the same age are compared in this table; the sheep with the moderate infestation being listed first.

Percentage take: With one exception (S30), the percentage take in moderate infestations was less than that in heavier infestations of the same age. Worm survival tended to decrease with increasing age of infestation.

Migration: In the three day old infestation a large percentage (42·86) of paramphistomes was present in the second three metre portion of the small intestine. These worms migrated forward during the next 11 days so that at 14 days most of them were present in the first three metres of small intestine.

Paramphistomes were present in the abomasum in three days, and the first worms recovered from the rumen within 10 days of infestation. Migration was almost complete at 36 days in a moderate infestation (S37), but incomplete at 50 days in a heavier infestation (S44). Paramphistomes were recovered from the gallbladders of 13 sheep, particularly those with heavy infestations (Table 7).

Worm measurements: The measurements of paramphistomes from 25 sheep and those of three hour old worms obtained after artificial excystation are given in Table 8. Where possible, sheep with moderate and severe infestations of approximately the same age are compared, with the moderate infestations given first.

Growth: Worms grew rapidly until eight days of age and thereafter growth rate declined with age of infestation. At eight days a three- to five-fold increase had taken place. The eight-day size was twice as large at 40 days and this doubled again at 86 days of age (Table 8).

Worm size: Although considerable variations in size were noted in infestations of the same magnitude and age (S12 and S11, and S39 and S55, Table 8), worms from heavily infested sheep were always smaller than those from sheep with moderate infestations.

This is dramatically illustrated in Fig. 10, in which the frequency distributions of the acetabular measurements of worms of approximately the same age and from the same organs are compared. The light infestations are abstracted from the comparative life cycle studies in sheep, goats and cattle (Table 3, Fig. 6) and the heavier infestations from Tables 7 and 8.

The disparity in worm size between light and heavy infestations became more marked as the worms grew older.

The effect of worm size on migration: As previously observed (Tables 2 and 3) migration from the small intestine commenced when the acetabular measurements approached 0·56 mm (S54, S16, S33 and S38, Tables 7 and 8). In one sheep, however, migration had already started when the acetabula of the paramphistomes were only 0·37 mm, but the percentage of worms that had migrated was small (S27, Tables 7 and 8). Only when the acetabular size exceeded 0·60 mm were the majority of worms recovered from the rumen (S37, Tables 7 and 8).

Thus the stunting effect of over-crowding had as a direct result a delay in migration because the worms took longer to grow to a size large enough to permit migration to the rumen.

Site of attachment: The sizes of worms of the same age obtained from different sites in the alimentary tract of seven sheep are summarized in Table 9.

The worms present in the rumen were considerably smaller than those in the other organs 24 days after infestation (S59). In older infestations the worms in the rumen were considerably larger (S40 and S57) or the same size as those at other sites of attachment.

FIG. 10.—A comparison of the frequency distributions of the acetabular measurements of paramphistomes from sheep with light and heavy infestations
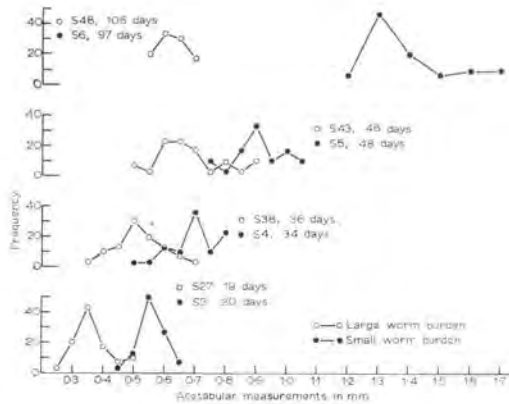
With the exception of two sheep (S59 and S60), the smaller size of the worms present in the first three metres of small intestine, was inversely related to the larger worm burdens present, when compared with the other sites of attachment. Once migration had taken place the few worms remaining in the first three metres of the small intestine were as large as those that had already migrated to the rumen (S44).

The worms attached to the anterior pillar of the rumen were slightly smaller than those attached to the posterior pillar, or the ruminal wall (S48).

Discussion

Migration and worm survival: In the comparative studies on the life cycle sheep were infested with small numbers of metacercariae and light worm burdens developed. These worms migrated from the intestine to the rumen between 20 and 34 days after infestation. During this migration large numbers were lost (Table 2).

In sheep with massive infestations migration on a large scale was delayed, only starting at 36 days and reaching completion 50 days after infestation. As in the comparative studies worm loss took place during migration, but commenced later and was more prolonged (Table 7). This explains why the percentage take in the more rapidly migrating populations, was lower than that in the slower migrating populations of the same age.

The worm distribution is dependent upon the age of infestation, distribution shifting anteriorly as the age of infestation increases (Table 7). The rate of migration is dependent upon the number of worms present, migration proceeding more rapidly in moderate or small populations than in large populations (Table 7). This confirms the observations of Thorpe (1965) that the migration of *F. hepatica* into the bile ducts of experimentally infested albino rats was delayed in severe infestations, when compared with lighter infestations.

The largest number of paramphistomes recovered from the rumen of a sheep irrespective of the method of infestation was 18,016 (S58, Table 8). It would appear as if there is a limit to the number of worms that can be accommodated in the rumen. Mukherjee & Deorani (1962), however, recovered 32,068 amphistomes from the rumen of a naturally infested sheep.

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Worm size: Size is extremely unreliable as it is affected by numerous factors. Factors which influence it are: the magnitude of the worm burden, although it may vary between hosts with similar burdens; the site of attachment and the number of worms present at a particular site (Tables 8 and 9, Fig. 10).

Size, therefore, cannot be used to estimate paramphistome age accurately.

C. *Goats*

From the comparative studies in sheep, goats and cattle it soon became evident that the life cycle of *P. microbothrium* in goats was not markedly different from that in sheep. For this reason the effects of massive infestation on the life cycle in this host were not investigated.

D. *The effect of massive infestation on the life cycle in cattle*

The life cycle, growth and prepatent period of *P. microbothrium* in cattle has been described by Dinnik & Dinnik (1954, 1962). Bennett (1936) described the life history of *C. cotylophorum* (considered identical to *P. microbothrioides* by Price & McIntosh, 1944), in cattle.

These workers infested cattle with moderate numbers of metacercariae, and found that the young conical fluke remained in the small intestine for a period of two and a half to six weeks before migrating to the rumen. In cattle with a severe, natural infestation, Butler & Yeoman (1962) found immature *P. microbothrium* in the intestine of an animal slaughtered 74 days after removal from the source of infestation.

An experiment to determine the effects of massive infestation on the life cycle of *P. microbothrium* in cattle was carried out.

Materials and methods

Eighteen cattle were each infested with a single dose of metacercariae varying from 2,000 to 305,000. These animals died or were slaughtered at various intervals after infestation. At autopsy the number, percentage take, distribution and size of paramphistomes were noted. In three cattle, worms from various sites of attachment were measured.

Results

The results of 15 cattle are summarized in Table 10.

Percentage take: With the exception of one animal (B23), in which the take was only 9·2 per cent, it varied between 21·7 and 67·5 per cent.

Migration: In massive infestations (50,000 or more worms) with the exception of one bovine (B13), a large percentage of paramphistomes was recovered in the second three metre portion of small intestine and occasionally in the third three metre portion up to 40 days after infestation. Worms were present in the abomasum and rumen eight and 14 days after infestation respectively (B11 and B12). Migration to the rumen was well advanced at 28 days in one bovine (B15), with 16,714 worms, while it had hardly commenced in another (B16), harbouring 102,103 worms. In two animals (B10 and B17) with more than 160,000 worms each, little migration had taken place at 33 and 40 days after infestation respectively.

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A bull and heifer twin (B20 and B21) were given practically identical numbers of metacercariae and slaughtered 52 days later. Migration was nearly complete in the one (B20) with 44,196 worms, whereas in the other (B21) with 72,252 worms it had just started.

These results confirm the observations in sheep that large worm burdens migrate more slowly than do moderate ones.

Worm measurements: The measurements of worms from 12 cattle are summarized in Table 11.

Similar comparisons to those made in sheep are illustrated in Fig. 11, in which the frequency distributions of the acetabular measurements of paramphistomes recovered from small and large populations are compared. The differences in size are even more marked than those in sheep.

The effect of worm size on migration: As previously shown the critical size that the acetabulum has to reach lies between 0·56 and 0·60 mm before migration from the small intestine can take place. In those infestations in which massive infestations suppressed growth, migration had hardly commenced (B13, B26 and B17, Tables 10, 11 and 12) despite the infestation in one bovine (B17) being 40 days old. In these animals the acetabular sizes varied between 0·52 and 0·60 mm.

FIG. 11.—A comparison of the frequency distributions of the acetabular measurements of paramphistomes from cattle with light and heavy infestations
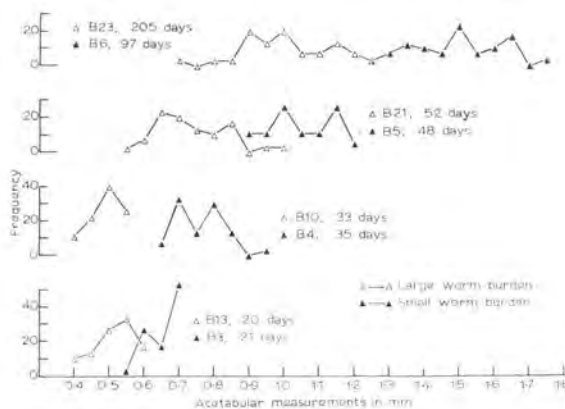
When the acetabular measurements exceeded 0·60 mm large numbers of worms migrated to the rumen (B15, Tables 10 and 11).

Site of attachment: The measurements of worms from different sites of attachment in three cattle are summarized in Table 12.

In a bovine (B15) the majority of worms had already migrated to the rumen and these were smaller than the few worms which remained in the small intestine. In another the worms were larger the more posteriorly they were situated in the small intestine and the number present decreased (B17). The worms attached to the anterior ruminal pillar were considerably larger than those attached to the posterior pillar (B9).

Discussion

Percentage take: With few exceptions the take after migration was higher in cattle than in sheep. In sheep there appears to be a limit to the number of worms that can be accommodated in the rumen, i.e. 18,016 (S58, Table 8). In cattle, if such a limit exists, it exceeds 40,000 worms (B20, Table 10). Therefore, the ruminal worm burden would appear to have less influence on the percentage take in cattle than in sheep. The largest number of *P. microbothrium* recovered from the rumen of a naturally infested bovine by Butler & Yeoman (1962) was 14,730 worms.

Ross (1965) artificially infested cattle with a single dose of metacercariae of *F. hepatica* varying from 1,300 to 15,000. He found that as the number of metacercariae increased the percentage take decreased. These observations were confirmed with *P. microbothrium* in those cattle in which migration was complete or nearly complete (compare B22 and B24 with B20 and B23, Table 10).

Migration: Dinnik & Dinnik (1962) recovered *P. microbothrium* from the rumen of an experimentally infested calf after 17 days, whereas in this experiment the first worms were recovered from the rumen 14 days after infestation. The rate of migration was generally slower than that recorded for cattle in the comparative studies (Table 2). This is similar to the observations made in heavily infested sheep. The rapid migration in light infestations (Table 2), is more likely to approximate the life cycle under natural conditions.

Worm size: The findings are similar to those in sheep, viz. that size is an unreliable characteristic. It is not only governed by the age of infestation, the magnitude of the worm population, and the site of attachment but also varies in individual hosts (Tables 11 and 12 and Fig. 11). It is interesting to note that in a bovine (B9) the worms attached to the anterior ruminal pillar were larger than those attached to the posterior pillar, while in a sheep (S48) the reverse was true.

The average length of *F. hepatica* from artificially infested cattle with large worm burdens was less than that from cattle with smaller worm burdens (Ross, Todd & Dow, 1966). They attribute these differences to over-crowding in the heavier infestations. It is obvious that both *P. microbothrium* and *F. hepatica* are extremely sensitive to the effects of over-crowding.

The host-parasite relationships as they affect *P. microbothrium* have now been recorded and these relationships as they affect the definitive host must be considered.

PART II

THE REACTION OF THE DEFINITIVE HOSTS

1. *Pathogenesis and symptoms*

A. *Pathological anatomy*

Simson (1926), Le Roux (1930) and Butler & Yeoman (1962) gave detailed accounts of the macroscopic pathology of paramphistomiasis. They described: congestion of the blood vessels on the peritoneal side of the affected intestine, hyperaemia, haemorrhages and thickening of the mucosa giving the internal surface a corrugated appearance; cachexia, hydropericardium, hydrothorax, ascites and oedema of the mesentery, abomasum and submandibular space.

Several authors have described the micro-pathology (Mudaliar, 1945; Boray, 1959; Varma, 1961; Mukherjee & Deorani, 1962; Canković & Batistić, 1963; Patnaik, 1964).

Their observations may be summarized as follows: infestation of the rumen causes oedema of the epithelial layer and lymphocytic infiltration in the propria and sometimes the submucous layer; in the vicinity of worms the mucosa is necrosed and sloughs with some hypertrophy of the stratum corneum; the tips of the papillae equently degenerate and slough. The immature worms in the small intestine are present not only on the mucosa, but embedded in the submucosa and some may reach the muscularis mucosa. Lymphocytic infiltration may occur around the worms.

The young paramphistomes attach themselves to the intestine by means of a plug of mucosa drawn into the acetabulum. This mucosa becomes strangulated and necrotic and severing takes place; some of the parasites may penetrate to the peritoneal cavity and cause haemorrhages on the serosa (Boray, 1959).

The author made the following observations on sheep, goats and cattle experimentally infested with *P. microbothrium*.

(i) *Macropathology*

The adult worms are attached to the epithelium and papillae of the ruminal pillars. If large numbers are present these areas appear anaemic, being white in colour when compared with the grey-green of the surrounding tissue. The papillae are often atrophied and their tips sloughed off due to pressure necrosis caused by the acetabula of the numerous paramphistomes attached at their bases.

When death or disease is due to infestation with immature paramphistomes, the carcass may be in good condition or severely emaciated depending on the duration of infestation. Invariably the hindlimbs and the peri-anal region are soiled with foetid, fluid faeces. Submandibular oedema is rare. If the animal is in good condition there may be fat necrosis, whereas in more chronic cases fatty tissues undergo serous atrophy.

Oedema of the lungs, hydrothorax, hydropericardium and ascites are frequently observed. In chronic cases splenic atrophy, ruminal atony and atrophy may be present. The mesenteric lymph glands are oedematous; the first two to three metres of the small intestine are hyperaemic and the larger vessels extremely congested; the mesenteric fat, at the site of attachment of the mesenterium to the affected intestine, is replaced by clear serous fluid (see Plate 1).

Young conical fluke may penetrate to just below the serosa and be observed from the peritoneal side of the intestine. The small intestine posterior to the affected portion is distended with fluid and the wall extremely thin. The bile-duct may be enlarged and the gall-bladder distended.

On opening the gastro-intestinal tract immature paramphistomes may be found attached to the mucosa of the rumen and omasum. The rumen contains little ingesta which is frequently very fluid. The walls and rugi of the abomasum are oedematous. In cattle the rugi may be so enlarged as to occlude the lumen (see Plate 2).

PLATE 1.—The serosal surface of paramphistome-infested small intestine showing congestion of the vessels and the replacement of mesenteric fat by clear serous fluid
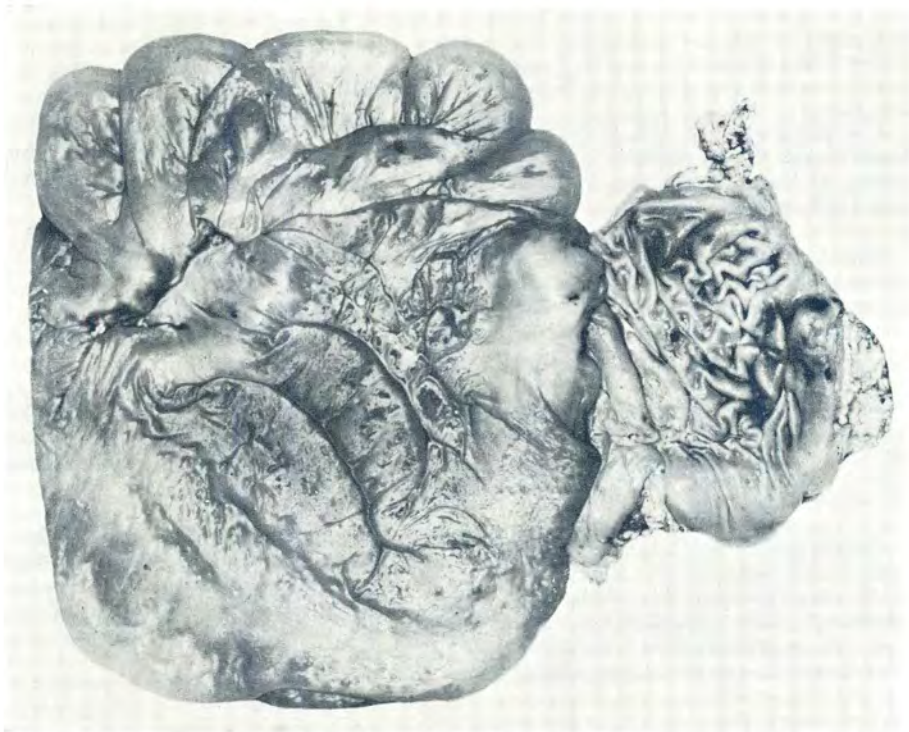
PLATE 2.—The abomasum of a paramphistome-infested bovine exhibiting marked oedema of the rugi

The abomasum of an infested bovine weighed 4·75 Kg compared to 1·27 Kg for the abomasum of a control bovine of the same size. Young paramphistomes are found attached in the abomasum and shallow erosions and petechiae may be observed.

The wall of the first two to three metres of the small intestine is thickened and friable, the mucosa corrugated and often covered by a catarrhal exudate. Large numbers of paramphistomes, similar in appearance to small pink millet seeds, are attached to the surface and deeply embedded in the mucosa (see Plate 3).

Numerous erosions, petechiae and ecchymoses are present in the intestine and the ingesta may be slightly haemorrhagic. Posterior to the first three metres few pramphistomes are found in sheep and goats, but large numbers may be present in cattle. Caecal and colonic ingesta are extremely fluid, and in cases that exhibit prolonged diarrhoea, rectal haemorrhages may be present.

On opening the gall-bladder a few paramphistomes may be found attached to the wall. The bile is frequently thick and viscous and superficial necrosis of the gall-bladder wall is noticeable.

(ii) *Micropathology*

The changes observed microscopically are similar to those described by Boray (1959) (see Plate 4); an additional observation was the almost complete absence of villi in massive infestations (Du Plessis, 1964).

B. *Clinical pathology*

The physiological and haematological changes in a sheep infested with 75,000 metacercariae of *P. microbothrium* were described by Lengy (1962). He found evidence of leucocytosis, eosinophilia and slight anaemia, but recorded no changes in the total plasma protein concentration.

Horak & Clark (1963) made a detailed study of acute paramphistomiasis induced by massive experimental infestations with 172,000 ± 3,000 metacercariae of *P. microbothrium* in six worm-free sheep. The effect of the anthelmintic Lintex* i.e. N-(2′ chlor-4-nitrophenyl)-5-chlor salicylamid on the course of the infestation and subsequent reinfestation of two sheep was also studied.

After massive infestation the sequence of changes observed were: a progressive anorexia commencing on the sixth or seventh day, while water consumption remained fairly constant; a fluid, foetid diarrhoea at 16 to 28 days and persisting until death, which occurred 22 to 36 days after infestation.

A marked drop in total plasma protein concentration, almost entirely due to a drop in plasma albumin occurred at 14 days, and persisted until death. Plasma calcium concentration decreased with plasma albumin concentration. This decrease in plasma protein concentration was frequently followed by a drop in plasma volume and a rise in haemoglobin concentration, packed cell volume, red cell count and total volume of circulating erythrocytes.

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*Lintex—Agro-Chem (Pty.) Ltd.

PLATE 3.—The internal surface of paramphistome-infested small intestine showing the corrugated appearance of the mucosa and the presence of numerous immature worms

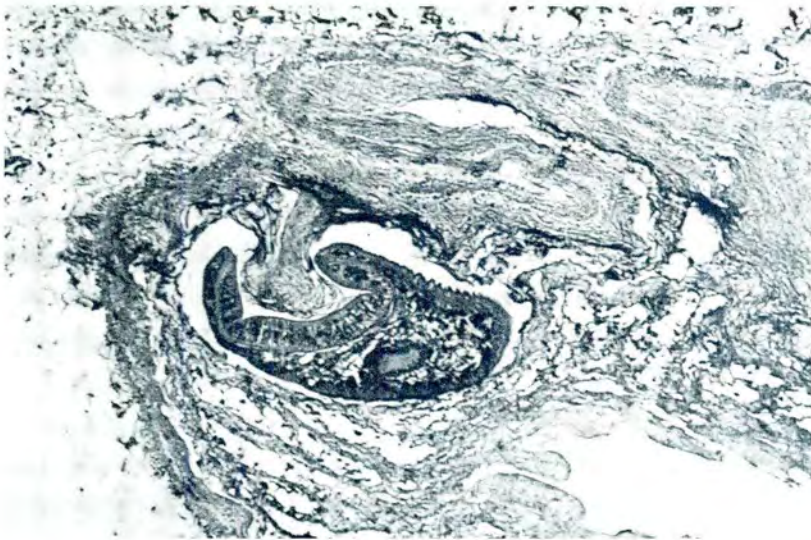PLATE 4.—Photomicrograph of a paramphistome deeply embedded in the mucosa of the small intestine with a plug of tissue drawn into its acetabulum (unfortunately autolysis of the mucosa has taken place *post mortem*) (×75)

Anthelmintic treatment with Lintex had a dramatic effect on the acute disease in two sheep. Appetite improved almost immediately, diarrhoea disappeared within three days and plasma protein concentration regained pre-infestation levels within 17 to 31 days of treatment. On reinfestation of these two sheep one was completely susceptible and was slaughtered *in extremis* 28 days later. The other exhibited a marked rise in plasma gamma globulin concentration 14 days after reinfestation, but no symptoms of disease were evident.

C. *Symptoms*

Symptoms in sheep, goats and cattle caused by infestations with paramphistomidae have been described (Simson, 1926; Le Roux, 1930; Srivastava, 1938; Edgar, 1938; Mudaliar, 1945; Ramakrishnan, 1951; Boray, 1959; Butler & Yeoman, 1962; Visnjakov & Ivanov, 1964).

Their observations may be summarized as follows: staring hair coat, anorexia, polydypsia, loss of condition, profuse, foetid diarrhoea sometimes containing blood or immature worms, anaemia and submandibular oedema.

The author noted the following symptoms in heavily infested animals:—

(1) A progressive decrease in appetite terminating in complete anorexia. Unweaned calves reject all solid feed but consume milk avidly.

(2) There is little reduction in the total water consumption; animals stand with their muzzles in the water troughs drinking small quantities of water frequently.

(3) The hair coat is staring in cattle and the animals are listless.

(4) Diarrhoea commences 16 to 28 days after infestation; faeces are extremely fluid, foetid and may contain immature worms. In particularly severe cases the diarrhoea is projectile, but as the disease progresses the rectal contents leak out involuntarily, soiling the hindlimbs. After multiple infestations prolonged diarrhoea, accompanied by straining, occurs and fresh blood may be seen in the faeces.

(5) Death can occur after a single massive infestation as early as 19 days in sheep and as late as 40 days in cattle. Multiple infestations can result in death in sheep 27 to 71 days after the commencement of infestation.

(6) If death does not occur, marked loss of condition and body weight persists for a long period. A sheep lost 11·4 Kg within 52 days, and a bovine 27·3 Kg in 140 days, while an uninfested bovine gained 50·9 Kg during the same period.

(7) Sub-mandibular oedema is rare and *anaemia has never been observed.*

Discussion

The effects of this worm on the host can only be based on a comprehensive study of the life cycle, pathological anatomy, clinical pathology and symptomatology.

The level of infestation necessary to produce clinical symptoms and death not only depends upon the host species but also on the conditions under which the animals are kept. Thus the availability of feed, its quantity and quality and the conditions of housing, obviously increase the housed animals' ability to withstand the pathogenic effects of the worm, when compared with animals in the field.

In sheep, housed under experimental conditions, worm burdens below 20,000 cause some pathological changes but symptoms are not evident. Worm burdens in excess of 40,000 however, invariably result in death in these animals. In cattle under experimental conditions, worm burdens in excess of 160,000 cause death.

Under pasture conditions Whitten (1955) found that a paramphistome burden of approximately 2,000 worms caused the death of a two-tooth ewe. Butler & Yeoman (1962) found that a worm burden as low as 23,703 caused the death of a calf in a natural outbreak of paramphistomiasis caused by *P. microbothrium.*

Pathogenesis: The author has evolved the following hypothesis on the pathogenesis of acute paramphistomiasis.

The immature worms excyst in the small intestine and attach to the intestinal wall by means of their acetabula. This causes strangulation, necrosis and sloughing of the intestinal mucosa (Boray, 1959), with the resultant development of erosions and petechiae. These lesions cause intestinal discomfort leading to reduced appetite and eventually complete anorexia.

The anorexia results in decreased total body weight, initially because of reduced ruminal and intestinal contents (Horak & Clark, 1966) and in time a reduction in carcass weight due to starvation atrophy. At the same time the function of food assimilation by the severely damaged small intestine is impaired resulting in further loss of body weight.

The severe hyperaemia and oedema of the small intestine lead to partial occlusion of the bile-duct, causing retention of bile with distention of the gall-bladder. The resultant increase in concentration of the bile-salts cause necrosis of the gall-bladder epithelium.

It is presumably through the erosions in the small intestine and abomasum caused by the young worms, that plasma albumin is lost. This is particularly marked three to four weeks after experimental infestation, and it is at this stage of the life cycle that massive migration of the worms from the small intestine to the rumen commences. This migration causes still further damage to the instestine resulting in the marked loss of albumin. Plasma calcium is bound to plasma albumin and its concentration is reduced at the same time.

Because of the low plasma protein concentration generalized oedema develops and the plasma volume is reduced. This oedema is seen as hydropericardium, hydrothorax, pulmonary oedema, ascites and oedema of the mesenterium and abomasum.

The reduced plasma volume leads to a decreased blood volume resulting in retarded circulation and anoxia. To combat the anoxia more erythrocytes are brought into circulation causing an increase in the total volume of circulating erythrocytes. This accounts for the thick, viscous appearance of the blood at slaughter.

The immediate cause of death would appear to be pulmonary oedema coupled with exhaustion and starvation.

The animal grazing in a paramphistome infested paddock presumably acquires infestation by the daily or irregular ingestion of metacercariae. If these numbers are excessively large death from paramphistomiasis results, whereas if they are small a balance between host and parasite develops.

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The objects of the following experiments are to investigate this " balance " and to determine the immunity, if any, that may develop.

2. *Immunity*

In an outbreak of paramphistomiasis in young cattle, Boray (1959) observed a large number of immature but no adult worms *post mortem*. Adult cows grazing the same pasture voided paramphistome eggs in their faeces, but showed no symptoms. He concluded that the disease rarely occurred in adult cattle, presumably because they had usually experienced an early infestation and developed some resistance.

Butler & Yeoman (1962) confirmed these observations. They recorded 73 deaths from paramphistomiasis in 76 calves; only six of 131 adult cows grazing the same swamp died.

In five outbreaks of paramphistomiasis in sheep, cattle seemed to have played a major role in infesting the intermediate snail hosts. In these outbreaks cattle either grazed with or preceded the introduction of sheep. The cattle showed no symptoms but the sheep died in considerable numbers (Le Roux, 1930; Whitten, 1955; Reinecke & Swart, 1958; Horak, 1963).

This does not imply that all sheep are entirely susceptible, as it has been shown experimentally that some sheep can develop resistance to reinfestation (Horak & Clark, 1963).

A more detailed study of immunity in the definitive hosts was undertaken, and the results published (Horak, 1965a). Further investigations were made and these are combined with previous experiments (Horak & Clark, 1963; Horak, 1965a).

Definition: Whenever the phrase " complete immunity " is used in the following experiments it implies that the immunized animal showed no symptoms after challenge and that there was a marked reduction in the percentage take of the challenge infestation, but not necessarily a complete elimination of the challenge worm burden.

" Partial immunity " means that no symptoms developed after challenge but nevertheless a large number of worms from the challenge infestation became established.

A. *Multiple infestations*

To simulate the manner in which animals would acquire infestation under field conditions metacercariae were dosed as follows:—

(*a*) in random numbers over varying periods of time to sheep, and

(*b*) to sheep and cattle according to a fixed infestation pattern.

Experimental observations

The results of the three experiments conducted are summarized in Table 13.

(i) *Random infestation of sheep over a short period*

Five sheep were infested with four to 13 doses of metacercariae each, over periods varying from six to 22 days. The number of metacercariae in each dose varied between 1,000 and 27,000. The sheep were slaughtered seven to 16 days after the last infestation.

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The percentage take varied between 30·8 and 50·3 per cent. This take was no different from that in sheep given a single massive dose of metacercariae and slaughtered soon after infestation (Table 7). In one sheep (S61), slaughtered seven days after the last dose of metacercarciae, 52 per cent of the worms were distributed posterior to the first three metres of small intestine; this is consistent with the distribution of worms after a recent single infestation (S21, Table 7); in the other sheep the majority of worms were present in the first three metre portion.

With the exception of one sheep (S64), the sheep in this experiment had worm burdens below the 40,000 lethal level. Sheep 64 had a burden of 74,820 worms but was slaughtered soon after the completion of infestation and no conclusions as to the eventual pathogenicity of this worm burden could be drawn.

(ii) *Regular prolonged infestation of sheep*

Six sheep were used in this experiment. One sheep was given 2,000, another 4,000 and a further two 6,000 metacercariae each, six times a week over a period of 35 to 458 days. Two sheep were either infested with 8,000 or 16,000 metacercariae daily for periods of 52 and 26 days respectively.

Mortality: The two sheep (S68 and S69) each dosed 6,000 metacercariae six times a week, died 71 and 63 days after the commencement of infestation respectively. The sheep dosed either 8,000 or 16,000 metacercariae daily, died 52 and 27 days after the commencement of dosing respectively (S70 and S71). The sheep infested with 2,000 metacercariae six times a week was slaughtered 36 days after the commencement of dosing as it had developed a severe respiratory stridor (S66). Four thousand metacercarciae dosed six times a week did not result in death and this sheep (S67) was slaughtered 487 days after the commencement of infestation. The sheep which died all exhibited diarrhoea starting on the 18th to 59th day after the commencement of infeststion.

Percentage take: With exception of one sheep (S66), the percentage take decreased as the length of the infestation period increased. The take in the three sheep (S66, S70 and S71) which died or were slaughtered within 27 to 52 days of the commencement of infestation varied between 28·4 and 45·4 per cent. This take is similar to that in sheep given a single massive infestation and slaughtered soon afterwards (Table 7) and was thus not affected by the method of infestation or period during which metacercariae were dosed. In the other three sheep the take was below 20 per cent and in one of them (S67) infested over a 458 day period it was only 1·4 per cent.

Worm distribution: In the five sheep, which died or were slaughtered one to two days after completion of infestation, a large percentage of worms was present posterior to the first three metre portion of small intestine. This percentage appeared to be directly related to the length of the infestation period; in those sheep in which the infestation period was relatively long (S68, S69 and S70) the percentage was large while in those in which it was short (S66 and S71) it was small.

This distribution may be due either to the persistent diarrhoea shifting the worms posteriorly or because the anterior three metres of small intestine is no longer a suitable habitat. A similar distribution, however, is also encountered in sheep slaughtered soon after a single massive infestation (S21 and S22, Table 7).

The other sheep (S67) in this experiment, was only slaughtered 29 days after the completion of infestation and most of the worms in the intestine were recovered from the first three metre portion. A total of only 10,631 worms was recovered from the rumen of this sheep, which was surprisingly low considering the large total number of metacercariae dosed.

(iii) *Regular prolonged infestation of sheep and cattle*

One sheep was dosed 500, and two sheep and two cattle were each dosed either 1,000 or 1,500 metacercariae three times a week for a period of 155 to 190 days. These animals were slaughtered one to four days after the last dose of metacercariae.

The percentage take in the sheep dosed 500 metacercariae three times a week was 30·5 per cent; in the two sheep dosed either 1,000 or 1,500 metacercariae three times a week, the take was 14·9 and 18·0 per cent respectively. The take in the two cattle infested either with 1,000 or 1,500 metacercariae three times a week, was 6·0 and 2·3 per cent respectively.

In the sheep worm distribution was practically identical; 70·9 to 77·9 per cent of the worms were present anterior to the pylorus and the total ruminal worm burdens of these sheep varied between 9,611 and 13,920.

In the cattle, with the exception of one worm in one animal (B28), all worms were anterior to the pylorus.

Discussion

Sheep

Three sheep in these experiments developed resistance to *P. microbothrium*. They exhibited no symptoms and at slaughter had reduced percentage takes (S67, S73 and S74, Table 13).

The reduced take was not caused by an inability of the metacercariae to excyst, nor was it due to the failure of the young worms to become established, because these sheep harboured considerable numbers of worms in their small intestines. Moreover, there was no diarrhoea which could have caused worm expulsion.

It was not possible to determine how or when the worms were expelled but various alternatives are suggested. The immature worms may be expelled after initial establishment and before migration, or they may be eliminated during migration towards the rumen. If, however, the worms do succeed in migrating they may be expelled on reaching the rumen or cause the expulsion of worms already established there.

The ruminal worm burdens of these sheep were reasonably similar despite the fact that the total number of metacercariae they had received varied considerably. It is possible that the ruminal saturation point had been reached and larger numbers of worms could not be accommodated in this organ.

Whatever the reasons for the reduction in percentage take, this is what probably occurs in the grazing animal, which regularly ingests metacercariae in insufficient numbers to cause disease. The gradual acquisition of infestation by the host eventually leads to a harmonious co-existence between host and parasite.

Cattle

Both animals (B27 and B28) developed a complete resistance to reinfestation. The percentage take was 6·0 per cent of 94,000 metacercariae dosed and 2·3 per cent of 139,500 dosed respectively. This resistance was directly proportional to the total number of metacercariae dosed.

The immune reaction in cattle interferes either with the excystation of metacercariae, or the attachment of the young worms. One bovine (B27) harboured no worms and the other (B28) only had one posterior to the pylorus despite each of these animals having received 3,000 metacercariae in the week preceding slaughter. Resistance was not due to ruminal over-population as both animals had few worms in the rumen.

From these results it is evident that cattle are more resistant to repeated infestation with *P. microbothrium* than are sheep.

Before proceeding with further studies on immunity it seemed advisable to study the effect of irradiation of metacercariae with X-rays, on the life cycle of *P. microbothrium* in a definitive host and because of their availability, sheep were used in the following experiments.

B. *X-Irradiation of metacercariae*

The effect of irradiation with X-rays on the viability of metacercariae of *F. hepatica* and the infectivity, longevity and fertility of the resulting liver fluke have been described (Wikerhauser, 1961b; Hughes, 1962; Lagrange, 1963; Dawes, 1963). Their findings may be summarized: irradiation of 3 to 20 kr reduced the infectivity of metacercariae; all mice died when infested with metacercariae exposed to 1 kr, whereas metacercariae treated with 4 kr resulted in the death of only some mice, and the majority of the flukes died in the livers of infested mice. The majority of liver fluke resulting from metacercariae exposed to 3 kr lived for 22 days; miracidia in eggs from worms derived from metacercariae exposed to irradiation ranging from 3 to 8 kr, took longer to develop and fewer developed than those in eggs from normal flukes.

Experiments to determine the effect of X-irradiation on metacercariae of *P. microbothrium* compared with normal (un-irradiated) metacercariae were carried out.

Experimental observations

The results are summarized in Table 14.

(i) *The effect of X-irradiation on the life cycle of P. microbothrium*

Four separate batches of metacercariae were exposed to X-irradiation of either 1, 2, 4 or 8 kr, at an irradiation tempo of 229 roentgen per minute.

Four sheep were each infested with 10,000 irradiated metacercariae and three sheep with a similar number of normal metacercariae. These animals were killed 20 and 21 days later, because little loss of worm burden due to migration has occurred at this stage in the normal life cycle and comparisons can be made as to the effect of irradiation on the viability of immature paramphistomes. Four sheep each received 5,000 irradiated metacercariae and a single sheep a similar number of normal metacercariae. These sheep were killed 189 and 190 days later respectively.

The percentage take and rate of migration 21 days after infestation were not affected by irradiation of 1 and 2 kr, but take was adversely affected by 4 and 8 kr. The migratory rate of small worm burden, after irradiation of 4 kr, was rapid (S78). No conclusions could be drawn from the migratory rate after 8 kr as only four worms were recovered (S79).

Irradiation of 1 kr did not affect the percentage take 189 days after infestation, but it was markedly reduced by 2 kr and no worms were recovered after irradiation of either 4 or 8 kr.

(ii) *First generation after X-irradiation*

Forty thousand metacercariae exposed to 2 kr were dosed to a bovine and the infestation allowed to mature. Paramphistome eggs derived from this infestation hatched normally within 14 days and the hatched miracidia were able to infest the intermediate snail host *Bulinus* (*Bulinus*) *tropicus* Krauss, 1848. Metacercariae were harvested and used to infest sheep. One sheep was given 10,000 of these metacercariae and slaughtered 20 days later; another two 5,000 each and slaughtered at 189 days; a fourth control sheep was given 5,000 normal metacercariae and slaughtered 189 days later.

Exposure of the metacercariae of the previous generation to 2 kr did not affect the percentage take or migration of paramphistomes (Table 14).

Size: The sizes of the worms recovered from some of the sheep in both experiments are given in Table 15.

There was no striking difference in the size of the 189 and 190-day old worms in either experiment. No comparisons are possible on the size of the 20 and 21-day old worms as some of them had been accidentally killed at 70°C and they are not included in Table 15.

Faecal worm egg counts: In those infestations in both experiments which were allowed to reach patency regular worm egg counts were made and the average egg counts until slaughter recorded. These results are summarized in Table 16.

The fertility of worms resulting from metacercariae treated with 1 or 2 kr was drastically reduced. Higher rates of irradiation further reduced fertility; prolonged the prepatent period to beyond 110 days; reduced the length of the patent period and no worms survived until the termination of the experiment at 189 days.

The egg counts in the sheep infested with first generation paramphistomes after irradiation, were similar to those of the control.

Discussion

The viability and fertility of *P. microbothrium* is adversely affected by irradiation of 2 to 8 kr. Similar observations have been made on *F. hepatica* (Wikerhauser, 1961b; Hughes, 1962; Lagrange, 1963; Dawes, 1963).

The degree of helminth infestation necessary to produce immunity to subsequent reinfestation often approaches the pathogenic level. Therefore, if the pathogenicity of the immunizing infestation can be reduced without limiting its antigenicity successful vaccination can be contemplated.

In attempts to establish a suitable level of irradiation which could be used in the development of a vaccine 1 kr was too low and 4 and 8 kr too high. Irradiation with 2 kr seemed to be optimal. Not only was the pathogenicity of the infestation

reduced because of the decreased percentage take after 21 days, but the number of worms still remaining may be sufficient to stimulate the development of resistance to reinfestation.

Experiments were set up in which normal and irradiated metacercariae were used to produce immunity in the definitive hosts.

C. *Immunization of sheep*

After a single initial infestation with metacercariae of *P. microbothrium*, Horak & Clark (1963) demonstrated partial immunity to reinfestation in three of five sheep. Horak (1965a) using 40,000 normal metacercariae as a vaccine, successfully protected adult sheep from the lethal effects of massive reinfestation. Applying the same technique he was unable to immunize suckling lambs.

Further investigations into this immunity in sheep were carried out. Various methods of immunization were attempted and the effects that the stress of pregnancy and anthelmintic interference may have on immunity investigated.

Experimental observations

Details of infestation, challenge and autopsy are given in Table 17.

Sheep reared, housed and maintained paramphistome-free were used throughout this experiment.

I. *Controls*

Challenge infestation: Twelve sheep were each given a single dose of meta-cercariae varying from 167,000 to 210,000. With one exception (S89), which died 36 days after infestation, all the sheep died or were slaughtered within 30 days.

The takes varied between $23 \cdot 6$ and $57 \cdot 6$ per cent and the worm burdens were all in excess of 40,000, which is the minimum lethal infestation for sheep maintained under experimental conditions.

These results confirmed the viability of the metacercariae and the susceptibility of the sheep and served as controls of the challenge infestations administered to immunized sheep.

Immunizing infestation: Two sheep were each infested with 40,000 metacercariae; one received these metacercariae as a single infestation and was slaughtered 106 days later; the other was given 20,000 metacercariae on two separate occasions 33 days apart, and slaughtered 73 days after the second infestation.

The worms recovered from these sheep were situated in the rumen and the numbers recovered were 10,246 and 9,462 respectively. These numbers were considerably below pathogenic levels and confirmed previous observations that this metacercarial dose might be suitable for use as an immunizing infestation. These sheep served as controls of the infestations used for immunization.

II. *Immunized sheep*

Immunization consisted of infesting sheep with sub-lethal doses of metacercariae; immunity to reinfestation was determined by challenging these sheep with a lethal number of metacercariae and recording their reactions and the number of worms recovered from the challenge infestation at death or slaughter.

Autopsy procedures: The worms recovered from the immunizing infestation were distinguishable from those of the challenge infestation by virtue of their greater age and size, and were counted separately. Worms recovered from the challenge infestation were counted and the percentage take and distribution calculated. The size of the worms from some of the challenge infestations was measured.

Certain fundamental principles such as the ability of sheep of various ages to develop immunity had to be established. The immunized sheep were, therefore, divided into two groups, one consisting of suckling and weaned lambs under one year of age and the other of adult sheep.

Suckling or weaned lambs under one year of age: Two suckling and four weaned lambs were each infested with 40,000 metacercariae, either as a single dose or as two equal doses of 20,000, 32 days apart. Two of the weaned lambs received metacercariae irradiated at 2 kr (S95 and S97, Table 17).

After periods varying from 43 to 80 days they were each challenged with a single dose of 169,000 to 207,000 normal metacercariae.

The suckling lambs died 31 and 33 days after challenge, with worm burdens resulting from the challenge infestation in excess of 40,000. The weaned lambs were killed 29 days after challenge. With the exception of one animal (S94), they had challenge worm burdens in excess of 40,000, but showed no symptoms.

The inability of these young sheep to develop immunity may be due to their inability to respond immunologically as well as adult sheep do (Manton, Peacock, Poynter, Silverman & Terry, 1962).

The number of worms resulting from the immunizing infestations in these sheep varied between 1,191 and 5,404 and the variation could not be ascribed to irradiation or non-irradiation of the metacercariae. These numbers were considerably smaller than those recovered from the control sheep (S48 and S91) which had received 40,000 metacercariae. This may be due to a partial " self-cure " reaction which will be discussed later.

Adult sheep: In adult sheep an initial infestation of 25,000 or 50,000 metacercariae produced a partial immunity to reinfestation, while 75,000 metacercariae caused disease and no immunity to challenge developed (Horak & Clark, 1963). Subsequently the ability of 40,000 metacercariae to produce immunity to reinfestation was investigated (Horak, 1965a). As neither of these reports included the complete range of metacercariae dosed to arrive at the latter number, this will now be presented.

(i) *Single or double immunizing infestations:* Ten adult sheep, i.e. over two years old, were each infested with a single dose of metacercariae varying between 500 and 75,000. Another adult sheep was given two equal doses of 20,000 metacercariae 32 days apart. These sheep were challenged with 198,000 ± 7,000 metacercariae 37 to 1,075 days later.

The sheep initially infested with 20,000 to 50,000 metacercariae developed resistance to reinfestation, the take on challenge varying between 0·04 and 18·7 per cent.

Those immunized with either 500, 1,000, 2,000 or 75,000 metacercariae died 22 to 26 days after challenge, with worm burdens resulting from challenge in excess of 70,000 and takes varying from 35·8 to 42·2 per cent.

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Two of the sheep (S104 and S105) which had received 40,000 metacercariae as double or single immunizing infestations harboured 2,353 and 815 worms from these infestations respectively. As was the case in the suckling and weaned lambs, these worm burdens were considerably smaller than those of the controls (S48 and S91).

(ii) *X-Irradiation of the immunizing infestation:* Four adult sheep were each infested with a single dose of 17,000 to 32,000 metacercariae which had been treated with X-rays at either 1, 2, 4 or 8 kr (S109 to S112, Table 17). They were challenged 74 days later with 205,000 $\pm$ 5,000 un-irradiated metacercariae and slaughtered 24 days after challenge.

The number of worms recovered from the immunizing infestation decreased progressively as the irradiation factor increased, confirming previous observations. With the exception of one sheep (S109), in which the take after challenge was 44·0 per cent, the susceptibility after challenge increased progressively with the increase in the dosage of irradiation of the immunizing infestation. None of these sheep were immune to challenge, and although no diarrhoea developed they all showed a marked loss in body weight.

These sheep probably did not develop immunity to reinfestation as the number of metacercariae used for immunization was below 40,000, which is regarded as the optimal immunizing dose and had resulted in complete immunity when un-irradiated metacercariae were used (*vide supra*).

(iii) *Multiple immunizing infestations:* (a) *Challenge immediately after immunization:* Two sheep were immunized by the administration of either 500 or 1,000 and another two with 1,500 metacercariae three times a week; a fifth sheep received 1,000 metacercariae six times a week. These infestations were continued for a period lasting several months and the total number of metacercariae dosed varied from 46,500 to 415,000.

The sheep were each challenged with 192,500 $\pm$ 9,500 metacercarie immediately on completion of immunization to three days later and slaughtered four to 27 days after challenge. At autopsy the worms in the rumen were regarded as resulting from the immunizing infestation. In the abomasum and small intestine no distinction could be made between the worms resulting from immunization or challenge, and these were regarded as originating from the challenge infestation.

Worms resulting from immunization were few to moderate in number, varying between 16 and 17,342. The percentage take of the challenge infestation in the sheep slaughtered after four days was 45·1, this fell to 1·3 per cent in the sheep killed 14 days after challenge and was low (0·4 to 1·8 per cent) in the sheep killed 24 to 27 days after challenge. It is clear that the worms were lost between four and 14 days after challenge. Excystation and initial attachment occurred but the latter was of a temporary nature and could not be maintained. Furthermore, the results show that as the number of metacercariae used for immunization increased, so did the immunity in the three sheep slaughtered 24 to 27 days after challenge (S115, S116 and S117, Table 17).

(b) *Pregnancy and delayed massive challenge:* Two adult ewes were immunized, the one receiving 500 metacercariae three times a week and the other 1,000 three times a week, until totals of 46,500 and 93,000 metacercariae had been dosed

respectively. Approximately 70 days after the last dose of metacercariae they were served and then challenged in an advanced state of pregnancy, 202 and 198 days after the completion of immunization.

The one ewe (S118) was challenged with 200,000 metacercariae, lambed 26 days later and four days after lambing was given a further 206,000 metacercariae. The ewe died 17 days after the second challenge infestation. Although the combined take of the challenge infestations was reduced to 14·4 per cent the number of worms recovered was 58,475 which is in excess of the lethal infestation of 40,000 worms. The other ewe (S119) was challenged with 408,000 metacercariae, lambed 19 days later and died 30 days after challenge with a massive challenge worm burden of 118,614.

Although both ewes died this was probably due to the massive challenge breaking down their immunity and not primarily caused by the stress of pregnancy. This is substantiated by the fact that the first ewe (S118) survived the initial challenge of 200,000 metacercariae for 47 days; the subsequent administration of 206,000 metacercariae four days *post partum* led to her death 17 days later.

(iv) *Immunization followed by anthelmintic treatment:* In outbreaks of paramphistomiasis in the field anthelmintics are used extensively. It seemed advisable to investigate the effects these anthelmintics might have on the development or maintenance of immunity.

Three adult sheep (S120, S121 and S122) were each infested with 50,000 metacercariae and treated with Bithionol* at 100 mg/Kg, 21 to 35 days later. Another adult sheep (S123) was infested with 40,000 metacercariae and treated with Freon† at 330 mg/Kg, 698 days later. These sheep were challenged with 170,000 to 205,000 metacercariae 41 to 54 days after anthelmintic treatment and slaughtered 22 to 29 days after challenge.

Anthelmintic treatment with Bithionol removed all the worms of the immunizing infestation and only 50 of these worms were left after treatment with Freon. The percentage take of the challenge infestation varied between 18·2 and 58·3. With the exception of one sheep (S120) which had 31,014 worms following challenge, the other three sheep had burdens in excess of the lethal level and would probably have died.

The removal of the adult worm burden, even after a period of 698 days, resulted in the loss of immunity (S123). In direct contrast an untreated sheep (S106), which had received the same immunizing infestation of 40,000 metacercariae, was still completely immune 1,075 days after immunization.

The *immunizing infestations* in the four sheep which were treated caused no symptoms. These sheep would not have been treated for paramphistomiasis in the field unless there was an acute outbreak of the disease in the flock.

Horak & Clark (1963) described the effect of anthelmintic treatment with Lintex at 50 mg/Kg on acute paramphistomiasis and on the development of subsequent immunity. Sheep No. 7 and 8 in their experiments are included in Table 17 and numbered Sheep 124 and 125. Their results showed that one of the sheep was completely susceptible to reinfestation (S124), while the other was partially immune

(S125). They ascribed this immunity to the number of worms of the immunizing infestation which had already migrated to the rumen at the time of treatment and were unaffected by the drug, and to the fact that this sheep had recovered from the disease at the time of challenge.

(v) *Other attempts at immunization:* Living, adult paramphistomes harvested from recently slaughtered sheep were administered orally to three adult sheep which were subsequently challenged 56 to 154 days later with 171,000 to 208,000 meta-cercariae and slaughtered 20 to 29 days after challenge. None of these sheep were immune (S126, S127 and S128, Table 17).

Immature paramphistomes recovered from the small intestine of an experi-mentally infested sheep were washed, weighed, $1 \cdot 5$ gram macerated, suspended in a $0 \cdot 85$ per cent NaCl solution and injected intraperitoneally into a worm-free weaned lamb under one year of age. Twenty days later the lamb was challenged with 179,000 metacercariae and it died 13 days after challenge.

This procedure had apparently sensitized the lamb as death occurred more rapidly than in any other animal. Moreover, worm migration was more rapid as $9 \cdot 94$ per cent of the worms had already migrated to the abomasum at the time of death. This worm distribution can be compared to that in a susceptible sheep (S35, Table 7) with a similar worm burden, where it took 27 days after infestation for $7 \cdot 73$ per cent of the worms to migrate to the abomasum.

Passive transference of immunity was attempted in a weaned lamb under one year of age (S130). Twenty ml of whole blood from immune sheep were injected intravenously three times a week, for three weeks. Simultaneously with the second injection of blood this lamb was challenged with 146,000 metacercariae, i.e. 18 days before immunization was complete, and slaughtered 20 days after challange.

The take of $73 \cdot 6$ per cent was considerably higher than that in any other control or immunized sheep in this experiment.

Worm distribution: (a) *Controls:* In 10 of the 11 control sheep which died or were slaughtered 19 to 36 days after infestation, less than eight per cent of the total worm burden was recovered posterior to the first three metres of small intestine. In the remaining sheep (S89), 75 per cent of the worm burden was recovered there. The worm distributions in six of these control sheep are given in Table 7.

(b) *Immunized sheep:* Of the 39 immunized sheep, 37 were slaughtered or died more than 14 days after challenge. Seven of these sheep had more than 20 per cent of their challenge worm burdens posterior to the first three metres of small intestine and a further three had more than 11 per cent in this locality (Table 18). Only one sheep (S114) was immune judging by the number of worms recovered from the challenge infestation.

Challenge infestation: (a) *Migration:* Most of the immunized sheep died or were slaughtered less than 35 days after challenge (Table 17), and no effect of immunization on the migratory rate of the challenge infestation was observed.

Six sheep, however, were slaughtered 35 to 55 days after challenge and the worm distributions in these and susceptible sheep with similar worm burdens are given in Table 19.

In two immunized sheep (S104 and S106) with very low worm burdens of 126 and 85, $15 \cdot 9$ and $9 \cdot 4$ per cent of these worms were still in the small intestine 35 and 48 days after challenge respectively. In the other four immunized sheep, which

had worm burdens between 1,073 and 38,329, 71·4 to 87·6 per cent of these worms were still in the small intestine. In the susceptible sheep, regardless of the number of worms present, migration to the rumen 34 to 50 days after infestation was more rapid, the maximum percentage still in the intestine being 11·8 in one sheep (S44).

(*b*) *Worm size:* Measurements were made on worms recovered from six immunized sheep with challenge worm burdens of less than 40,000. These are compared with the measurements of worms recovered from susceptible sheep with burdens of a similar magnitude or age (Table 20).

The size of the worms recovered from the immunized sheep was considerably smaller than that of worms from susceptible sheep. Where worm burdens exceeded 40,000, however, no difference in size was noted between immunized and susceptible sheep.

(*c*) *The effect of worm size on migration:* The delay in migration was due to the retarded growth of the paramphistomes. In the normal life cycle in sheep the average acetabular measurement reaches 0·56 mm at 20 days and migration commences (Tables 2 and 3). In the immunized sheep migration was delayed as the acetabular measurements had not reached this size by 48 days (Table 20).

Discussion

Methods of immunization

In adult sheep complete immunity to paramphistomiasis is dependent upon a single previous infestation between the limits of antigenic insufficiency and pathogenic embarrassment. A single infestation with 40,000 metacercariae not only produces no clinical signs of paramphistomiasis, but also a complete immunity. It may, therefore, be considered as an ideal immunizing infestation for adult sheep kept under laboratory conditions. Although multiple infestations with small numbers of metacercariae also fulfil these requirements, immunization by this method is not practical because of the time and the labour involved. All other methods attempted were unsuccessful.

The effects of immunity on the worms

(1) Excystation of the challenge infestation is not inhibited. This was demonstrated in a sheep (S113) which was slaughtered four days after challenge and harboured large numbers of recently excysted paramphistomes.

(2) Soon after challenge large numbers of immature worms are expelled as shown in sheep (S114), which had already expelled the major portion of its worm burden 14 days after challenge.

(3) Worm burdens resulting from challenge, are markedly reduced in immune sheep. Similar findings in numerous nematode species have been recorded (for review see Urquhart, Jarrett & Mulligan, 1962).

(4) In some immunized sheep many immature worms are attached posterior to the first three metres of small intestine which is their normal site of attachment. This may be due to an unsuitable environment in the anterior portion of the small intestine, or an inability of the worms to migrate anteriorly, so that their distribution as late as 31 days after challenge, is similar to that occurring in susceptible sheep within five days of infestation (S21 and S22, Table 7).

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(5) The growth of worms in immune sheep is severely retarded (Table 20). This is similar to retarded larval development in parasitic nematodes (Urquhart et al., 1962).

(6) Migration from the small intestine to the rumen is delayed (Table 19), which can be compared with the delayed larval migration of nematode species (Urquhart et al., 1962). This delay in migration is due to the retarded growth of the paramphistomes in immunized sheep.

(7) On challenge, part of the initial worm burden is expelled. The average worm burden in the controls (S48 and S91) dosed 40,000 metacercariae each, was 9,854, compared with an average worm burden of 2,769 in immunized sheep, which had immunizing worm burdens of approximately the same age, also resulting from 40,000 metacercariae (S92, S93, S94, S96, S104 and S105, Table 17).

This reaction would seem to correspond to the " self-cure " phenomenon in Haemonchus contortus Rudolphi, 1803, infestation, first observed by Stoll (1929) and later studied by Stewart (1953) and others (for reviews see Urquhart et al., 1962; Soulsby, 1965).

The mechanism of elimination of the initial and challenge paramphistome burdens would appear to be complex and was not investigated. All that is known is that the challenge infestation excysts and attaches in the small intestine. Subsequently it may be partially eliminated together with a similar reduction in the immunizing infestation (Table 17).

(8) The pathogenic effects of the challenge infestation are reduced. The controls usually died within 30 days of infestation, while many of the immunized sheep, although harbouring large worm burdens, exhibited no symptoms and survived for 29 days or longer after challenge (Table 17).

Factors governing immunity

(1) Immunity is dependent upon the number of metacercariae dosed initially. In single immunizing infestations 40,000 is optimal; lower or higher doses do not produce the same degree of immunity. In contrast when multiple infestations over a period of months are used as an immunzing procedure, the larger the number of metacercariae dosed to produce immunity, the more complete is the immunity (S115, S116 and S117, Table 17).

(2) Immunity is not dependent upon the number of worms which reach the rumen. One sheep (S105), which only had 815 worms present in the rumen was completely immune, while another (S109) was susceptible in spite of having 5,136 ruminal parasites (Table 17).

(3) Immunity is dependent upon the continued presence of worms. When the worms originating from the immunizing infestations were removed by anthelmintic treatment sheep were susceptible or only partially immune to challenge (S120 to S125, Table 17).

Somewhat similar observations were made by Roberts & Keith (1959) in calves experimentally infested with *Haemonchus placei* (Place, 1893), Ransom, 1911. They found that after the administration of a therapeutic dose of phenothiazine and the expulsion of the adult immunizing infestation, superimposed infestations developed.

(4) Immunity is dependent upon the sheep having recovered from the pathogenic effects of the immunizing infestation. This was demonstrated in a sheep which had a low plasma protein concentration and had lost considerable weight as a result of the immunizing infestation. On challenge this animal was susceptible and subsequently died (Horak & Clark, 1963).

(5) Immunity is dependent upon the worm completing its normal life cycle, i.e. excystation, attachment and migration. If these stages of the life cycle are circumvented by dosing adult paramphistomes *per os*, immunity does not develop (S126, S127 and S128, Table 17).

(6) Immunity can be broken down by massive doses of metacercariae during the stress of pregnancy and parturition. The percentage take of the challenge infestation in pregnant ewes (S118 and S119) was comparatively low, indicating some immunity, but both sheep succumbed to massive challenge (Table 17).

Immunity in sheep is dependent upon numerous factors. If these requirements are fulfilled the sheep will become immune and remain immune, if not it will succumb to infestation resulting in the death of host and parasite.

D. *Immunization of goats*

The successful immunization of two adult goats and one suckling kid has been described by Horak (1965a). He showed that 40,000 metacercariae either as a single dose or two equal doses of 20,000 each were a suitable immunizing infestation. His results are reproduced here without further comment. Additional findings on the migratory rates and worm sizes in susceptible and immunized goats are also recorded.

Experimental observations

Details of controls, immunization, challenge and slaughter are presented in Table 21.

At autopsy the worms resulting from the immunizing infestations were counted separately from those originating from the challenge infestations by virtue of their greater size. The percentage take, worm distribution and worm size of the challenge infestations were recorded.

Challenge infestation

(a) *Migration:* In the controls 91·6 to 97·2 per cent of the worms were recovered from the first three metres of small intestine. The worm distributions in immunized and susceptible goats, with worm burdens of a similar magnitude and age, are given in Table 22. One immunized goat (G13) is not included as its intestine was not divided into separate portions.

In two immunized goats (G14 and G16) few worms had migrated anterior to the pylorus despite worm burdens being small and the goats slaughtered 46 and 42 days after challenge respectively. Another goat (G15) had a total challenge burden of only 84 worms; less than 50 per cent of these worms had migrated from the small intestine 42 days after challenge. In the susceptible goats migration had already commenced at 34 days (G4) and was virtually complete 48 days (G5) after infestation.

(b) *Worm size:* Worm size was not measured in the kids. Samples of three and 30 worms from the adult immunized goats were measured and compared with the sizes of 30 worms obtained from susceptible goats harbouring small worm burdens (Table 23).

The worm measurements in the immunized goats, 42 days after challenge, were smaller than those from a susceptible goat with an infestation 20 days old; and considerably smaller than those from a goat with an infestation 34 days old. Moreover, the worm burdens in the immunized goats were smaller than those in the susceptible goats.

Discussion

As in adult sheep a single infestation with 40,000 metacercariae or two equal infestations of 20,000 metacercariae produced a complete immunity in adult goats. Unlike sheep in which no immunity developed in suckling lambs, a complete immunity developed in one of two suckling kids.

Large numbers of worms originating from the challenge infestation were eliminated. Those worms which remained were severely retarded in their rate of growth and as a result their migration to the rumen was delayed.

Judging by the small numbers of worms recovered from the immunizing infestations in all the goats it would appear that " self-cure " had taken place causing a reduction in the number of these worms.

E. *Immunization of cattle*

The results obtained by Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp & Urquhart (1958) using X-irradiated larvae of *Dictyocaulus viviparus* (Bloch, 1782) Railliet & Henry, 1907 as a vaccine, and the subsequent success of this vaccine, prompted similar work on *P. microbothrium* in cattle. The results of this work were published in a preliminary report (Horak, 1965a). These results and additional observations are presented below.

Experimental observations

The cattle used in these experiments were reared, housed and maintained paramphistome-free. Details of infestation, challenge and slaughter are summarized in Table 24.

I. *Controls*

Challenge infestation: Twin weaned calves, five months of age, were each infested with a single dose of 202,000 ± 2,000 metacercariae and slaughtered 52 days later.

Both animals developed anorexia and diarrhoea after infestation and the percentage takes at slaughter were 21·7 and 36·1.

Four adult cattle were each given 253,000 ± 3,000 metacercariae as a single dose and one animal was infested with 305,000 metacercariae. All the animals exhibited symptoms of paramphistomiasis. Three were killed 23 to 29 days after infestation and the percentage take varied between 37·1 and 43·3. The other two died 33 and 40 days after infestation with takes of 67·5 and 53·0 per cent respectively and worm burdens exceeding 160,000. This appears to be the lethal level of infestation for adult cattle under experimental conditions.

All the percentage takes were within the normal range, confirming the viability of the metacercariae and the susceptibility of the cattle.

Immunizing infestation: Two adult cattle were each infested with 40,000 metacercariae, and slaughtered 46 and 53 days later. This level of infestation resulted in worm burdens of 21,246 and 17,908 but produced no symptoms.

II. *Immunized cattle*

In this experiment worms originating from the immunizing infestations were counted separately from those resulting from the challenge infestations. The number. percentage take and distribution of the worms of the challenge infestations were recorded. The worms from some of the challenge infestations were measured.

It seemed advisable to establish the earliest age at which cattle can develop immunity to *P. microbothrium* and an attempt was made to immunize bucket-reared 14-day old calves.

Fourteen-day old calves: One calf was infested with two equal doses of 20,000 metacercariae 28 days apart and another with a single dose of 40,000 metacercariae. They were challenged with 202,500 ± 1,500 metacercariae, 35 and 63 days after their last doses of metacercariae, and slaughtered 42 and 44 days after challenge respectively.

The calves were in poor condition and had symptoms of pneumonia and diarrhoea at the start of the experiment. Both developed symptoms of paramphistomiasis after immunization and again after challenge.

At autopsy severe pneumonic lesions in the cardiac lobes of the lungs were evident. Only four and 12 worms of the immunizing infestations were recovered; the percentage takes of the challenge infestations were 23·7 and 16·8 per cent respectively (Table 24).

These calves were not suitable experimental animals as their poor state of health probably interfered with the development of immunity.

Adult cattle: (i) *Single immunizing infestations:* Two adult cattle were each infested with 2,500 metacercariae, three with 40,000 and one with 100,000. These animals were challenged with 101,000 to 261,000 metacercariae 28 to 924 days later and were slaughered 17 to 43 days after challenge.

Immunizing infestations of 2,500 metacercariae resulted in worm burdens of 756 and 1,121 respectively. Although the percentage take of the challenge infestation was below that of similar challenge infestations in susceptible cattle, a considerable number of paramphistomes was recovered and both animals exhibited symptoms of paramphistomiasis (B32 and B33, Table 24).

The number of worms recovered from single immunizing infestations of 40,000 metacercariae varied between 10,698 and 20,569 while 17,889 worms were recovered from the immunizing infestation of 100,000 metacercariae. These initial infestations resulted in complete immunity to reinfestation within 28 and for as long as 279 days after immunization. Not only did these animals exhibit no symptoms after challenge but remarkably few or no worms of the challenge infestations were recovered (B34 to B37, Table 24).

(ii) *X-Irradiation of the immunizing infestation:* In the experiments on X-irradiation of metacercariae in sheep it was shown that 2 kr was the most suitable level of irradiation; in cattle an infestation of 40,000 normal metacercariae was successful

in producing complete immunity to reinfestation (*vide supra*). For these reasons infestations of 40,000 metacercariae treated with 2 kr of X-rays were used throughout the present group of seven adult cattle.

One animal was immunized with two equal doses of 20,000 metacercariae 32 days apart and the other six, single doses of 40,000 metacercariae each. They were challenged with 250,000 un-irradiated metacercariae 42 to 279 days later and killed 22 to 34 days after challenge.

The numbers of worms recovered from the immunizing infestations were remarkably similar, varying between 766 and 1,490. The percentage take of the challenge infestations was insignificant, the total number of worms recovered varying from 3 to 622, confirming the results obtained after immunization with 40,000 normal metacercariae.

(iii) *Multiple immunizing infestations:* Four animals were immunized by repeated administration of un-irradiated metacercariae; two at a level of 500, one at 1,000 and another at 1,500 three times a week until a total of 46,500 to 631,500 metacercariae had been given.

Three of these animals (B45, B46 and B47) were challenged with 211,000 to 298,000 metacercariae 10 to 161 days after the last metacercarial dose, and slaughtered eight to 33 days after challenge. The take of the challenge infestation was negligible (0·02 to 0·4 per cent).

The other animal (B48) was challenged 376 days after the last dose of metacercariae and received a massive dose of 792,000 metacercariea irregularly administered over a period of 18 days. Thirty-eight days after the last dose of these metacercariae it was given a single dose of 781,000 metacercariae and killed 22 days later.

The youngest worms from the immunizing infestations in this animal were at least 454 days old (Table 24), they were large and with one exception present in the rumen. The worms from the challenge infestations were 22 to 78 days old, small to medium in size and present in the rumen and intestine. Thus the immunizing and challenge infestations could readily be distinguished on size. The total number of worms recovered (1,066 and 182) is negligible. Even if the author's interpretation of the original and challenge infestations is open to question, this animal received a tremendous challenge of 1,573,000 metacercariae and was obviously completely immune.

The numbers of worms resulting from the multiple immunizing infestations varied considerably. Two of the cattle (B46 and B48) were each dosed a total of 46,500 metacercariae, but the one (B48) was challenged and slaughtered more than a year after the other, and had an immunizing burden of 1,066 worms compared to 10,314 in the animal slaughtered soon after immunization (B46).

(iv) *Immunization followed by anthelmintic treatment:* One adult bovine (B49) was infested with 176,000 metacercariae and treated with Bithionol at 40 mg/Kg, 294 days later. This animal was challenged with 272,000 metacercariae 259 days after treatment and slaughtered 51 days after challenge. Faecal samples were collected for three days after challenge and on examination showed that 85 per cent of the 522 metacercariae recovered in the faeces had excysted.