Studies on Bunostomum trigoncephalum,

The Hookworm of Sheep

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J.M. GRAHAM, 1969

ABSTRACT

This thesis records a series of experimental observations on Bunostomum trigonocephalum. The studies were made under the following broad headings:

a) pre-parasitic development

b) comparison of routes of infection and studies of the mechanism of infection including exsheathment of the infective larvae

c) the pathogenesis and pathogenicity of the parasite for the sheep host.

The morphology and development of the first, second and third larval stages was examined. The moult of the first to the second stage was observed and the first stage cuticle appeared to disintegrate rather than be cast as an intact sheath. This moult occurred after about 5 days incubation at 27°C. Little larval growth occurred after the first moult and the second moult followed comparatively quickly. 80% of the larvae were in the infective stage after 7 days incubation.

Survival of infective larvae was compared at temperatures of 0°C, 10°C, 20°C, 27°C and 37°C. Larvae survived longest at 10°C.

Infective larvae were administered to sheep intravenously, subcutaneously, intraruminally, orally and percutaneously. The largest infections were established after percutaneous administration.

Infective larvae applied to the skin of sheep exsheathed rapidly and penetrated the keratinised layers of the skin. They were also found to exsheath on and penetrate into the skin of calves, guinea pigs and rabbits. There was evidence that larvae migrated to the lungs in the guinea pigs and patent infections were established in some calves.

During exsheathment the anterior portion of the sheath appeared to disintegrate allowing the larvae to emerge. This is unlike the processes which have been described in other strongylate nematodes.

A variable proportion of the infective larvae were found to exsheath "spontaneously" in faecal cultures.

Attempts were made to find what stimulates the infective larvae to exsheath. The results were inconclusive. The highest rate of exsheathment was obtained by exposing larvae to buffer of pH 2. The addition of CO_2 enhanced the exsheathment rate but only at pH 2. Even so, the results were variable and difficult to reconcile with conditions on the skin surface.

After infection of sheep the prepatent period was found to range from 54 to 60 days. The 24 hour egg-output per female worm was estimated in 5 infected sheep and was found to range from 2579 to 10480 eggs. Most of the Bunostomum were found in the intestine between 5 and 35 feet posterior to the gastric pylorus. Numbers of Bunostomum in excess of 2-300 caused a significant and progressive decline in haemoglobin, packed cell volume and total serum protein levels over observation periods of up to 16 weeks after infection. The serum protein decline was primarily due to a fall in albumin concentration. The animals so affected also showed evidence of anorexia, retarded growth rate and loss of weight. Diarrhoea occurred sporadically.

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Index to Figures

Figure		Facing page
1	Graph showing egg production of Donor sheep 272	6
2	Graph showing egg production of Donor sheep 273	7
3	Photomicrograph of the anterior end of a 1st stage larva of *B. trigonocephalum*.	17
4	Photomicrograph of the posterior end of a 2nd stage larva of *B. trigonocephalum*.	18
5	Photomicrograph of the anterior end of a 2nd stage larva of *B. trigonocephalum*.	19
6	The growth pattern of the preparasitic stages of the larvae of *B. trigonocephalum*.	21
7	Photomicrograph of a late 3rd stage larva of *B. trigonocephalum* recovered from the lung of a sheep.	22
8	Photomicrograph of the buccal capsule of an adult *B. trigonocephalum*.	24
9	Male and female *B. trigonocephalum* in process of copulation.	25
10	Stages in the development of the free living stages of *B. trigonocephalum*.	26
11	The effect of temperature on the survival of the 3rd stage larvae of *B. trigonocephalum*.	28
12	Colour photograph of the lung of a sheep showing focal traumatic damage caused by the migrating larvae of *B. trigonocephalum.*	35
13	Sheep skin tensed across an Agee lid.	38
14	Water bath with funnels containing skin in contact with 0.85% saline.	39
15	Petri dish being placed in a pocket under the skin of an anaethetised sheep.	40

Figure		Facing page
16 (a and b)	Photomicrograph of 3rd stage larvae of B. trigonocephalum migrating through the epidermis and superficial layers of the dermis.	42
17	Colour photograph of the lungs of a guinea pig showing focal traumatic damage caused by migrating larvae of B. trigonocephalum.	46
18	Photomicrograph of the anterior end of a 3rd stage larva of B. trigonocephalum showing anterior portion of the sheath disintegrating.	71
19	Photomicrograph of an empty cast sheath of a 3rd stage larva of B. trigonocephalum.	72
20	Photomicrograph of the anterior end of a 3rd stage larva of B. trigonocephalum showing the cuticle markedly distended in the region of the excretory pore.	73
21	Photomicrograph of the 3rd stage larva of B. trigonocephalum retracting within the sheath.	74
22	Test tubes containing ensheathed larvae of B. trigonocephalum being gassed with 20% CO_2 and 80% air.	81
23	Electrophoretic patterns of serum samples taken from sheep before and after infection with B. trigonocephalum.	105
24	The haemoglobin level in blood samples taken from sheep in Experiment II, Chapter 9.	106
25	The packed cell volume percentage of blood samples taken from sheep in Experiment II, Chapter 9.	107
26	The estimated total serum protein in samples taken from sheep in Experiment II, Chapter 9.	108

Figure		Facing page
27	The haemoglobin level in blood samples taken from sheep in Experiment III, Chapter 9.	109
28	The packed cell volume percentage of blood samples taken from sheep in Experiment III, Chapter 9.	110
29	The estimated total serum protein in samples taken from sheep in Experiment III, Chapter 9.	111
30	Photograph showing 3 sheep from Group A, Experiment III together with one control sheep from Group C, Experiment III, Chapter 9.	112
31	Colour photograph of the small intestine showing B. trigonocephalum attached to the mucosa and subsequent petechiae on the surface of the epithelium.	113

CHAPTER 1 GENERAL INTRODUCTION

B. trigonocephalum is a hookworm belonging to the family Ancylostomidae and is found in the adult stage in the small intestine of sheep and goats.

Nematodes belonging to the family Ancylostomidae have long been recognised as serious pathogens in man but it is only since the early part of this century that detailed studies have been made on Ancylostomes of both animals and man. Hookworms invariably have direct life cycles. As adults they inhabit the small intestine of the host. The adults produce eggs which are voided in the faeces. In the egg a larval nematode develops which hatches and then grows and moults until the third larval instar is reached. This third larva is enclosed in the cuticle of the second larval stage and is the infective stage. The infective larvae, free in the environment, gain entry to the host animal either by being ingested or by penetrating through the host's integument. Different species within the family show varying abilities to establish themselves in a host following entry by one or other of these routes. After percutaneous infection, and usually after oral infection of the host, the developing larvae migrate through the lungs before returning to the intestine. In some cases, however, infection of the host by ingestion is not followed by migration, the larvae developing entirely within the alimentary tract. Some species can pass across the placenta and infect the young *in utero*.

The adult nematodes range from 5-15 mm long in the male and 5-30 mm long in the female. Invariably they are equipped with a large buccal capsule which is usually armed with teeth and marginal cutting plates which may be smooth or toothed. This buccal apparatus is associated with an invariable habit of hookworms namely, the ingestion of blood. In some cases it is known that this habit is also facilitated by the secretions of anticoagulants. Anaemia

is the usual sequel to hookworm infection in both man and animals. It seems clear however, that the anaemia is not simply a post-haemorrhagic one but is complicated by an induced iron deficiency and perhaps by hypoproteinaemia. Much of the evidence on the pathogenesis and pathogenicity of hookworms is fragmentary and very little of it relates directly to B. trigonocephalum. As a consequence estimates of the numbers of B. trigonocephalum that are of pathological significance to a single sheep range from 50 to 5000.

The lack of information on B. trigonocephalum is surprising in view of the fact that the nematode is of world wide distribution in sheep and goats and has been shown to have a significant effect on its host. In New Zealand there is evidence of its importance in hill country sheep, particularly ewes (Tetley and Langford 1966, Personal Observations) where it may be responsible for serious loss of condition and deaths. Its significance in hill country ewes has also been commented on in Scotland (Cameron 1923a, Morgan, Parnell and Rayski, 1950) and Russia (e.g. Sarimsakov, 1959). The parasite causes less comment in lowland sheep although it may sometimes be present in large numbers. Nothing is known of the reasons for these differences.

The nematode has a comparatively long generation time and this, may account, in part, for the fact that populations of B. trigonocephalum are slow to build up in lambs only reaching a peak late in the autumn. Observations by Tetley and Langford (1966) suggest that at least the Bunostomum egg output of Hoggets declines in mid-winter, but that the egg output of ewes does not vary throughout the year; on the other hand Brunsdon (1968), Soulsby (1965) and personal observations (1968) indicate that the egg output of B. trigonocephalum in certain ewes does increase during the late spring and in fact may make a substantial contribution to the spring rise phenomena. There is however virtually no satisfactory work on the seasonal pattern of infection or on the epidemiological events that give rise to it.

In an attempt to investigate at least some aspects of the biology of the parasite and its relationship with the sheep host, studies were made under the following broad headings;

(1) the preparasitic development.

(2) comparison of routes of infection together with studies on the exsheathment of infective larvae and the mechanism by which infection of the host is achieved.

(3) the pathogenesis and pathogenicity of this infection.

The appropriate literature is reviewed at the beginning of each chapter.

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CHAPTER 2 GENERAL MATERIALS AND METHODS

Only materials and methods related to all experiments are mentioned here. Those pertaining to particular experiments are described in detail in the relevant chapter.

Rearing Worm Free Sheep

The Lambs:- Romney and Perendale lambs were removed from their mothers 24 hours after birth having received a feed of colostrum.

Housing:- The lambs were housed in separate indoor pens to each of which was attached an outdoor run enclosed with bird-proof netting. Fresh straw was placed in each pen each day. The pens and outdoor runs were also cleaned out every 24 hours. Once a week the concrete floors were scrubbed with a dilute solution of "Nudane", an iodophor based disinfectant. Initially eight lambs were run in each pen but as they grew older the number was reduced to four. Infected donor sheep were always housed individually in order to reduce any risk of cross infection.

Feeding:- In the early stages each lamb was fed four times a day with pasteurised milk to which had been added lime water and glucose. The lambs continued to receive 30-40 oz of milk per day, but the number of feeds per day was gradually reduced until just prior to weaning when they were fed twice daily. Weaning usually took place 12-14 weeks after birth. As soon as the lambs were placed in the pens they had unlimited access to both sheep nuts* and lucerne chaff. After weaning the diet of each lamb was restricted to approximately ½ lb of sheep nuts* and 600 gms of lucerne chaff per day.

* Sheep nuts - Supplied by Manawatu Mills contained:-

60% dried lucerne meal

20% Barley meal

20% Wheat tailings (mill Husks etc.)

additives	2 kg Sodium sulphate and 1.8 gm anhydrous sodium molybdate plus standard mineral and vitamin A + D_3 premix	per 1000 lb of feed

Production of Infective Larvae

First culture of Infective Larvae:- Female B. trigonocephalum were collected from the intestines of ewes killed at the Longburn Freezing Works abbatoirs Palmerston North. The nematodes were washed twice in 0.85% saline and individually picked out and placed in a mortar with 2-3 mls 0.85% saline. They were then ground and the resultant suspension added to 20 gms of a mixture containing 3 parts sterilized sheep faeces and 1 part vermiculite*. The mixture was placed in an open 2 oz jar which was subsequently put inside a 1 pint preserving jar containing approximately 100 mls water. The lid of the jar was screwed loosely on and the jar held at 25°-27°C for 7 days. After this period the larvae were recovered from the culture by the baermann technique viz:- A small wire mesh basket lined with a double layer of Snowtex tissue was put in a filter funnel. The faecal culture was placed on the Snowtex tissue. The funnel was filled with distilled water at 37°C so that the water was in contact with the faecal sample. The funnel was kept in a water bath at 37°C. The lower portion of the funnel protruded through a seal in the bottom of the water bath. A piece of rubber tubing was attached to the lower end of the filter funnel and this was closed by means of a spring clip. After a period of 3 hours had elapsed 10-15 ml of fluid was drawn from the filter funnel and collected in a 1 oz universal bottle. The larvae were allowed to settle and the supernatant fluid plus faecal debris siphoned off.

* Mortaflex vermiculite grade 2.

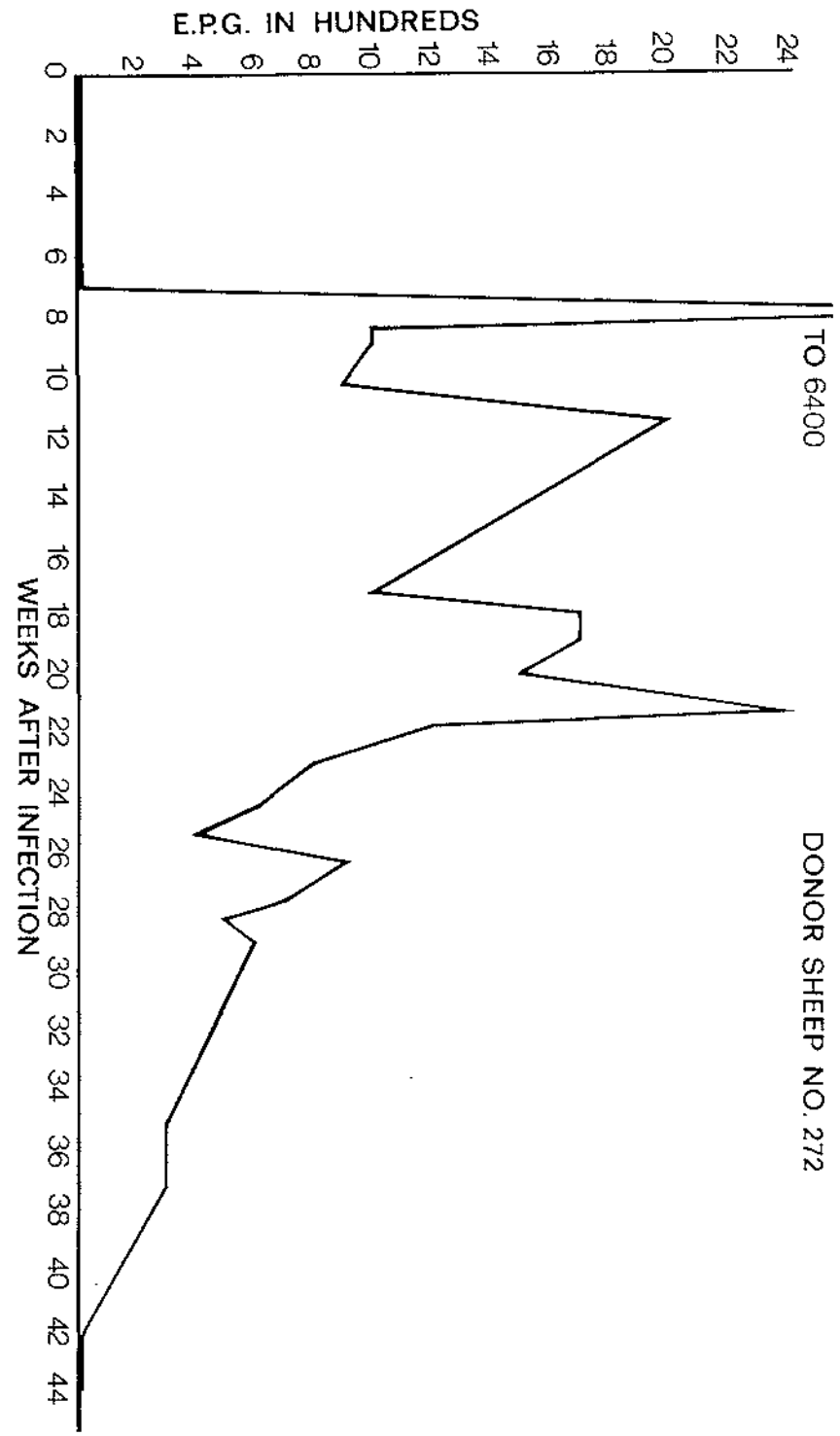

Fig. 1 Graph showing egg production of Donor sheep 272

The supernatant fluid which was removed was replaced with distilled water.

It was usually necessary to repeat this procedure four times in order to obtain a relatively clean suspension of larvae.

The Establishment and Maintenance of pure infections of B. trigonocephalum:-

Eight thousand infective larvae of B. trigonocephalum in 1 ml of water were placed on a clean area of skin over the ribs of sheep selected as donor animals. In the first instance two male Hoggets, Nos 272 and 273 respectively, received infective larvae. It was found that although the donor sheep became patent 53-54 days after infection and continued to produce viable eggs for up to 50 weeks p.i. as illustrated by donor sheep 272 and 273, (figs 1 and 2) the egg counts were sufficiently high for culturing purposes for a much more limited period. Bags were placed on donor sheep and the faeces collected only between the 8th-20th week after infection. The purity of the experimental infection in the donor animals was checked frequently. Male sheep were used throughout to ensure that the faeces were not contaminated with urine. Sarimsakov (1961) found that urine and excess moisture impeded the development of the eggs of B. trigonocephalum. Faeces collected from donor sheep were mixed with vermiculite in ratio 3:1 as previously mentioned, moistened and the mixture put into a two pint preserving jar. The lid was screwed loosely into position and the jar held at 25°-27°C for 7 days.

Infective larvae were then recovered by the baermann technique described earlier in this chapter. In the majority of experiments, unless otherwise stated, the baermannised infective larvae were used on the same day, generally within 6 hours of being recovered. Larvae were always examined prior to use, identified and the percentage of exsheathment recorded. Exsheathment was invariably found to have taken place during the culturing process; it ranged from 2%-20%. Unless specifically mentioned

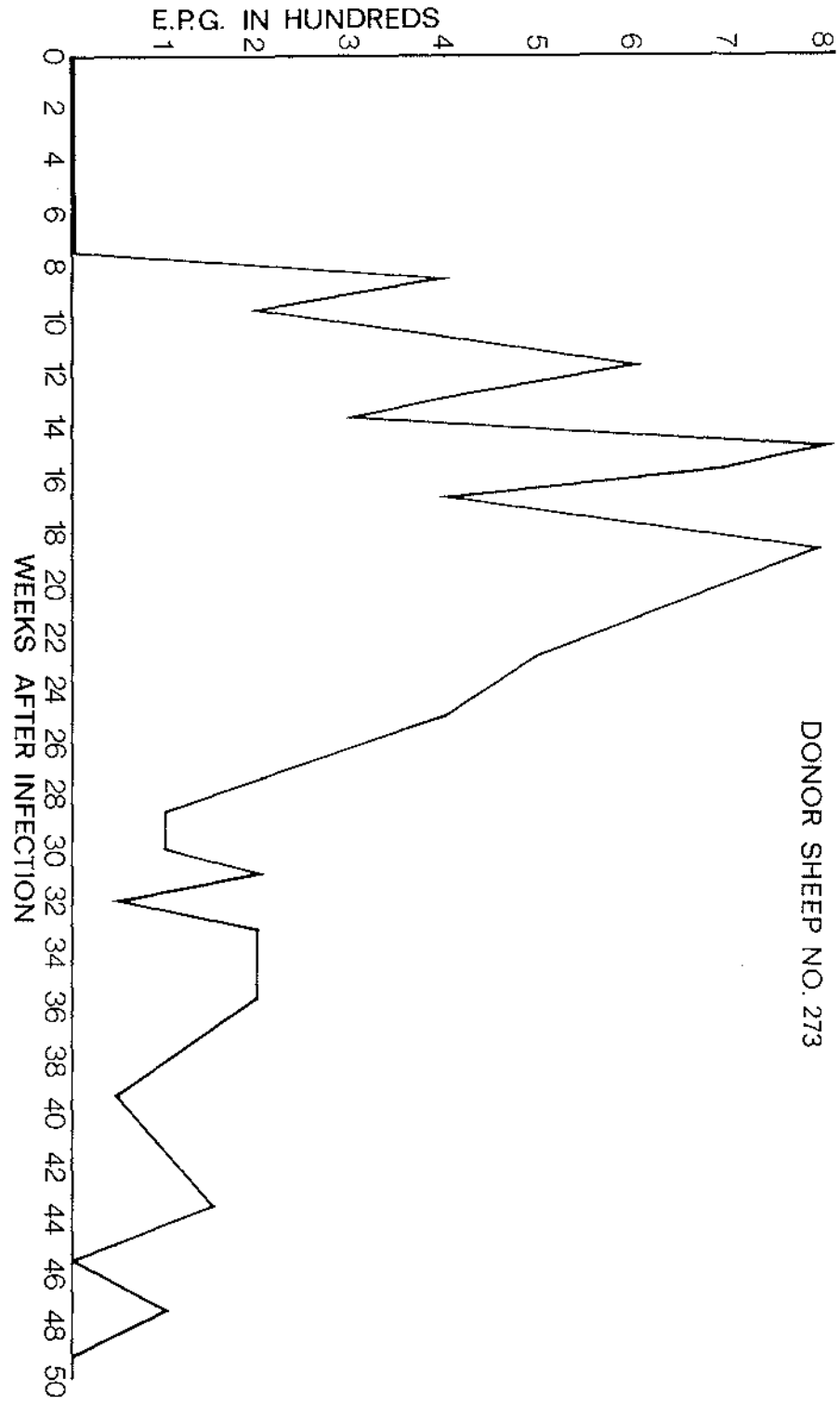

Fig. 2 Graph showing egg production of Donor sheep 273

in the text only those cultures in which less than 10% of the larvae had exsheathed were used in the experiments conducted in this thesis. The 'spontaneous' exsheathment observed during culture is discussed in more detail in chapter 8.

Egg counting technique

Quantitative Count:- In preliminary experiments several techniques were evaluated. The method found to give the most consistent results was that of Brunsdon (1966). 1.7 gm of faeces were placed in a small square sided bottle (capacity 51 cc). The faeces mixed with 20 ml of water to which was added 31 ml of saturated saline. The faecal suspension then stirred with a paddle attached to an electric drill for approximately 30 seconds. Two aliquots were then taken, placed under McMaster slide and egg counts recorded. Various suspending agents were also evaluated; these included saturated Zn SO_4 and saturated sugar solutions. Neither were as practical to use as saturated NaCl.

Qualitative count:- Ether-Acetic acid method. This technique was used to determine the presence of B. trigonocephalum eggs in the faeces.

A 1 gm faecal sample was mixed with 5 ml 5% Acetic acid in a mortar The resultant suspension was passed through a sieve and then placed in a centrifuge tube. 5 ml of ether was added and the tube shaken vigorously for two minutes.

The suspension was then centrifuged (2000 r.p.m.) for 5 minutes, the supernatant fluid poured off and the small residue in the bottom of centrifuge tube smeared onto a slide, coverslipped and then examined microscopically for the presence of eggs.

Larval counting technique

An estimation of the numbers of larvae used for each experiment was made prior to use. Larvae were suspended in 10 ml of water and the

universal container gently shaken. 0.5 ml was transferred with a pipette to a bijou bottle. 4.5 mls distilled water were added to bring the volume to 5 mls and to give a 1 in 10 dilution. Larvae were evenly suspended by mixing and 0.5 ml was withdrawn, transferred drop by drop by drop to a petri dish with numbers 1-10 around its perimeter. 0.01 ml of iodine was added to each drop and the number of larvae per drop were counted and totalled.

This procedure was replicated four times and the total count averaged, then multiplied by 20 which gave an estimation of the number of larvaer per ml of original suspension. This figure was then recorded to the nearest 1000/ml.

Killing Larvae of B. trigonocephalum

There are two generally accepted methods for killing larvae

(1) The use of solutions containing iodine.

(2) By the application of heat

(a) by immersing test tubes containing suspensions of larvae in boiling water.

(b) the direct flaming of a slide containing a suspension of larvae.

In a set of preliminary experiments on the infective larvae of B. trigonocephalum it was found that heat caused a marked increase in the degree of exsheathment whereas iodine caused no significant change in the degree of exsheathment. Iodine was therefore selected throughout this study as the routine method for killing the larvae of B. trigonocephalum. The Iodine solution used in all the experiments was made up as follows

30 gms Iodine

40 gms Potassium Iodide dissolved in 100 ml distilled water

Preliminary experiments showed that a small drop (0.01 ml - 0.02 ml) of this iodine solution was sufficient not only to kill larvae suspended in 0.05 ml water but also kill larvae suspended in 5 ml.

The Administration of Infective Larvae to Sheep

The number of larvae administered to sheep in different experiments varied. The number used in each individual experiment is recorded in the relevant chapter.

Just prior to administration the selected number of larvae to be used on each sheep were placed in individual bijou bottles. One bijou bottle was allocated at random to each sheep which had previously been grouped on a random basis. The contents of each bijou bottle were drawn up into a separate 2 ml disposable syringe and the larval suspension administered to the sheep according to the design of the particular experiment involved (percutaneously, *per os* or parentally). The bijou bottles were rinsed out with 1 ml of distilled water drawn up by the syringe which corresponded to each bottle. The washings from each bottle were then administered to the relevant sheep. Finally 1 ml of water was drawn up by each syringe and put into its corresponding bottle. The contents of each bottle were then examined for the presence of larvae. In no case were more than 0.5% of the initial number of larvae found to have been left in each bijou bottle.

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CHAPTER 3

MORPHOLOGICAL STUDIES ON THE DEVELOPMENT OF THE FREE LIVING AND PARASITIC STAGE OF B. TRIGONOCEPHALUM

Introduction

The morphological development of B. trigonocephalum has been described by several workers (Cameron 1923 and 1927, Hesse 1923, Ortlepp 1939, Soulsby 1965, Monnig 1968, Lapage 1968).

The fullest account of the morphology of the parasitic stages is that of Cameron (1927). On the other hand the free living stages have been studied more closely by Ortlepp (1939). However, little precise information is given on the morphology and development of B. trigonocephalum; Ortlepp merely states that they are similar to those of Gaigeria pachyscelis described in a previous paper (Ortlepp, 1937). Hesse (1923) supposedly gives details on both the morphology and development of the earlier stages of B. trigonocephalum, but Cameron (1923) was of the opinion that the larvae described were not Bunostomum but Nematodirus. The drawings by Hesse (1923) of 3rd stage larvae however, show that it is a trichostrongylid with 16 intestinal cells - presumably Haemonchus contortus. It is certainly not the larvae of Nematodirus as Cameron (1923) assumed since the 3rd stage larvae of Nematodirus Sp. have 8 not 16 intestinal cells (Eckert 1960).

It is evident from a perusal of the available literature that although there is agreement amongst workers on morphological and developmental details of the later parasitic stages, this is not so in regard to the free living stages. Measurements made on the overall length of 3rd stage larvae of B. trigonocephalum by Dickman and Andrews (1933), Monnig (1931), and Ortlepp (1939) differ one from the other. Whitlock (1960) in a discussion on Bunostomum Sp. states that the free living stages do not feed but exist during this time on the food stored in the larva. He also goes on to say that the infective larvae are enclosed in two sheaths. Other workers, notably Cameron (1923)

and Ortlepp (1939), describe a 1st ecdysis taking place. Cameron's accounts on this particular stage though are both brief and superficial and Ortlepp's observations relate in the main to G. pachyscelis.

In view of these existing anomalies it was felt that prior to studying other aspects of B. trigonocephalum an attempt should be made to clarify the situation. A thorough study was therefore made on the morphology of the different stages of development of this particular parasite, greater emphasis though being placed on the earlier rather than the later phases of the life cycle.

Materials & Methods

(a) Studies on the morphology and development of the Egg

Faeces from a sheep with a pure infection of B. trigonocephalum were collected and an estimate made of the number of eggs per gram (see General Methods) 10 gram aliquots of faeces were placed in Petri dishes with a moistened filter paper beneath the lid of each dish. Since Belle (1959) found that the highest percentage of larvae hatched at 25°C, the cultures were maintained at this temperature.

Immediately after collection of the faeces from the donor animal, eggs were examined after flotation in saturated salt solution. Eggs were similarly examined from the cultures at six hour intervals from the time of being placed in the incubator for a period of 54 hours. One culture was examined 78 hours after collection. All examinations and measurements were made at a magnification of 400 x.

1: At each examination 10 eggs were selected at random, their length and width measured. Observations were made on the stage of development that the embryo had reached within these eggs. This stage of development was classified as follows:-

(i) In the early stages of development the number of

cells was counted.

(ii) When the number of cells exceeded 14 it was difficult to assess the exact number. This stage was recorded as the blastula stage.

(iii) There was then a marked reduction in the size of the cells and the cellular mass resembled a "bunch of grapes". This was designated the gastrula stage.

(iv) Following the gastrula stage, a tail-like appendage was seen developing from the cellular mass. This was designated the early tadpole stage.

(v) The final stage of development was the appearance of the motile larva within the egg. This was described as the tadpole stage.

2: Measurements on the length and the width of 55 eggs in the morula stage and on 55 eggs in the tadpole stage were made to provide data on egg size.

3: A record was kept of the time when each of the five mentioned stages of development was first noticed in the cultures.

4: A series of four egg counts was done on each of the cultures at the time each one was being examined. Four separate 1 gram samples being taken from each culture.

(b) Studies on the morphology of the free living larval stages

Faeces were collected from donor sheep and eggs per gram estimated. Seven bottles containing faecal material were placed in an incubator at 27°C and cultured in the usual manner (see General Methods). 48 hours after culturing had commenced one bottle was removed once every 24 hours for a period of eight days. Larvae were recovered from each culture by the baermann technique and their morphology and development studied. Larvae were

examined at a magnification of 400 x.

1: A portion of the larvae collected from each culture were killed with aqueous iodine (see General Methods). 50 larvae were examined at random and their length and width at the level of the junction of the oesophagus and intestine were measured.

2: The remaining live larvae were examined on slides which had been cooled by placing over "dry ice" - care being taken not to actually freeze the larval suspension. Initially the larvae were stationary and it was possible to study their morphology in detail. Measurements of certain internal organs were taken. As the temperature of the larval suspension rose towards room temperature the motility of the larvae gradually increased. During this stage observations were made on the manner in which they moved and fed. A small drop (approx .05 ml) of .02% "Coomassie"* Blue dye, was added to the suspension of larvae on some slides , - this dye did not immediately stain the larvae but gave a contrasting background.

3: 20 third stage larvae were killed with iodine. Measurements were made of the distance between

(1) the anterior end and
   (a) Excretory pore
   (b) Nerve ring
   (c) Posterior end of the oesophagus

(2) the Anus and
   (a) Posterior tip of the tail
   (b) Posterior tip of the sheath

* "Coomassie" Blue (Medical) Sodium Anoxynapthonate B.P. I.C.I.

(c) Studies on the Parasitic Stages from the definitive Host (Sheep)

1: 3rd and 4th stage larvae - Four sheep were each infected with 4,000 viable ensheathed 3rd stage larvae - 2 sheep by the percutaneous route, 2 sheep by subcutaneous injection of the larvae. 1 sheep of each pair was slaughtered six days after infection and the remaining 2 sheep were slaughtered eight days after infection. The lungs from these sheep were minced through a household mincer, placed in a filter funnel containing 0.85% saline and held for 6 hours in a water bath at 37°C. Samples were taken at two hourly intervals, placed in petri dishes and examined for the presence of larvae under a dissection microscope. Any larvae found were transferred to a slide and a more detailed examination undertaken at a magnification of 400 x.

2: The 5th stage of B. trigonocephalum - The morphological study on this stage was conducted on specimens obtained from sheep slaughtered in association with other experiments relating to this thesis.

Results & Discussion

Preparasitic Stages

1: The Egg:-

The sizes of eggs of B. trigonocephalum quoted by various authors are as follows:-

length (microns)		width (microns)	reference
75u-83u	x	38u-45u	Hall (1924)
84u-96u	x	44u-53u	Wood (1931)
75u-85u	x	38u-45u	Ross & Gordon (1936)
82u-97u	x	47u-57u	Shorb (1939)
88u-104u	x	44u-56u	Kates (1947)
80u-102.5u	x	35u-50u	Cunliffe & Crofton (1953)
79u-97u	x	47u-50u	Lapage (1968)

In the present study the eggs were observed to vary between 77u-99u in length and 45u-54u in width. The mean length for those in the morula stage was 88.04u S.E. 0.4988, width 49.6u S.E. 0.2786. The mean length for those in the tadpole stage was 87.9u S.E. 0.4641, width 49.4u S.E. 0.3372 (see table No. II in Appendix).

When passed in the faeces the eggs contained between 6-10 cells (see table No. I in Appendix). However, in spite of the fact that initially all eggs were at a similar stage of development and all were cultured at the same temperature, their subsequent development varied considerably. Silverman and Campbell (1959) in a study on the effect of temperature on hatching demonstrated that eggs from an individual worm varied markedly in the time required for hatching at any one temperature and they quoted median and mean times but Crofton and Whitlock (1965a) point out that these are not satisfactory statistics in a system where variation is high. It would appear from the work of Crofton (1965) and Crofton and Whitlock (1965 b,c) that observations on the minimum hatching time are the most useful. Not only is this technically the most simple observation to make but it has the merit in enabling a biological boundary to be described. Particular note was therefore taken on this aspect in this study of B. trigonocephalum.

In some eggs cellular cleavage took place quickly, and after 6 hours incubation, eggs containing 10-14 cells were seen. 6 hours later eggs in both the blastula and gastrula stage were noted and 18 hours after culturing had commenced some eggs had reached the early-tadpole and tadpole stage. Hatching was seen to commence at 24 hours. This is in agreement with Sprent's (1946b) findings on B. phlebotomum cultured at 21°-27°C.

However, the majority of eggs took 36 hours to reach the blastula stage after which time development to the tadpole stage proceeded rapidly. In some cases eggs appeared to remain in this latter stage (tadpole) for a

further 24-36 hours, the embryo moving spasmodically within the shell. In others the embryo moved actively thrusting its anterior extremity against the shell which eventually ruptured, allowing the larvae to uncoil and emerge. The majority of those eggs which had not hatched 78 hours after culturing had commenced, were seen to be in either the early tadpole or tadpole stage, (see table No. I in Appendix).

It was difficult in this particular experiment to assess the time when the majority of larvae hatched. An attempt was made to deduce this from a series of egg counts recorded at intervals during culture, but the variation in these counts was too great for them to be of any value. In preliminary experiments relating to the study of the free living larvae of B. trigonocephalum it was found that the greatest number of 1st stage larvae were obtained by the baermann technique 48 hours after culturing had commenced. This confirmed the work of Belle (1959) who stated that eggs hatched in less than 48 hours when cultured at temperatures between 21°-27°C. Ortlepp (1937), working with G. pachyscelis incubated at 26°C, also found that the majority of eggs hatched 36-48 hours after culturing had commenced and in 1939 he inferred that his findings on B. trigonocephalum were similar.

2: Free living larvae

(i) 1st stage larvae - The morphogenesis of Bunostomum trigonocephalum larvae which emerge from the egg is similar to the 1st stage larvae of many other nematodes which infect the gastro-intestinal tract of sheep. However, it was found from observations made in this study that they were slightly larger than those sizes of 1st stage larvae quoted by Whitlock (1959). Unfortunately he did not include B. trigonocephalum in his paper.

The larvae of B. trigonocephalum were measured just after hatching and their length was found to vary from 416-496u and width 22-26u (mean length 454.2 S.E.2.5226, width 22.3u, S.E. 0.1227). The body was widest at

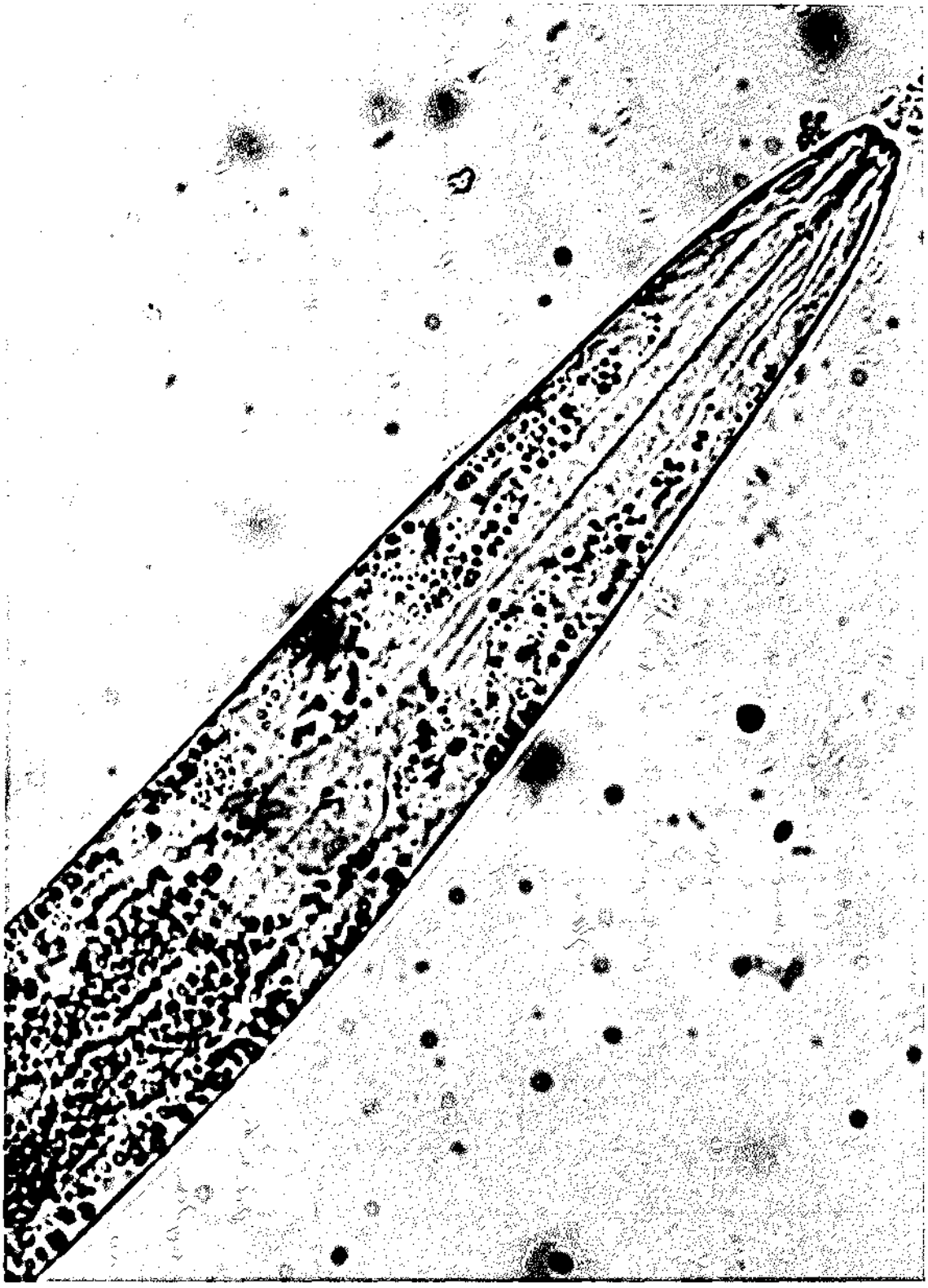

Fig. 3 Photomicrograph of the anterior end of a 1st stage larva of B. trigonocephalum.

the beginning of the intestine and from this point tapered in an anterior and posterior direction. Around the mouth small refractile points together with transparent protusions were noted. The mouth itself was less than 5u in length ending abruptly in the buccal tube which extended for approximately 10u and led into a typically rhabditoid oesophagus. This began with a transparent fusiform swelling which narrowed to an isthmus and then terminated in a slight bulb. The overall length of the oesphagus was approximately 100u. The structure of oesophagus was clearly discernible, particularly in those larvae examined against a contrasting background of Coomassie blue. Fine transverse striations were seen anteriorly and the 'valve flaps' of the posterior bulb (haustrulum) identified (fig 3).

It was difficult, in these early 1st stage larvae, to define any structures occuring posterior to the oesophagus as the larvae appeared to be full of opaque globules. The zig-zag course of the intestine was however, visible.

The newly hatched larvae did not seem to be highly active but progressed slowly with an unsteady wavering motion, often attaching themselves with their whip like tails to each other or to small particles of debris. At intervals movement ceased and the oesophagus was seen to contract and dilate rapidly for a few seconds. This was followed by a period of rest and then the cycle was repeated again. These short bursts of pharyngeal pump like action continued for two to three minutes, after which the larvae would commence to 'swim' again. The high frequency intermittent action of the oesophagus seen to occur in these larvae gives additional support to Crofton's (1966) theory on the manner in which the oesophagus 'pumps' material through to the intestine.

On the third day after culturing had commenced the larvae had grown to approximately 500 u in length (mean 505.8u, S.E. 2.8915), and there had been a slight increase in width (see table III in Appendix). It was possible to see

Fig. 4 Photomicrograph of the posterior end of a 2nd stage larva of B. trigonocephalum.

the nerve ring and distinguish the intestinal cells. The rectum was also visible running in a slanting direction towards the anus and the tail was seen to be long and thin ending in a whip like filament.

After a further 24 hours the majority of larvae were over 600u in length (mean 634.4u and S.E. 4.7653) 27u in width (see table III in Appendix). They appeared to be entering a phase of lethargus prior to moulting from Stage 1 to Stage 2.

(ii) 2nd stage larvae - A few 2nd stage larvae encased in the first stage cuticle were first noticed on the 4th day, but they were not seen in any numbers until the 5th day after culturing had commenced. The 2nd stage ensheathed larvae were more slender than 1st stage larvae. Their measurements ranged from 512-672u in length and 22-29u in width. (mean length 636.3u S.E. 4.0806, mean width 23.3u, S.E. 0.2586), (see table III in Appendix). The anterior protusions seen on the mouth of 1st stage larvae had disappeared and the anterior end had become more rounded. The oesophageal bulb was less rounded

The intestinal cells were difficult to count since the mid portion of the larvae contained globules. The tail was bluntly rounded (fig 4), but the tail sheath still retained its whip like character. At this time (day 5) 90% of the 2nd stage larvae seen were still encased in the cuticular sheath of the 1st stage which, although separated from the 2nd stage body, was closely applied to it except at the anterior end where a space of approximately 5u separated it from the anterior tip. (fig 5).

Although detailed observations were made on the cultures, the "typical" 1st ecdysis of strongyloid larvae as described by Soulsby (1965) was not seen to occur. Soulsby (1965) states that the anterior cap is "virtually" knocked off and Ortlepp (1937) mentions 2nd stage larvae of G. pachyscelis breaking through the cuticle of the 1st stage by forcibly

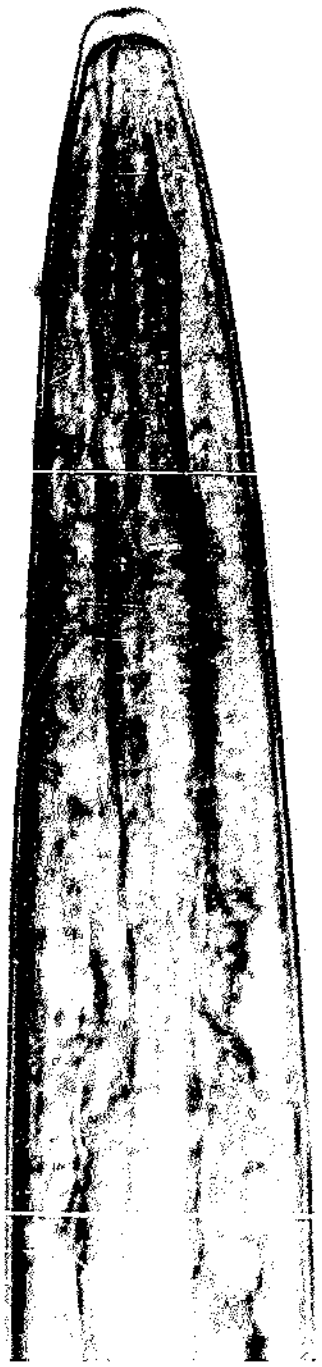

Fig. 5 Photomicrograph of the anterior end of a 2nd stage larva of B. trigonocephalum.

pushing off the anterior end of the cuticle which comes off like a cap. Sprent (1946) in his paper on B. phlebotomum says that "the site of emergence is not known", but added that "the anterior end of the cast sheath is undamaged" and that the sense organs of the 1st stage are still visibly on it.

There is little reference in the literature to the manner in which the 1st ecdysis of B. trigonocephalum takes place. Most workers (Cameron, Ortlepp), except for Whitlock (1960), state that exsheathment at this phase in the life cycle occurs. The present observations have shown that this is undoubtedly the case as from the 5th day on 2nd stage larvae were seen without the ensheathing cuticle and moving actively and feeding.

A careful examination of suspensions containing 2nd stage larvae moulting, failed to reveal any empty sheaths. It was noticed however, that small particles were adhering to the surface of the moulted larvae. It is suggested that the sheath disintegrates rather than being cast off in one piece, and that this particulate matter adhering to the larvae may be fragments of the 1st stage cuticle.

The time interval between 2nd and 3rd stage was short. After six days culturing some ensheathed 3rd stage larvae together with partially and completely moulted 2nd stage were seen.

Measurements of 2nd stage larvae with and without their sheaths were made. The dimensions of the ensheathed 2nd stage were slightly longer mean length 652.5 S.E. 3.0456 but width was similar to that obtained 24 hours previously. The exsheathed larvae were 80-100u shorter than their ensheathed counterparts (this being the length that the sheath extended beyond the tail of the ensheathed 2nd stage). Once the 2nd stage larvae had completed their moult the majority did not appear to remain in the culture for more than 24 hours before moulting to the 3rd stage.

(iii) 3rd stage larvae - Seven days after culturing had commenced the culture contained 80% ensheathed 3rd stage larvae. According to Soulsby (1965) the 3rd

stage larvae of B. trigonocephalum are the smallest of the 'strongyle' larvae found in sheep and he gives their length as varying between 514-678u after Dickmann Andrews (1933). He also quotes measurements made by other workers, 560-637u (Monnig 1931), 450-700u (Hesse 1923). This latter reference should be deleted from the literature as Hesse's measurements were certainly not made on B. trigonocephalum. Ortlepp (1939) in his study found that the length varied from 563-632u.

In this study the 3rd stage larvae measured 7 days after culturing had commenced (see Table No III in Appendix), had a mean length of 649.2u (S.E. 3.3747) and mean width of 23.8u (S.E. 0.1743).

The morphology of the 3rd stage larvae was quite distinct from that of the 1st stage, however care was required in differentiating ensheathed 2nd from ensheathed 3rd stage larvae. The buccal tube of the 3rd stage was similar to that of the 2nd stage. It was approximately 15u in length and was dilated at its anterior end. The oesophagus of the 3rd stage though was more filariform and did not appear to be as thick as that of the 2nd stage. Both 2nd and 3rd stage larvae possessed a posterior oesophageal bulb but it was more conspicuous in the latter than the former. The nerve ring and excretory pore were also more easily seen in 3rd stage larvae than 2nd stage larvae; similarly, the intestine, their cells and the anus were more distinct. The granules and globules that were seen in the 2nd stage were not as noticeable in the 3rd stage larvae. The tail of the 3rd stage was however similar to that of the 2nd stage being blunt and stumpy.

The following measurements were made on 20 third stage larvae (see table IV in appendix).

The distance between:-

1: the anterior end and

	Range u	Mean u	S.E.
the end of the oesophagus	163-192	175.2	1.6118

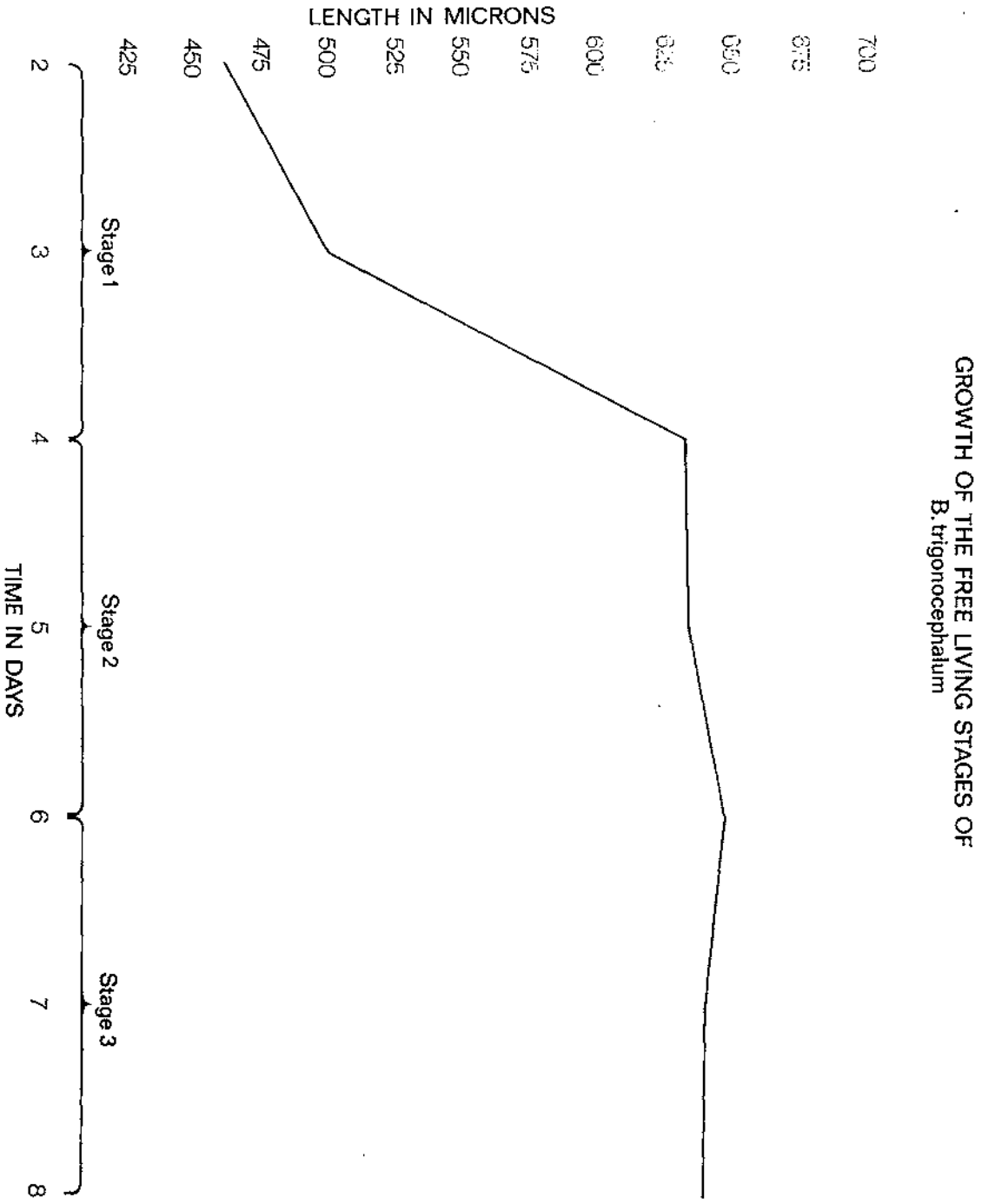

Fig. 6 The growth pattern of the preparasitic stages of the larvae of B. trigonocephalum.

the excretory pore	96-112	106.8	1.1859
the nerve ring	83-96	91.1	0.8334
2: the anus and			
the posterior tip of the tail	61-77	68.3	0.9200
the posterior tip of the sheath	144-182	165	2.3963
3: the posterior tip of the tail and			
	Range u	Mean u	S.E.
the posterior tip of the sheath	67-112	96.7	2.9497

The foregoing measurements are in most cases similar to those recorded by other workers. Ortlepp (1939) gave the distance between the anterior end and the excretory pore as 99-102u and the distance between the anterior end and the nerve ring as 85-87u. Soulsby (1965) quotes the length of the larval tail as being 55-68u in length. Unfortunately he does not mention the anterior location from which the measurements were taken, one can only assume that it was the anus. The distance found in this study between the anus and the tip of the sheath was closer to measurements made by Dickmans and Andrews (1933) who gave it as varying from 153-183u, than to Monnig (1931) who gave the distance as being 133-158u. In this study the mean distance recorded between the tip of the tail and the tip of the sheath was much less than the figures quoted by Soulsby (1965). He states that "the tail sheath projects a distance of 85-150u from the end of the larva".

The growth pattern (1st stage larvae through to ensheathed 3rd stage larvae).

The mean size of the 1st stage larvae of B. trigonocephalum was observed in this study, to be greater than that of the 1st stage larvae of most other nematodes infecting sheep. The 3rd stage larvae of B. trigonocephalum on the other hand was found to be smaller than the corresponding stage of other nematodes infecting sheep. The larvae of B. trigonocephalum appeared to grow rapidly during their 1st stage, but once moulting to the 2nd stage had occurred, little increase in length was noted. (fig 6). This finding is contrary to

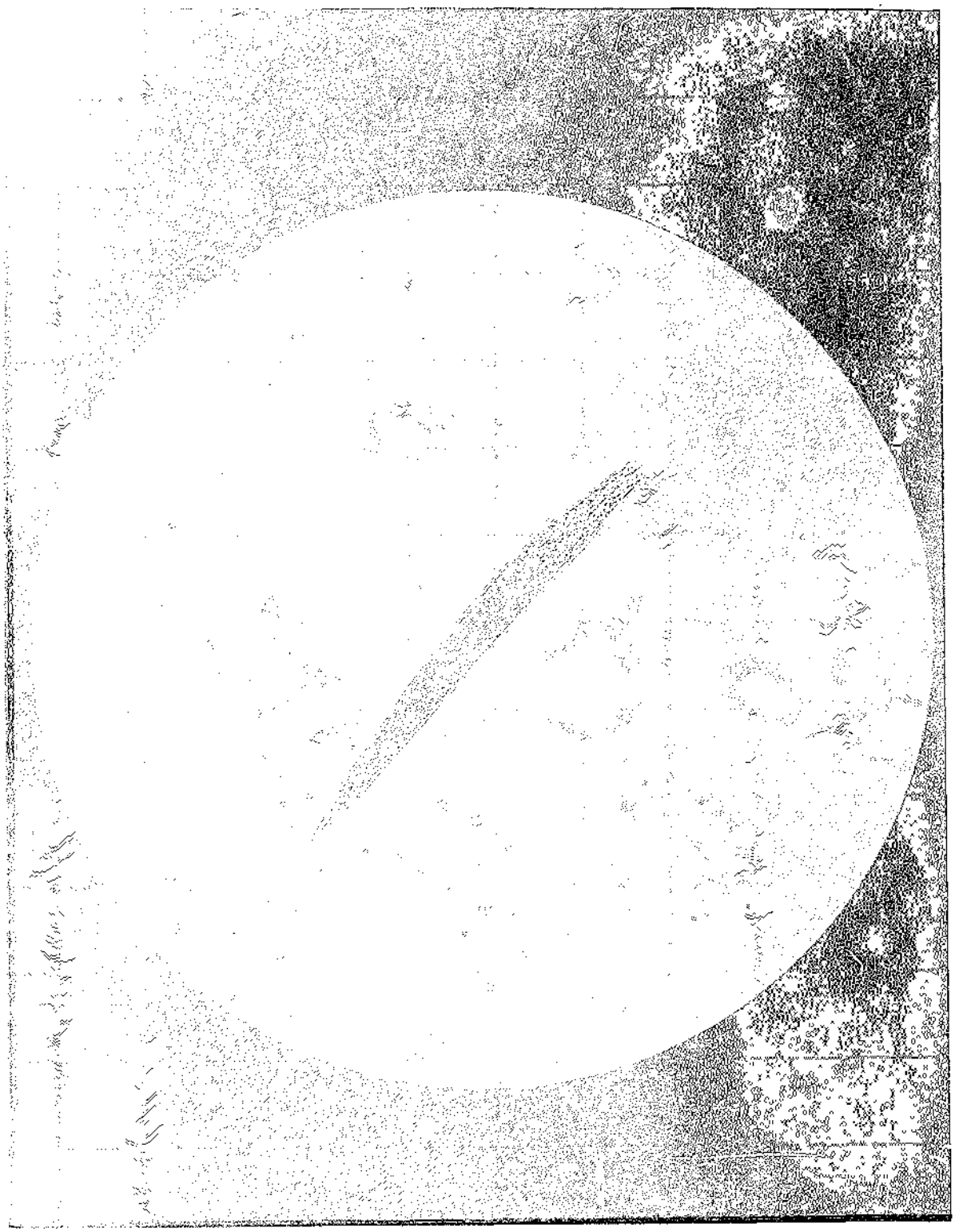

Fig. 7 Photomicrograph of a late 3rd stage larva of *B. trigonocephalum* recovered from the lung of a sheep.

the typical growth pattern mentioned in the literature (Rogers 1961, Soulsby 1965) for the free living larval stages of the majority of nematodes infecting sheep. Both the 1st and 2nd larval stages of these nematodes are reported to grow in length.

Observations made on the 2nd stage larvae of B. trigonocephalum indicated that once they had completely moulted they did not remain long in this phase before becoming ensheathed 3rd stage larvae. These completely moulted 2nd stage larvae consequently had little time to feed. This fact may account for the lack of growth, which was recorded in these experiments, between the 2nd and 3rd stage.

(iv) Exsheathed 3rd stage larvae. 8 days after culturing approximately 8% of the 3rd stage larvae obtained had exsheathed. In cultures incubated at 27°C a certain number of 3rd stage infective larvae were invariably seen to have exsheathed at this time. Since exsheathment is so closely associated with the process of infecting the host, a special study of this aspect was made and is described in chapter 8.

Parasitic Stages:-

Both 3rd and 4th stage larvae were obtained from the lungs of sheep slaughtered 6 days after being percutaneously or subcutaneously infected with B. trigonocephalum. The majority of larvae found were still in the 3rd stage. Some were morphologically similar to the exsheathed 3rd stage larvae seen in cultures, except that they were reddish in colour, as were all the larval recovered from the lung. But most 3rd stage larvae observed were much thicker though no longer than the earlier 3rd stage larvae - (500-530u in length). (fig 7).

In the sheep slaughtered 8 days after infection, a higher proportion of 4th stage larvae were seen together with numbers of late 3rd stage larvae which appeared lethargic and in the process of moulting to the 4th stage.

During and following this moult the buccal tube of the 3rd stage was seen to be replaced by a provisional buccal capsule; the formation of this capsule being preceded by an increase in width of the anterior end. The 4th stage larvae at this time were encased in the cuticle of the 3rd stage. No 4th stage larvae which had moulted completely were observed in this particular experiment. The early parasitic stage of B. trigonocephalum was intensively studied by Ortlepp (1939) he also did not see any evidence of a completed 3rd ecdysis taking place in the lungs but he did recover ensheathed 4th stage larvae from the lungs. However, Sprent (1946) mentions 4th stage larvae of B. phlebotomum, moulting in the lungs 10 days after skin penetration and suggests that migration to the intestine probably occurs as soon as ecdysis is complete. Failure to find 4th stage larvae free of the 3rd cuticle may have been due to the fact that either:-

(1) The 3rd ecdysis may take place later than 8 days after infection.

or (2) The exsheathed 4th stage larvae may migrate immediately after moulting and as only a relatively small number of larvae (100-150) were recovered from some of the lungs, the chance of missing this stage was high.

A further study of this particular phase in the life cycle of B. trigonocephalum is warranted. Demidowa (1957 & 1958) found 4th stage larvae in the lungs 6-10 days after percutaneous infection. Ortlepp (1939) recovered ensheathed 4th stage larvae 8 days after infection; the length of these 4th stage larvae, varied from 551-592.

The youngest forms obtained by Cameron (1927) were completely moulted 4th stage larvae from the intestine, he failed to observe any larvae in transition from stages 3 to 4. Had Cameron examined the lungs he would undoubtedly have observed the earlier stages. The morphology of the later

Fig. 8 Photomicrograph of the buccal capsule of an adult *B. trigoncephalum*.

4th and 5th stages though have been adequately described by Cameron (1927).

Exsheathed 4th stage larvae have been found in the small intestine 11 days after infection. According to Ortlepp (1939) the most striking feature of these larvae as compared with those 4th stage larvae recovered from the lung was the presence of a dorsal and two sub-ventral lancets, these were absent in the lung forms. Demidowa (1957 & 1958) obtained larvae from the small intestine 12-17 days after infection. Fifteen days after infection sexual differentiation commences and is completed by the 35th day. Ortlepp (1939) found that the 4th ecdysis occurred during the 5th week after infection and that the parasites attain sexual maturity 4-5 weeks later. Observations associated with this thesis indicate that sexual maturity occurred much earlier than recorded by Ortlepp (1939) and was reached in the majority of cases 8 weeks after percutaneous infection.

The morphology of both male and female adults was studied in detail and the anatomical findings were in general agreement with Cameron (1927) and Ortlepp (1939). The large buccal capsule was seen to contain a pair of subventral lancets in its base, and on the dorsal aspect the duct of the dorso-oesophageal gland was seen in the dorsal gutter and extending on to the dorsal cone (fig 8).

Measurements were made on 100 adult males and 100 adult females. The overall length of the male was found to vary between 12-17 mm mean 15.4 mm S.E. 0.0973 and that of the female 17-25 mm mean 20.8 S.E. 0.1682 (see tables V and VI in Appendix). The length of the male spicules were also measured and were within the range quoted by Soulsby (1965) 600-645u.

When copulating the male was observed firmly attached to the female, the pair lying at right angles to one another. The bursal lobes of the male appeared to completely encircle and enclose the vulva and the twisted male spicules were seen passing through the cloacal aperture and into the vagina (fig 9).

Fig. 9 Male and female B. trigonocephalum in process of copulation.

Prepatent period:- Information was derived from faecal egg counts conducted on sheep used in experiments throughout this thesis. Faeces were collected on a daily basis and examined by Ether/Acetate method. (See General Methods).

The eggs of B. trigonocephalum were never found in samples until at least 52 days had elapsed from the time of infection. In the majority of cases eggs were first noticed in the faeces 53-56 days after infection. The prepatent period observed in these experiments did not appear to be influenced by the method of infection (percutaneous, subcutaneous, intraruminal or oral). Stoye (1965) also found no correlation between prepatent period and route of infection.

The prepatent periods recorded in the literature for B. trigonocephalum vary considerably.

Prepatent period in Sheep	Reference
17-24 days	Beller (1928)
63-70 "	Ortlepp (1939)
53-60 "	Lucker & Neumayer (1946)
40-46 "	Demidowa (1958)
49-53 "	Stoye (1965)
51-54 "	Westen (1968)
28-42 "	Soulsby (1965)

The figures given by both Beller (1928) and Soulsby (1965) are open to question. It appears from a study of the literature that the eggs which Beller observed, although of B. trigonocephalum, were in fact from infections gained prior to the commencement of his experiments. Soulsby, (1965) in his textbook, incorrectly quotes Ortlepp (1939) saying that maturity is reached in 5 weeks, Ortlepp (1939) actually states that "the larvae moult after the 5th week and grow to attain sexual maturity in from 9-10 weeks". Secondly, Soulsby mentions that Sprent (1946b) noted that the adults were mature 30-56

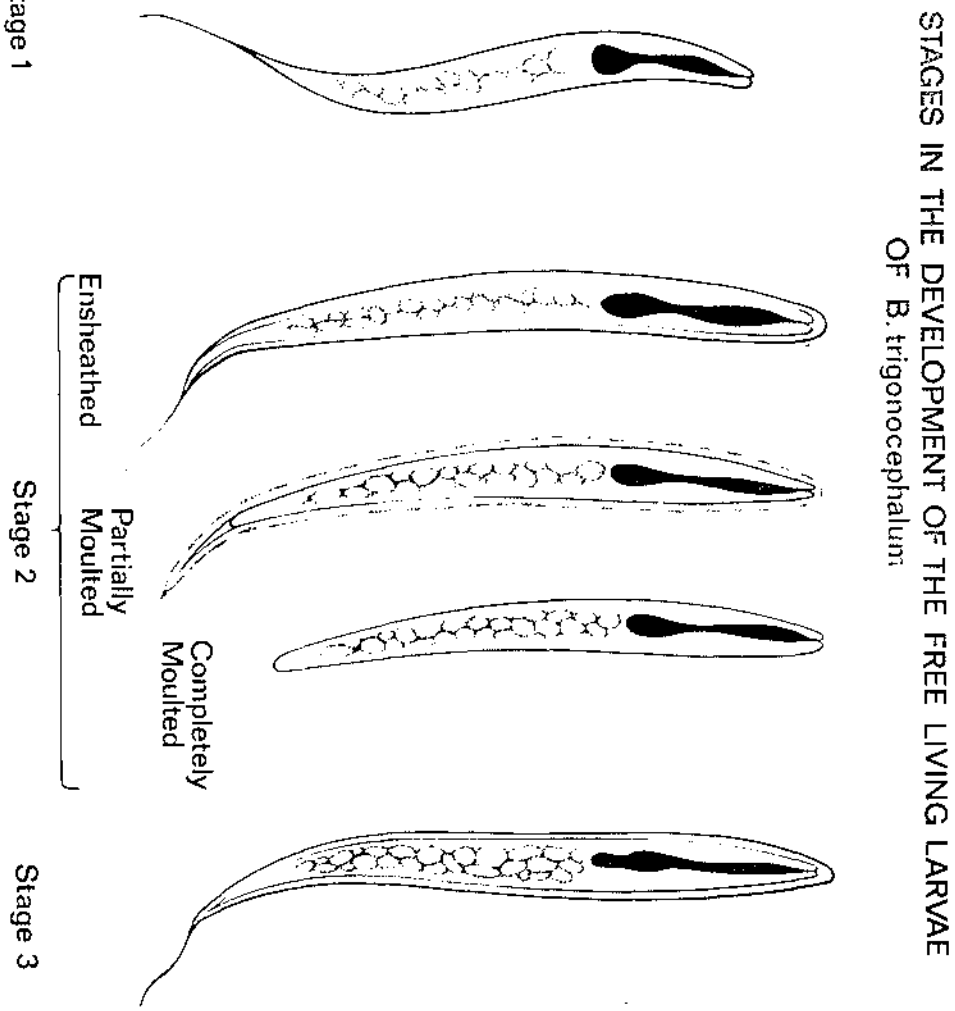

Fig. 10 Stages in the development of the free living stages of B. trigonocephalum.

days after penetration of the skin, whereas Sprent's (1946) figures refer to B. phlebotomum not B. trigonocephalum.

Even dismissing the two foregoing references the variation between Demidowa's findings and those of Ortlepp is still considerable. It has been suggested by Stoye (1965) that age may influence the prepatent period, he considers that the development of B. trigonocephalum may be retarded in older animals. This phenomenon is seen to occur in the case of Ancylostoma caninum (Herrick 1928), (Kotlan 1960). However, this seems unlikely in the case of B. trigonocephalum. Ortlepp's experiments were undertaken on young lambs which had previously not been exposed to the infective larvae of B. trigonocephalum. The existence of strain differences within the species could possibly account for this variation, on the other hand less sensitive methods of faecal examination, particularly that used by Ortlepp, could also be a major factor contributing to this variation.

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CHAPTER 4
EXPERIMENTS ON THE EFFECT OF TEMPERATURE ON THE SURVIVAL OF INFECTIVE LARVAE

Introduction

Many workers have shown that temperature and humidity are the two most important factors governing the development and survival of the "free living" stages of those Zooparasitic nematodes infecting sheep. Detailed experimental studies have recently been undertaken on the viability of the infective stages of Trichostrongylus colubriformis (Andersen et al 1966; Andersen and Levine 1968). The survival of the infective larvae of Bunostomum trigonocephalum has however only been studied by a few workers. Early investigators (Cameron 1923[c]; Hesse 1923) did not critically study the effect of the microenvironment but made observations of a general nature. Later workers (Demidowa 1957; Sarimsakov 1959; Belle 1959; Narrain 1965) carried out more carefully controlled observations on the biology of these larvae. However, as the findings of these later workers differed one from another it was felt that further investigation on this aspect was warranted. This aspect was of particular importance as it was necessary to find out the optimum temperature for the survival of the infective larvae so that they could be kept in a viable state for experimental purposes.

An experiment was therefore designed to study the survival of infective larvae at various temperatures. At the same time the effect that these various temperatures had on the exsheathment of these particular larvae was also observed.

Materials & Methods

1 ml of a suspension containing approximately 1000 larvae/ml was placed in a McCartney bottle. To this was added 2 mls distilled water. The lids were loosely screwed on. Groups of six McCartney bottles containing larvae were put inside a quart preserving jar containing approximately 1" of

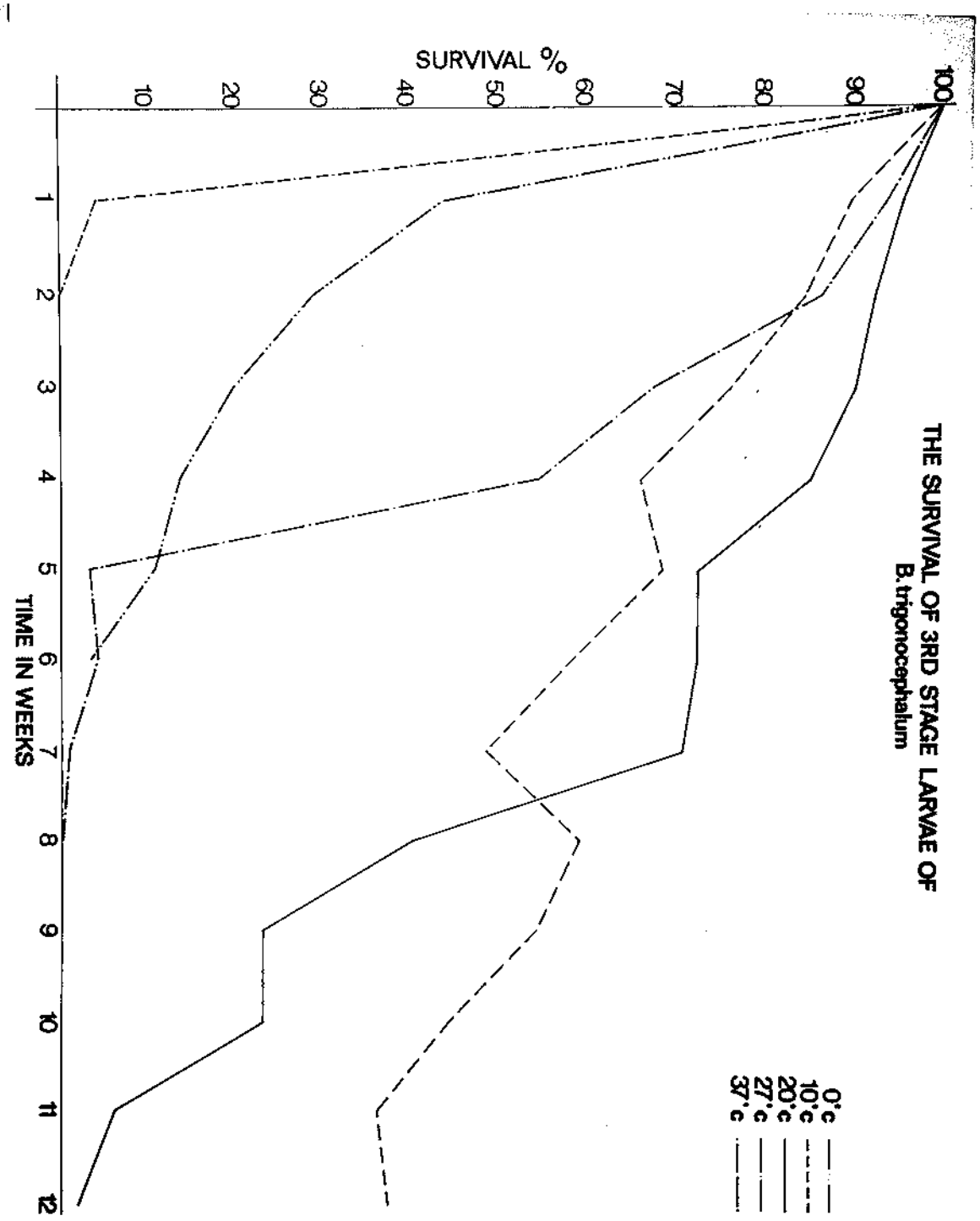

Fig. 11 The effect of temperature on the survival of the 3rd stage larvae of B. trigonocephalum.

distilled water and the lids of these jars were again screwed loosely into position.

The jars containing the McCartney bottles were then held at the following temperatures:

Area	Temperature
Refrigerator	0°C
Cool Room	10°C
Animal House	Approx. 20°C
Incubator	27°C
Incubator	37°C

A maximum/minimum thermometer was placed beside the jars in each area and read at weekly intervals. The temperature did not fluctuate by more than $\pm 0.75^{\circ}C$ in any of the selected environments except in the "Temperature regulated" Animal House where the maximum total variation recorded was $4.5^{\circ}C$ (see table No VII in Appendix).

Once a week one McCartney bottle was removed from each of the controlled environments. The suspensions of larvae were allowed to reach room temperature and 100 larvae were examined at random. The percentage alive was recorded. The remaining larvae were then killed with iodine (see General Methods) and a further 100 larvae examined for presence or absence of a sheath. Twelve weekly examinations were conducted on those larvae held at $10^{\circ}C$ and $20^{\circ}C$, but on those held at $0^{\circ}C$, $27^{\circ}C$ and $37^{\circ}C$, studies were terminated 6, 9 and 3 weeks after the commencement of the experiment.

The larvae throughout this experiment were kept in water. Preliminary experiments had shown that larvae died rapidly in the absence of free water.

Results

1: Survival of larvae - At the commencement of the experiment all the larvae were alive. The survival of these larvae at different temperatures

BUNOSTOMUM TRIGONOCEPHALUM VIABILITY STUDY :

% LIVE LARVAE

	WK. 1	WK. 2	WK. 3	WK. 4	WK. 5	WK. 6	WK. 7	WK. 8	WK. 9	WK. 10	WK. 11	WK. 12
$0^{o}C$	44	28	20	13	11	3	-	-	-	-	-	-
$10^{o}C$	90	85	77	67	69	59	49	60	55	45	37	38
$20^{o}C$	96	93	91	86	73	73	72	41	23	23	6	2
$27^{o}C$	94	87	68	55	3	4	1	0	0	-	-	-
$37^{o}C$	4	0	0	-	-	-	-	-	-	-	-	-

Table No 4.1

BUNOSTOMUM TRIGONOCEPHALUM VIABILITY STUDY :

%/EXSHEATHED LARVAE

	WK.1	WK.2	WK. 3	WK. 4	WK. 5	WK.6	WK. 7	WK.8	WK. 9	WK. 10	WK. 11	WK. 12
0°C	9	7	9	6	6	10	-	-	-	-	-	-
10°C	3	5	11	10	23	18	13	32	24	26	13	30
20°C	7	15	22	28	19	26	18	27	27	30	40	41
27°C	22	30	30	33	42	54	58	77	66	-	-	-
37°C	14	16	25	-	-	-	-	-	-	-	-	-

Table No 4.2

is shown in table 4.1 and fig 11.

2: Exsheathment of larvae - table 4.2 shows the percentage of exsheathed larvae at the various temperatures. At 0°C there was no significant change in the ratio of exsheathed:ensheathed larvae. However, at temperatures of 10°C and above, the longer the storage time the greater was the percentage of exsheathed larvae.

Discussion

Survival of infective larvae

Of the various temperatures used in these observations 10°C gave the best survival rate. Unfortunately facilities were not available to make a more precise estimate of the optimum temperature for survival. In general these results are in agreement with those of Belle (1959). However, Belle obtained 100% survival after 18 weeks at temperatures between 10° and 20°C. Sarimsakov (1959) found that larvae "lived well at room temperature (8°-25°C)".

At 0°C there was a rapid loss of viability although some larvae survived for six weeks. Belle (1959) obtained no survival at this temperature beyond fifteen days. However, Narrain (1965) records survival rates almost identical with the ones described here, a small proportion surviving for 40 days.

In preliminary experiments which are not recorded in this thesis, it was found that the mortality rate at 5°C was much greater than at 10°C. Belle obtained a maximum survival of four months at this temperature whilst Narrain (1965) observed only a two and a half month survival.

In the present experiments some larvae remained alive for seven weeks at 27°C and less than two weeks at 37°C. But Belle (1959) found that at temperatures of 30°C and 35°C some larvae survived for 84 days and 34 days respectively. On the other hand Narrain (1965) found that the maximum survival time at 30°C was 38 days and at 35°C less than six days.

In the absence of more precise information it is difficult to explain the various findings of different workers. Both Belle and Narrain kept their larvae in water (Narrain used tapwater) but it is possible that the depth of water could have a bearing on the results, through the existence of comparatively lower oxygen tensions in deeper water. It is possible too that, as with other nematodes (Das & Whitlock 1960; Crofton, Whitlock & Glazer 1965) different strains have evolved adapted to different ranges of temperatures. But in this case it is difficult to explain the similarity of Narrain's (1965) findings in India to the present ones.

It is also possible that the culture condition in which strongylid nematodes are produced may affect their subsequent viability.

Although it is conceivable that there are different strains it is difficult to compare and correlate the findings of various workers in this field in order to substantiate this point. The observations of Cameron's (1923) and Hesse's (1923) were comparatively superficial. But even the papers of later workers leave much to be desired. Belle (1959) did not tabulate his results, so that comparison with those obtained in this study is not possible. Narrain (1965) who did tabulate his results used tap water, whilst distilled water was used in the present experiments. Sarimsakov's (1959) experiments were carried out at room temperature which fluctuated and Narrain (1965) has shown that fluctuating temperatures are more lethal to these larvae than constant temperatures. Although no reference in the literature could be found to the effect of temperature on the exsheathment of B. trigonocephalum. Workers have investigated the relationship between temperature and the exsheathment of certain trichostrongylids. In the presence of the "correct" stimuli infective larvae of H. contortus exsheath readily at temperatures 35-37°C. but to a much lesser extent at temperatures below this. The findings in this particular study indicate that the infective larvae of B. trigonocephalum. follow a similar pattern. Exsheathme is discussed in greater detail in chapter 8.

CHAPTER 5

COMPARISON OF ROUTES OF INFECTION IN THE SHEEP

Introduction

The route of hook worm infection was first studied in dogs by Leuckart (1876) and in man by Leichtenstern (1886). Looss in a series of experiments between 1886 and 1904 found that the 3rd stage larvae of Ancylostoma duodenale were infective both per os and percutaneously. In 1911 Looss stated that the 3rd stage larvae of A. duodenale when administered per os developed immediately in the intestine and did not migrate via the lungs. Myagaw (1913, 1916) disagreed with Looss on this point, but further studies by Yokogawa and Oiso (1926), Foster and Cross (1934), Schwartz and Alicata (1936) and Rohde (1959) confirmed the findings of Looss.

Cameron (1923) was the first to study the mechanism of infection of B. trigonocephalum and he concluded that sheep became infected per os rather than percutaneously. In 1927 he conducted further experiments, the results of which, in the light of the findings of Yokogawa and Oiso (1926), he considered, gave additional support to his hypothesis that the larvae of B. trigonocephalum did not infect the host by the percutaneous route. Beller (1928) on the other hand proved conclusively that sheep could be infected both per os and percutaneously. Ortlepp (1939) confirmed Beller's findings. Further workers (Lucker and Neumayer 1946, Demidowa 1957, 1958, Stoye 1965, Westen 1967) not only showed that percutaneous infection was possible but that higher worm burdens were found in sheep infected by this route compared with those infected per os. In addition Habermann (1946) and Sarimsakov (1959 a & b, 1961) were of the opinion that most 'natural' infections were acquired percutaneously rather than orally.

In the present investigation, experiments were undertaken to study the effect that the route of administration had on the subsequent adult worm

burden.

EXPERIMENT 1

Materials and Methods

Fifteen 9 month old worm free sheep were divided into four groups (A, B, C and D). Each group contained 4 sheep, except D which contained 3 sheep. Four thousand infective larvae of B. trigonocephalum (92% ensheathed) were administered, intravenously to each of the sheep in group A, subcutaneously to each of the sheep in group B and percutaneously to each of the sheep in group C. (See General Methods). Group D remained as the control group.

Six days after infection 4 sheep were slaughtered, one from each of groups A, B, C and D. 48 hours later 3 more sheep were slaughtered, one from each of groups A, B and C. Immediately after slaughter the lungs were removed, examined macroscopically, then minced and baermanised as described in Chapter 3. The remaining sheep, except for two which died during the experiment, were slaughtered 14 weeks after the experiment commenced. Sheep number 3 in the contol group D died from pregnancy toxaemia 11 weeks after the commencement of the experiment, whilst sheep number 3 in group C died 13 weeks after the commencement of the experiment. Its' death was attributed to a heavy infection of B. trigonocephalum. The small intestine of all these sheep, including the two which died, were examined and the number of male and female B. trigonocephalum found in each intestine recorded.

Results

Table No 5.1 shows the number of B. trigonocephalum larvae recovered from the lungs of sheep slaughtered 6 and 8 days after infection, and Table No 5.2 shows the number of male and female B. trigonocephalum recovered from the intestines of the remaining 8 sheep.

Group	A	B	C	D
Route of Infection	Intravenous	Subcutaneous	Percutaneous	Control
Sheep 1 (slaughtered 6 days after infection)	Nil	11	132	Nil
Sheep 2 (slaughtered 8 days after infection)	Nil	8	156	-

Table No 5.1 The number of larvae recovered

Group	A		B		C		D	
Route of Infection	Intravenous		Subcutaneous		Percutaneous		Control	
	Male	Female	Male	Female	Male	Female Note 1	Male	Female Note 2
Sheep 3	3	7	49	90	249	623	Nil	Nil
Sheep 4	Nil	Nil	1	2	71	235	Nil	Nil

Table No 5.2 The number of worms recovered

Note 1 - Sheep No 3 group C died 13 weeks after commencement of the experiment.

Note 2 - Sheep No 3 group D died 11 weeks after commencement of the experiment.

EXPERIMENT II

Materials & Methods

Fourteen 6 month old worm-free sheep were divided into four groups. Groups E, F and G each contained four sheep whilst group H contained two sheep. Four thousand infective B. trigonocephalum larvae were administered

intraruminally to each of the sheep in group E, orally to each of the sheep in group F and percutaneously to each of the sheep in group G. The two sheep in group H remained as controls. All the sheep, with the exception of one sheep in group G, which was kept as a donor animal, were slaughtered 12 weeks after the commencement of the experiment. The intestine of each sheep was examined and the number of male and female B. trigonocephalum present recorded.

Results

Table No 5.3 shows the number of male and female B. trigonocephalum recovered.

Group	E		F		G		H
Route of Infection	Intraruminal		Oral		Percutaneous		Control
Sheep 1	Male 2	Female 6	Male 3	Female 3	Male 312	Female 498	Nil
Sheep 2	5	12	1	4	409	652	Nil
Sheep 3	9	11	81	95 Note 1	166	157	-
Sheep 4	9	16	2	3	Not Slaughtered		-

Note 1 - This sheep coughed whilst the dose of infective larvae was being administered.

Discussion

Although many workers have administered infective larvae of B. trigonocephalum percutaneously to sheep, no reference in the literature could be found to these larvae being given either subcutaneously or intravenously. In this study no larvae were recovered from the lungs of the

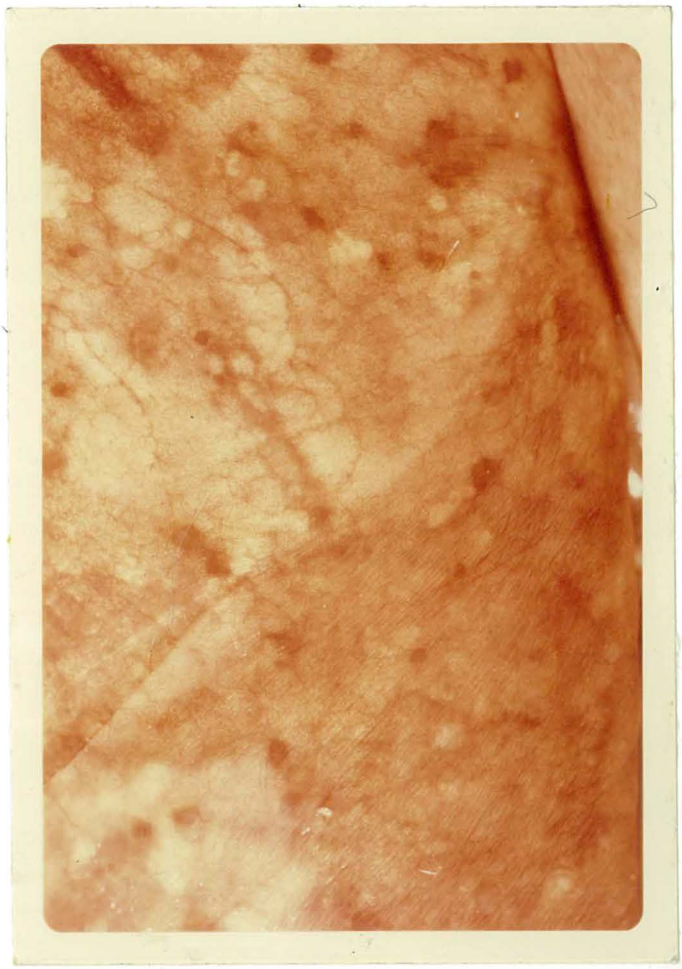

Fig. 12 Colour photograph of the lung of a sheep showing focal traumatic damage caused by the migrating larvae of *B. trigonocephalum*.

sheep given infective larvae intravenously and comparatively few larvae were recovered from those sheep which received infective larvae subcutaneously. There was however, both macroscopic and histological evidence of focal traumatic damage to the lungs of all the sheep slaughtered 6 and 8 days after infection, which could have been due to the activities of migrating larvae. (fig 12). Whilst there was obviously a clear cut difference in the number of larvae recovered between the various groups, this difference could only be assessed on a qualitative basis. The commonly accepted baermann technique employed in this experiment does not allow a quantitative estimation to be made of the actual number of larvae present in the lung. In those sheep examined 13 and 14 weeks after infection, fewer adults were found in groups A and B compared with group C.

Subsequent experiments showed that prior to penetrating the skin, the larvae exsheathed (see Chapt. 6). In experiment I 92% of the infective larvae administered were ensheathed, and those infective larvae which migrated through the skin of the sheep in group C would undoubtedly have all exsheathed. A possible explanation for the differences seen between groups is that those ensheathed larvae which were injected intravenously did not exsheath or develop further and that the few adults which were found in one of the sheep at the termination of the experiment developed from the 8% of infective larvae which had exsheathed prior to administration. In those sheep inoculated subcutaneously it is conceivable that some of the ensheathed larvae exsheathed subcutaneously prior to their entry into the vascular system and this could account for the greater number of larvae and adults found in these sheep compared with those recovered from the sheep infected intravenously.

An additional or alternative explanation consistent with the findings is that the free living infective larvae may require time to adapt to their new environment within the host. The physiology of the early parasitic stage

of B. trigonocephalum has not been investigated but that of Schistosoma mansoni has been intensively studied (Lewert and Lee 1954, 1957). The physiology of the schistosomule has been found to differ markedly from that of the cercaria. It is similarly quite logical to expect the physiology of the 3rd stage free living larvae of B. trigonocephalum to be different to that of the 3rd stage parasitic larvae and it may take time for the free living stage to adapt to a parasitic existence. Those larvae injected intravenously would have had little time to do this.

The results obtained in experiment II are in agreement with those of other workers who found that higher worm burdens followed percutaneous infection with B. trigonocephalum than after oral infection.

In this study, the percentage of the infecting dose recovered as adults following percutaneous infection varied between 7.4% and 26.5%. Lucker and Nuemayer (1946) obtained a lower percentage recovery varying from 0.3% in one experiment to 3-10% in another. However, the results may not be directly comparable because in the first experiment Lucker and Nuemayer used a dose of 500,000 larvae and in the second experiment doses ranged from 5,000-50,000. One of the sheep infected orally in the present study gave a 4.4% recovery, whilst the recovery in the other sheep was much lower, (av. recovery 0.15%). The sheep with the higher worm burden coughed when the larvae were being administered and it is possible that some of the larvae may have been inhaled. These may have penetrated through the bronchial mucosa and this may account for the higher worm burden.

It is apparent that the behaviour of the infective larvae of B. trigonocephalum and their subsequent development is quite different to that of the larvae of hookworm infecting the dog. Foster and Cross (1934) studying Ancylostoma sp. in the dog found that higher worm burdens occurred after oral infection than after percutaneous infection. This is undoubtedly

associated with the fact that Ancylostoma larvae develop immediately in the intestine after oral infection. Fulleborn (1926, 1927) also found this to be true for Uncinaria stenocephala. Whereas experiments conducted by Stoye (1965) proved conclusively that the larvae of B. trigonocephalum would not develop to the adult stage unless they migrated through the lung.

Stoye (1965) and Westen (1967) introduced the infective larvae of B. trigonocephalum directly into the abomasum and the duodenum respectively. In both cases the workers were able to induce patent infections. The percentage of adults recovered in relation to the larvae administered however, was low, much lower than when larvae were given per os.

In the present study the percentage of adults recovered after intraruminal injection, although slightly higher (0.2% to 0.625%), was similar to that obtained after oral infection. No reference could be found in the literature to B. trigonocephalum, being administered intraruminally. However, it is reasonable to expect that the percentage of larvae developing via this route would be similar to the percentage developing after oral administration, particularly in view of the fact that ruminal fluid does not appear to influence exsheathment.

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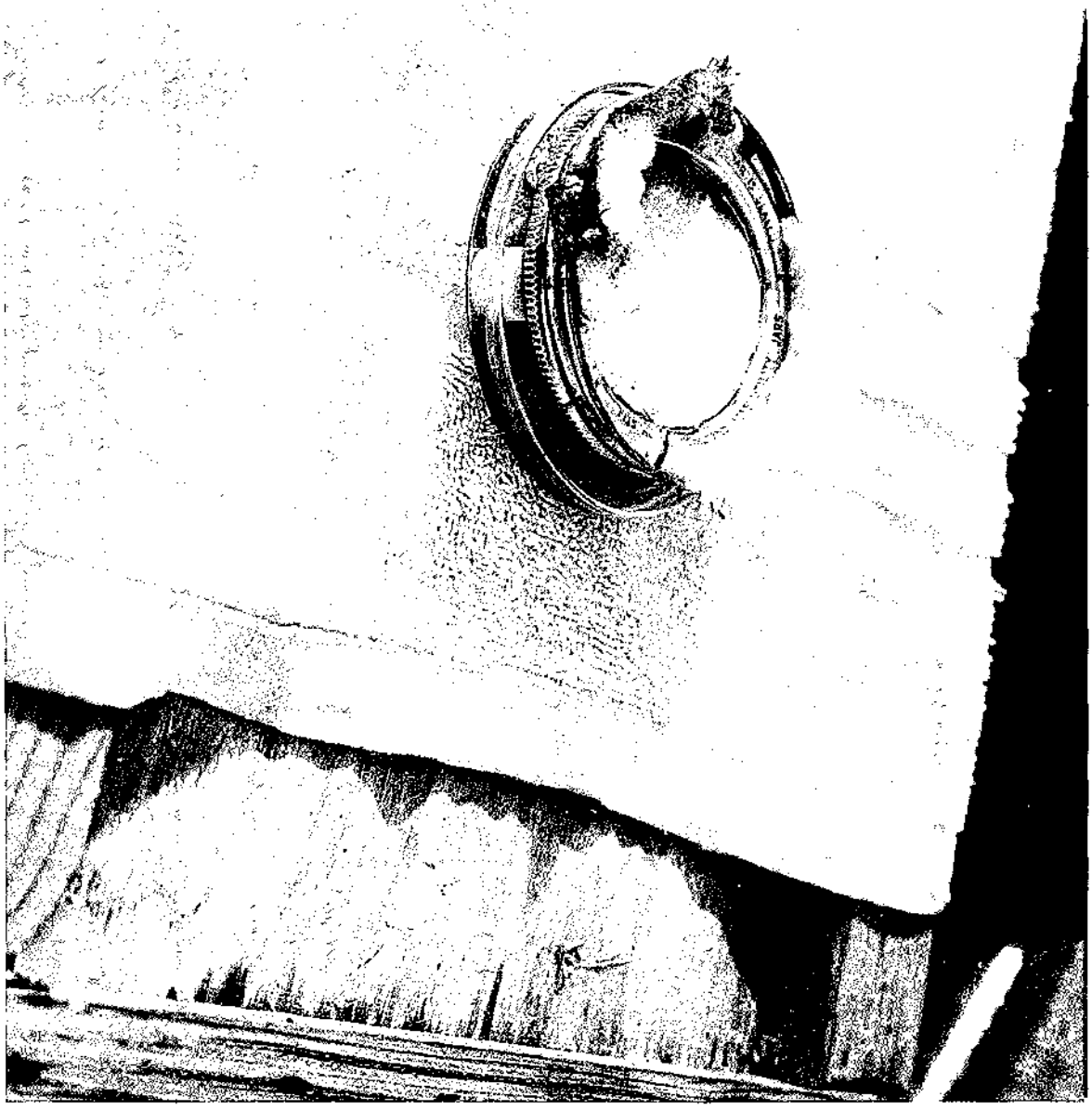

Fig. 13 Sheep skin tensed across an Agee lid.

CHAPTER 6 SKIN PENETRATION

Introduction

Most of the early work on the Ancylostomidae relating to the skin penetration of infective larvae was done by Cort et al (1922), Goodey (1922, 1925), Augustine (1923), Cameron (1927). These workers were mainly concerned with the behaviour of the larvae on skin. Later workers studied the skin lesions associated with the penetration of the larvae. Skin lesions in man due to A. duodenale, characterised by intense pruritis are well authenticated (Soulsby 1965). Similarly, marked dermatitis has been recorded by Christensen and Roth (1949) as a feature hookworm infection in dogs and reputed to be due to repeated percutaneous infection. Little work has however been carried out on this aspect in relation to B. trigonocephalum. Cameron (1927) did conduct a series of experiments using Goodey's (1922) technique in an attempt to determine whether or not the infective larvae of B. trigonocephalum were able to infect the sheep via the skin. He used rat skin and freshly stripped buccal mucous membrane of the sheep. His findings caused him to exclude the possibility of percutaneous infection. Had he used sheep skin in his experiments his conclusion may well have been different.

In view of the fact that penetration of the skin by the infective larvae of B. trigonocephalum had not been investigated in detail and that the nature of the stimuli which bring it about was unknown, a set of experiments was set up to study this aspect.

Materials & Methods

Experiment I

A recently shorn worm free sheep was slaughtered and two areas of skin approximately 6" x 4" were removed from over the ribs. The two pieces of skin were then taken and tensed across the lid of an Agee jar and held in position by means of a tight inner ring (fig 13). Surplus skin from around the

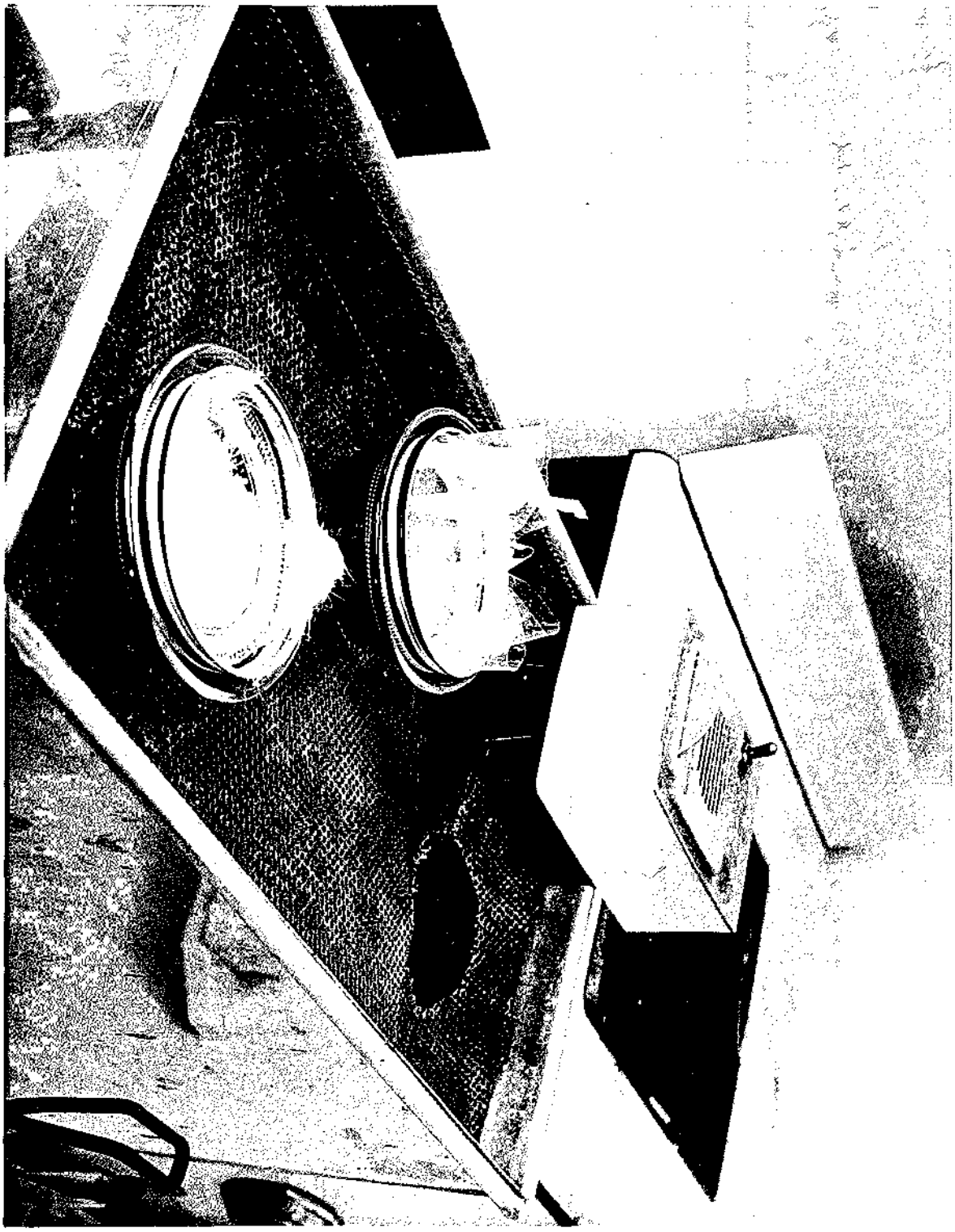

Fig. 14 Water bath with funnels containing skin in contact with 0.85% saline.

edge was trimmed off. One piece of skin was placed in a filter funnel containing 0.85% saline so that the saline was in contact with the inner surface of the skin. The filter funnel was kept in a water bath at 37°C (fig 14). The second piece of skin was similarly held in an Agee lid but was placed over a petri dish which contained 0.85% saline at 37°C.

Fifty infective larvae in 1 ml of water were pipetted on to the skin in the filter funnel. At intervals of 5, 15 and 30 minutes after the commencement of the experiment, a small sample (approximately 0.1 ml) of the suspension was removed, dropped on to a slide and examined under a microscope (magnific x 400). Fifty larvae in 1 ml were also put on to the skin held over the petri dish. The behaviour of these larvae was observed constantly for 30 minutes under a dissection microscope.

This experiment was repeated 3 times with skin taken from different worm free sheep and with suspensions containing 500, 2000 and 4000 infective larvae per ml.

At the conclusion of each of these experiments (i.e. 30 minutes after commencement), the saline from both the petri dish and the filter funnel was examined for the presence of larvae.

Experiment II

A worm free sheep was anaethetised with pentobarbitone sodium and an incision 6" in length was made over and parallel to the ribs. Cranial to the incision a piece of skin approximately 6" x 4" was removed, set over a petri dish and examined in a similar manner to that described in Experiment I. Caudal to the incision the skin was lifted from the subcutaneous tissue to form a pocket. A petri dish containing 0.85% saline was introduced into this pocket and positioned so that the saline was in direct contact with the under surface of the skin. (fig 15).

4,000 infective larvae in 1 ml of water were placed on the skin of the anaethetised sheep diretly above the centre of the petri dish.

Fig. 15 Petri dish being placed in a pocket under the skin of an anaethetised sheep.

A further 4,000 larvae were placed simultaneously on the skin which had been removed from the sheep. The behaviour of these larvae were examined under a dissection microscope for 30 minutes. 0.1 ml samples of the suspension containing the infective larvae were removed 5, 15 and 30 minutes after the suspension had been placed on the skin over the petri dish. After the period of 30 minutes had elapsed the saline from both petri dishes was examined for the presence of larvae.

The sheep then was allowed to recover, given 2.5 cc of Streptopen* and slaughtered ten weeks after the commencement of the experiment at which time the intestine was examined for the presence of B. trigonocephalum.

Experiment III

Pieces of skin approximately 6" x 4" area were taken from recently killed guinea pigs, rabbits and from two anaesthetised worm free calves. The pieces of skin were stretched across Agee lids as described in Experiment I and placed in contact with 0.85% saline at 37°C in filter funnels. Numbers of B. trigonocephalum infective larvae ranging from 50 to 4000 were placed on replicate skin samples as shown in table 6.1.

Nos. of Larvae	50	500	1000	4000
Source Rabbit replicate	4	4	4	4
Source Guinea Pig replicate	4	4	4	4
Source Calf replicate	2	2	2	2

Table 6.1

* Streptopen Injection, Glaxo, N.Z.

Five, fifteen and thirty minutes after the commencement of the experiment approximately 0.1 ml of the suspension containing the larvae was removed from the skin and placed on a slide and examined under a microscope (magnific x 400).

Results & Discussion

According to Rogers and Sommerville (1963), the nature of events associated with skin penetration probably involve firstly some form of orientation to the skin which may be influenced by the depth of the water film and temperature. Secondly the actual movement of the larvae may lead to penetration of the skin. Observation made in this study support the view held by Rogers and Sommerville. In all experiments the larvae tended to gravitate towards the skin surface. Once close to it they orientated themselves vertically and their activity increased markedly. It was noticed though that the activity of those larvae placed on the skin held over the petri dishes decreased in time. This may have been due to the fact that the temperature of the saline below the skin and consequently the skin itself, gradually dropped from 37^{o}C to room temperature (approximately 20^{o}C) during the 30 minute period in which the observations were made. It is also possible however that as the majority of larvae had disappeared 30 minutes after the experiment commenced these remaining larvae may have been initially less active and the marked activity seen at the beginning of the experiment may have been due to those which penetrated the skin during this 30 minute period.

In experiments I and III, samples of larvae recovered five minutes after the experiment commenced comprised a few cast sheaths and, exsheathed and ensheathed larvae. Fifteen and 30 minutes after commencement larger numbers of cast sheaths and many fewer exsheathed and ensheathed larvae were recovered. The results were the same whatever the source of the skin and with calf skin no larvae at all were recovered at the 30 minute sampling, only

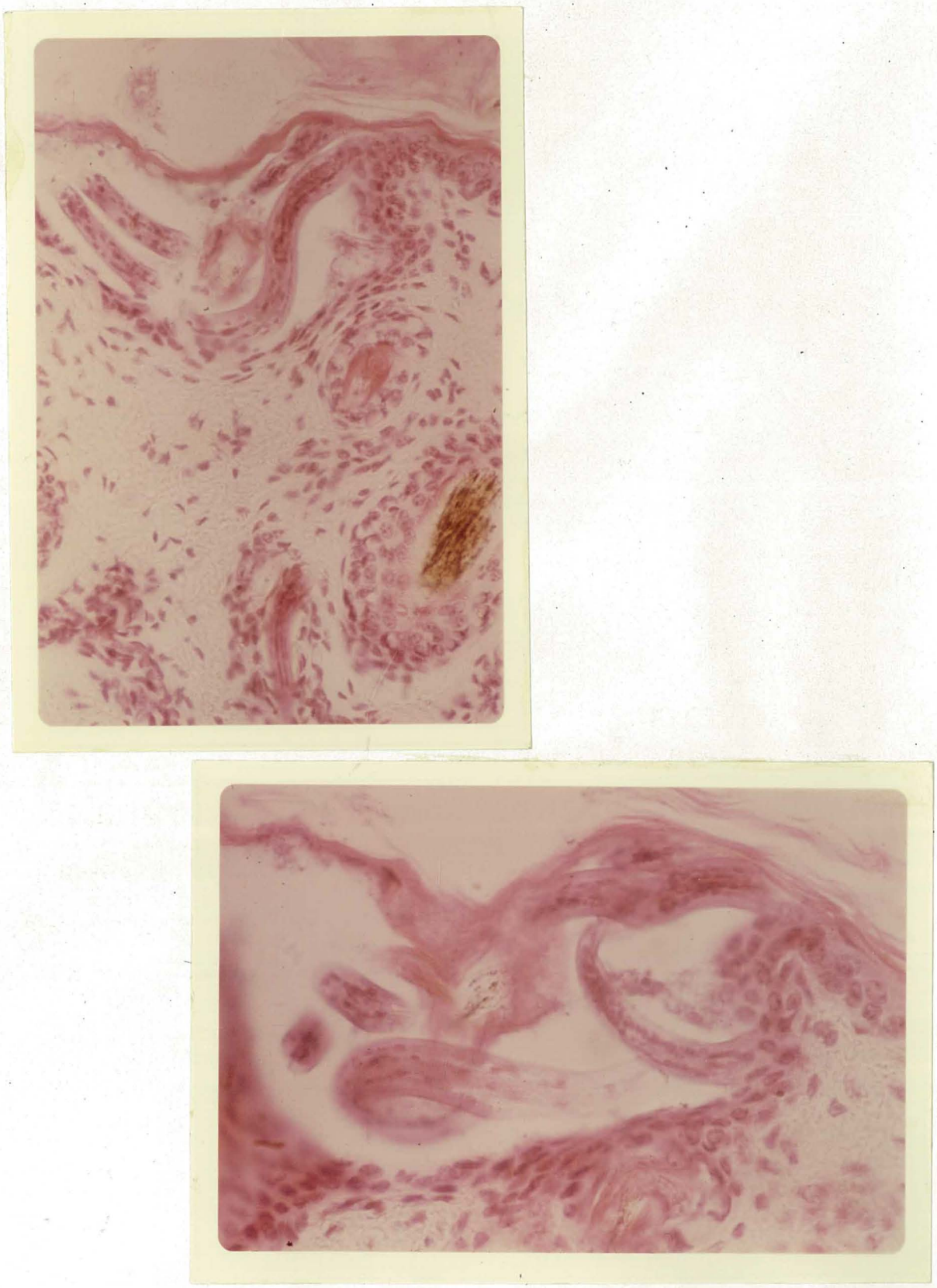

Fig.16 (a & b) Photomicrograph of 3rd stage larvae of B. trigonocephalum migrating through the epidermis and superficial layers of the dermis.

cast sheaths.

Although neither Cameron (1923c) nor Goodey (1922) could find any evidence that the infective larvae of B. trigonocephalum penetrated the skin Beller (1928) excised a portion of sheep skin 30 minutes after infective larvae had been applied to the skin surface and found the larvae penetrating the dermis. In the present investigation the results of all the experiments certainly indicated that B. trigonocephalum not only penetrated the skin of the sheep but also that the guinea pig, rabbit and calf. Histological examination of a skin biopsy taken from a calf 15 minutes after infective larvae of B. trigonocephalum had been pipetted on to the surface revealed larvae migrating through the epidermis and superficial layer of the dermis (fig 16). It is of interest to note that the larvae seen in sections were migrating directly through the St. corneum and not through hair follicles.

The saline on which the skin in all these experiments was resting was examined for the presence of larvae and on no occasion were any larvae seen in it. Goodey (1922) and Cameron (1923c) carried out a set of similar experiments with the infective larvae of B. trigonocephalum and they also found no larvae in the fluid on which the skin was resting. However, both these workers and Ortlepp (1937) in his early experiments with G. pachyscelis considered it necessary for larvae to migrate through the skin to below the dermis for patent infection to occur.

In experiment II adult male and female B. trigonocephalum were found when the sheep was slaughtered 10 weeks after the experiment was commenced. But no larvae were recovered from the petri dish placed beneath the skin suggesting that infection may take place without the larvae migrating to a subcutaneous level. Presumably larvae leave the skin in blood or lymph vessels and the results indicate that they enter these vessels within the dermis.

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CHAPTER 7

EXPERIMENTS ON THE INFECTION OF ABNORMAL HOSTS WITH B. TRIGONOCEPHALUM

Introduction

In the previous chapter it was shown that 3rd stage larvae of B. trigonocephalum penetrated into the skin of rabbits, guinea pigs and calves. As these animals are considered 'abnormal' hosts for this nematode, a further set of experiments was set up to investigate whether or not a patent infection could be induced in these animals.

Various workers have reported finding B. trigonocephalum in animals other than the normal host, the sheep. Lane (1917) described a parasite in goats, B. kashinathi, which Cameron (1923b) declared identical to B. trigonocephal Cameron (1923a) reported finding this parasite in a number of goats. Westen (1967) studied the life cycle and pathogenicity of B. trigonocephalum in the goat, and found that the goat could be infected per os, percutaneously and intraintestinally. B. trigonocephalum has also been recorded from Roe deer (Capreolus capreolus) (Hübner, 1937, 1939; Kock, 1942; Welker, 1964), Red deer (Cervus elephus) (Ross and Gordon, 1936; Lapage, 1962; Borchert, 1962), Chamois (Rupicara rupicara) (Wedl, 1861 cited by Gebauer, 1932; Molin, 1861) and Mouflon (Ovis ammon musimon) (Bürger, 1961). This nematode has also been recorded from cattle (Cameron, 1923a) though the accuracy of this finding has been questioned (Soulsby 1965). No reference could be found to adult B. trigonocephalum being recovered from either guinea pigs or rabbits. Demidowa (1958) attempted to infect these animals but failed to induce a patent infection and did not indicate the route of infection used.

Experiment I - Percutaneous infection of rabbits

Materials & Methods

Five castrated bucks approximately 2 months old were infected percutaneously with 3rd stage larvae of B. trigonocephalum. Rabbit Nos. 1

and 2 each received 2,200 larvae whilst Nos. 3, 4 and 5 were each given 10,000 infective larvae.

Rabbit Nos. 1, 2 and 3 were killed 8, 12 and 10 days respectively after infection. Immediately, the lungs were removed, examined macroscopically, minced and baermanised, as described in chapter 3.

Rabbit No. 4 died 39 days after infection from an enteritis of unknown origin. Rabbit No 5 was killed 56 days after infection. The intestinal tracts of both rabbits 4 and 5 were examined for the presence of B. trigonocephalum.

Results

Table No 7.1 shows the number of B. trigonocephalum larvae recovered from the lungs of rabbits slaughtered 8, 10 and 12 days after infection. Focal traumatic damage similar to that seen in sheep lungs was noticed in the lungs removed from these rabbits.

Rabbit No.	Days after administration of larvae	No. of larvae administered	No. of larvae recovered
No. 1	8	2,200	6
No. 2	12	2,200	Nil
No. 3	10	10,000	1

Table 7.1

Number of larvae recovered from lungs of rabbit

No adult or immature B. trigonocephalum were recovered from the intestinal tracts of rabbits 4 and 5.

Experiment II - Percutaneous infection of guinea pigs

Materials & Methods

18 young adult guinea pigs were divided into 3 equal groups A, B and C. Each guinea pig was infected percutaneously with the number of 3rd

stage larvae of B. trigonocephalum shown in table No 7.2

Group	No. of larvae Administered
Group A 6 guinea pigs	10,000
" B " " "	20,000
" C " " "	40,000

Table No 7.2

One guinea pig from Group A was slaughtered 4 days after infection, one from Group B on the 5th day and one from Group C on the 6th day. A similar sequence was carried out on days, 7, 8 and 9. The remaining 12 guinea pigs were slaughtered 64 days after infection and the intestinal tract of each one examined for the presence of immature and adult B. trigonocephalum.

Results

Table no 7.3 shows the number of larvae recovered from those guinea pigs slaughtered 4-9 days after infection.

No. of days after larvae administered	No. of larvae administered	No. of larvae recovered
Day 4 (guinea pig from Group A)	10,000	7
Day 5 (guinea pig from Group B)	20,000	1
Day 6 (guinea pig from Group C)	40,000	153
Day 7 (guinea pig from Group A)	10,000	26
Day 8 (guinea pig from Group B)	20,000	42
Day 9 (guinea pig from Group C)	40,000	8

Table No 7.3

Number of larvae recovered from guinea pig lungs

Fig. 17. Colour photograph of the lungs of a guinea pig showing focal traumatic damage caused by migrating larvae of B. trigonocephalum.

Focal traumatic damage, similar to that seen in sheep and rabbit lungs was noticed in the lungs removed from these guinea pigs (fig 17). Examination of the intestinal tracts of the remaining 12 guinea pigs did not reveal any evidence of infection with B. trigonocephalum.

Experiment III - Percutaneous infection of calves

Experiment (i) - Materials & Methods

Infective 3rd stage larvae of B. trigonocephalum were administered percutaneously to two 14 day old worm-free calves. Calf No 1 received 6,000 infective larvae. Calf No 2 received approximately 3-4000 (some of the suspension containing the larvae being administered to Calf No 2 was spilt and it only received an estimated 3-4000 larvae instead of the intended 6000).

Calf No 1 was slaughtered 5 days after the administration of the larvae. Calf No 2 was slaughtered 7 days after the commencement of the experiment. Immediately after slaughter the lungs were removed, examined macroscopically, minced and baermannised (see chapter 3).

Results

There were no visible lesions on the lungs, nor were any larvae recovered from the lungs of these two calves.

Experiment (ii) - Materials & Methods

10,000 3rd stage infective larvae of B. trigonocephalum were administered percutaneously to each of two 7 day old worm-free calves (nos. 3 and 4) which had been used in experiment III described in previous chapter.

45 days after the larvae had been administered, a faecal sample was taken from each calf and was examined, using the ether/acetic acid method, for the presence of B. trigonocephalum eggs. Further faecal examinations were made 52, 54, 59 and 62 days after the experiment had commenced. On the 62nd day both calves were slaughtered, and the small intestines removed and examined for the presence of immature and mature B. trigonocephalum.

Results

1. Eggs of B. trigonocephalum were seen in the faeces of calf No 3 fifty two days after the administration of the larvae and at each subsequent faecal examination. No eggs however were found in the faeces of calf No 4 at any of the examinations.

2. 8 adult female and 5 adult male B. trigonocephalum were recovered from the small intestine of calf No 3. No B. trigonocephalum were found in the small intestine of calf No 4.

Experiment (iii) - Materials & Methods

Six 7 day old worm free calves (Nos 5-10) were each given percutaneously 50,000 3rd stage larvae of B. trigonocephalum. Calves 8, 9 and 10 were anaethetised the day before the larvae were administered and a piece of skin, approximately 6" x 4" was removed from over the ribs of each of these calves and used in an experiment on exsheathment, described in Chapter 8.

46 days after the administration of the larvae a faecal sample was taken from each of the calves and examined for the presence of B. trigonocephalum eggs using the ether/acetic acid method. Further samples were taken and similarly examined at 3 day intervals to the 56th day from calves 5, 6 and 7 and until the 59th day from calves 8, 9 and 10.

Calves 5, 6 and 7 were slaughtered on the 57th day and calves 8, 9 and 10 on the 60th day after infection. The small intestine of each calf was examined for the presence of B. trigonocephalum. Measurements were made of the length of both the male and female B. trigonocephalum found in these calves.

Results

B. trigonocephalum eggs were first noticed in samples taken from calf No 6 fifty three days after larvae had been administered. Samples from

this calf examined on the 56th day were also positive. Samples from the other calves were negative throughout the experiment. Live 3rd stage B. trigonocephalum larvae were cultured from eggs recovered from the faeces of calf 6. Fifteen hundred of these larvae were administered percutaneously to a worm free sheep, but no infection was established.

Table No 7.4 shows the number of adult male and female B. trigonocephalum recovered from the calves.

Calf No.	No. of worms recovered	
	Male	Female
5	1	1
6	48	58
7	2	2
8	Nil	Nil
9	1	Nil
10	Nil	Nil

Table No 7.4

B. trigonocephalum recovered from the intestines of calves

The length of the female worms ranged from 10.0-20.5 mm (mean length 18.1 mm). The lengths of the male spicules were found to be within the normal range quoted by Soulsby (1965) for B. trigonocephalum.

Discussion

The findings from both the rabbit and the guinea pig experiments are in agreement with those of Demidowa (1958) in that no adult B. trigonocephalum were recovered from either species. However, larvae were recovered from the lungs of both rabbits and guinea pigs. These larvae had provisional buccal capsules and were considered to be late 3rd stage larvae.

No fourth stage larvae were recovered.

It was clearly established that B. trigonocephalum could infect calves and that the infection could mature and reach patency, although the proportion of worms recovered compared to the number of larvae administered was much lower than that recorded from sheep for this route of infection.

Although no B. trigonocephalum were found in calves Nos. 4, 8 and 10 the larvae which were applied to skin removed from these calves (see experiment III Chapter 6 and experiment VIII Chapter 8), were seen to exsheath and subsequently penetrate it.

The mean length of the B. trigonocephalum recovered from calves in this study was less than the mean length of those recorded for sheep. This difference may be a chance observation but could be due to the relative unsuitably of the calf as a host for this nematode.

In studies on calves grazing for six weeks on a pasture previously grazed by parasitized sheep, Roberts (1942) found that although calves became infected with many of the nematodes found in the sheep, they exhibited a degree of resistance to infection with these species, and amongst those species which did not 'transfer' to calves was included the hookworm B. trigonocephalum. Porter (1953), in a set of similar experiments, also found that B. trigonocephalum was not transmitted to calves. The only reference in the literature to B. trigonocephalum being isolated from cattle is that of Cameron (1923a) who conducted a survey on the intestinal parasites of ruminants in Scotland. He recorded the occurrence of B. trigonocephalum in cattle, at the same time noting the absence of B. phlebotomum. Because of this latter statement and since he made no reference to these findings in his textbook in 1951, later workers (Lapage, 1962, Westen, 1967, Soulsby, 1965) have tended to discredit his earlier observations. However, in the light of the experiments described here Cameron (1923a) could have been correct, particularly as at the time he

conducted his survey he recorded infections of B. trigonocephalum in both sheep and goats in the surveyed areas. This would no doubt increase the likelihood of cross transmission occurring.

The prepatent period in the calf was found to be similar to that of the sheep, though patency was only observed in two animals. In the goat the prepatent period appears to be longer. Westen (1967) found it to vary between 58-67 days whereas in the sheep he found it to range between 51-54 days.

No explanation can be offered for the failure to successfully infect a sheep with the larvae cultured from the faeces of calf 6. However, in view of the variability of worm recovery after infecting sheep it seems likely that this failure could have been due to chance.

CHAPTER 8 STUDIES ON EXSHEATHMENT

Introduction

During the development of nematodes parasitic in animals there are four moults. The cuticle of the preceding stage is shed at the end of each moult. In most nematodes belonging to the sub-order Strongylata, the third larval stage, which develops outside the host, remains enclosed in the cuticle of the preceding stage. In these nematodes this ensheathed larvae is the infective stage and development proceeds no further until the retained cuticle is lost. This normally occurs following direct contact between the host and the nematode i.e. during or after infection of the host by the nematode larva. Exsheathment must occur before infection can be established.

The physiology of the process of infection of animals by nematodes has been studied in comparatively few species and it is not possible to make broad generalisations about the mechanisms involved (Rogers and Sommerville 1963). From the work that has been done it is apparent that the actual establishment of infection by those nematodes studied involves the provision of stimuli by the host which, on being received by the nematode, set in train processes which lead to the establishment of infection. Rogers (1961) has suggested that the infective stage is a resting stage in which development has been suspended.

The host must provide the correct stimuli for development to be resumed and the parasitic stage to be established. Within the framework of this hypothesis Rogers (1966) has suggested that the mechanisms involved in the exsheathment of ensheathed strongylate larvae comprise the following three steps:-

(1) The host supplies a stimulus that acts on a "receptor" in the infective larva.

(2) As the result of the stimulus, changes take place in the

infective stage which lead to the resumption of development and the first obvious indication of this is the secretion of "exsheathing fluid".

(3) This fluid attacks the sheath; the infective agent emerges and starts developing as the parasitic stage.

A knowledge of the mechanism of exsheathment is essential to the understanding of the process of infection; it is also pre-requisite for any investigations concerned with the controlled "in vitro" development of parasitic nematodes.

Most of the work on exsheathment has related to trichostrongylid nematodes (see for example Sommerville 1957). Poynter (1954) has studied it in some strongylids from horses; Silverman and Podger (1964) in Dictyocaulus viviparus and Rogers (1966) in Nematospiroides dubius. Apart from some superficial observations by Looss (1911) Goodey (1922) there does not appear, however, to be any reference in the literature to the mechanism of exsheathment of nematodes belonging to the family Ancylostomidae.

The earliest experiments on exsheathment were conducted by Lapage (1933) who showed that "Milton" could induce ecdysis in 3rd stage infective larvae of Trichostrongylus retortaeformis. Glaser and Stoll (1938, 1940) reported successful results using a similar "Labarrague" solution. Lapage, Glaser and Stoll considered the use of "Milton" as an abnormal and artificial procedure but nevertheless felt that it was an extremely useful technique which produced viable sterile larvae. In 1935 Lapage extended his work to include H. contortus, O. circumcincta and Trichostrongylus sp. He demonstrated the ecdysis of the infective larvae of these species in solutions of HCl and NaOH (pH 9-10) in the absence of the host's digestive enzymes, but the average time required was 8 days at 38°C.

Sommerville (1957) carried out a series of classical experiments on the 2nd ecdysis of nematode larvae in the sheep. He showed that exsheathment

in vivo for *H. contortus*, *T. colubriformis*, *T. axei*, *N. spathiger*, *N. abnormalis*, *Oe. columbianum* and *O. circumcincta* was dependent upon the presence of a dialyzable factor(s) in the alimentary tract. Infective larvae of some species responded initially to these factors in the rumen whereas others responded in the abomasum or in the small intestine.

Further studies (Rogers and Sommerville 1957, 1960 and Rogers 1960) showed that the exsheathing activity of rumen fluid was impaired by centrifuging, filtering, boiling and aeration but activity could be restored by the addition of a reducing agent. The activity *in vitro* of either fresh rumen fluid or freeze dried rumen fluid after the addition of a reducing agent was comparable with that obtained *in vivo*. The ability of this fluid to stimulate larvae was found to be dependent on temperature, pH and Eh. It was also shown that once the stimulation had been applied exsheathment continued in the absence of the stimulating agent. The whole process of exsheathment took about 3 hours but the action of the stimulus was completed after 15 minutes. Larvae exposed to the stimulus from the host for 15 to 30 minutes and then removed from the stimulus, continued to exsheath in water at 38°C.

In 1960 Rogers stated that one of the chief factors in the stimulus provided by the host for those larvae infecting it via the alimentary canal was CO_2 or a related compound. About this time Taylor and Whitlock (1960) also showed that CO_2 caused the exsheathment of *H. contortus* and that exsheathment was enhanced by various salts (NaCl, KCl, NaBr and $NaHCO_3$). In these experiments CO_2 was present in the gaseous phase but no reducing agent was added. At concentrations of 0.1% to 0.8% NaCl exsheathment occurred freely; below 0.1% activity fell. The action of salts was not simply an osmotic effect, osmotically equivalent solutions of glucose and lactose were ineffective (Rogers and Sommerville 1963). Neither were all the salts equally effective, Taylor and Whitlock (1960) considered that the variation observed was in part a reflection of the ability of buffer oxy-acid salts to catalyze

the reaction:-

$$H_2O + CO_2 \longrightarrow H_2CO_3$$

In their studies Taylor and Whitlock found that reducing agents were not required for *in vitro* exsheathment. Other workers however, considered that exsheathment was enhanced by the presence of reducing agents such as sodium dithionite, cysteine and ascorbic acid. Rogers (1960) found sodium dithionite to have the most effect though its efficacy was sometimes influenced by pH. It seems reasonable to suppose that these reducing agents act by lowering the redox potential of the medium. Rogers (1960) found that the effect of reducing agents on the exsheathment of larvae of *T. axei* and *H. contortus* was appreciable at the higher end of the pH range (pH 6-8) and when $[H_2CO_3]$ was relatively low. Whilst with the larvae of *T. colubriformis* which require acid conditions and a relatively high $[H_2CO_3]$ reducing agents had no effect. (Rogers and Sommerville 1963). This may explain at least some of the results obtained by Taylor and Whitlock (1960) who showed that reducing agents had no effect on exsheathment of *H. contortus*. In their experiments the pH was about 4.5 and the $[H_2CO_3]$ relatively high.

The exsheathment of *T. axei* and *H. contortus* increases as the concentration of $[H_2CO_3]$ increases. *T. axei* and *H. contortus* normally exsheath in the rumen and the maximum exsheathment and has been shown to take place between pH 7 and pH 8 when the amount of dissolved gaseous CO_2 and $[H_2CO_3]$ are above 0.5×10^{-3} M. However, the ensheathed larvae of *H. contortus* need about three times the concentration of $[H_2CO_3]$ required by *T. axei* to bring about exsheathment but the pH range is the same. This is of interest as it may explain why *H. contortus* has such a narrow host range, for the high concentration of $[H_2CO_3]$ necessary for exsheathment is found in relatively few situations other than the rumen of ruminants. Rogers (1960) found that the exsheathment of the infective larvae of *T. axei* and *H. contortus* at a given $[H_2CO_3]$ was increased as the pH was raised from 6.0 to 8.0. This

indicates that the hydrogen ion concentration may have an independent effect on exsheathment in addition to its effect via $[H_2CO_3]$ and redox potential.

Other factors such as the addition of sodium taurocholate horse serum and "Tween 80" have been shown to increase exsheathment rates but the effect of these substances was small (Rogers 1960). Simple organic acids in the absence $[H_2CO_3]$ have also given rise to exsheathment (Taylor and Whitlock 1960). Their action however was slight. Though Taylor and Whitlock referred to a similarity between carbonic acid and these acids they also considered that their actions were different.

Crofton (1947) studied the 2nd exsheathment of T. retortaeformis and found that its completion could be initiated by pepsin and hydrochloric acid at pH 4-5, 60 hours being required for exsheathment to occur. Silverman and Podger (1964) investigating the various factors which induce optimal exsheathment of the infective larvae of D. viviparus, T. colubriformis and H. contortus demonstrated three distinct types of reaction to pepsin.

(a) D. viviparus had an absolute requirement for pepsin, which could be presented in an inactive state. The exsheathing stimulus of the enzyme being activated by placing larvae which had previously been immersed in an aqueous solution of pepsin in HCl solution (pH 1-2).

(b) T. colubriformis showed a relative requirement for pepsin although it could be induced to exsheath in a buffer solution (pH 1.7) under 100% CO_2, it underwent a more rapid and complete ecdysis when pepsin was present in the solution.

(c) H. contortus infective larvae were quite indifferent to pepsin.

Sommerville (1954) and Bird (1955) had already shown though that in the sheep, the digestive enzymes played no part in the ecdysis of H. contortus.

Although Christie and Charleston (1965) state CO_2 cannot be considered as an important component of the stimulus to the exsheathment of N. battus and Silverman and Podger (1964) have shown the importance of the enzyme pepsin in the exsheathment of D. viviparus, investigations to date, indicate that for the majority of parasites which infect the host via the alimentary canal, dissolved gaseous CO_2 and/or undissociated carbonic acid seem to be required to trigger the development of the parasitic stage. This is true not only for the exsheathment of infective larvae but also for the hatching of the infective eggs of certain nematodes and the excystment of metacercariae and coccidial sporozoites. Rogers (1966) suggest that CO_2 sets in train a variety of developmental changes in addition to the more immediate processes of exsheathment, hatching and excystment. Work done by Rogers (1966) on the reversible inhibition of both exsheathment and infection supports this view. He concludes that it is reasonable to suppose that CO_2 reacts with a "receptor" in the infective stage which affect various target organs on tissues concerned not only with exsheathment but also with the general developmental processes of the parasitic stages.

The activation of the exsheathing mechanism appears to be a stimulus for the release of a preformed exsheathing fluid. Rogers and Sommerville (1960) prepared and partially characterized this fluid which they found to be antigenic. They presumed it to be a protein which requires for its action a low molecular weight co-factor which is heat stable and which is replaceable by manganese or magnesium ions. They considered that the co-factor may be one of these ions.

The active substance in exsheathing fluid is extremely labile. It will attack sheaths from the inside only, and only in a special region. The nature of the substrate in the sheath which is attacked is at present unknown (Rogers 1966). However, the enzyme concerned in the process is a leucine

aminopeptidase. Rogers (1965) found this enzyme to be present in the exsheathing fluid released as the result of stimulating infective larvae of H. contortus and T. colubriformis. These leucine aminopeptidases appear to be highly specific and attack the substrates only in their own sheaths.

Because of the importance of the mechanism of exsheathment in relation to the process of infection and as little investigation has been done in this field with the skin penetrating larvae of the Ancylostomidae a series of experiments were designed to study

(1) Section A - The process by which the larvae of B. trigonocephalum exsheath.

(2) Section B - The nature of the stimuli which lead to their exsheathment.

Section A

The manner in which B. trigonocephalum larvae exsheath in various circumstances:

Studies were made on the 3rd stage infective larvae of B. trigonocephalu H. contortus, O. circumcincta, and T. colubriformis. The three trichostrongylids were included in these experiments to see whether or not there were any marked differences between the manner in which they and B. trigonocephalum exsheathed. Infective larvae were cultured from the faeces of sheep infected with a single nematode species and recovered by the baermann technique.

The criterion for exsheathment was similar to that used by other workers, (Lapage 1933, Silverman and Podger 1964, Christie and Charleston 1965), i.e. exsheathment was considered to have taken place when the 3rd stage larvae had actually freed themselves from the 2nd stage cuticle. This criterion was different to that described by Rogers and Sommerville (1960) in whose experiments larvae were classified as exsheathed when the refractile ring had commenced to form (Lapage 1935; Sommerville, 1957).

Throughout the experiments on exsheathment results were assessed on the basis of samples of 100 individual larvae. It was found that successive samples of this size from a simple larvae pool could show variations of the

order of 5% of larvae exsheathed. For this reason it was considered that changes of less than 10% of larvae exsheathed should not be regarded as significant.

A series of experiments was undertaken to observe the process of exsheathment in the following circumstances:

(1) After hypochlorite solution ("Milton")* had been added to suspensions containing larvae.

(2) After 3rd stage infective larvae had been applied directly to

(i) the skin of a living sheep

(ii) areas of skin removed from freshly killed sheep, calves, guinea pigs and rabbits.

Additional observations were also made on the 'spontaneous' exsheathment of B. trigonocephalum larvae which was seen to occur during the culturing process. The trichostrongylid nematodes did not undergo spontaneous exsheathment in faecal cultures.

Exsheathment associated with Milton

Preliminary experiments had shown that when undiluted Milton was added to suspensions containing B. trigonocephalum larvae, their sheaths disappeared within 1-2 minutes and the larvae themselves disintegrated within 4-5 minutes. Further experiments were set up to study the effect that various concentrations of Milton had on

(1) exsheathment of live larvae

(2) the exsheathed larvae

(3) killed ensheathed larvae

Experiments I and II were designed to study the exsheathment of larvae in Milton.

* Milton - Active ingredients Sodium Hypochlorite 1%, Sodium chloride 16.5%, Richardson-Merrel inc., New York.

Experiment I - Materials and Methods

Approximately 200 ensheathed 3rd stage larvae of B. trigonocephalum in 0.2 ml water were placed on a slide and 0.2 ml of 50% Milton solution was added. The larvae were examined microscopically for a period of 15 minutes after the addition of Milton. This experiment was repeated with approximately 200 ensheathed 3rd stage larvae of H. contortus, O. circumcincta and T. colubriform

Results

B. trigonocephalum:- Within 5 minutes 100% of the larvae had exsheathed. However, no cast sheaths were seen. The majority of larvae at this time appeared to be alive and moving; mobility gradually decreased until, 15 minutes after the Milton had been added, only approximately 15% showed some movement which consisted of a slow coiling and uncoiling, approximately 70% were motionless and coiled and approximately 15% were motionless and extended and appeared to have died.

H. contortus:- Within 5 minutes the majority of larvae had exsheathed. Cast sheaths were seen together with may cap-like fragments of cuticle from the anterior end of the sheath. The larvae moved vigorously and continued to do so throughout the 15 minute observation period.

O. circumcincta:- Exsheathment was slower; 60%-70% had exsheathed after 10 minutes and 100% after 15 minutes. The larvae were active both before and after exsheathment. Cast sheaths were seen but no anterior caps.

T. colubriformis:- Exsheathment took place with extreme rapidity; within 2 minutes 100% exsheathment was recorded. The larvae moved vigoroursly throughout the 15 minute period of observation. Cast sheaths were seen during this time but no anterior caps were seen.

As exsheathment took place rapidly in most of the above cases, it was difficult to observe the manner in which exsheathment took place. A further experiment was therefore set up using Milton at a lower concentration.

Experiment II - This was carried out in the same way as Experiment I except that a 5% solution of Milton was used instead of a 50% solution.

Results

B. trigonocephalum

Even at this lower concentration exsheathment was still rapid. 75-80% had exsheathed within 5 minutes and at the end of 15 minutes 90% had exsheathed. No cast sheaths were seen at any time. Milton appeared to cause the whole sheath to disintegrate rather than to rupture at any specific region. The released larvae were active and moved freely. At the end of 15 minutes the larvae were still active but their movement slower than at the 5 minute interval.

H. contortus

Shortly after the addition of Milton the larvae were seen to move vigorously. They appeared to become separated from the internal surface of the 2nd cuticle, and retracted within it - particularly from the anterior end. Within 3-4 minutes some larvae were seen to burst through the anterior end, lash vigorously and extricate themselves from the sheath. In others a refractile ring was seen approximately 20u from the anterior end. The sheath frequently ruptured at this point, the larvae propelling themselves into the surrounding fluid leaving behind the empty sheath and anterior cap. In certain instances the sheath became distended in the region of the excretory pore and subsequently split at this point releasing the larva. Within 5 minutes all of the larvae counted, had exsheathed. Both the ensheathed larvae and those that exsheathed were highly active.

O. circumcincta

The exsheathment process was very similar to that of H. contortus. Shortly after the addition of Milton the larvae moved vigorously within the sheath, refractile rings were seen and the sheaths ruptured at this point.

The exsheathed larvae appeared to move freely and were still active at the end of the 15 minute observation period. Exsheathment did however seem to take place more slowly, the majority of the larvae did not extricate themselves until 5-10 minutes had elapsed. The anterior sheath caps seen in the suspension containing exsheathed H. contortus larvae were not seen.

T. colubriformis

Exsheathment took place rapidly. The larvae burst through the anterior end and there was no sign of any free anterior caps. Most of the larvae had exsheathed within 2 minutes and 100% had exsheathed 5 minutes after the experiment had commenced. The refractile ring noticed in the sheaths of both H. contortus and O. circumcincta was not seen in the sheaths of these larvae. The larvae themselves appeared to move actively throughout the 15 minute observation period.

Experiment III was designed to examine the effect of increasing dilutions of Milton on the larval exsheathment and viability, and to see whether or not exsheathment continued in larvae killed with iodine and in the presence of Milton.

Materials and Methods

In each of 5 test tubes 1500 ensheathed 3rd stage larvae of B. trigonocephalum were suspended in 10 ml of Milton at the following concentrations:-

Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
5%	1%	0.5%	0.25%	0% (Control)

The tubes were held in a water bath at 37°C for 15 minutes. During this time the larvae settled to the bottom of the tubes. The tubes were removed from the water bath and 9 ml of the supernatant fluid siphoned off.

From each of the tubes 0.75 ml containing approximately 750-1000 larvae was taken and 0.25 ml placed on each of three slides allocated to each tube.

The slides derived from each tube were treated as follows:-

Slide 1 - 100 larvae were counted, the percentage of live larvae recorded.

Slide 2 - The larvae were killed with 1 drop of iodine immediately; 100 larvae were counted and the percentage of exsheathed larvae recorded.

Slide 3 - The larvae were killed with 1 drop of iodine, and 15 minutes later 100 were counted and the percentage of exsheathed larvae recorded.

This experiment was repeated in a similar manner with the ensheathed 3rd stage larvae of H. contortus, O. circumcincta and T. colubriformis except that a 5% solution of Milton only was used together with a control tube containing the larvae in distilled water.

Results

B. trigonocephalum

Table No 8.1 shows the percentage of live larvae and the percentage exsheathment of the 3rd stage larvae of B. trigonocephalum in varying concentrations of Milton. Milton did not appreciably affect the viability up to the time the larvae were killed. A lower percentage of exsheathment was recorded at lower concentrations of Milton. There was no evidence of continuing exsheathment after the larvae had been killed with iodine.

Concentration of Milton	5%	1%	0.5%	0.25%	0%
Slide 1 percentage of live larvae after 15 mins in Milton	92%	97%	92%	92%	99%
Slide 2 initial count percentage of exsheathment after 15 mins in Milton larvae killed with iodine	90%	90%	66%	18%	11%
Slide 3 percentage exsheathment 15 mins after killing	96%	83%	64%	22%	8%

Table No 8.1

H. contortus, O. circumcincta, T. colubriformis

All the 3rd stage larvae of these three trichostronglids were alive and had exsheathed after being suspended in a 5% solution of Milton for 15 minutes.

Experiment IV was carried out to see whether infective larvae killed with iodine or by heating differed in their response to Milton.

Materials and Methods

1500 ensheathed 3rd stage larvae of B. trigonocephalum in one ml water were placed in each of four tubes (Nos. 1-4). The larvae in tubes 1 and 2 were killed by adding one drop of iodine. The larvae in tubes 3 and 4 were killed by immersing the tubes in boiling water.

Immediately after the larvae had been killed 0.25 ml containing approximately 150-200 larvae was pipetted out of each tube and placed on a slide; 100 larvae were counted and the percentage exsheathment recorded. 1.25 ml of 10% Milton was then added to tube Nos. 1 and 3. After a further

two minutes samples of larvae were removed from each tube and placed on a slide. 100 larvae were counted from each sample and the percentage exsheathment recorded.

This experiment was repeated with H. contortus, O. circumcincta and T. colubriformis.

Results

The examination of samples of B. trigonocephalum larvae immediately after killing showed 4% and 5% exsheathed in the samples killed with iodine and 37% and 42% in the samples killed by heat. No exsheathed larvae were found at this time in the samples of the three trichostrongyle larvae killed by either method.

Examination of samples taken after a further two minutes but without further treatment showed that the percentage of larvae exsheathed in the heat - killed sample of B. trigonocephalum larvae had increased from 42% to 56%. None of the other samples showed any change from the situation found immediately after killing.

Treatment of iodine-killed larvae of all species with Milton caused no exsheathment. However, treatment of heat killed larvae with Milton produced rapid and, in most cases, complete exsheathment of the larvae.

The results of the Milton treatment are summarised in table 8.2.

	Larvae killed with iodine			
	Initial count	2 mins after addition of Milton	Initial count	2 mins after addition of Milton
B. trigonocephalum	4%	4%	37%	100%
H. contortus	0%	0%	0%	100%
O. circumcincta	0%	0%	0%	100%
T. colubriformis	0%	0%	0%	88%

Table 8.2. The effect of Milton on the percentage exsheathment of larvae killed with iodine or heat.

The process of exsheathment in Milton following heat treatment in all cases took the following form:

(1) The sheath ruptured and seemed to peel off the larvae. In most instances the exsheathed larvae appeared to be stiff and straight, but in others they were twisted and shortened.

(2) The sheath rapidly disintegrated and disappeared.

(3) The larvae became transparent, then disintegrated and disappeared

Exsheathment on the Skin - The larvae in many cases penetrated the skin rapidly and it was not always possible to recover and count 100 larvae. Empty cast sheaths were counted in order to assist in subjectively assessing the degree of exsheathment. The figures tabled relating to the percentage of exsheathment occurring on the skin must be interpreted in the light of the fact that progressively fewer larvae were often recovered towards the end of many experiments.

Experiment V

Materials and Methods

A 6 month old hogget was shorn and anaesthetised with sodium pentothal. Five thousand ensheathed 3rd stage larvae of B. trigonocephalum in 0.5 ml water were placed on the skin surface over the ribs. Five thousand ensheathed 3rd stage larvae of B. trigonocephalum in 1 ml water were also placed on a piece of polythene sheet suspended over a glass funnel containing 0.85% saline so that the saline was in contact with the lower side of the polythene sheet. The funnel was kept in a water bath at 37°C (fig 14). At 5, 15, 30 and 45 minute intervals after the commencement of the experiment 0.1 ml of the suspension containing the larvae was pipetted from both the skin of the sheep and from the polythene sheet. In each case the 0.1 ml suspension was placed on a slide to which a drop of iodine was added. 100 larvae were counted (unless otherwise stated) and the percentage exsheathment, and the number of cast sheaths present in the area containing the 100 larvae counted, were recorded.

Results

Table No 8.3 shows percentage exsheathment and number of cast sheaths recorded from the skin of the sheep and the polythene control.

	Sheep Skin		Polythene Control
Time	% exsheathed	No. cast sheaths	% exsheathed
5 min	32%	-	1%
15 min	18%	18	2%
30 min	35%*	30	1%

Table No 8.3

* Only 20 larvae recovered and counted (13 ensheathed, 7 exsheathed) after 30 minutes many of the larvae had disappeared from the skin surface.

Experiment VI

Materials and Methods

A recently shorn worm-free sheep was slaughtered and an area of skin 6" x 4" was removed from over the ribs. The piece of skin was placed in a filter funnel over 0.85% saline as described on page 39 and held in a water bath at 37°C. A piece of polythene was also suspended over a filter funnel in a similar manner. Five thousand ensheathed 3rd stage larvae of B. trigonocephalum in 1 ml water were placed on the piece of skin and the same number were placed on the polythene sheet. 0.1 ml larval suspension was pipetted from both the skin and polythene sheet 5, 10, 30 and 45 minutes after commencement of the experiment. In each case the 0.1 ml suspension was placed on a slide, the larvae killed with iodine and 100 larvae counted unless fewer than 100 were recovered. The percentage exsheathment and the number of cast sheaths present in the area containing the larvae counted were recorded.

This experiment was replicated 4 times using skin from different worm-free sheep.

Results

Table No 8.4 shows the variations in percentage exsheathment and the variation in number of cast sheaths recorded from both the skin and the polythene sheet.

Time	Sheep skin		Polythene control
	% exsheathed	No. cast sheaths	% exsheathed
5 min	0% - 10%	3*	2% - 5%
10 min	32% - 48%	22 - 28	3% - 6%
30 min	72% - 83%	18 - 30	6% - 8%
45 min	91% - 92%	32 - 47	5% - 8%

Table No 8.4

* 3 cast sheaths were seen in one of the replicate experiments.

Experiment VII

Materials and Methods

Experiment VI was repeated using a similar number of 3rd stage ensheathed larvae of O. circumcincta, T. colubriformis and H. contortus.

Results

No exsheathment was recorded with the 3rd stage larvae of O. circumcincta or T. colubriformis on either sheep skin or polythene sheet during the period of observation. However, exsheathment was seen to occur with the 3rd stage ensheathed larvae of H. contortus on both skin and polythene sheet. Table No 8.5 shows the percentage of exsheathment and the number of cast sheaths seen on the skin and polythene sheet.

Time	Sheep Skin		Polythene control	
	% exsheathment	No. cast sheaths	% exsheathment	No. cast sheaths
5 min	8%	1	6%	2
15 min	13%	2	0%	2
30 min	7%	3	12%	1
45 min	18%	10	11%	10

Table No 8.5

The experiment involving H. contortus was repeated. Table No 8.6 shows the percentage exsheathment and number of cast sheaths seen during the replicate observations.

Time	Sheep Skin			Polythene Sheet		
	% exsheathment	No. of cast sheaths	Refractile rings	% exsheathment	No. of cast sheaths	Refractile rings
5 min	2%	0	0	1%	0	0
15 min	5%	0	5%	4%	0	0
30 min	3%	0	0	7%	0	15%
45 min	4%	0	10%	6%	5	20%
60 min	6%	0	0	13%	5	0
120 min	2%	0	10%	22%	0	10%

Table No 8.7

Time	Sheep skin		Polythene control	
	% exsheathment	No. cast sheaths	% exsheathment	No. cast sheaths
5 min	0%	2	0%	0
15 min	1%	2	3%	1
30 min	7%	1	12%	10
45 min	12%	9	34%	45

Table No 8.6

The infective larvae of H. contortus used in these experiments had been stored for 11 weeks at 10°C. Prior to the commencement of this experiment they were examined. No refractile rings were observed in the larvae and they were totally ensheathed. 10% appeared to be dead.

As these results were totally unexpected the experiment was repeated using a fresh batch of larvae and period of observation was extended to 120 minutes. The presence of refractile rings was also recorded. Table No 8.7 shows the percentage exsheathed, number of cast sheaths and refractile rings seen during the 120 minute observation period.

Experiment VIII

Materials and Methods

This experiment was conducted in a similar manner to Experiment VI using ensheathed 3rd stage larvae of B. trigonocephalum except that:-

(1) the skin of the guinea pig, rabbit and calf was used instead of the sheep.

(2) the numbers of ensheathed 3rd stage larvae applied to skin also differed. Table No 8.8 shows the number of larvae used in this experiment.

(3) in the case of the rabbit and calf skin observations were only continued for 30 minutes. After this time

majority of larvae had penetrated the skin and insignificant numbers were recovered.

	No. of larvae applied to skin	No. of larvae applied to polythene sheet
Guinea Pig experiment	5000	5000
Rabbit experiment	6000	6000
Calf experiment	10,000	10,000

Table No 8.8

Results

Table Nos 8.9, 8.10, 8.11 show the percentage exsheathment and the number of cast sheaths recorded from the skin of the guinea pig, rabbit and calf respectively, together with the polythene controls.

Guinea Pig experiments

Time	Guinea pig skin		Polythene control
	% exsheathment	No. cast sheaths	% exsheathment
5 min	7%	0	3%
15 min	9%	0	8%
30 min	37%	33	5%
45 min	44%	32	5%

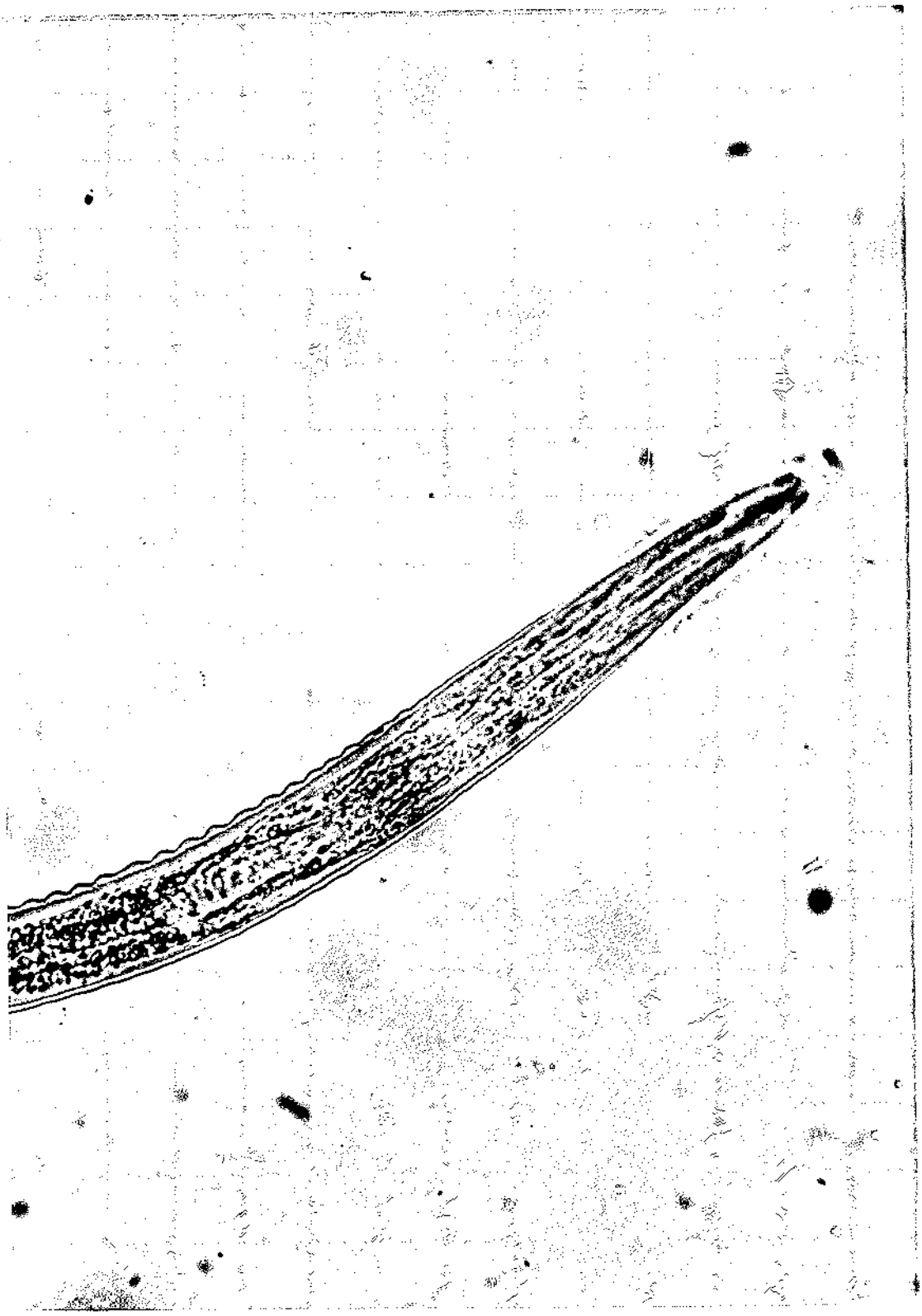

Fig. 18 Photomicrograph of the anterior end of a 3rd stage larva of B. trigonocephalum showing anterior portion of the sheath disintegrating.

Time	Guinea pig skin		Polythene control
	% exsheathment	No. cast sheaths	% exsheathment
5 min	17%	0	20%
15 min	50%	35	19% (17 sheaths)
30 min	73%	84	24% (8 sheaths)
45 min	68%	164	19% (22 sheaths)

Table No 8.9

Rabbit experiments

Time	Rabbit skin		Polythene control
	% exsheathment	No. cast sheaths	% exsheathment
5 min	2%	0	3%
15 min	8%	0	2%
30 min	91% *	0	10%
45 min	No count	-	-

* 25 larvae counted

Time	Rabbit skin		Polythene control
	% exsheathment	No. cast sheaths	% exsheathment
5 min	3%	0	4%
15 min	15%	0	4%
30 min	17%	47	14%
45 min	No count	-	-

Table No 8.10

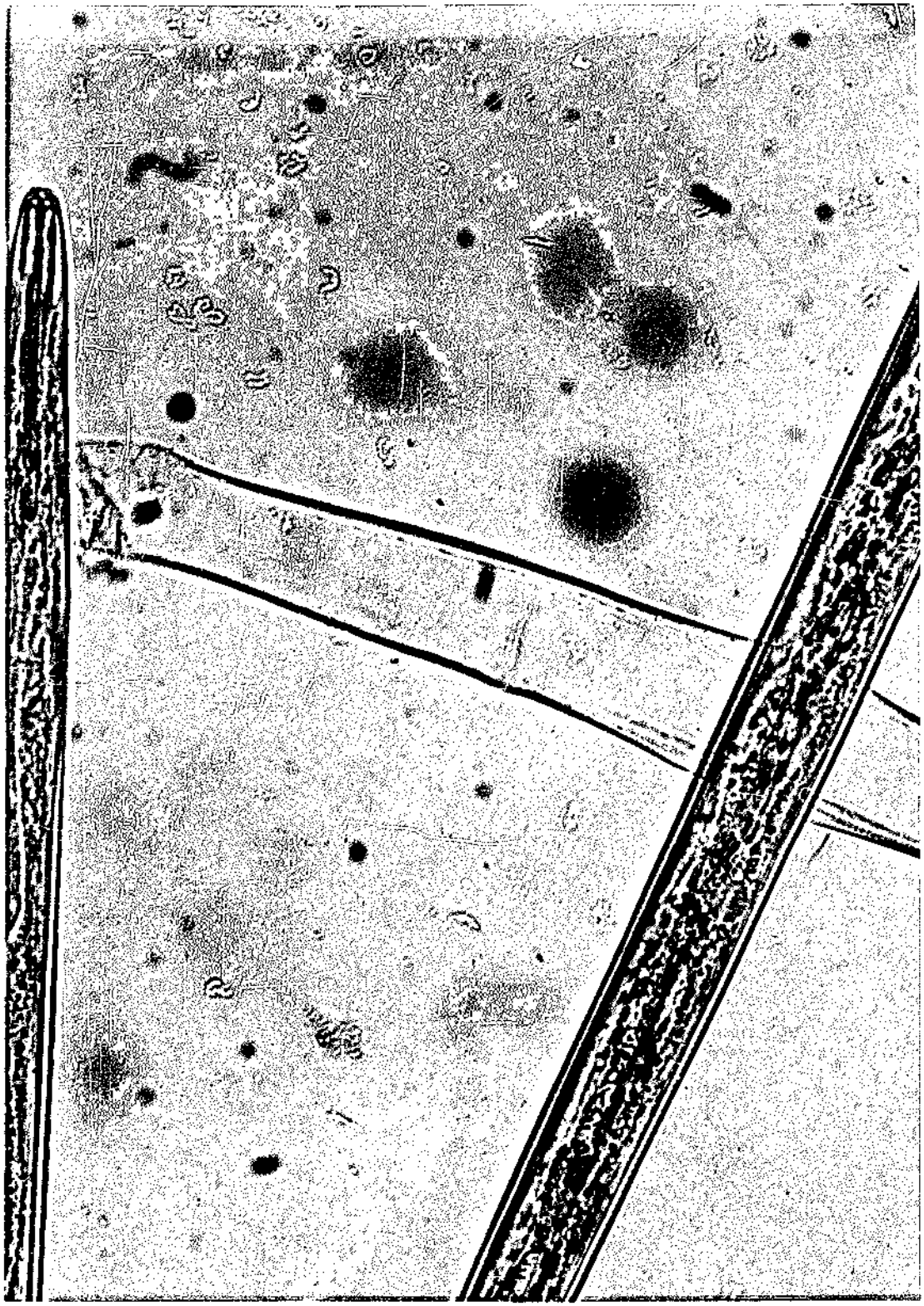

Fig. 19 Photomicrograph of an empty cast sheath of a 3rd stage larva of B. trigonocephalum.

Calf experiments

Time	Calf skin % exsheathment	No. cast sheaths	Polythene control % exsheathment
5 min	49%	12	17%
15 min	46% *	25	11%
30 min	No count	-	-

* 96 larvae counted

Time	Calf skin % exsheathment	No. cast sheaths	Polythene control % exsheathment
5 min	10%	1	2%
15 min	15%	50	4%
30 min	13%	77	6%

Table No 8.11

Observations on the manner of exsheathment on skin

The exsheathment of B. trigonocephalum and H. contortus in the previous set of experiments was seen to occur in the following manner:-

B. trigonocephalum

The larvae exsheathed in the same way on all the skin types tested.

(1) The anterior tip of the sheath enclosing the 3rd stage larva became swollen.

(2) The anterior portion of the sheath for a distance of approximately 30-40u from the anterior end appeared to disintegrate (fig 18).

(3) The larva moved actively within the remaining posterior portion of the sheath. It gradually freed itself and worked its way out leaving behind the empty sheath (fig 19).

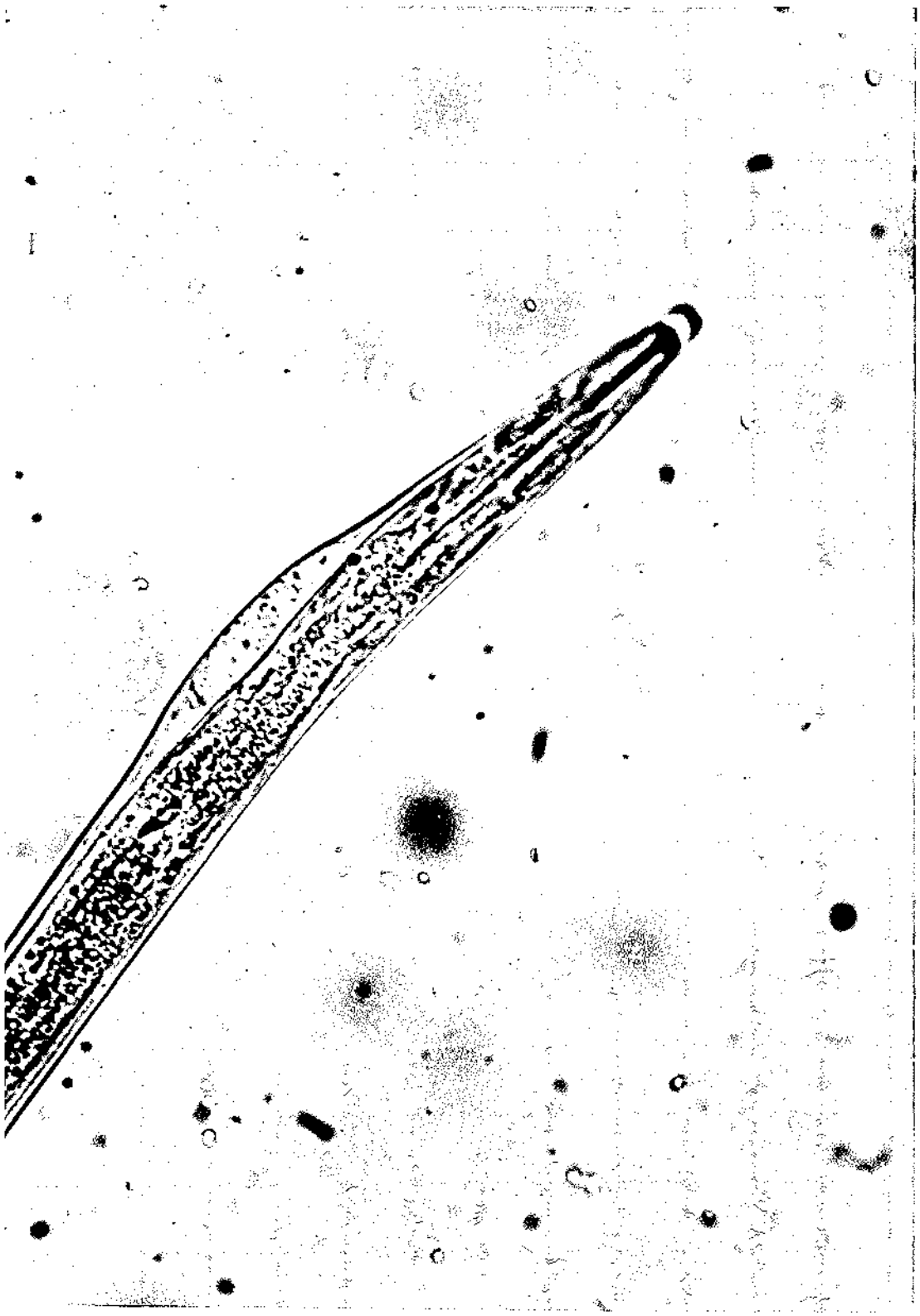

Fig. 20 Photomicrograph of the anterior end of a 3rd stage larva of B. trigonocephalum showing the cuticle markedly distended in the region of the excretory pore.

In a few instances the anterior portion did not disintegrate but the cuticle became markedly distended in the region of the excretory pore. (fig 20). The larvae were seen to move vigorously within the sheath (fig 21). The sheath eventually ruptured close to the excretory pore and the larvae then freed themselves by active movement. Once this had occurred the anterior portion of the sheath collapsed and disintegrated quickly.

H. contortus

(1) The sheath enclosing the 3rd stage larva was seen to swell near the anterior end and separate into two layers.

(2) A refractile ring was noticed approximately 20u from the anterior end.

(3) The anterior tip of the sheath broke off in the form of a cap and the larvae "wriggled" free.

Occasionally the sheath was seen to distend just posterior to the refractile ring and subsequently split longitudinally. The larvae by their active movement freed themselves through this opening.

Exsheathment during Culture

Experiment IX

Materials and Methods

3rd stage infective larvae of B. trigonocephalum were recovered by the baermann technique from 6, 7, 8 and 9 day cultures of a single faecal collection. 4000 larvae in 2 ml of water were taken from each culture, placed in bottles and held at room temperature. The larvae were then examined as follows:-

(1) 0.1 ml of the suspension containing the infective larvae was pipetted from each bottle and placed on a slide. The larvae were killed with a drop of iodine, 100 counted and the percentage exsheathment recorded.

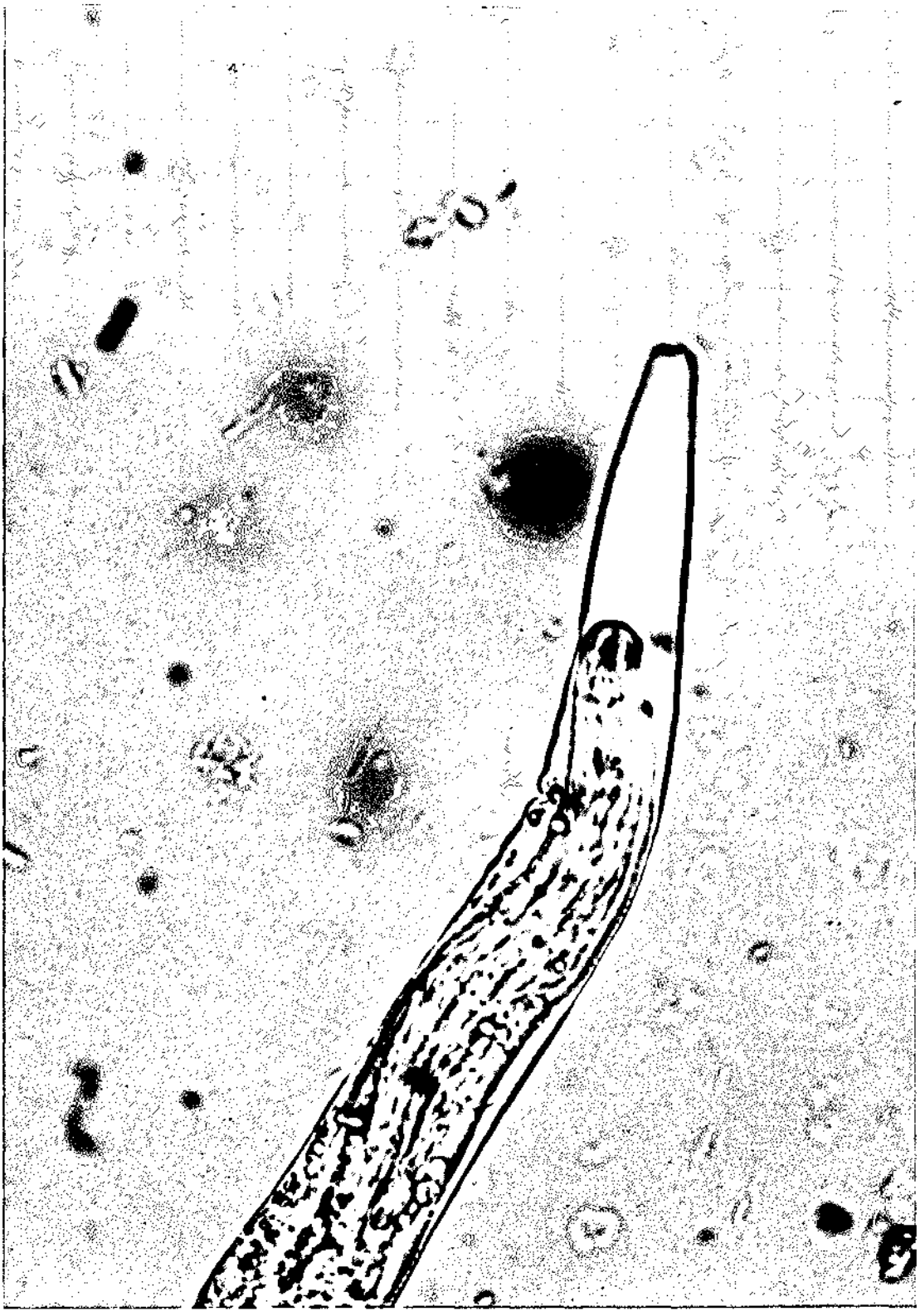

Fig. 21 Photomicrograph of the 3rd stage larva of B. trigonocephalum retracting within the sheath.

(2) A further aliquot of 0.1 ml was taken from each bottle and placed on a second slide. This slide was cooled as described in chapter 3 page 13. Larvae in various stages of exsheathment were observed. The manner in which exsheathment took place was recorded and compared with that seen to occur on the skin.

This experiment was replicated 5 times using cultures of larvae derived from five different donor animals.

Results

(1) The percentage exsheathment found in the 6, 7, 8 and 9 day cultures varied considerably. Table No 8.12 shows the variation in percentage exsheathment recorded.

Time after commencement of culturing	% exsheathment
6 days	2% - 10%
7 and 8 days	5% - 20%
9 days	15% - 20%

Table No 8.12

Although a higher percentage of exsheathment was generally recorded in the older cultures it could not be directly correlated with the time after the culturing had commenced. The degree of exsheathment was so variable that it appeared to be more closely connected with some unknown factor or factors associated with the individual cultures.

(2) The manner in which exsheathment took place appeared to be similar in all respects to that occurring not only on the skin of the normal host the sheep, but also on the skin of the guinea pig, rabbit and calf.

Discussion

Milton caused the 3rd stage larvae of B. trigonocephalum, H. contortus, O. circumcincta and T. colubriformis to exsheath. The form of exsheathment though in each case appeared to differ. No cast sheaths were seen in suspensions of B. trigonocephalum larvae treated with Milton whereas they were present after H. contortus, O. circumcincta and T. colubriformis had been induced to exsheath. The anterior cap like structures seen after the exsheathment of H. contortus were not seen after the other nematodes had exsheathed, and the refractile rings noticed in sheaths of H. contortus and O. circumcincta just prior to exsheathment were not observed in the sheaths of B. trigonocephalum or T. colubriformis.

The manner in which the 3rd stage larvae of H. contortus exsheathed in Milton, on skin and polythene sheets was similar to that described by Lapage (1935). It was also similar to the description given by Sommerville (1957) of the exsheathment of this particular nematode, in vivo. On the other hand the 3rd stage larvae of B. trigonocephalum were seen to exsheath in quite a different manner in Milton to that observed in vivo (i.e. on the skin). Milton appeared to cause the whole of the sheath to disintegrate rather than rupture at a specific point. Whereas in vivo the anterior portion of the sheath of B. trigonocephalum disintegrated whilst still closely adhering to the body of the larvae (fig 18). The larva the moved actively and freed itself leaving behind the empty sheath (fig 19). In a few instances the anterior portion did not disintegrate but became markedly distended in the region of the excretory pore just prior to exsheathment (fig 20). The sheath subsequently ruptured at this point, but no empty anterior portions of sheath were found. This form of exsheathment however did seem to be atypical and was not recorded on many occasions. Poynter (1954) mentioned this latter type of exsheathment in a paper on the 2nd ecdysis of some parasitic nematodes found in the horse. He

described two distinct methods. This first was by the detachment of a cap at the anterior end of the sheath. He stated that this occurred in Strongylus vulgaris, S. edentatus and T. axei 60 minutes after the addition of duodenal contents. The second method he described was similar to the atypical form of exsheathment recorded for B. trigonocephalum and did not involve the shedding of a cap. Poynter (1954) stated that "the larvae of Trichonema spp. Triodontophorus spp. and Oesophagodontus robustus develop a bulge at the anterior end in the region of the oesophagus, lashing movements enlarge this and eventually it ruptures to product a split through which the larvae escapes".

Many workers (Lapage 1935, Glaser and Stoll 1940, Crofton 1954, Bird 1955, Sommerville 1957, Taylor and Whitlock 1960, Rogers and Sommerville 1963, Silverman and Podger 1964, Christie and Charleston 1965), have described the process of exsheathment of trichostrongylids and other nematodes under both in vivo and in vitro conditions; but the manner in which B. trigonocephalum was normally seen to exsheath in this study does not appear to be mentioned in the literature. It is suggested that the process of exsheathment of B. trigonocephalum as described in this chapter could well be added as yet one more example of the methods by which the exsheathment of nematode larvae take place.

A curious finding in these set of experiments was the exsheathment of H. contortus on both skin and polythene sheet. No reference in the literature could be found to the 3rd stage larvae of H. contortus exsheathing on skin. Although in the initial experiments exsheathment was recorded in larvae which had been stored for some time, it nevertheless was observed, particularly on the polythene sheet, to also occur with freshly baermannised larvae. The results of these experiments are difficult to interpret and the reason for the exsheathment is not clear. It is possible that sufficient exsheathing fluid may have accumulated during the 11 week storage period to trigger off the exsheathing

process when the ambient temperature was raised to 37°C. However, Silverman and podger (1964) found no significant differences in exsheathment rates in tests which compared larvae stored between 1 week and 3 months although a tendency to increased exsheathment rates after storage longer than 3 months was observed.

This does not explain though, the exsheathment of the freshly baermannised larvae on the polythene sheet. It is conceivable that certain chemicals notably chlorine may have been present on the surface of the polythene sheet, however had this been so one would have expected the larvae of O. circumcincta and T. colubriformis also to have exsheathed.

The process of exsheathment induced by Milton after the larvae had been killed by heat, although abnormal, was of interest in that the process of exsheathment in all the four nematodes was similar. The fact that Milton did not increase exsheathment after the larvae had been killed with iodine indicated that heat treatment must affect the larvae in a different manner to iodine. It seems too that heat treatment affected the larvae of B. trigonocephalum in a different way to that of the trichostrongylids. This may be due to the fact that the larvae of B. trigonocephalum are extremely sensitive to factor(s) which induce exsheathment. If this is so then it could explain why a certain degree of exsheathment was invariably recorded during the culturing process of these larvae and not with the trichostrongylids.

This "spontaneous" exsheathment of 3rd stage larvae during culture has also been recorded for A. caninum by Clark (1969). There is the possibility that the factor(s) responsible for exsheathment during culture could be transferred with the larvae at the time of baermannising and may in part be responsible for the variation and increase in exsheathment which occurred in certain control samples.

It does appear that the mechanism, or at least part of the mechanism involved in the exsheathment of B. trigonocephalum, differs quite markedly

from that associated with the trichostrongylids. The following evidence derived from these experiments support this view.

(1) It was found that when the infective larvae of B. trigonocephalum were killed by immersion in boiling water many exsheathed, whereas boiling water did not affect the exsheathment rate of trichostrongylids studied.

(2) The infective larvae of the trichostrongylids did not exsheath during culture, but it was quite common to find that 5% - 10% of the 3rd stage larvae of B. trigonocephalum had exsheathed during culture.

(3) The 3rd stage larvae of B. trigonocephalum appear to exsheath far more rapidly under natural conditions i.e. on the skin than do the trichostrongylids in vivo.

Section B - Experiments on stimuli which may induce exsheathment

Materials and Methods

In this study suspensions of the 3rd stage larvae of B. trigonocephalum, H. contortus and T. colubriformis, recovered by baermann technique, were subjected to different physico-chemical conditions in test tubes held in a water bath at 37°C. The nature of these conditions, and the number of larvae used, is described in the design of each separate experiment. At stated intervals larvae were removed from the tubes, placed on a slide and killed with iodine; 100 were counted and the percentage exsheathment recorded. The two buffers used in several of these experiments consisted of:

(1) Citric acid and disodium phosphate. Range pH 2 - pH 8.

(2) Sorenson-Walburn (glycine, Sodium Chloride and Sodium Hydroxoide), buffer range pH 8 - pH 11.

In this study the period during which observations were made in each experiment did not exceed one hour for two reasons;

(1) the ensheathed 3rd stage larvae of B. trigonocephalum were found to exsheath rapidly in vivo i.e. on the skin of sheep.

(2) Silverman and Podger (1964) noticed that once the exsheathment of H. contortus had been iniated all those larvae capable of exsheathment eventually underwent ecdysis if sufficient time was allowed, and that relative exsheathment rates induced by various stimuli were obscured if exposure times exceeded several hours.

Experiment I - The effect of pH on exsheathment

Expt. design: Each of 10 test tubes was filled with 4 mls of a buffer solution and the pH of the tubes adjusted between pH 2 and pH 11 to give a series of values within this range. One additional control tube was filled with distilled water. 1000 ensheathed 3rd stage larvae of B. trigonocephalum in one ml of

water were added to each tube. Fifteen minutes later the percentage exsheathment was recorded. This experiment was replicated once. A similar study was carried out on the 3rd stage larvae of H. contortus and T. colubriformis, except that the percentage exsheathment was recorded 60 minutes after the experiment had commenced.

Results

B. trigonocephalum - Table No 8.13 shows the percentage exsheathment recorded 15 minutes after the experiment had commenced.

Tube	1	2	3	4	5	6	7	8	9	10	11
pH	2	3	4	5	6	7	8	9	10	11	Control
% exsheathment Replicate I	13%	13%	8%	6%	5%	6%	5%	3%	12%	12%	6%
% exsheathment Replicate II	24%	18%	6%	6%	11%	11%	8%	10%	11%	17%	4%

Table No 8.13

H. contortus - No exsheathment was seen blow pH 5 or above pH 8 and no refractile rings were seen below pH 5 or above pH 9.

Table No 8.14 shows percentage exsheathment and percentage refractile rings seen between pH 5 - pH 9.

pH	5	6	7	8	9
% exsheathment	1%	7%	6%	3%	0%
% refractile rings	12%	16%	8%	8%	4%

Table No 8.14

T. colubriformis - No exsheathment occurred nor were any refractile rings seen in any of the tubes.

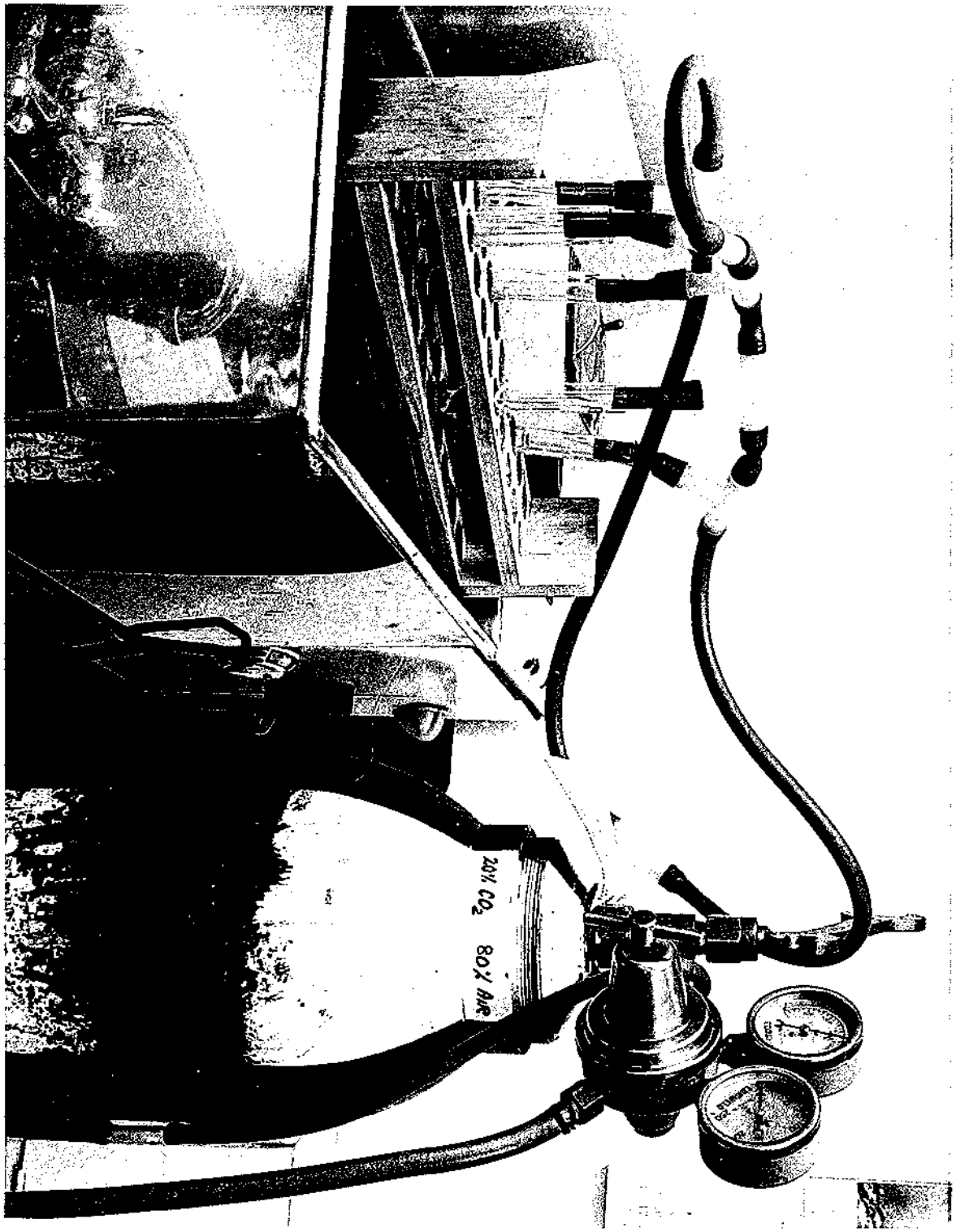

Fig. 22 Test tubes containing ensheathed larvae of B. trigonocephalum being gassed with 20% CO_2 and 80% air.

Experiment II - The effect on exsheathment of pH and CO_2

Expt. design - Each of 10 test tubes was filled with 4 ml of a buffer solution and the pH of each tube adjusted in a similar manner to that described in experiment I. A further tube was filled with 4 ml of distilled H_2O. The solution in each of the tubes was then gassed with 20% CO_2 and 80% air for a period of 30 minutes (fig 22). After which 2000 3rd stage infective larvae of B. trigonocephalum in 1 ml of water were added to each tube. All the tubes were continuously gassed for a further 15 minutes, the larvae removed and the percentage exsheathment recorded.

A similar study was also carried out on the 3rd stage larvae of H. contortus and T. colubriformis, except that percentage exsheathment was recorded 60 minutes after the experiment had commenced. i.e. after the larvae had been added.

Results

B. trigonocephalum - Table No 8.15 shows the percentage exsheathment recorded 15 minutes after the experiment had commenced.

Tube	1	2	3	4	5	6	7	8	9	10	11
pH	2	3	4	5	6	7	8	9	10	11	Control
% exsheathment	61%	11%	3%	6%	4%	3%	3%	4%	5%	9%	5%

Table No 8.15

H. contortus - Table No 8.16 shows the percentage exsheathment and percentage refractile rings seen 60 minutes after experiment had commenced.

Tube	1	2	3	4	5	6	7	8
pH	2	3	4	5	6	7	8	9
% exsheathment	0%	0%	0%	7%	53%	29%	49%	72%
% refractile rings	8%	4%	4%	48%	15%	15%	0%	0%

Table No 8.16

T. coiubriformis - No exsheathment nor refractile rings were recorded below pH 4 or above pH 5 but 1% of the larvae were seen to exsheath at pH 4.

Experiment III

This experiment was carried out in a similar manner to experiment II except that the percentage exsheathment of 3rd stage larvae of B. trigonocephalum was recorded 15, 30 and 45 minutes after commencement of the experiment.

Results

No increase in exsheathment was recorded at pH values greater than pH 2. Table No 8.17 shows the percentage exsheathment at pH 2 recorded 15, 30 and 45 minutes after the commencement of the experiment.

Time	% exsheathment
15 mins	37%
30 mins	47%
45 mins	46%

Table No 8.17

Experiment IV - The effect of pH 2 gassed with CO_2 on exsheathment

Expt. design:- This experiment was set up in a similar manner to the previous experiments except that all 10 tubes were buffered at pH 2. Percentage exsheathment recorded 15 and 30 minutes after the commencement of the experiment,

and only the 3rd stage larvae of B. trigonocephalum were examined.

Results

Table No 8.18 shows percentage exsheathment recorded 15 and 30 minutes after commencement of the experiment.

Tube	1	2	3	4	5	6	7	8	9	10	11 (Control)
% exsheathment 15 mins	30%	39%	26%	23%	-	45%	-	74%	56%	72%	10%
% exsheathment 30 mins	39%	45%	48%	44%	-	40%	-	66%	51%	74%	20%

Table No 8.18

Experiment V - The effect of various concentrations of NaCl on exsheathment

Expt. design - 9 mls of 5%, 2%, 1%, 0.5% and 0.25% NaCl were respectively placed in five separate tubes. A further tube was filled with 9 mls of distilled water. 3000 3rd stage larvae of B. trigonocephalum were added to each tube. Larvae were removed from each tube 5, 15, 30, 45 and 60 minutes after the experiment had commenced and the percentage of exsheathment recorded.

This experiment was replicated once. The experiment was also repeated using the 3rd stage larvae of H. contortus and T. colubriformis.

Results

B. trigonocephalum - The various concentrations of saline did not appear to cause any significant increase in exsheathment (see table No. VIII in appendix).

H. contortus - 2% exsheathment was recorded 60 minutes after experiment commenced in concentrations of 5% saline.

T. colubriformis - No exsheathment was recorded at any of the concentrations of NaCl used in this experiment.

Experiment VI - The effect of saline gassed with CO_2 on exsheathment

Expt. design - This experiment was carried out in a similar manner to experiment V except that all the tubes were gassed with CO_2 for 30 minutes prior to the addition of larvae and gassing was continued for the duration of the experiment.

Results - The findings were similar to those recorded for experiment V. The percentage exsheathment recorded for H. contortus in 5% saline was higher. (12%).

Experiment VII - The effect of Sodium selenite or exsheathment

Expt. design - 9 mls of 0.1% M solution of Sodium selenite was added to 4 tubes (Nos 1-4). Two further tubes were filled with 9 ml distilled H_2O. Tubes 1, 2, 3 and 5 were all gassed with 20% CO_2 for a period of 30 minutes. 6000 3rd stage larvae of B. trigonocephalum in one ml were then added to each tube. The tubes numbered 1, 2, 3 and 5 were then gassed with CO_2 for a further 30 minutes. Larvae were removed from each of the tubes 15 and 30 minutes after the experiment had commenced.

This experiment was replicated once. The experiment was also repeated using the 3rd stage larvae of H. contortus and T. colubriformis.

Results

B. trigonocephalum and T. colubriformis - 0.1% M Sodium selenite did not appear to stimulate exsheathment, nor did it when gassed with CO_2.

H. contortus - Table No 8.19 shows the percentage exsheathment recorded 30 minutes after the commencement of the experiment.

Tube	1	2	3	4	5	6	control
% exsheathment	18%	52%	16%	6%	16%	9%	10% *

Table No 8.19

* No explanation can be offered for the high exsheathment rate seen in control tube beyond that already given in earlier discussion.

Experiment VIII - The effect of 0.1% M Sodium tetraborate on exsheathment

Expt. design - This experiment was carried out in a similar manner to experiment VII except that 0.1% M Sodium tetraborate was used instead of 0.1% M Sodium selenite.

Results

B. trigonocephalum, H. contortus and T. colubriformis - The findings were similar to those recorded for experiment VII.

Experiment IX - The effect of organic acids (proprionic, butyric and caproic) on exsheathment

Expt. design - 9 mls of 0.2% M proprionic, butyric and caproic acid were put respectively in 3 separate tubes, a fourth tube was filled with 9 mls of distilled water. 1000 3rd stage larvae of B. trigonocephalum in 1 ml water were added to each tube. Larvae were removed 30 and 60 minutes after the experiment had commenced and the percentage of exsheathment recorded.

This experiment was replicated three times.

Results

These acids did not appear to cause any increase in the rate of exsheathment of 3rd stage larvae of B. trigonocephalum.

Experiment X - The effect of Ammonia on exsheathment

Expt. design - 9 mls of 0.5%, 0.25%, 0.125% concentration of .88 ammonia were put in three separate tubes, a fourth tube was filled with 9 mls of distilled water. 2500 3rd stage larvae of B. trigonocephalum in 1 ml of H_2O were added to each tube. Larvae were removed 5 and 15 minutes after the experiment commenced and the percentage exsheathment recorded. As it was considered that ammonia could be toxic to larvae even in low concentrations. The percentage of dead larvae at 15 minute interval was recorded.

Results

Table No 8.20 shows the percentage exsheathment and the percentage of dead larvae recorded 15 minutes after the experiment had commenced.

Concentration of Ammonia	0.5%	0.25%	0.125%	Control
% exsheathment at 5 minutes	4	9	8	9
% exsheathment at 15 minutes	1	4	12	9
% dead at 15 minutes	100	100	28	1

Table No 8.20

Experiment XI - The effect of an aqueous extract of wool on exsheathment

Expt. design - 4 oz of wool were clipped from a hogget and soaked for two hours in 400 ml of warm water.

The wool was removed and 0.5 ml of the remaining suspension was placed in each of ten tubes. 4 mls of buffer were added to each of these tubes. A control tube was filled with 4.5 ml distilled water. 800 3rd stage larvae of B. trigonocephalum in 1 ml of H_2O were added to each tube. The pH of tubes 1-10 ranged from pH 2 to pH 11. The percentage of larvae which exsheathed after 15 minutes was recorded.

Results

Table No 8.21 shows the percentage exsheathment 15 minutes after the commencement of the experiment.

Tube	1	2	3	4	5	6	7	8	9	10	11
pH	2	3	4	5	6	7	8	9	10	11	Control
% exsheathment	9%	15%	6%	5%	6%	8%	3%	6%	8%	6%	8%

Table No 8.21

Discussion

In general the findings on the exsheathment of H. contortus and T. colubriformis were similar to those of other workers. The degree of exsheathment though did not appear to be as high as that recorded by Sommerville (1957). This may have been due in part to a different criterion being used to define exsheathment in these experiments compared with those of Sommerville (1957).

In this study two physico-chemical stimuli were found to affect the percentage exsheathment of the 3rd stage larvae of B. trigonocephalum. Firstly an increase in the percentage exsheathment was observed at pH 2 compared with higher values for pH. Secondly the addition of CO_2 to suspensions containing 3rd stage larvae markedly enhanced the percentage of exsheathment at pH 2 but not at higher values for pH. In the replicate experiments carried out at pH 2 a wide variation in the percentage of exsheathment was recorded (23%-74%). This occurred in spite of the fact that in this particular experiment the larvae were all from the same batch and each of the replicate tubes was held under the same conditions. No obvious explanation can be offered to account for this variation, but it is conceivable that once exsheathment has commenced the secretion of exsheathing fluid by a few stimulated larvae may, under certain circumstances as yet unidentified, produce a cumulative result which is to some extent independent of the adequacy of the initial trigger stimulus.

The findings in both the experiments associated with the process of exsheathment and those involved with stimuli suggest that the balance between the ensheathed 3rd stage larva of B. trigonocephalum and the exsheathed larva is very precarious and once it is stimulated, exsheathment proceeds rapidly.

Although other factors responsible for initiating exsheathment have yet to be identified, it seems reasonable to suppose that undissociated carbonic acid together with low pH should be important components of the

environment which start infection in the alimentary canal of animals. These properties distinguish regions in the alimentary tract from many other habitats and they ensure that development from the infective stage will not start until the infective agent is eaten by the host.

In some preliminary experiments the 3rd stage larvae of B. trigonocephalu were found to exsheath on pieces of abomasum stretched across funnels containing 0.85% NaCl at 37°C. These findings confirmed those of Camerən (1923) who conducted a series of similar observations. In 1965 Stoye managed to successfully infect two out of three sheep by injecting the 3rd stage larvae of B. trigonocephalum into the abomasum. The resulting infection though was considerably lighter than after the administration of equivalent doses of larvae per os and percutaneously. He does point out that although the larvae were injected into the lumen of the abomasum, infection of the host may not necessarily have taken place via the abomasal mucosa. However, the fact that the larvae of B. trigonocephalum require a low pH in order to exsheath certainly indicates that exsheathment after ingestion takes place in the abomasum rather than the rumen or duodenum. This also lends further support to the view associated with most nematodes infecting the gut, that the adult worm can be expected to settle in the region of the alimentary tract posterior to that where conditions necessary for exsheathment are found. It is however difficult to reconcile the fact that a low pH is necessary when exsheathment is seen to take place so rapidly on the skin which is alkaline. It was also observed that in faecal cultures where a large proportion of Bunostomum larvae had exsheathed the pH was strongly alkaline (approx. pH 9). Undoubtedly there are factors yet to be found which will induce the 3rd stage larvae of this particular nematode to exsheath.

Taylor and Whitlock (1960) reported that CO_2 gas in suitable salt solutions acted as an adequate stimulus for the exsheathment of H. contortus.

They demonstrated that all salts were not equally effective and that there was a greater tendency for the oxy-acid buffer salts to be effective at markedly lower concentrations. Some of the salts discussed by Taylor and Whitlock (1960) and Whitlock (1966) were used in experiments associated with this thesis. The 3rd stage larvae of H. contortus were seen to exsheath in solutions of sodium, chloride, selenite and tetraborate gassed with CO_2, but the 3rd stage larvae of T. colubriformis and B. trigonocephalum were unaffected by the various salt solutions. The pH of the solutions in these experiments was in the region pH 6-7 and it must be conceded that had these experiments been conducted at a lower pH than the results could have been different. On the other hand it is possible that saline may even prevent the exsheathment of these particular larvae. Whitlock (1966) found that 0.5% NaCl inhibited the exsheathment of O. circumcincta.

Taylor and Whitlock (1960) also showed that the organic acids (proprionic, butyric and valeric) caused the exsheathment of the 3rd stage larvae of H. contortus. As fatty acids or their salts are present in skin secretions it was felt that there may be a relationship between them and the exsheathment of the 3rd stage larvae of B. trigonocephalum on the skin. However, the low chain fatty acids used in this study did not influence the percentage of exsheathment of B. trigonocephalum.

Similarly, it was considered that as NH_3 is commonly present in skin secretions, it too may influence the exsheathment of B. trigonocephalum. Silvermann and Podger (1964) found 1.0% solution of NH_4OH (sp. gravity 0.880) together with CO_2 caused H. contortus to exsheath. However NH_4OH in this study was found to be toxic for the larvae of B. trigonocephalum in a concentration as low as 0.125% and although there was a slight increase in the percentage of exsheathed larvae following treatment with NH_4OH the results were not considered reliable and could not be interpreted.

The work to date on the mechanism of the exsheathment of 3rd stage larvae of B. trigonocephalum suggests that the manner in which these larvae exsheath and the stimuli which induce it are probably different to those of the other strongylates which have been studied. Because of the precarious balance between ensheathed larvae and those that have exsheathed it is possible that the techniques employed have not been sufficiently refined. Further detailed study on this aspect is undoubtedly warranted.

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CHAPTER 9 PATHOGENESIS

Introduction

Although the association between anaemia and infection of man with "abdominal worms" has been recognized for as long as 3500 years (Ebers papyrus dated in the 18th Egyptian dynasty 1500 B.C. translated 1873). It is only in the last 15 years that the pathogenicity of hookworms, particularly in relation to anaemia has been elucidated. (Roche et al 1957; Foy et al 1958; Clark et al 1961; Roche and Layrisse 1966). Apart from anaemia many clinical accounts of severe hookworm disease have been described these include oedema of the body and serous cavities and the impairment of intestinal absorption. The disease in man has been well reviewed by Layrisse and Roche (1964); Woodruff (1965; and Ball (1966).

In animals the pathogenicity of hookworm infection has in the main been restricted to studies in the ox and the dog. Little detailed work is available on the pathogenicity of the sheep hookworm. From the beginning of the century reports of bunostomiasis in cattle have appeared in literature. Stiles (1901) found the parasite to be present in 50% of cattle in a survey conducted in the U.S.A. Conradi and Barnette (1908) mentioned a number of cases of infection all of which were characterised by a typical anaemic picture. Other workers in many parts of the world (Boynton and Wharton 1916, Reisinger 1916, 1924; Poisson and Buck 1938; Roberts 1939) have described a clinical syndrome in cattle involving emaciation, anaemia, oedema, diarrhoea and death which they attributed to infection with B. phlebotomum. Sprent (1946) carried out a detailed study upon an "anaemic disease" occurring in young zebu cattle. He found that the incidence bore some relationship to infection by the bovine hookworm and that the lesions resembled those described by other observers in cases of heavy infection by this parasite. Soulsby (1965) bases his description on the gross pathology of bunostomiasis on Sprent's work,

although Sprent himself was careful in his paper to point out that the evidence he presented was only circumstantial. Mayhew (1939, 1946, 1947, 1949) conducted a series of experimental infection in calves with B. phlebotomum his findings though were somewhat inconclusive. It seems that in spite of all the various studies undertaken the genesis of hookworm anaemia in cattle is still not clear.

In the dog the investigations have been more objective and the findings have to some extent paralleled those of hookworm infection in man. The anaemia recorded in the dog being characterised by microcytosis and hypochromia, both of which responded well to iron therapy. As early as 1934 Foster and Landsberg undertook work to determine the type of anaemia and whether sufficient blood was removed to account for the degree of anaemia or whether it was necessary to postulate additional causal factors. They concluded that toxins were not responsible for the anaemia of hookworm disease in dogs and that it was of a haemorrhagic type. Later workers supported this view and it appears that the damage done by the worms to their host is a consequence of their extraction of red cells and protein containing fluid from the small intestine. Roche and Martinez-Torres (1960) showed that the adult A. caninum pumped blood actively and enclosed much of a villus as it did so, red cells passing at least partly undamaged through the worm. However, what the worm actually feeds upon is still an open question.

The severity of the clinical condition depends upon various factors such as the age and nutritional status, the number of parasites present and the resistance of the animal as a result of previous infection. In addition to anaemia a mild to severe enteritis has been found in dogs (Soulsby 1965), and this may have a marked influence on intestinal absorption.

There are undoubtedly significant differences in the epidemiology and the biology of the Ancylostominae and the Necatorinae however, reports in

the literature indicate that the pathogenesis of both sub-families may be similar. During the last 60 years there has been considerable controversy about the relative importance of intestinal haemorrhage, intravascular haemolysis, and depressed erythropoiesis in the pathogenesis of hookworm disease in both animals and man. It is now generally accepted though that the prime cause of hookworm anaemia, at least in man and the dog, is directly attributable to the blood sucking activities of the worms.

The importance of B. trigonocephalum as a pathogen in sheep is however still somewhat obscure. Although earlier parasitologists such as Curtice considered this parasite to be a serious problem other workers in the early 1900's particularly French and German, tended to hold its pathogenicity in doubt and in many cases regarded it as a harmless commensal. It was not until the mid 1920's that workers (Cameron 1923; Beller 1926; and Velu and Balozet 1926) independently observed severe outbreaks of bunostomiasis. They described an anaemia together with marked loss of condition which led progressively to paralysis and death. Beller (1926) stated that the degree of anaemia observed by him did not sufficiently explain the severity of the disease and he postulated that death was associated with a toxin which affected the central nervous system. Beller (1928) together with later workers Ortlepp (1939), Lucker and Neumayer (1946, 1947), Demidowa (1958), Westen (1967) experimentally infected sheep with the 3rd stage larvae of B. trigonocephalum in an attempt to establish the pathogenesis of this parasite. Unfortunately most of their observations were subjective and of limited scope. Beller's experiments concerned chiefly the routes of infection. The sheep and goats used by him, as Ortlepp has pointed out were already infected with Bunostomum when the larvae were administered to them. Aside from this restriction of their validity, Beller's observations were very limited. Ortlepp's study was concerned mainly with the life history of the species but he briefly

described the post mortem findings in one experimental lamb. Lucker and Neumayer did carry out a more extensive, thorough and controlled study but their experimental sheep were consistently infected with Strongyloides. Westen's investigations were detailed but his work was concerned mainly with goats and only a few sheep were used for comparative purposes. The results of the initial experiments carried out by Lucker and Neumayer were at variance with those of earlier workers.

Lucker and Neumayer found the pathogenesis to be much lower than had been previously reported. In view of these apparent discrepancies they carried out a further set of small experiments (Lucker and Neumayer 1947) on the affect of diet on the severity of this condition. The results of these experiments showed that well fed lambs survived from the effects of infections resulting from exposure to exactly the same numbers of larvae that induced infections which were fatal to lambs on a low plane of nutrition. They concluded that the drastic effects seen in the poorly fed lambs were due to the fact that

(1) a higher percentage of the larvae administered developed in these lambs.

(2) The ability of lamb on a low plane of nutrition to compensate for blood loss was inferior to that of the well-fed lambs.

These conclusions are similar to those of Foster and Cort (1931, 1932a, 1932b, 1935) who found that in ancylostomiasis in dogs diet markedly affected the severity of the infection and that a greater percentage of larvae developed in the poorly nourished animals compared with the well fed ones.

It is apparent after reviewing the literature on the pathogenicity of B. trigonocephalum that there are still many aspects on the pathogenesis of this parasite which require further investigation. There is considerable diversity of opinion as to the number of worms which may be pathogenic. The New Zealand Department of Agriculture handbook 1960 quotes a figure of 50

as pathogenic to sheep whilst Gordon (1967) suggested 400 and certain Russian workers 5000. Riek (1956) and Wetzel and Riek (1962) consider B. trigonocephalum a severe pathogen in deer and that 50-70 worms may cause a clinical syndrome. They support the findings of Hübner (1937, 1939) who stated that approximately 100 worms were sufficient to cause the death of a deer, on the other hand Welker (1964) considered that 200 specimens had little affect on the deer.

The worm burden found in sheep under natural conditions is usually low, although certain workers have recorded excessively high counts. Lenshin mentions 5000-6000 worms being recovered at post mortem, and Habermann (1946) recovered numbers varying between 555-932. Morgan et al (1951) noted a high incidence in Scottish hill sheep. Parnell et al (1954) considered it was probably one of the most pathogenic nematodes to be found in hill sheep, as did Tetley and Langford (1966). In surveys carried out in different parts of the world though the numbers recorded are much lower. Demidowa (1958) gives a mean recovery figure of 112 per sheep, Sarimsakov (1959) a mean of 162 per sheep, whilst Cameron (1923b) found an average of 10.5 hookworms in 38 infected sheep, the highest number in one sheep was 31. None of the three latter authors however relate pathogenicity to the number of worms recovered.

Little reference in the literature could be found to the distribution of B. trigonocephalum in the small intestine of sheep. Tetley (1937) did examine this question but the numbers he recovered were insufficient for him to draw any definite conclusions, Lucker and Neumayer (1946) mentioned that the first 10 to 15 feet and last 30 feet of the small intestine even in the heavily infected lambs, were free of worm lesions; in the intervening region both worms and lesions were well distributed but tended to be more numerous anteriorly, similarly little investigation has been done on the egg output of the adult female B. trigonocephalum.

Soulsby (1965) admits that no detailed work is available on the pathogenesis of B. trigonocephalum. However, he does add that "it is likely the parasites affect the host in a manner comparable to the dog and human hookworms".

A series of experiments were therefore designed in an attempt to investigate further, certain aspects of the pathogenesis of B. trigonocephalum.

These include:-

(1) The relationship between the number of worms recovered at post mortem and pathogenicity particularly in relation to (a) Haematological picture
(b) Serum protein level

(2) The distribution of these worms in the small intestine.

(3) Clinical signs and gross lesions due to infection with B. trigonocephalum.

(4) The egg production of the adult female worm.

Materials and Methods

Egg counts:

Faeces were examined for the presence of eggs at weekly intervals except at the expected end of the prepatent period when daily examinations were made. The Ether/Acetate method was used prior to eggs being recorded thereafter counts were carried out by the modified McMaster method at weekly intervals or as specified in the individual experiment.

Worm counts:

At necropsy the whole gastro-intestinal tract including the large intestine was examined for the presence of worms. The viscera were not subjected to a digestion technique.

Distribution of worms in the small intestine:

At necropsy ligatures were tied around the small intestine, close to the pyloric sphincter, approximately 5 feet, 20 feet and 35 feet from pylorus and also just prior to the ileocaecal valve. Each portion was examined separately for the presence of B. trigonocephalum, the number counted and recorded. The ratio of male:female was also noted.

Blood samples:

These were taken from the jugular vein at weekly intervals or as stated in the particular experiment. Approximately 20 ml were removed from each sheep at each sampling. About 2 ml were placed in a small 3 ml plastic bottle containing anticoagulant (Disodium E.D.T.A.). This sample was subjected to a haematological examination. The remaining 18 ml portion of the blood sample was allowed to clot overnight, centrifuged and the serum removed. Total serum protein estimations were made on this sample.

Haematological examination:

(i) Packed cell volume percentage was estimated using a micro-haematocrit method.

(ii) Haemoglobin was estimated using a standard cyanmethaemoglobin method.

(iii) Total and differential white cell counts were done with the usual haemocytometer and staining techniques.

Total serum protein estimation:

Total serum protein concentrations were estimated by measuring the U.V. absorption at a wavelength of 280μ of replicate serum samples diluted 1:100 in distilled water. The concentrations were then obtained using a standard line prepared with bovine serum albumin dissolved in distilled water.

Electrophoretic studies

Selected serum samples were subjected to electrophoresis on cellulose acetate strips. The strips were stained with amido black.

Experimental design

Four separate experiments were conducted. Experiments I, II and III involved different groups of sheep and related directly to the study of the pathogenesis of B. trigonocephalum. Clinical observations, gross pathological examinations, haematological studies and total serum protein estimations were made on these sheep. Experiment IV was concerned with the estimation of the egg output of the adult female B. trigonocephalum.

Experiment I

Four 9 month old worm free hoggets were selected at random from the worm free flock. Two were infected percutaneously with 4000 3rd stage larvae of B. trigonocephalum and two were left as controls. All four sheep were run together for a period of 13 weeks after which time one of the infected sheep died. The remaining three sheep were held for a further week then slaughtered. Necropsies were performed on all the sheep and the total number of adult B. trigonocephalum recovered from each sheep recorded. Faecal and blood samples were taken at the commencement of the trial from each sheep and thereafter at weekly intervals throughout the experiment.

Faecal samples - These were examined for the presence of eggs as described earlier.

Blood samples - Total and differential white cell counts were undertaken and the total number of circulating eosinophils estimated. The P.C.V. percentage and haemoglobin also estimated on each sample.

Serum samples - Total serum protein was estimated on each sample and electrophoretic studies undertaken on the initial sample and the final sample of the two infected sheep.

Experiment II

This was carried out in a similar manner to Experiment I except that ten sheep were used. Eight were infected with the 3rd stage larvae of B. trigonocephalum and two remained as controls. The experiment was continued for 112 days. Blood samples were taken at approximately fortnightly intervals until the 86th day after which the sheep were bled on a weekly basis. Faecal samples were taken twice weekly. Haematological studies were as in Experiment I and the total serum protein was estimated on each sample. No electrophoretic studies were undertaken in this experiment.

Experiment III

This was again similar to Experiments I and II. Thirteen sheep were involved, eleven were infected with the 3rd stage larvae of B. trigonocephalum and two remained as controls. The experiment was continued for 85 days. Blood samples were taken at approximately 2 weekly intervals until patency was observed thereafter the sheep were sampled on a weekly basis. Total serum protein was estimated on each sample as was the P.C.V. percentage and the haemoglobin. Total and differential white cell counts however were not done as in both the previous experiments it was found that infections with B. trigonocephalum appeared to have had little or no effect on the white cell picture.

Experiment IV

This experiment was concerned with the estimation of average daily egg output of the adult female B. trigonocephalum. Faecal collecting bags were placed on five sheep infected solely with B. trigonocephalum. The faeces were collected from each of the bags 6 hours, 14 hours and 24 hours after they had been placed in position. The total faecal output of each sheep for the 24 hour period was weighed and thoroughly mixed. Five aliquots were taken from the samples collected from each sheep and the mean E.P.G. estimated.

The number of eggs passed by each sheep during the 24 hour period was calculated. All the sheep were then slaughtered and the total number of adult female B. trigonocephalum present in each sheep recorded. The number of eggs passed by each sheep in the 24 hour period was then related to the number of adult females found in each sheep.

Results

Experiment I

The number of adult B. trigonocephalum recovered from the sheep at necropsy are shown in table 9.1.

Sheep No. for each Group	Number of B. trigonocephalum			
	Infected Sheep			Control Sheep
	Male	Female	Total	Total
No. 1	249	623	862	Nil
No. 2	71	235	306	Nil

Table No 9.1

A decline in the haemoglobin levels, haematocrit values and the total serum protein concentrations in the animals infected with B. trigonocephalum occurred throughout the course of this experiment, whereas the values recorded in the control animals were not depressed and remained within the normal range quoted for sheep, (Schalm 1961). The haemoglobin level, P.C.V. % together with the total serum protein concentration estimated in each blood sample are recorded in tables 9.2, 9.3, 9.4 respectively.

There did not appear to be any significant difference in the total white cell counts of the infected sheep compared with those of the control sheep during this experiment. Neither, with the possible exception of the eosinophils any apparent difference between the differential white cell count of the infected sheep and the control sheep. The total number of circulating

HAEMOGLOBIN LEVEL GMS/100 ML BLOOD

DAY p.i.		0	7	14	21	28	35	42	49	56	63	70	77	84	91	98
Infected Sheep	Sheep No. 1	13.2	13.7	12.0	11.9	11.0	10.2	8.9	8.4	8.7	8.0	7.6	7.8	7.7	-	-
	Sheep No. 2	13.6	13.2	12.7	13.25	12.9	11.6	10.7	10.0	9.6	9.9	11.1	10.1	10.25	8.9	9.7
Uninfected Controls	Sheep No. 1	31.2	11.4	11.5	12.6	12.7	12.0	12.4	12.9	13.6	12.2	11.6	13.0	13.0	12.0	11.5
	Sheep No. 2	12.5	11.2	10.0	11.25	11.7	10.6	11.2	11.9	11.2	11.7	11.2	11.9	11.7	10.7	10.8

Table No. 9.2

P.C.V. %

DAY p.i.		0	7	14	21	28	35	42	49	56	63	70	77	84	91	98
Infected Sheep	Sheep No. 1	43	42	40	39	34	32	29	25	27	24	22	21	21	-	-
	Sheep No. 2	42	42	37	42	39	35	33	29	31	30	30	30	30	27	29
Uninfected Controls	Sheep No. 1	42	36	37	40	39	39	39	37	38	37	36	37	37	35	35
	Sheep No. 2	39	37	31	35	34	33	35	32	34	35	34	35	36	33	33

Table No. 9.3

TOTAL PROTEIN CONCENTRATION GMS/100 ML SERUM

DAYS p.i.		0	7	14	21	28	35	42	49	56	63	70	77	84	91	98
Infected Sheep	Sheep No. 1	6.2	6.0	6.2	6.0	5.9	5.3	4.9	4.6	4.5	4.6	5.0	5.1	N/S	-	-
	Sheep No. 2	6.2	6.2	6.0	6.2	5.8	5.7	4.9	N/S	4.4	4.6	4.5	5.2	4.4	4.9	5.2
Uninfected Controls	Sheep No. 1	7.0	6.5	6.0	6.2	6.5	6.0	6.2	6.5	6.9	6.8	6.0	6.9	6.2	6.2	6.5
	Sheep No. 2	5.6	6.0	5.8	5.7	5.6	6.0	6.3	6.0	6.0	5.6	5.9	5.9	5.6	5.9	5.9

Table No. 9.4

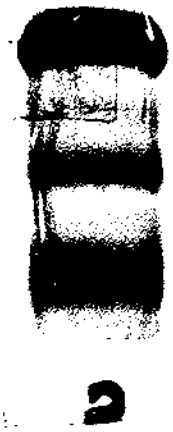
TOTAL NUMBER CIRCULATING EOSINOPHILS PER CMM

DAYS p.i.		0	7	14	21	28	35	42	49	56	63	70	77	84	91	98
Infected Sheep	Sheep No. 1	-	-	128	150	-	98	61	90	392	294	234	-	59		
	Sheep No. 2	-	48	-	-	72	71	-	268	85	208	44	156	212	237	50
Uninfected Controls	Sheep No. 1	-	-	-	51	-	-	140	-	-	-	-	-	-	70	-
	Sheep No. 2	-	-	-	-	-	-	-	168	71	-	-	-	-	-	-

Table No. 9.5

Electrophoretic Pattern of Serum Samples

Sheep 1



Day 0

Sheep 1

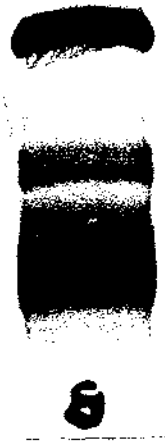
Day 84

Sheep 2

Day 0

Prior to the administration of infective larvae of B. trigonocephalum.

Sheep 2



Day 84

84 days after the establishment of infection with B. trigonocephalum.

Fig. 23 Electrophoretic patterns of serum samples taken from sheep before and after infection with B. trigonocephalum. The figure shows the drop in serum albumin which occurred 84 days after infection.

eosinophils per cmm estimated from each sample is recorded in table 9.5.

Egg counts

Eggs were first noticed in the faeces 53 and 60 days after infection in Sheep No. 1 and No. 2 respectively, but the counts did not rise substantially until the 70th day p.i.

Table 9.6 shows the mean E.P.G. of the two infected sheep from the 70th day until the experiment ended.

Sheep No.	E.P.G.				
1	950	2400	2800	-	-
2	1150	650	350	1150	1250
Day p.i.	70	77	84	91	98

Table No. 9.6

Electrophoretic studies conducted on the initial and final serum samples taken from the two infected sheep indicated that the depression in total serum protein concentrations were due mainly to a drop in circulating albumin. (fig 23).

Experiment II

At the end of this experiment the worm burden in the 8 infected sheep was found to vary widely (1305-1). However, it was possible to arrange the infected animals into two groups according to the total number of worms recovered at necropsy from each sheep, Group A harbouring 1305-335, Group B 74-1. The two uninfected controls forming the 3rd Group C.

Table No. 9.7 shows the manner in which the 10 sheep are grouped.

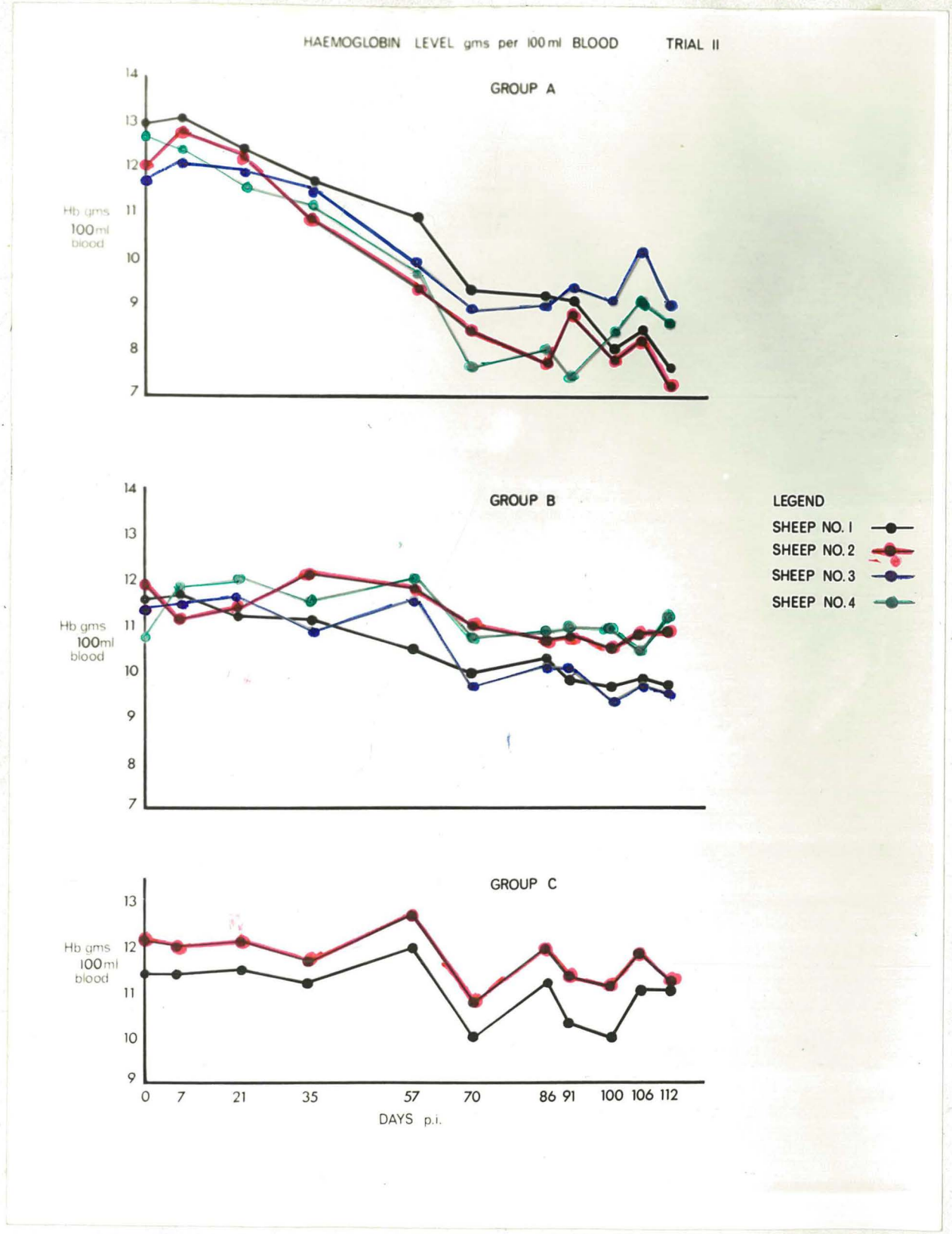


Fig. 24 The haemoglobin level in blood samples taken from sheep in Experiment II, Chapter 9.

4 sheep in Group A, 4 sheep in Group B, 2 sheep in Group C.

Sheep No. for each group	Number of B. trigonocephalum						
	Group A			Group B			Group C
	Male	Female	Total	Male	Female	Total	Total
Sheep 1	608	697	1305	32	42	74	Nil
Sheep 2	555	627	1182	32	26	58	Nil
Sheep 3	165	172	335	19	24	43	
Sheep 4	244	281	525	1	-	1	

Table No. 9.7

A marked decline occurred in the haemoglobin level, haematocrit value and the total serum protein concentration during the course of the experiment in all the sheep in Group A. Whereas the values recorded from the samples taken from the sheep in both Groups B and C remained within the normal range. (figs 24, 25, 26) based on tables IX, X and XI in the appendix). Table XII in the appendix shows the total serum protein concentration in gms/100 ml of serum in each sample taken during the course of the experiment.

There appeared to be a slight downward trend in the haemoglobin levels of sheep Nos. 1, 2 and 3 of Group B (fig 24) but it is questionable whether this trend is of significance. The total white cell count of all the samples lay within the normal range and even in those sheep more heavily infected the white cell count did not appear to be affected. Similarly the differential white cell counts for each of the sheep in all the groups were also within the normal range, although again there did appear to be a slight increase in the total number of circulating eosinophils in the heavily infected group compared with the more lightly infected groups and the controls (see Table XIII in the appendix).

Egg counts

These are recorded in Table XVIII in the appendix from 61st day after infection and thereafter at approximately 3 day intervals. The E.P.G. of the

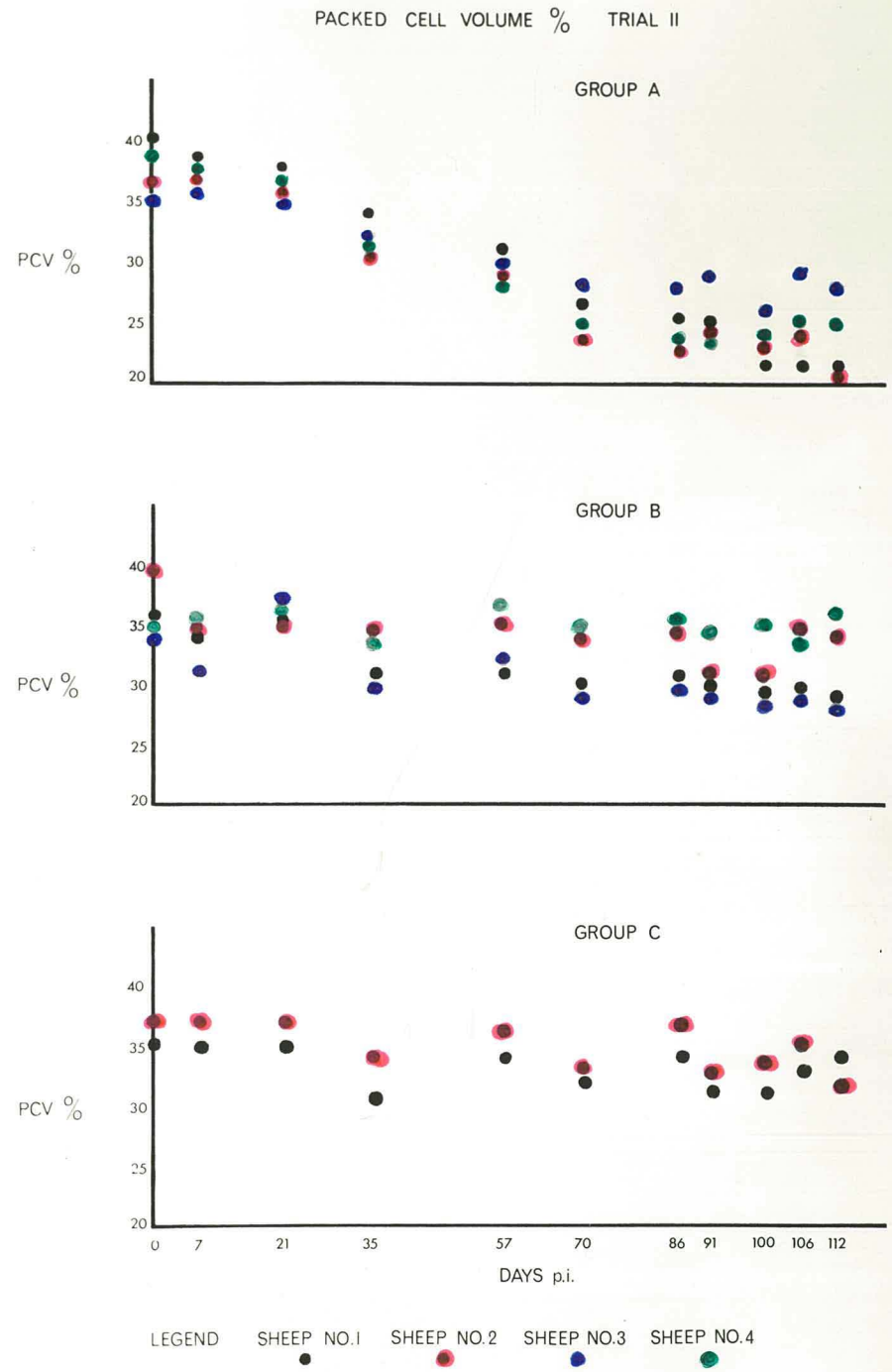


Fig. 25 The packed cell volume percentage of blood samples taken from sheep in Experiment II, Chapter 9.

4 sheep in Group A, 4 sheep in Group B, 2 sheep in Group C.

Sheep No. for each group.	Number of B. trigonocephalum									
	Group A			Group B			Group C			Group D
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Total
Sheep No. 1	312	498	810	5	12	17	3	3	6	Nil
Sheep No. 2	409	652	1061	9	11	20	1	4	5	
Sheep No. 3	166	157	323	9	16	25	2	6	8	
Sheep No. 4	95	81	176				2	3	5	

Table No. 9.8

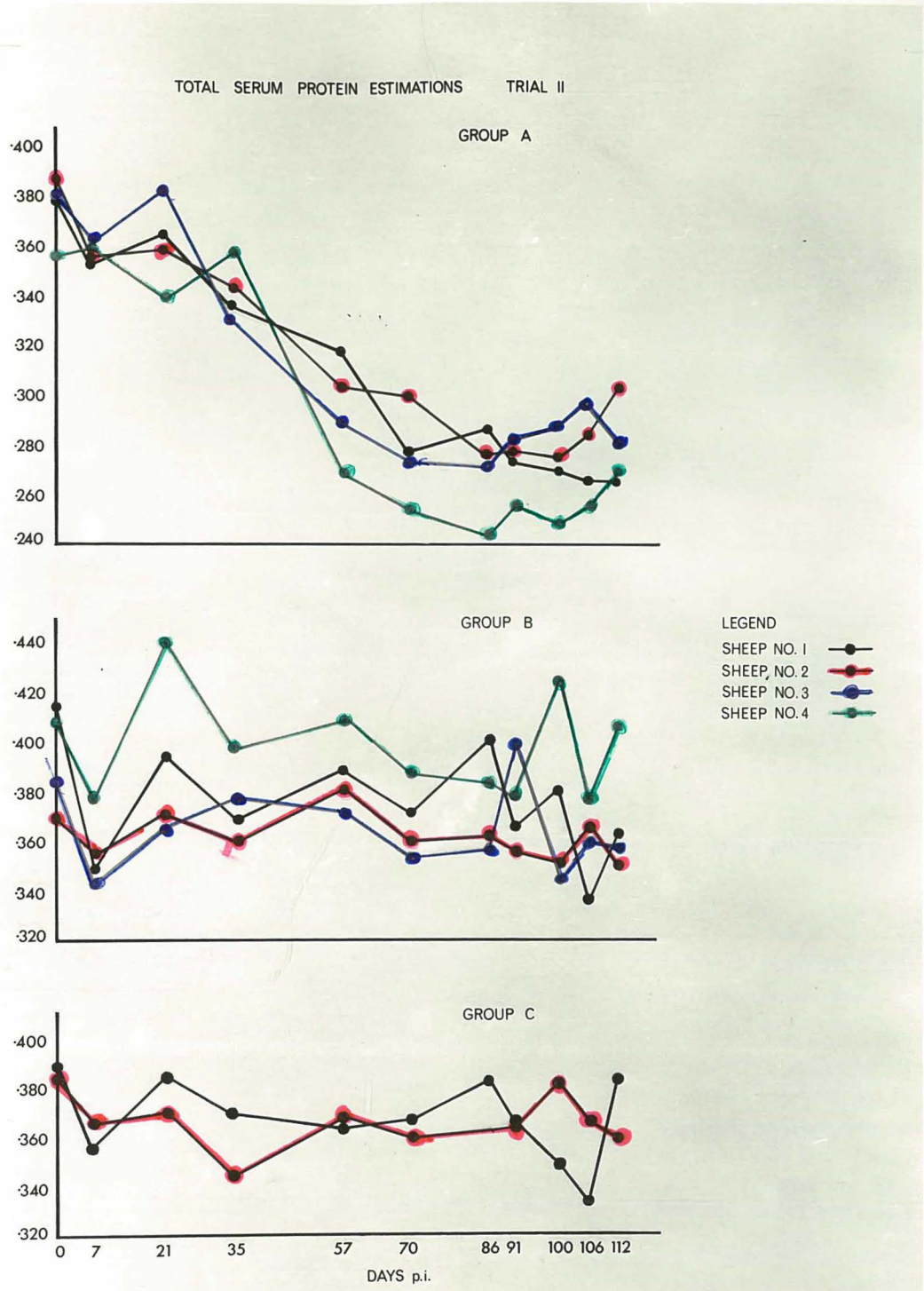


Fig. 26 The estimated total serum protein in samples taken from sheep in Experiment II, Chapter 9.

Scale on ordinate gives spectrophotometer reading.

Table XII in appendix shows readings converted to total serum protein concentration in gms/mls.

sheep in Group A were considerably higher than those of the other groups. Sheep Nos. 1 and 2 of Group A showed consistently higher egg counts than any of the other sheep. In Group B Sheep No. 1 had the most consistently high egg counts for any of the sheep in that group.

Experiment III

For the purposes of discussion the sheep were grouped in a similar manner to that described in Experiment II.

Group A consisted of 4 sheep whose worm burdens varied from 176 to 1061.

" B " " 3 " " " " " " 17 to 25.

" C " " 4 " " " " " " 5 to 6.

" D " " 2 sheep which were the uninfected controls.

Table No. 9.8 shows the manner in which the 13 sheep in this experiment were grouped.

A marked decline in the haemoglobin level, haematocrit value and the total serum protein concentration was recorded during the experiment in all the sheep in Group A, whereas the values obtained from the samples taken from sheep in Groups B, C and D appeared to remain within the normal range (figs 27, 28, 29, based on tables XIV, XV , XVI in the appendix). Table XVII in the appendix shows the total protein concentration in gms/100 ml of serum in each sample taken during the course of the experiment.

Egg counts

Eggs were first noticed in the faeces 54 days after infection in all the sheep in Group A, 56 day p.i. in Sheep Nos. 2 and 3 in Group B and 60 days p.i. for the remainder of Group B and all of Group C. An estimate of the E.P.G. on all sheep was started on the 65th day after the commencement of the experiment. Table 9.9 shows the estimated E.P.G. for all the sheep between day 65 and 85 p.i.

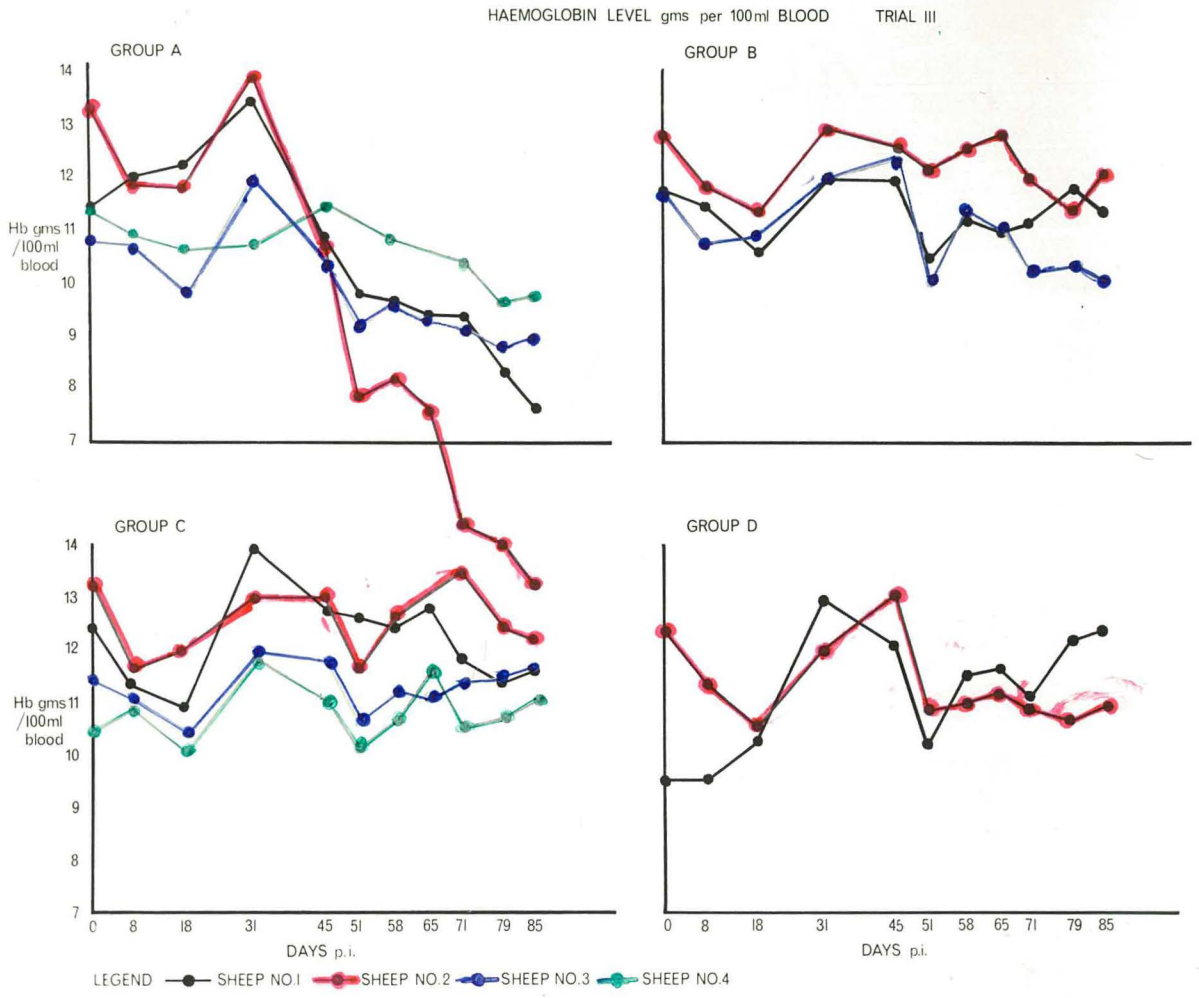


Fig. 27 The haemoglobin level in blood samples taken from sheep in Experiment III, Chapter 9.

4 sheep in Group A, 3 sheep in Group B, 4 sheep in Group C, 2 sheep in Group D.

ESTIMATED E.P.G. EXPERIMENT III

Days p.i.	65	71	79	85
Group A				
Sheep No 1	500	1900	1800	2900
2	300	4100	3900	7000
3	500	800	700	1300
4	-	400	600	1500
Group B				
Sheep No 1	-	-	200	100
2	100	100	100	100
3	-	-	100	100
Group C				
Sheep No 1	-	200	-	-
2	-	-	-	-
3	-	-	-	100
4	-	-	100	-
Group D				
Sheep No 1	-	-	-	-
2	-	-	-	-
Days p.i.	65	71	79	85

Table No. 9.9

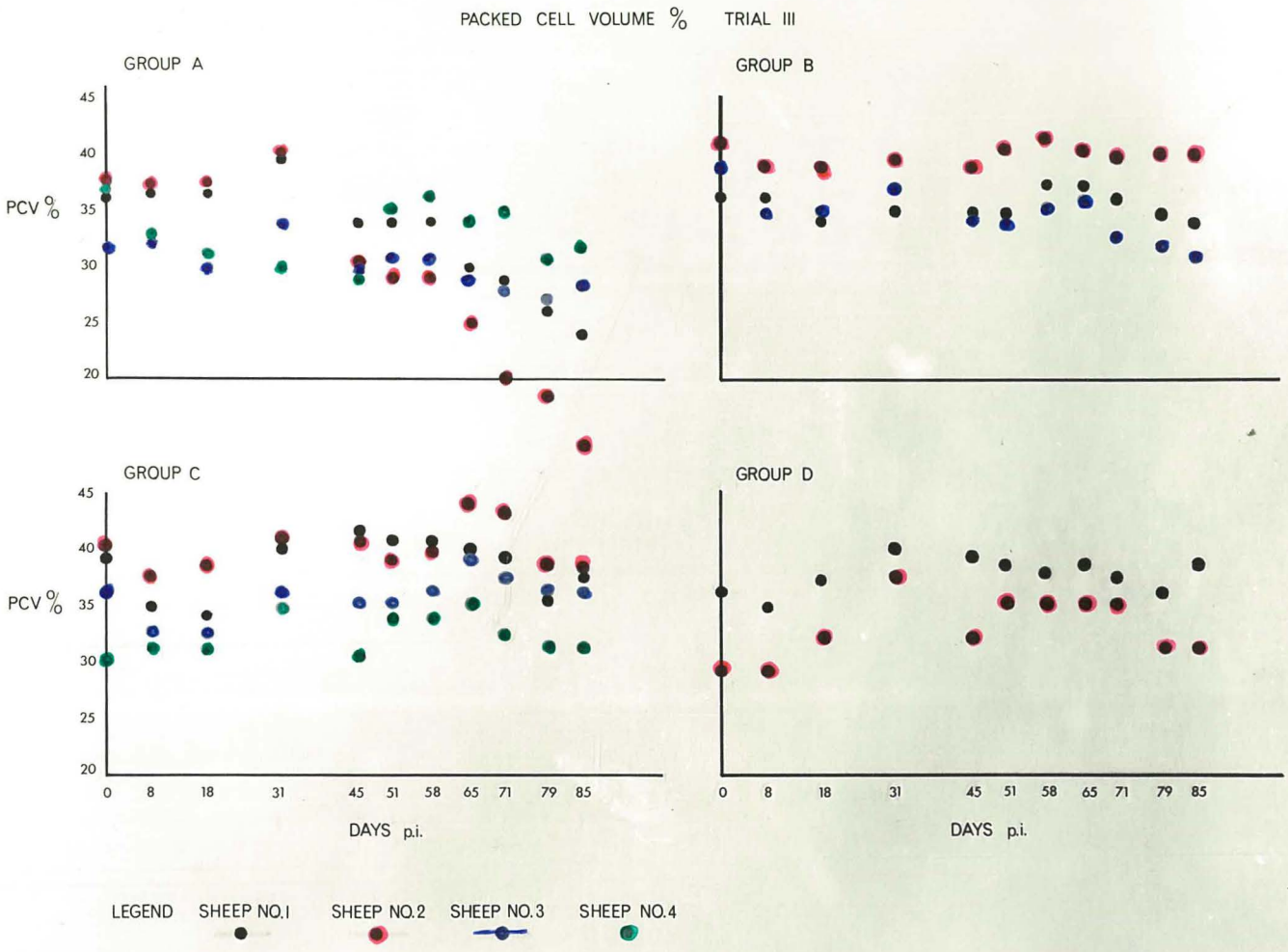


Fig. 28 The packed cell volume percentage of blood samples taken from sheep in Experiment III, Chapter 9.

4 sheep in Group A, 3 sheep in Group B, 4 sheep in Group C, 2 sheep in Group D.

Clinical signs

Although the sheep in these experiments were not weighed the infected sheep in Experiment I and the more heavily infected sheep (Group A) in Experiments II and III were seen to lose condition, whereas the controls and remaining sheep did not deteriorate. Observations indicated that this loss of condition commenced 2-3 weeks after the administration of the infective larvae of B. trigonocephalum and was most marked from the 8th week onwards. Figure 30 taken 2 days before the termination of Experiment II shows 3 infected sheep from Group A together with one of the control sheep from Group C.

None of the sheep in any of the experiments exhibited acute diarrohea but those sheep referred to in the previous paragraph which lost condition did on occasions produce soft loose faeces. These soft faeces not seen were until 8th-10th week after infection and did not occur in the affected sheep at the same time. The syndrome appeared to be intermittent and in each case only lasted for two or three days after which time the faeces returned to their normal consistency (i.e. pelleted).

Gross pathology

At necropsy the lungs, abomasum, small and large intestine together with the other visceral organs were all examined for any macroscopic lesions. The appearance of all the organs with exception of the small intestine appeared normal. There was no evidence of any lesions in lung similar to those described in Chapter 5, page 35 which were seen to occur shortly after infection. Small haemorrhagic lesions about 1 mm in diameter (fig 31) were seen in the mucosa of the small intestine of all hoggets from which hookworms were recovered. These lesions obviously marked the recent sites of attachment of these worms; they were identical in appearance to those observed when the parasites were detached from the mucosa.

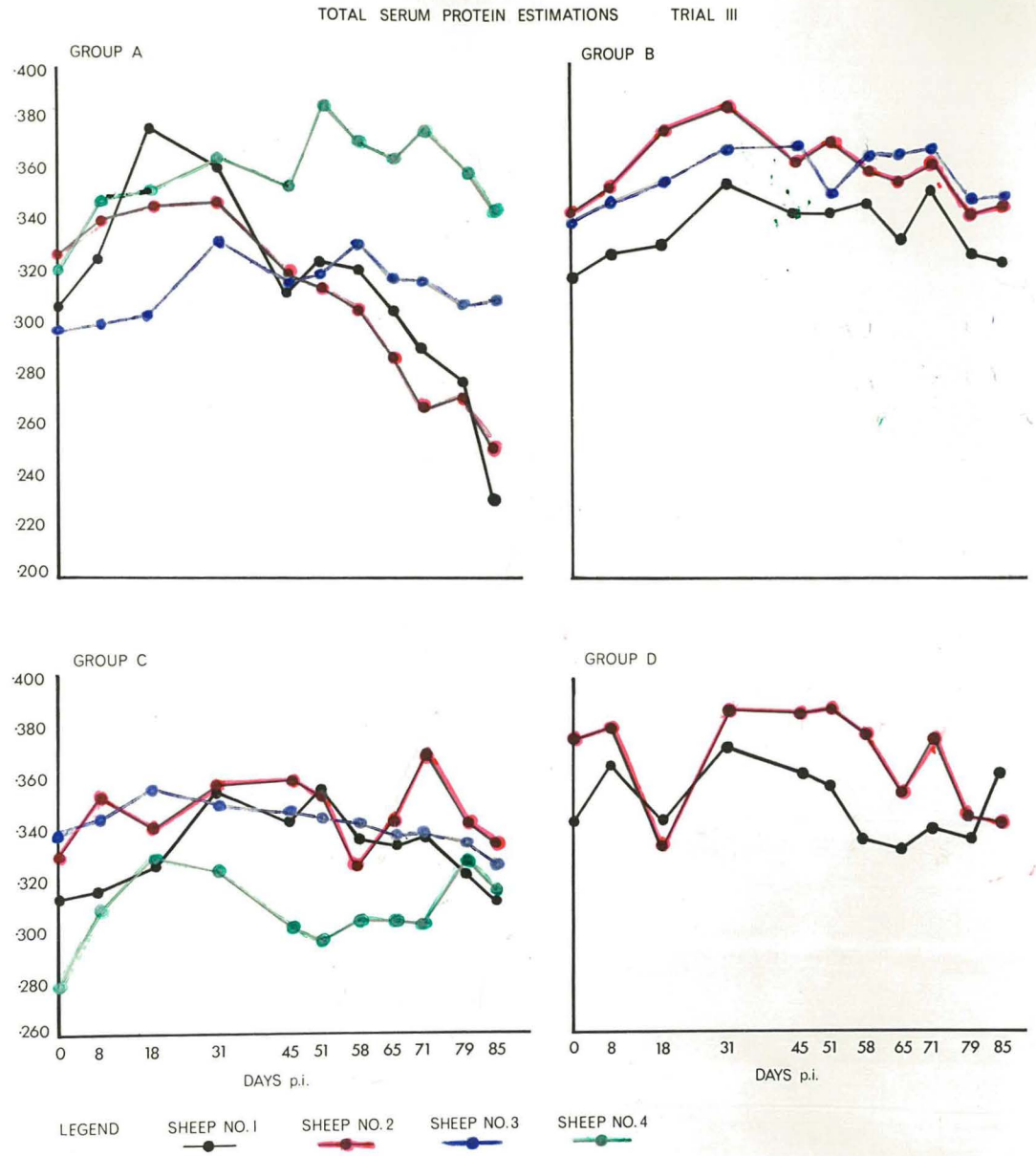


Fig. 29 The estimated total serum protein in samples taken from sheep in Experiment III, Chapter 9.

Scale on ordinate gives spectrophotometer reading.

Table XVII in appendix shows readings converted to total serum protein concentration in gms/100 mls.

NO. OF ADULT B. TRIGONOCEPHALUM RECORDED FROM VARIOUS SECTIONS OF THE SMALL INTESTINE

Section of Intestine	1st Section Pylorus - 5 feet		2nd Section 5 - 20 feet		3rd Section 20 - 35 feet		4th Section 35 feet - ileocaecal valve	
	Male	Female	Male	Female	Male	Female	Male	Female
Sheep No. 1	54	93	137	347	213	199	5	13
2	18	42	140	258	111	154	43	44
3	2	-	467	489	137	205	2	3
4	-	-	461	495	94	130	-	2
5	-	-	139	132	22	35	2	5
6	-	-	105	121	139	159	-	1
7	-	-	138	113	24	33	4	11
8	-	4	76	95	1	-	-	-
9	-	-	14	20	5	4	-	-
10	-	-	32	41	-	1	-	-
11	-	-	30	23	2	2	-	1
12	-	-	5	12	-	-	-	-
13	-	-	9	11	-	-	-	-
14	-	-	9	16	-	-	-	-
15	-	-	2	5	-	-	-	-

Table No. 9.10

Fig. 30 Photograph showing 3 sheep from Group A, Experiment III together with one control sheep from Group C, Experiment III, Chapter 9.

Photograph taken 12 weeks after the administration of infective larvae to Group A.

Sheep Number	wt.of faeces in gms collected in 24 hrs.	Mean egg count/gm.	Total No. of eggs passed in 24 hrs.	Total No. of females recovered	Average No. of eggs per female/24 hrs.
1	552	3,500	1,932,000	498	3,880
2	328.8	4,800	1,578,240	612	2;579
3	446.4	1,033	461,131	44	10,480
4	345.6	983	339,725	95	3,576
5	357.6	266	95,122	16	5,945

Table No. 911.

Fig. 31 Colour photograph of the small intestine showing B. trigonocephalum attached to the mucosa and subsequent petechiae on the surface of the epithelium.

These lesions were most numerous in those areas where the greatest number of nematodes were found (see table 9.10). Many of the lesions were visible through the wall of the unopened intestine.

Distribution of B. trigoncephalum throughout the small intestine.

Table 9.10 shows the number of adult male and female B. trigonocephalum recovered from various sections of the small intestine. The majority of worms were found in the section 5 - 20 feet from the pylorus, but in the more heavily infected animals large numbers were also found in the section 20 - 35 ft caudal to the pylorus.

Experiment IV

The average egg output of the adult female B. trigonocephalum during the 24 hour period of examination was found to range from 2599-10,480. Table 9.11 shows the estimated number of eggs found in the faeces of these sheep during the 24 hours period and is related to the number of adult female B. trigonocephalum recovered from each of five sheep.

Discussion

The distribution of B. trigonocephalum in the small intestine in many ways resembled that of other nematodes found in this area in that the shape of the distribution approximates to a normal frequency curve. However, the location of B. trigonocephalum recorded in this thesis does differ from the findings of Lucker and Neumayer (1946), who had previously reported that the first 10-15 feet and the last 30 feet of the small intestine even in heavily infected lambs were free of worms and lesions. In the present study the majority of worms were recovered from the sections 5-20 feet and 20-35 feet from the pylorus. In a few instances both worms and lesions were recorded within 5 feet of the pylorus and just anterior to the ileocaecal valve.

In most cases in these experiments more female than male worms were recovered. Several workers have studied the female to male ratio (F.M.R.)

in hookworm infections of both the dog and man. Roche and Patrzek (1966) in reviewing the literature invariably found that the F.M.R. recorded was greater than unity. Crofton and Whitlock (1969) in a study on H. contortus report a predominance of female worms in experimental and natural infections of this particular nematode. No reference in the literature though could be found to the F.M.R. of B. trigonocephalum. The departure from a 1:1 ratio recorded in this study appeared to be greater than could be expected by chance and is yet another example of an elevated F.M.R. occurring in nematode infections. Although Roche and Patrzek (1966) found a higher F.M.R. in the anterior portion than in the posterior portion of the small intestine and a significant correlation between increased F.M.R. and duration of infection, the data presented in this thesis is insufficient to confirm these findings. There did however appear to be a trend towards a lower ratio in the posterior portion of the intestine and a higher F.M.R. in those animals harbouring a lower worm burden. Roche and Patrzek (1966) suggest that in the case of the Ancylostomidae the female worms hold on more readily to the upper reaches of the intestine while the males travel downwards and are hence more easily lost. Crofton and Whitlock (1969), on the other hand, in their study of H. contortus have shown that the shorter life span of the male accounts in the main for the general observation that there is normally a preponderance of females in natural infections of this nematode.

Little critical work has been done to estimate the egg output of the sheep hookworm. Crofton (1963) suggests that the egg productivity of B. trigonocephalum is similar to that of H. contortus. Westen (1967 from observations on one infected sheep, records an average output of 1100 eggs per 24 hours per female worm, but this is surprisingly low in view of Crofton's remarks. Soulsby (1965) makes no mention of the egg production of the females of Bunostomum sp., but does imply that the female hookworm of dogs may produce 10,000-30,000 eggs per day. This figure is based on the figure quoted for

the hookworms in man. The results of Experiment IV, in which adult females were estimated to produce between 2579-10,480 eggs per day confirm Crofton's suggestion.

The results of Experiments I - III associated with pathogenicity indicate that numbers of B. trigonocephalum in excess of 200-300 cause significant changes in the haemoglobin level, haematocrit and the total protein concentration of the serum over a comparatively short period of time. The maximum period of observations in these experiments was however only 16 weeks and it is conceivable that a lower worm burden over a longer period may significantly affect the blood picture.

In the literature severe outbreaks of bunostomiasis have been recorded in sheep with infections of B. trigonocephalum which were light compared to some induced in these experiments. Beller (1928) conducted an unconvincing experiment and stated that a hogget given only 100 larvae and from which 42 worms were recovered at necropsy died of bunostomiasis. Similarly Velu and Balozet (1926) regarded bunostomiasis as the cause of death in flocks in which less than 100 worms were recovered at necropsy. Ortlepp (1939) reported that a lamb which died of bunostomiasis harboured 200 B. trigonocephalum. Apparently it was 'very' anaemic and showed degenerative changes of the liver and marked gelatinization of the fatty tissues.

Haberman (1946) reported an outbreak of bunostomiasis in which two sheep were infected with 634 and 982 B. trigonocephalum respectively. His findings are consistent with those of this present study. Lenshin (1950) on the other hand recovered as many as 5,000 - 6,000 B. trigonocephalum from sheep in the Chelyabinsk area of the Soviet Union. The mortality rate in this instance was in the region of 60%-80%.

Although the findings of earlier workers differ markedly from those of Lenshin and from those in this thesis, it is quite feasible that under certain

conditions comparatively light infections may be associated with clinical disease.

It is likely that long standing infections with small numbers of hookworm in sheep induce an iron deficiency due to continuing blood loss. This phenomenon occurs in haemonchosis in sheep and in hookworm infections in man and other animals.

In such cases the degree of anaemia is only indirectly related to the numbers of worms present and the duration of infection assumes greater significance. Other factors may affect the occurrence of disease such as inadequate diet, and concomitant disease due to other pathogens. The effect of diet and the importance of various minerals on parasitism has been studied by many workers. In general the more deficient the diet is in its various components the more serious is the parasitism which arises. Lucker and Neumayer (1947) regarded diet as the cause of the differing severity of hookworm disease induced in experimental lambs equally exposed to infection.

The clinical appearance of affected sheep observed in this study resembled that described by various authors cited previously (Lucker and Neumayer 1946, Lenshin 1950, Soulsby 1965). The outstanding clinical features were progressive anaemia, anorexia retarded growth rate and loss of weight, together with sporadic digestive upsets and intermittent diarrhoea. In both heavy and light infections petechiae were noticed in the small intestine. These marked the site of attachment and were usually more numerous than the number of B. trigonocephalum recovered. The fact that there were more lesions than worms suggests that movement and reattachment may be common.

The haematological findings were similar in many ways to those observed by Lucker and Neumayer (1946) and Westen (1967). No evidence was obtained to indicate that B. trigonocephalum affected the total number of leucocytes or the differential white cell counts except for the eosinophils. Even in this case although there appeared to be an increase in the circulating number of eosinophils

in the more heavily infected sheep the numbers were still within the normal range quoted for sheep (Schalm, 1961). The anaemia in these experiments tended to be microcytic and hypochromic, whereas that recorded by Lucker and Neumayer (1946) in their investigation although hypochromic was normocytic. In this study as in that of Lucker and Neumayer (1946) the degree of anaemia appeared to be directly correlated to the magnitude of the hookworm burden.

In addition to anaemia the heavily infected animals showed a progressive diminution of total serum proteins. From the preliminary studies made this drop seemed to be entirely due to a decrease in the serum albumin, for serum alpha, beta and gamma globulin showed an increase, thus further depressing the albumin/globulin ratio. Mulligan et al (1963) working with Ostertagia ostertagi in calves recorded a hypoalbuminaemia. These workers suggested that it was associated with loss of serum albumin into the gastro-intestinal tract by leakage through the damaged abomasal wall. It is possible that a similar mechanism operates and that the hypoalbuminaemia observed during the course of bunostomiasis is due to albumin seepage into the intestinal lumen via the lesions produced by the worms in the intestinal epithelium. A low rate of albumin synthesis may though be partly responsible for the hypoalbuminaemia and could be associated with:-

(1) Anaemia affecting liver cell function; when the haemoglobin level is low the reserve capacity of the liver to synthesis albumin is lost, (Ball, 1966).

(2) Anorexia - there is little doubt that an inadequate intake of food can contribute to albumin depletion in an anaemic animal, (Ball, 1966

The results of the experiments described confirm the importance of anaemia in disease due to B. trigonocephalum. Diarrhoea does not invariably occur. A marked hypo-albuminaemia accompanied the anaemia. Whilst it seems obvious that the hookworms will cause a post-haemorrhagic anaemia the quantitative aspects of the

blood loss have yet to be studied. Further work is clearly indicated on the cause and extent of the serum protein changes, on the significance of long term infections with small numbers of worms and, in more general terms, on the effect of B. trigonocephalum on weight gains, food intake and related phenomena.

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Table I — Observations and the development of B.trigonocephalum eggs

0 hours - at time of collection			6 hours after culturing commenced			12 hours after culturing commenced		
Stage of Development			Stage of Development			Stage of Development		
6-7 cells			10-13 cells			11-12 cells		
7	E.P.G.		6	E.P.G.		8-9	E.P.G.	
7-8	5,500)		7-9	4,500)		11-12	2,200)	
8	4,600)	Mean: 4,950	9	3,400)	Mean: 3,875	gastrula	3,400)	Mean: 2,975
9-10	5,600)		6	3,500)		9-10	3,200)	
6	5,100)		9-11	3,875)		blastula	3,100)	
8-9			10			11-12		
6-7	SE+		8			8-9		
7			8			8-9		
7			9			11-12		

18 hours after culturing commenced			24 hours after culturing commenced			30 hours after culturing commenced		
early tadpole			12-13 cells			7-8 cells		
6-7 cells	E.P.G.		9-10	E.P.G.		8-9	E.P.G.	
10-11	2,700)		gastrula	4,800)		10-12	2,200)	
11-13	5,000)	Mean: 3,675	9-10	3,000)	Mean: 4,025	8-9	2,300)	Mean: 2,275
9-10	3,400)		9-10	3,200)		blastula	2,600)	
tadpole	3,600)		12-14	5,100)		8-9	2,000)	
11-12			9-10			blastula		
10-11			9-10			10-13		
4			9-10			10-12		
8-9			12-14			8-9		

Table I cont. Observations and the development of B.trigoncephalum eggs

36 hours after culturing commenced			42 hours after culturing commenced			48 hours after culturing commenced		
Stage of Development			Stage of Development			Stage of Development		
8-9 cells			blastula			10-12 cells		
10-12	E.P.G.		9-10 cells	E.P.G.		12-14	E.P.G.	
gastrula	5,100)		tadpole	3,900)		gastrula	3,900)	
gastrula	5,000)	Mean: 4,875	12-13	3,600)	Mean: 3,950	8-9	5,200)	Mean: 4,075
10-12	5,000)		blastula	4,900)		blastula	4,000)	
gastrula	4,400)		11-12	3,400)		9-10	3,200)	
10-12			9-10			11-12		
8-9			7-9			11-12		
gastrula			6-7			blastula		
tadpole			early tadpole			blastula		

54 hours after culturing commenced		78 hours after culturing commenced	
Stage of Development		Stage of Development	
gastrula		early tadpole	
blastula	E.P.G.	tadpole	E.P.G.
gastrula	2,600)	gastrula	1,600)
gastrula	2,800)	gastrula	2,700)
gastrula	2,500)	tadpole	2,800)
10-11 cells	3,400)	10-12 cells	2,700)
gastrula		tadpole	
8-9		gastrula	
gastrula		blastula	
tadpole		tadpole	

Table II — Dimensions: Bunostomum trigonocephalum eggs - measured in Microns

1. Morula Stage

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
86		48	86		51	86		51	86		48	86		48
86		48	86		51	83		50	86		48	96		50
86		51	88		48	83		51	91		48	93		51
83		51	90		51	90		48	90		45	82		51
83		53	93		48	85		51	90		51	93		48
83		48	86		51	91		50	86		54	93		48
85		48	90		50	93		50	91		48	83		54
86		51	91		50	88		48	96		48	90		50
90		50	88		50	90		48	86		48	90		48
83		50	86		53	90		48	90		53	90		45

Range: length 83 - 96; width 45 - 54

Mean: length 88.04 S.E. 0.4988; width 49.6 S.E. 0.2786

2. Tadpole Stage

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
86		45	90		51	83		51	90		48	86		50
86		48	86		50	88		50	86		50	90		48
85		53	88		50	85		51	88		48	88		50
90		53	88		50	90		48	93		46	86		51
86		51	85		50	96		51	86		54	86		54
90		51	93		48	86		51	86		48	83		50
93		45	90		50	93		48	90		48	93		45
91		48	83		51	90		50	86		48	82		56
90		50	83		51	90		46	90		48	90		46
93		45	85		51	82		50	86		48	86		48

Range: length 82 - 96; width 45 - 56

Mean: length 87.9 S.E. 0.4641; width 49.4 S.E. 0.3372

Table III — Measurements: Bunostomum trigoncephalum larvae

Overall length x width (just posterior to end of oesophagus). Measured in Microns

1. 2 days after culturing had commenced: - 1st Stage Larvae.

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
440		22	472		22	464		22	440		22	456		22
416		22	448		22	448		22	480		22	448		22
440		22	432		22	456		22	464		22	456		22
448		22	440		22	480		24	432		22	464		22
456		22	464		22	496		22	440		22	448		22
464		22	464		22	440		22	480		22	480		22
440		22	472		24	440		22	448		22	440		22
456		22	456		22	416		22	464		22	480		27
448		22	464		22	480		24	480		24	416		22
448		22	440		22	448		22	472		22	448		22

Range: length 416 - 496; width 22 - 274

Mean: length 454.2 S.E. 2.5226; width 22.3 S.E. 0.1227

2. 3 days after culturing had commenced: - 1st Stage Larvae.

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
496		24	528		24	552		29	520		24	496		26
528		24	496		26	480		22	512		26	488		24
480		24	488		26	480		24	504		22	512		24
528		26	520		24	528		24	496		24	520		24
480		22	480		26	512		24	520		24	496		24
480		22	496		24	504		22	480		24	520		24
440		22	504		24	512		26	504		24	496		26
520		22	512		24	504		24	512		22	544		24
496		24	520		24	536		22	504		22	496		22
504		26	520		24	528		26	536		22	480		22

Range: length 440 - 552; width 22 - 29

Mean: length 505.8 S.E. 2.8915; width 24.0 S.E. 0.2163

Table III cont. Measurements: Bunostomum trigoncephalum larvae

Overall length x width (just posterior to end of oesophagus). Measured in Microns

3. 4 days after culturing had commenced: - 1st Stage larvae.

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
640		26	648		26	600		26	640		26	640		26
600		27	640		26	604		36	632		35	624		32
640		26	648		26	664		26	648		26	640		26
632		26	520		27	648		26	648		27	632		27
568		24	608		26	624		26	608		27	616		27
640		29	624		27	608		26	640		27	648		27
600		27	680		27	664		26	632		26	584		26
560		26	664		35	632		26	632		29	692		35
648		32	664		35	664		35	680		32	696		35
680		35	616		35	696		32	648		35	616		32

Range: length 560 - 696; width 24 - 36

Mean: length 634.4 S.E. 4.7653; width 28.7 S.E. 0.5185

4. 5 days after culturing had commenced: - 2nd Stage larvae. (ensheathed)

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
648		22	640		26	648		22	664		22	632		22
640		22	656		26	584		22	672		24	624		24
624		24	656		22	656		22	648		22	640		24
656		22	512		22	624		22	640		24	632		26
656		22	616		22	640		24	624		22	632		22
664		26	656		26	608		22	640		22	640		22
648		22	640		22	640		22	640		22	664		22
648		29	640		26	632		22	640		22	640		26
648		26	640		24	672		24	640		22	656		22
536		26	600		26	608		22	656		26	656		22

Range: length 512 - 672; width 22 - 29

Mean: length 636.3 S.E. 4.0806; width 23.3 S.E. 0.2586

Table III cont. Measurements: Bunostomum trigoncephalum larvae

Overall length x width (just posterior to end of oesophagus). Measured in Microns

5. 6 days after culturing had commenced: - 2nd Stage larvae. (ensheathed)

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
680		26	672		24	656		26	640		24	600		22
640		24	656		24	664		22	640		24	600		22
664		22	640		22	624		26	688		24	640		24
680		26	664		22	664		22	680		26	656		26
664		26	680		24	648		24	624		24	680		24
680		24	640		24	664		24	632		26	640		26
648		22	648		26	640		24	656		24	600		24
664		24	672		26	640		26	640		24	648		24
656		24	640		26	624		22	640		27	672		22
640		22	680		22	680		22	664		22	672		26

Range: length 600 - 688; width 22 - 27

Mean: length 652.5 S.E. 3.0456; width 24.1 S.E. 0.2198

6. 7 days after culturing had commenced: - 3rd Stage larvae. (ensheathed)

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
640		24	688		24	640		24	646		24	632		24
648		24	656		27	696		24	656		24	640		22
640		24	640		24	648		24	600		24	656		22
656		22	688		24	608		22	680		24	608		24
616		24	592		24	696		26	664		22	656		22
664		24	640		26	640		26	640		22	648		22
680		24	640		22	640		24	664		22	640		24
624		26	648		24	656		24	648		26	648		24
664		24	656		24	656		24	600		22	680		24
648		24	664		24	696		24	640		25	648		22

Range: length 592 - 696; width 22 - 27

Mean: length 649.2 S.E. 3.3747; width 23.8 S.E. 0.1743

Table III cont. Measurements: Bunostomum trigoncephalum larvae

Overall length x width (just posterior to end of oesophagus). Measured in Microns

7. 8 days after culturing had commenced: - 3rd Stage larvae (ensheathed)

length	x	width	length	x	width	length	x	width	length	x	width	length	x	width
640		24	648		24	600		24	660		24	672		24
632		22	648		24	640		22	632		22	648		26
680		24	616		24	632		24	672		26	648		24
632		24	632		22	640		26	624		22	632		22
648		22	648		22	632		22	648		24	640		22
664		22	584		22	648		22	648		24	648		22
668		24	648		22	656		27	688		26	632		26
680		26	656		22	592		24	640		24	640		24
640		24	624		24	640		26	632		24	656		24
664		26	664		26	608		26	624		24	640		22

Range: length 584 - 688; width 22 - 27

Mean: length 642.6 S.E. 2.9389; width 23.8 S.E. 0.2141

Table IV

3rd Stage Larvae (ensheathed)

Measurements (in microns) on distance between :

Anterior End & Excretory pore	Anterior End & Nerve Ring	Anterior End & Post.end of oesophagus	Anus & Tip of tail	Anus & Tip of sheath	Tip of tail & Tip of sheath
112	96	173	74	170	96
112	93	176	67	166	99
112	90	173	70	147	77
102	90	189	67	176	109
112	93	176	70	157	87
112	86	170	77	150	73
102	93	179	64	173	109
106	96	192	70	157	87
112	93	179	67	166	99
109	90	166	61	160	99
102	93	163	64	166	102
109	93	163	67	157	90
96	86	182	67	179	112
99	83	179	77	144	67
109	86	173	64	176	112
99	93	173	70	160	90
102	96	173	70	176	106
112	93	170	70	182	112
112	93	179	67	163	96
106	86	176	64	176	112
Total 2137	1822	3504	1367	3301	1934
Mean: 106.8	91.1	175.2	68.3	165.0	96.7
Range: 96-112	83-96	163-192	61-77	144-182	67-112
S.E. 1.1859	0.8334	1.6118	0.9200	2.3963	2.9497

Table V Length of Adult Male B. trigonocephalum - measured in mm.

16	16	16	17	16
17	16	15	16	16
16	16	16	15	15
16	16	16	16	16
16	15	15	16	16
16	16	16	16	16
16	17	17	16	16
15	16	15	15	14
15	15	15	15	15
13	15	14	17	16
15	16	16	13	15
15	16	16	15	15
15	16	15	15	16
17	16	14	15	16
12	16	16	15	14
15	15	14	16	17
15	16	12	15	15
16	15	17	16	13
15	16	15	15	16
15	14	16	16	15

Range: 12 - 17

Mean: 15.44 S.E. 0.0973

Table VI Length of Adult Female B. trigonocephalum - measured in mm.

23	19	21	21	22
22	22	24	23	24
20	22	17	22	18
22	18	19	23	23
22	21	24	17	25
24	20	21	23	20
23	21	20	22	24
20	22	20	21	20
18	17	22	18	19
22	20	22	21	17
21	22	21	18	18
18	21	22	21	22
21	22	21	20	21
22	21	22	21	20
21	22	20	21	21
21	21	19	20	19
22	18	21	20	22
22	20	21	20	20
21	21	21	21	19
21	22	21	21	20

Range: 17 - 25

Mean: 20.85 S.E. 0.1682

Table VII Temp.Variation Recorded in Temp.Controlled Animal House

		Min.Temp.	Max.Temp.	Variation
Week	1	16.5°C	20°C	3.5°C
	2	16.5°C	21°C	4.5°C
	3	16.5°C	19.5°C	3°C
	4	16.5°C	21°C	4.5°C
	5	16.5°C	20.5°C	3.5°C
	6	16.5°C	21°C	4.5°C
	7	17°C	21.5°C	4.5°C
	8	18°C	22°C	4°C
	9	20°C	21.5°C	1°C
	10	18°C	21.5°C	3°C
	11	20°C	22°C	2°C
	12	18.8°C	21°C	2.2°C

Table VIII Expt.V - effect of Saline on the exsheathment of B. trigonocephalum.

Saline Conc.	% exsheath. at 5 mins.	% exsheath. at 15 mins.	% exsheath. at 30 mins.	% exsheath. at 45 mins.	% exsheath. at 60 mins.
5%	7	12	9	13	26
2%	9	7	16	18	11
1%	7	11	13	7	8
.5%	11	6	8	5	7
.25%	13	7	12	10	4
Dist.H_2O Control	12	11	12	13	9
Replicate					
5%	0	4	3	3	12
2%	3	7	7	4	7
1%	4	3	3	6	2
.5%	2	8	4	12	8
.25%	6	3	8	5	6
Dist.H_2O Control	7	11	14	29 *	36 *

* The percentage of exsheathment recorded was unusually high. This particular tube may have been contaminated with a small quantity of Milton.

HAEMOGLOBIN LEVEL in gms.per 100 ml.blood

Table No. IX — Trial II

DAY p.i.	0	7	21	35	57	70	86	91	100	106	112
GROUP A											
Sheep No.											
1	12.9	13.0	12.4	11.75	10.9	9.3	9.25	9.1	8.0	8.4	7.6
2	12.0	12.75	12.2	10.9	9.4	8.4	7.75	8.8	7.8	8.3	7.2
3	11.75	12.1	11.9	11.75	9.9	8.9	9.0	9.4	9.1	10.1	9.0
4	12.6	12.4	11.6	11.2	9.75	7.6	8.0	7.4	8.4	9.1	8.6
GROUP B											
Sheep No.											
1	11.6	11.75	11.25	11.1	10.5	9.9	10.3	9.9	9.6	9.8	9.75
2	11.9	11.1	11.3	12.1	11.9	10.9	10.75	10.8	10.5	10.8	10.9
3	11.4	11.6	11.6	10.9	11.6	9.9	10.1	10.0	9.4	9.8	9.6
4	10.75	11.75	12.0	11.5	12.0	10.9	10.8	10.9	10.9	10.4	11.25
GROUP C											
Sheep No.											
1	11.4	11.4	11.5	11.25	12.0	10.0	11.25	10.3	10.0	11.1	11.0
2	12.1	12.0	12.1	11.75	12.75	10.8	11.9	11.3	11.1	11.75	11.2
DAY p.i.	0	7	21	35	57	70	86	91	100	106	112

PACKED CELL VOLUME %

Table No. X — Trial II

DAY p.i.	0	7	21	35	57	70	86	91	100	106	112
GROUP A											
Sheep No.											
1	40	39	38	34	32	27	26	25	22	22	22
2	37	37	36	31	28	23	23	25	23	24	21
3	35	37	35	32	29	27	27	28	26	29	28
4	39	37	36	32	27	24	23	24	23	25	25
GROUP B											
Sheep No.											
1	36	33	36	31	32	30	31	30	29	29	28
2	40	34	36	34	35	33	34	32	32	34	33
3	33	32	36	30	32	28	29	30	27	28	27
4	35	35	36	33	37	34	34	34	35	33	36
GROUP C											
Sheep No.											
1	35	35	35	31	34	32	34	31	31	33	34
2	37	37	37	34	36	33	36	33	34	35	32
DAY p.i.	0	7	21	35	57	70	86	91	100	106	112

PROTEIN ESTIMATIONS

Table No. XI (Spectrophotometric readings) Trial II

DAY p.i.	0	7	21	35	57	70	86	91	100	106	112
GROUP A											
Sheep No.											
1	.379	.354	.365	.335	.317	.277	.285	.274	.269	.267	.267
2	.384	.357	.360	.342	.301	.300	.277	.279	.277	.285	.304
3	.381	.361	.382	.355	.289	.276	.273	.281	.288	.297	.280
4	.355	.358	.340	.355	.266	.251	.241	.252	.248	.257	.269
GROUP B											
Sheep No.											
1	.409	.348	.399	.369	.390	.371	.399	.363	.380	.336	.363
2	.369	.351	.372	.359	.380	.359	.362	.357	.347	.367	.350
3	.383	.345	.368	.379	.373	.352	.357	.400	.341	.361	.359
4	.403	.376	.439	.397	.410	.386	.381	.377	.423	.376	.405
GROUP C											
Sheep No.											
1	.388	.358	.386	.370	.361	.365	.382	.364	.349	.330	.384
2	.384	.365	.371	.341	.364	.359	.361	.364	.379	.366	.360
DAY p.i.	0	7	21	35	57	70	86	91	100	106	112

TOTAL SERUM PROTEIN MEASURED IN GMS/100 MLS

Table No. XII — Trial II

DAY p.i.	0	7	21	35	57	70	86	91	100	106	112
GROUP A											
Sheep No.											
1	6.35	5.90	6.10	5.60	5.30	4.65	4.75	4.60	4.50	4.45	4.45
2	6.40	5.95	6.00	5.70	5.00	5.00	4.65	4.65	4.65	4.75	5.05
3	6.35	6.05	6.35	5.90	4.85	4.65	4.55	4.70	4.85	5.00	4.65
4	5.90	5.95	5.65	5.90	4.45	4.20	4.05	4.20	4.15	4.30	4.50
GROUP B											
Sheep No.											
1	6.85	5.85	6.65	6.20	6.55	6.20	6.65	6.10	6.35	5.65	6.05
2	6.20	5.90	6.25	6.00	6.35	6.00	6.05	5.95	5.80	6.15	5.85
3	6.40	5.80	6.20	6.35	6.25	5.85	5.95	6.70	5.70	6.05	6.00
4	6.75	6.30	7.35	6.60	6.90	6.45	6.35	6.30	7.10	6.30	6.80
GROUP C											
Sheep No.											
1	6.50	5.95	6.45	6.20	6.05	6.10	6.40	6.10	5.85	5.50	6.40
2	6.40	6.15	6.20	5.70	6.10	6.00	6.05	6.10	6.35	6.15	6.00

DAY p.i.	0	7	21	35	57	70	96	91	100	106	112

TOTAL NUMBER OF CIRCULATING EOSINOPHILS PER CMM OF BLOOD

Table No. XIII — Trial II

DAY p.i.	0	7	21	35	57	70	86	91	100	106	112
GROUP A											
Sheep No.											
1	720	560	1414	477	387	1350	1272	848	124	56	524
2	924	296	860	300	528	504	756	616	431	513	210
3	225	348	65	130	294	1018	754	312	318	978	968
4	180	94	67	142	73	340	360	588	140	73	511
GROUP B											
Sheep No.											
1	415	246	305	769	-	530	86	59	67	-	237
2	309	1075	67	285	824	1520	784	510	78	540	1962
3	428	348	1776	150	1440	1581	450	-	609	261	280
4	-	85	-	-	-	210	-	-	-	-	-
GROUP C											
Sheep No.											
1	116	60	76	80	-	410	-	-	153	49	325
2	390	58	70	198	180	360	56	-	-	62	280
DAY p.i.	0	7	21	35	57	70	86	91	100	106	112

HAEMOGLOBIN LEVEL gms/100 ml.blood

Table No. XIV — Trial III

DAY p.i.	0	8	18	31	45	51	58	65	71	79	85
GROUP A											
Sheep No.											
1	11.4	12.0	12.2	13.5	10.8	9.8	9.75	9.4	9.4	8.2	7.6
2	13.4	11.9	11.8	14.0	10.7	7.8	8.2	7.6	5.4	5.00	4.2
3	10.8	10.7	9.8	12.0	10.4	9.1	9.6	9.3	9.2	8.8	9.00
4	11.4	10.9	10.6	10.7	11.5		10.8	10.6	10.4	9.6	9.8
GROUP B											
Sheep No.											
1	11.8	11.5	10.6	12.00	12.00	10.4	11.3	11.0	11.2	11.9	11.4
2	12.8	11.9	11.4	13.0	12.6	12.2	12.6	12.9	12.0	11.4	12.2
3	11.8	10.8	10.9	12.0	12.4	9.9	11.4	11.0	10.2	10.3	10.1
GROUP C											
Sheep No.											
1	12.4	11.3	10.9	14.0	12.7	12.6	12.4	12.8	11.8	11.4	11.6
2	13.4	11.6	12.0	13.0	13.0	11.6	12.6	13.1	13.5	12.4	12.2
3	11.4	11.0	10.3	12.0	11.8	10.6	11.2	11.1	11.4	11.4	11.6
4	10.4	10.8	10.0	12.0	11.0	10.1	10.6	11.6	10.5	10.7	11.0
GROUP D											
Sheep No.											
1	9.5	9.5	10.2	13.0	12.0	10.1	11.5	11.6	11.1	12.2	12.4
2	12.4	11.4	10.5	12.0	13.1	10.8	11.00	11.2	10.8	10.6	10.9
DAY p.i.	0	8	18	31	45	51	58	65	71	79	85

PACKED CELL VOLUME %

Table No. XV

Trial III

DAY p.i.	0	8	18	31	45	51	58	65	71	79	85
GROUP A											
Sheep No.											
1	36	36	36	40	34	34	34	30	28	26	24
2	37	37	36	40	30	29	29	25	20	17	14
3	32	33	30	34	30	31	31	29	28	27	28
4	36	33	31	30	30	35	36	34	35	31	32
GROUP B											
Sheep No.											
1	36	36	34	35	35	35	37	37	36	35	34
2	41	38	38	39	38	41	42	41	40	40	40
3	38	35	35	37	35	34	35	36	33	32	31
GROUP C											
Sheep No.											
1	38	35	34	40	42	41	41	40	39	35	37
2	40	37	38	41	41	39	40	44	43	38	37
3	36	33	33	36	35	35	36	39	37	36	36
4	30	32	32	35	31	34	34	35	33	32	32
GROUP D											
Sheep No.											
1	36	35	37	40	39	38	37	38	37	36	38
2	29	29	33	37	33	35	35	35	35	32	32
DAY p.i.	0	8	18	31	45	51	58	65	71	79	85

PROTEIN ESTIMATIONS

Table No. XVI (Spectrophotometric readings) Trial III

DAY p.i.	0	8	18	31	45	51	58	65	71	79	85
GROUP A											
Sheep No.											
1	.305	.324	.379	.363	.312	.321	.319	.304	.289	.277	.231
2	.324	.340	.344	.348	.317	.314	.303	.286	.264	.271	.251
3	.295	.299	.303	.333	.316	.320	.333	.319	.319	.306	.308
4	.322	.349	.351	.363	.354	.386	.373	.365	.377	.361	.343
GROUP B											
Sheep No.											
1	.319	.327	.329	.356	.341	.342	.349	.330	.353	.328	.326
2	.342	.355	.379	.384	.362	.374	.361	.357	.362	.342	.345
3	.342	.349	.357	.370	.372	.351	.365	.365	.368	.345	.345
GROUP C											
Sheep No.											
1	.313	.317	.324	.353	.343	.357	.337	.335	.338	.325	.310
2	.330	.353	.340	.355	.360	.356	.324	.344	.369	.342	.336
3	.338	.344	.358	.356	.346	.345	.341	.339		.338	.327
4	.278	.309	.325	.322	.301	.299	.306	.307	.307	.330	.315
GROUP D											
Sheep No.											
1	.340	.364	.340	.373	.362	.358	.336	.331	.340	.336	.361
2	.375	.380	.330	.387	.385	.388	.379	.355	.378	.344	.343
DAY p.i.	0	8	18	31	45	51	58	65	71	79	85

TOTAL SERUM PROTEIN MEASURED IN GMS/100 MLS

Table No. XVII — Trial III

DAY p.i.	0	8	18	31	45	51	58	65	71	79	85
GROUP A											
Sheep No.											
1	5.10	5.40	6.30	6.05	5.20	5.35	5.30	5.10	4.85	4.65	3.85
2	5.40	5.65	5.75	5.80	5.30	5.25	5.05	4.80	4.40	4.55	4.20
3	4.95	5.00	5.05	5.55	5.30	5.35	5.55	5.35	5.35	5.10	5.15
4	5.40	5.80	5.85	6.05	5.90	6.45	6.25	6.10	6.30	6.00	5.75
GROUP B											
Sheep No.											
1	5.35	5.45	5.50	5.95	5.70	5.70	5.80	5.50	5.90	5.50	5.45
2	5.70	5.90	6.30	6.40	6.05	6.25	6.05	5.95	6.05	5.70	5.75
3	5.70	5.85	5.95	6.20	6.20	5.85	6.10	6.10	6.15	5.75	5.75
GROUP C											
Sheep No.											
1	5.25	5.30	5.40	5.90	5.70	5.95	5.65	5.60	5.65	5.45	5.20
2	5.50	5.90	5.65	5.90	6.00	5.95	5.40	5.75	6.15	5.70	5.60
3	5.65	5.75	5.95	5.95	5.80	5.75	5.70	5.65	-	5.65	5.45
4	4.65	5.20	5.40	5.35	5.05	5.00	5.10	5.15	5.15	5.50	5.25
GROUP D											
Sheep No.											
1	5.65	6.10	5.65	6.20	6.05	5.95	5.60	5.50	5.65	5.60	6.00
2	6.25	6.35	5.70	6.45	6.45	6.50	6.35	5.95	5.35	5.75	5.70

DAY p.i.	0	8	18	31	45	51	58	65	71	79	85

EGGS PER GRAM

Table No. XVIII — Trial II

DAY p.i.	61	65	70	72	76	79	82	86	91	97	98	100	103	106	109	112
GROUP A																
1	-	500	600	600	1800	2000	2100	NS	2800	3100	3000	5000	2400	4400	2900	4400
2	400	800	1400	1100	1000	1800	2400	2500	4400	4800	5400	6100	1000	2600	2600	6600
3	100	200	200	200	700	900	900	600	1000	800	800	900	900	400	500	800
4	-	-	400	300	500	100	500	700	1100	1300	1100	1900	1000	800	1100	900
GROUP B																
1	200	-	100	100	200	400	400	400	300	200	400	800	700	1100	1000	900
2	-	-	100	-	-	100	400	-	-	-	100	200	100	200	-	100
3	100	-	-	-	200	100	-	100	200	200	100	-	100	200	300	700
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GROUP C																
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DAY p.i.	61	65	70	72	76	79	82	86	91	97	98	100	103	106	109	112