A STUDY OF THE DEVELOPMENT AND HOST-PARASITE RELATIONS OF A NEMATODE, TRICHOSTRONGYLUS RETORTAEFORMIS (ZEDER)

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CONTENTS

SUMMARY

1. The development of Trichostrongylus retortaeformis in rabbits was studied. Third-stage larvae of this species do not start to grow in length until they have been in the host for at least 24 hours. The third moult occurs about 60 hours after ingestion and the fourth moult about 70 hours later. Both these moults are preceded by a lethargus, during which growth ceases; they are followed immediately by a period of rapid growth.

2. There were no apparent morphological changes in larvae during the first 24 hours in the host. After this, renette cells developed and the number of nuclei in the intestine increased. No cell division was apparent in the genital primordium, but the germinal and epithelial cells were rearranged.

3. Fourth-stage larvae grow considerably. Cells in the genital primordium divide and differentiate and, when the final moult occurs, the regions of the adult reproductive system are recognisable.

4. Larvae at different stages of development were incubated with serum conjugated with a fluorochrome. It was expected that larvae would ingest the conjugated serum if they had been feeding in the host. The results indicate that third-stage larvae start to feed after they have been in the host for about 14 hours, but do not feed during the lethargus when the mouthparts of the fourth-stage larvae are being formed; similarly, fourth-stage larvae feed continuously until they enter the lethargus before the final moult.

5. The osmotic pressure of fluid from the stomach, duodenum and remaining small intestine (jejunum and ileum) of rabbits was measured. The rabbits were not fasted. The total osmotic pressure in all three regions was about 300 milliosmols. The osmotic pressure due to electrolytes was approximately 230 milliosmols in the duodenum and small intestine.

6. The concentrations of sodium and potassium in the duodenum and the remainder of the small intestine did not differ significantly. The concentrations of these ions in the stomach was significantly lower, fluid from the stomach contained 26.2 $\pm$ 11.0 mmol/l of sodium and 18.2 $\pm$ 7.2 mmol/l of potassium whereas fluid from the duodenum contained 88.8 $\pm$ 15.7 mmol/l of sodium and 32.5 $\pm$ 6.4 mmol/l of potassium.

7. The process of exsheathment of infective larvae was studied. It seems that the host provides a stimulus for exsheathment and the larvae release the substance which attacks the sheath. Larvae were stimulated to exsheath *in vitro* if they were incubated at 37-40^{o}C in a medium which contained concentrations of dissolved gaseous carbon dioxide or undissociated carbonic acid greater than 6 x 10^{-3}M at pH 5 or less. Although high concentrations of undissociated carbonic acid were necessary to stimulate larvae, they inhibited the actual casting of the sheath. A similar stimulus probably induces exsheathment in the stomach of rabbits.

This thesis contains no material previously submitted by me for a degree in any University. I believe that it contains no material written or published by other people except when due reference is made in the text of the thesis.

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I. INTRODUCTION

1.1 The scope of the study

Trichostrongylus retortaeformis is a parasite of the alimentary canal of rabbits. The development and ecology of free-living stages in its life cycle have been studied, but virtually nothing is known about the parasitic stages. This is perhaps surprising, for its host can be kept easily in a laboratory and, although Trichostrongylus retortaeformis itself is not of economic importance, it is closely related to species which are parasites of sheep and cattle.

The life cycle of nematodes has six stages : the egg, four larval stages and the adult. A moult terminates the development of each larval stage. Trichostrongylus retortaeformis infects the host as a third-stage larva. As in other trichostrongyles, the second moult is incomplete; a new cuticle forms and the old cuticle parts from it, but is not cast until after the larva has been ingested by the host. The outer cuticle is termed the sheath and growth, feeding and development are suspended in the ensheathed larva.

Infective larvae of several trichostrongles are stimulated to exsheath by the environment in the gut of their host (Rogers, 1960; Rogers and Sommerville, 1960). The process of exsheathment in Trichostrongylus retortaeformis was studied to find out whether it was induced similarly by conditions in the gut of rabbits.

I could find no literature on the development of Trichostrongylus retortaeformis in the host and studied this for three reasons: firstly to find out when the third and fourth moults occur; secondly, to look for indications that the stimulus which induces exsheathment also stimulates the resumption of development, and thirdly, to find morphological changes which take place within a few hours so that, in future experiments in vitro, the influence of a particular treatment could be assessed by its influence on development. The influence of components of the environment on parasites is usually assessed by measuring the time the parasites survive in different media. This method has obvious limitations: the parasites usually survive for at least 2 days and the medium they are in probably alters considerably within this time; some substances may be removed from it by the parasites and others, which they excrete or which result from autolysis, may accumulate. Consequently a number of factors may cause the death of the parasites. Hobson (1948) has reviewed the literature on survival of parasitic nematodes in artificial media. It would be preferable to assess the influence of the environment on parasitic larvae more quickly by observing its influence on the rate of development.

The environment of a gut parasite is complex and many of its features may determine whether it is favourable for a parasite. Read (1950) has reviewed the available information on aspects of the physiology of the vertebrate small intestine which may be important for parasites.

The activity of the environment which stimulates exsheathment of several trichostrongyles depends on its physico-chemical properties. These properties of the environment may also influence other stages in development of nematodes; for example, the development *in vitro* of parasitic stages of *Haemonchus contortus* and *Trichinella spiralis* is influenced by the temperature, hydrogen ion concentration and partial pressure of carbon dioxide in the gas phase of the medium (Sommerville, 1964, 1966; Berntzen, 1965).

To obtain information about physico-chemical characteristics of the environment of *Trichostrongylus retortaeformis*, the osmotic pressure of gut contents of rabbits was measured. Read and Simmons (1963) have pointed out that parasites may be permeable to some constituents of the medium which surrounds them. These substances would contribute to the total osmotic pressure of the medium, but would not exert an osmotic pressure across the body wall of the parasite. *Trichostrongylus retortaeformis* is probably more permeable to ions of electrolytes than to substances of high molecular weight; an attempt was therefore made to differentiate between osmotic pressure due to electrolytes and that due to non-electrolytes.

The amount of sodium and potassium in the gut contents was also measured. Sodium is probably the anion present in the greatest concentration in gut contents and there is evidence that potassium stimulates the metabolic rate of *Eustrongylides ignotus* (von Brand, 1943), is necessary for development of third-stage larvae of *Haemonchus contortus* (Sommerville, personal communication) and aids the survival

of Ascaris lumbricoides if the medium is gassed with a mixture containing 5% carbon dioxide and 95% nitrogen (Harpur, 1963).

1.2 Information from the literature which formed a basis for the study

No intermediate host is involved in the life cycle of Trichostrongylus retortaeformis; eggs pass to the ground in the faeces of the host where they hatch and develop to the infective stage.

The influence of climate on the hatching of Trichostrongylus retortaeformis has been studied by Crofton (1948b), Prasad (1959) and Gupta (1961). The rate of hatching is influenced by temperature. Eggs hatch at temperatures between 5°C and 30°C; higher temperatures are lethal. Increase in temperature decreases the time taken for eggs to hatch and at optimum temperatures between 25°C and 30°C, eggs hatch within a day. Crofton found that eggs passed by the host in autumn do not hatch during winter but can survive and hatch in spring; those passed during very cold periods die.

Eggs are resistant to desiccation but unless they are in a moist environment few hatch.

Wilson (1958) studied the process of hatching of Trichostrongylus retortaeformis. He found that immediately before hatching the permeability of an egg to water increases. He inferred that the fluid in the egg contains an emulsifying agent which attacks the inner lipid membranes of the egg shell when the larva moves actively and agitates the fluid. When the lipid membrane ceases to be impermeable to water, the difference in osmotic pressure between the external medium and the larval fluids causes the larva to take up water and exert pressure

on the outer, protein layer of the egg shell. Wilson thought that the outer layer was also weakened chemically but did not suggest how this was accomplished.

In solutions of electrolytes, eggs remained impermeable to water and hatching was delayed. Wilson concluded that ions delay the breakdown of the lipid membrane and pointed out that this would increase the chance of survival of emerging larvae. Larvae will be more resistant to desiccation when they are surrounded by the impermeable lipid membrane of the egg shell. If faeces containing eggs lose water, the concentration of any electrolytes present in the faecal fluid will increase and inhibit hatching. When the faeces are moistened by rain or dew, the concentration of ions in the faecal fluids will decrease and eggs will hatch in conditions which favour the survival of emerging larvae.

Wilson suggested that in the absence of ions the rate of hatching is controlled by the rate at which the outer, protein layer of the shell is weakened.

The rate at which infective larvae develop increases with temperature up to 30°C. At optimum temperatures between 20°C and 30°C, larvae undergo the first moult 23-41 hours after they emerge from the egg (Gupta, 1961) and are ensheathed, infective larvae after 3-4 days (Prasad, 1959, Gupta, 1961).

Infective stage larvae are resistant to desiccation and when the relative humidity is less than 66%, they may survive for up to 7 weeks. This is not true of younger larvae (Crofton, 1948b; Prasad, 1959).

Infective larvae will be ingested by a host only if they are on herbage. Crofton (1954) has shown that larvae of Trichostrongylus retortaeformis move randomly in vertical and horizontal planes and their distribution on herbage is influenced by humidity, temperature and light intensity (Crofton, 1948a). Larvae tend to congregate in areas near the base of blades of grass, where climatic change is least.

Bull (1964) found this parasite in all the populations of rabbits he examined in New Zealand, even those from small islands. Between 72 and 98% of full grown rabbits were infected. Similarly, Mykytowycz (1956) found it in 79% of the rabbits he examined from 29 localities in Australia which had a moderate rainfall. It was not present, or present only in small numbers, in rabbits from semi-arid or subtropical regions in Australia. Probably the environment in these regions was unfavourable for the development of infective larvae (Dunsmore, 1966).

A smaller percentage of the rabbits examined by Evans (1940) in Wales were infected. These rabbits were also infected with a tapeworm of the genus Clittotaenia which does not occur in Australia or New Zealand. Bull (1964) has suggested that rabbits which are infected with Clittotaenia may be less susceptible to Trichostrongylus retortaeformis. There is some evidence that female rabbits are very susceptible to infection with Trichostrongylus retortaeformis during the breeding season but that during the rest of the year they are less susceptible than male rabbits (Bull, 1964; Dunsmore, 1966).

Michel (1952a, 1952b, 1953) described three mechanisms which limit the size of an infection in a rabbit.

i. Self cure: this is the sudden elimination from the host of all adult worms. Sometimes this is evoked if an uninfected rabbit is given a single, large does of infective larvae. It also occurs when a rabbit with a light infection is given a large number of infective larvae. The duration of the infection bears an inverse relationship to the number of worms in the rabbit; adults may be expelled after a few weeks or they may remain in the duodenum for several months.

ii. Inhibition of larval development: when a large number of infective larvae are given to an infected rabbit, many larvae burrow into the intestinal mucosa and remain dormant. They resume development when the resistance of the host declines. These dormant larvae are not affected by the self-cure mechanism.

iii. Protection: if rabbits are repeatedly infected, they become resistant and infective larvae which are ingested are unable to establish themselves in the intestine.

Although the principal hosts of Trichostrongylus retortaeformis are rabbits and hares (Oryctolagus cuniculus, Lepus europaeus and Lepus timidus), it has also been found in rodents of the genera Sciurus, (Oldham, 1961) Viscaccia and Galea (Skrjabin, Shikhobalova and Schulz, 1954). There are reports that it has been found in sheep, cattle and goats, but the parasite observed was probably Trichostrongylus cohibriformis (Skrjabin, Shikhobalova and Schulz, 1954).

Although the development of Trichostrongylus retortaeformis in the host has not been studied, that of several other species of the Trichostrongylidae has been described (Theiler and Robertson, 1915; Veglia, 1915, 1924; Mönnig 1926; Alicata, 1935; Andrews, 1939; Sommerville, 1960; Haley, 1962). Development in all species studied seems to follow the same pattern. When infective larvae are ingested by the host, the sheath is cast, but no obvious changes occur in their morphology during the first 24 hours in the host. After this, larvae grow and the number of nuclei in the intestine increases. Two very large cells, the renette cells, start to develop and lengthen so that they lie ventral to the anterior half of the intestine. These cells have been called ventral or cervical glands, but the term "renette cell" is preferable as their function is unknown. Little, if any, cell division occurs in the genital primordium of third-stage larvae, but its cells are rearranged. Third-stage larvae of Haemonchus contortus are exceptional for they do not grow and the number of nuclei in the intestine does not increase (Veglia, 1915).

Fourth-stage larvae grow considerably. Many cells in the genital primordium divide and differentiate so that immediately before the final moult the reproductive system has its adult form.

Larvae stop growing and are quiescent for a few hours before both the third and fourth moults. This period of inactivity is termed a lethargus.

It seems that the environment in the duodenum is more favourable for Trichostrongylus retortaeformis than that in other regions of the gut; Bull (1953) found that 98% of the worms in a rabbit occurred in this region, although some worms were found in the remainder of the small intestine and, occasionally, in the stomach. The environment in the stomach is probably also important for the parasite; Sommerville (1957) found that infective larvae of all the trichostrongyles he studied were stimulated to exsheath in the region of the alimentary canal immediately anterior to that which the adults inhabit. This suggests that Trichostrongylus retortaeformis may be stimulated to exsheath in the stomach of rabbits. Contents of the stomach and duodenum were therefore collected separately and examined. The contents of the remainder of the small intestine were also examined to see if they differed markedly from those of the duodenum.

Some information about the physico-chemical characteristics of the stomach and small intestine of rabbits has been published. The hydrogen ion concentration of fluid from these regions has been measured (Redman, Willimott and Wokes, 1927; Beauville and Raynaud, 1963; Griffiths and Davies, 1963) and Campbell (1933) has measured the tensions of oxygen and carbon diocide in the mucosa. The body temperature of rabbits has also been recorded (Kanitz, 1925).

The process of exsheathment has been studied in a number of trichostrongyles which inhabit the gut. The earliest work was mainly descriptive and larvae were exsheathed in a variety of unphysiological media (Lapage, 1935a, 1935b; Looss, 1911). Attempts to exsheath larvae

in natural or artificial digestive juices were not very successful (Goodey, 1922; Lapage, 1935c). Crofton (1947) induced infective larvae of Trichostrongylus retortaeformis to exsheath in solutions containing hydrochloric acid and pepsin but, although 90% of the larvae exsheathed, the process took 24-60 hours; it seems unlikely that the process is as slow as this in the host.

Recently, more has been learnt about the physiology of exsheathment. It seems that the host stimulates larvae to secrete a substance which attacks the sheath. Infective larvae of several species exsheath if they are incubated in fluid from the gut of the host (Poynter, 1954; Sommerville, 1954, 1957; Whitlock, Taylor and Conway, 1959). Larvae exsheath even if they are enclosed in a dialysis sac when they are incubated in the fluid. This indicates that the host does not induce exsheathment by providing an enzyme, or other substance of high molecular weight, which acts directly on the sheath; it seems rather that the physico-chemical conditions in the gut act as a stimulus for exsheathment. Studies of exsheathment in vitro indicate that the main components of the stimulus are carbon dioxide or its derivatives, the oxidation-reduction potential, hydrogen ions, temperature and salts (Rogers, 1960; Rogers and Sommerville, 1960; Taylor and Whitlock, 1960). It was thought that Trichostrongylus retortaeformis would respond similarly to a stimulus from the host and the nature of the stimulus was investigated.

Parasitologists usually refer to immature nematodes which have not undergone the final moult as larvae. I have used this term although, as Hyman (1951) points out, it is erroneous, for the juveniles do not differ markedly from adults in morphology.

II MATERIALS AND METHODS

2.1 Source of infective larvae

Throughout this investigation rabbits were infected with Trichostrongylus retortaeformis so that three or four with patent infections were always available. The rabbits were bred in the animal house and were infected when they were 8-12 weeks old. They were given approximately 2,500 larvae in 0.5 ml distilled water orally. These were administered by means of a hypodermic syringe fitted with an inch long, 18 gauge needle enclosed in polythene tubing. The tubing was 1 mm in diameter and projected for about 1 cm beyond the tip of the needle.

Two weeks after the rabbits had been infected, samples of their faeces were examined for eggs using the method of Gordon and Whitlock (1939). For the following two weeks, faeces were collected daily from the rabbits with patent infections and ground up with a pestle and mortar. They were moistened with distilled water and then incubated in glass jars at 25°C in a constant temperature room, lit by artificial light for approximately 8 hours each day. After about a week, infective larvae migrated up the walls of the jars and were washed off with distilled water twice a week. This method was based on that of Whitlock (1942). Figure 2.1 shows a typical culture. If great numbers of larvae were required on a particular day, they were recovered from cultures by the technique of Roberts and O'Sullivan (1950). The jars were filled with distilled water and inverted in petri dishes con-

Figure 2.I A typical culture of Trichostrongylus retortaeformis; infective larvae have migrated up the walls of the jar.

Scale represents 5 cm.

taining distilled water. Overnight the larvae migrated into the petri dishes.

Infective larvae were washed with distilled water and concentrated to approximately 5,000 per ml. They were stored at 5°C in McCartney bottles. The bottles were never filled completely, nor were their caps screwed on tightly. Larvae were discarded after they had been stored for two months and usually those used for experiments, or to infect rabbits, had been collected during the previous week.

2.2 Recovery of parasitic larvae and adults

Various methods were tried in attempts to recover the maximum number of larvae from the duodenum with the minimum associated debris. The method described by Silverman, Poynter and Podger (1962) was useful for recovering larvae that had been in a rabbit for 5 days or more, but frequently yielded very few younger larvae. Mucinase obtained from cultures of Vibrio cholerae was used to break down the mucus surrounding larvae, but unfortunately it also damaged larvae. Similarly, acid solutions containing pepsin appeared unfavourable for the larvae. Finally a satisfactory method was devised using a Vibro-mixer model EI (Chemap AG., Männedorf - Zurich, Switzerland). This shook the larvae out of the mucosa. The duodenum was removed from the rabbit and evaginated on a rod 25 cm long and 7 mm in diameter. It was tied to the rod at each end and the exposed mucosa was briefly washed with 0.9% sodium chloride. The rod was then attached to the Vibro-mixer and vibrated at maximum speed in a vertical plane. During vibration, the rod was positioned inside a glass tube filled with Tyrode's solution at

40°C (see Figure 2.2). Vibration was continued until the mucosa began to slough off. This took about 10 minutes. By this time, the saline in the tube contained many larvae but little debris. A sieve 2 inches in diameter and with 100 meshes per square inch was placed on top of another with 200 meshes per square inch and the saline was poured through. The larvae were retained by the sieves but most of the debris was small enough to pass through. Larvae which were washed off the sieves did not seem to have been adversely affected by the treatment. Not all of the larvae or adults in the duodenum were recovered by this method, but it yielded enough clean larvae for most investigations. One advantage of the method was that it took little time, the whole process taking less than 30 minutes.

2.3 Histological techniques

The nematodes were fixed by heat, glacial acetic acid or acetic alcohol (1 part of glacial acetic acid and 3 parts 70% alcohol). Aceto-carmine or aceto-orcein was used to stain specimens for examination of the reproductive system. These stains acted erratically, taking much longer to stain some specimens than others. However, good preparations were obtained in which the nuclei were stained but not the cytoplasm. Third-stage larvae generally became stained only slowly; they were left in stain for at least 24 hours whereas fourth-stage larvae or adults were stained within an hour. A 10% solution of acetic acid was used to remove stain from the cytoplasm when necessary.

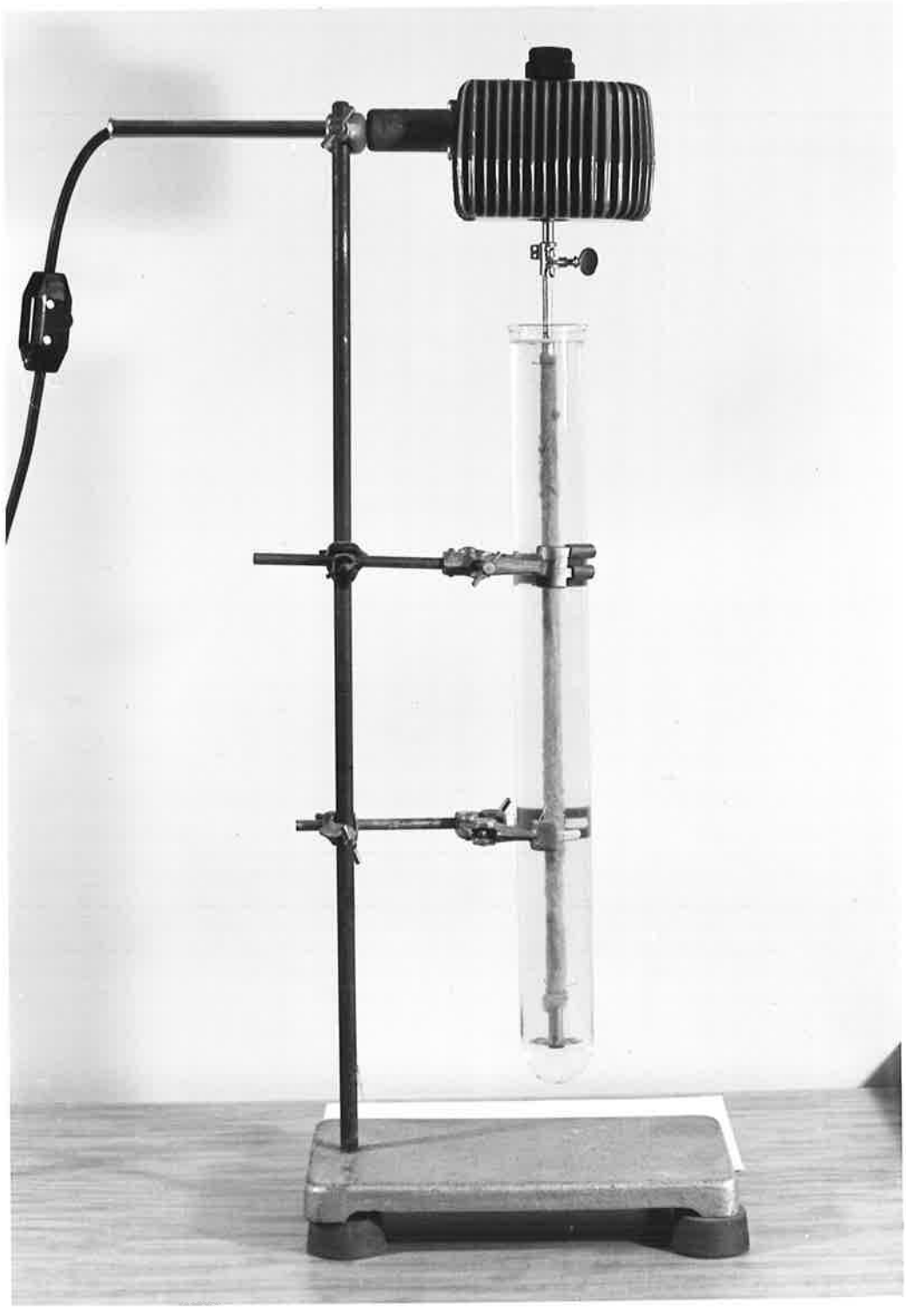


Figure 2.2 The apparatus which was used to recover parasitic larvae and adults. The duodenum of a rabbit is evaginated on the rod attached to the Vibro-mixer.

2.4 Measuring

An ocular micrometer was calibrated and used to measure the parasites. The specimens were killed by heat as this usually made them straighten out.

2.5 Drawing.

Drawings were made of larvae which had been stained with either aceto-carmine or aceto-orcein. A Wild projecting mirror was used in conjunction with a monocular Wild M20 microscope to project an image of the specimen and this was traced. The magnification of images traced was either 560 or 900 diameters.

2.6 Photomicrography

Photomicrographs were taken with a Wild camera and M20 microscope. Ilford films and developers were used. Most photomicrographs were taken on Pan F or Micro-neg Pan 35 mm film, but some were taken on FP3 size 120. Films were developed in ID2 or Hyfin.

Fluorescence photomicrographs were taken with a Leitz Orthomat camera attached to an Ortholux microscope. Kodak High speed Ektachrome film was used and processed by a commercial firm.

2.7 Physiological salines

Larvae and adults recovered from rabbits were usually incubated in Tyrode's physiological saline. This contained per litre:

NaCl	8.0 gm
KCl	0.2 gm
$CaCl_2$	0.2 gm
$MgCl_2$	0.2 gm
$NaHCO_3$	1.0 gm
NaH_2PO_4	0.04 gm
glucose	1.0 gm
water	1000 gm

If larvae were incubated in saline overnight, saline containing 1,000 units of streptomycin and penicillin per ml was used.

The modified Krebs' bicarbonate Ringer used to dilute serum conjugated with a fluorochrome contained

183.0 ml	4.5% NaCl
7.3 ml	5.75% KCl
5.5 ml	6.1% $CaCl_2$
1.8 ml	19.1% $MgSO_4 \cdot 7H_2O$
92.7 ml	0.05M $NaHCO_3$
709.7 ml	water

Glass electrodes were used to check the pH of solutions and their osmotic pressure was measured with a Fiske model H osmometer. The osmometer measured freezing points with an accuracy of ± 0.001% by means of a thermistor. It was calibrated with solutions of sodium chloride to give readings in milliosmols.

2.8 Preparation of gas mixtures

Gas mixtures containing 5, 10 or 40% (vol/vol) carbon dioxide in nitrogen were obtained commercially. These had a tolerance of up to ± 0.05%. Other mixtures were made up in the laboratory by displacing water in a graduated flask with carbon dioxide, nitrogen or oxygen. The apparatus used is shown in Figure 2.3.

When calculating the total concentration of carbon dioxide in solution, it was assumed that carbonic acid is undissociated in solutions with a pH of 4 or below (Davis, 1961). The solubility of carbon dioxide in solutions of hydrochloric acid was taken from graphs of Van Slyke, Sendroy and Hastings (1928). The concentration of dissolved gaseous carbon dioxide and undissociated carbonic acid in bicarbonate-carbon dioxide buffers was calculated from the formula

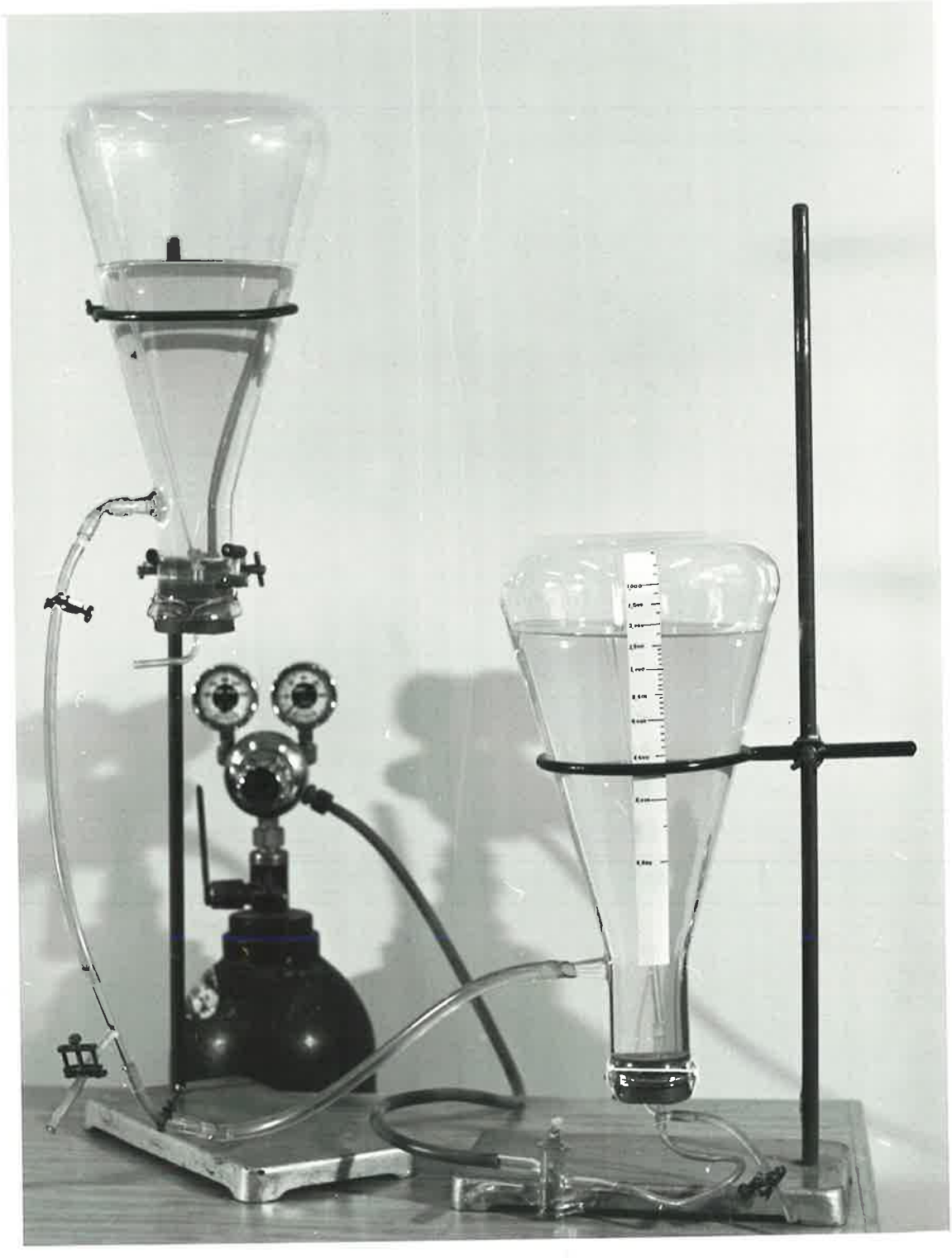


Figure 2.3 The apparatus which was used to prepare gas mixtures

$$\left[H_2CO_3\right]\left[1+\frac{K_1}{[H^+]}\left(1+\frac{K_2}{[H^+]}\right)\right] = \frac{P_{CO_2}\ 55.51}{K_H}$$ (Rogers, 1960)

where H_2CO_3 = the molar concentration of undissociated carbonic acid plus dissolved carbon dioxide;

Kh = Henry's law constant;

P_{CO_2} = the partial pressure of carbon dioxide in the gas phase in mm. of mercury,

and K_1 and K_2 are the first and second dissociation constants respectively. The effect of ionic strength on the dissociation constants was neglected. The value of pK at 37°C was taken as 6.317 (Shedlovsky and McInnes, 1935).

2.9 Fluorescence microscopy

Rhodamine B isothyocyanate, obtained from G.T. Gurr Ltd., London, England, was conjugated with sheep serum by a method similar to that described by Nairn (1964). One volume of serum was mixed with 2 volumes of 0.5M carbonate-bicarbonate buffer, pH 9.0. Rhodamine B isothiocyanate was stirred into this and stirring was continued in a cold rood for 20 hours. The amount of fluorochrome added was 3 mg for each ml of serum. The conjugate was purified by gel filtration using a Sephadex C-25 column. Specimens which had been treated with the fluorochrome conjugated serum were examined most easily using a Leitz Ortholux microscope fitted with an HBO, 200 watt mercury vapour lamp, but satisfactory observations were also made with a Wild M20 microscope and mercury vapour lamp set up as described by Gray (1953). The rhodamine B emitted orange-yellow fluorescence when stimulated with ultra-violet-blue light.

2.10 Anaesthesia and surgery

Rabbits were anaesthetised with Nembutal (pentobarbitone sodium). The solution, which contained 60 mg per ml., was injected into the marginal ear veins. The dose given was approximately 1 ml per kg of body weight, but the response of individual rabbits varied greatly and Nembutal was injected until either the pedal or eye reflex disappeared; if the anaesthetic were injected until both reflexes disappeared, the rabbit was liable to die (Croft, 1960). Anaesthesia was maintained by injecting further doses of Nembutal through the ear veins. Occasionally, during an acute operation lasting for 3-4 hours, the ear veins collapsed and Nembutal was then injected into a cannulated jugular vein.

Incisions in the peritoneum and musculature were closed with simple continuous sutures of chromic catgut, size 00, those in the wall of the alimentary canal with a Lembert suture of size 0000. Initially a continuous suture of sterile cotton was used to close skin incisions, but the rabbits frequently removed this by scratching or biting. An interrupted mattress suture was then used and this did not seem to cause irritation.

The operating table incorporated a thin steel plate, which was warmed by a 75 watt light bulb mounted underneath it. Rabbits were kept in a warm room until they regained consciousness. They were given intramuscular injections of 300,000 units of procaine penicillin daily for three days and the wound was treated with Neosporin ointment (Burroughs Wellcome & Co.Ltd., Sydney) twice each day until it healed.

III THE DEVELOPMENT OF PARASITIC LARVAE IN THE HOST

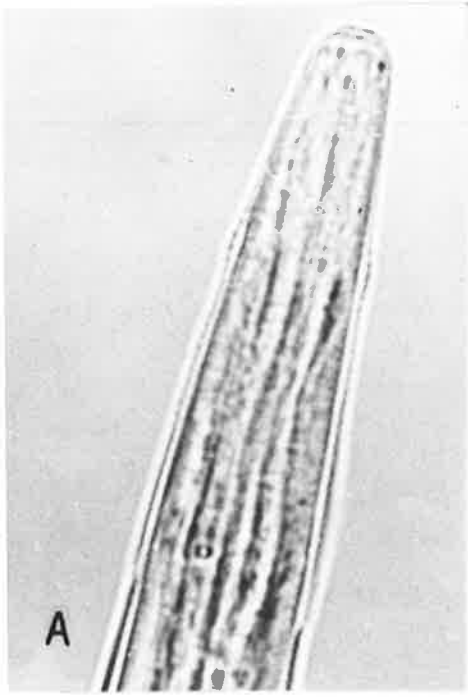
3.1 Exsheathment and moulting

The morphological changes that take place during exsheathment of Trichostrongylus retortaeformis infective larvae are similar to those described in other species (Veglia, 1915; Lapage, 1935a; Rogers and Sommerville, 1960). The larvae lie loosely inside the sheath. Frequently the excretory and anal apertures of the sheath lie dorsal to those of the larva (Figure 3.5A, page 29). When the larvae are stimulated by conditions in the stomach of the host or in vitro, a "refractile ring" (Lapage 1935a) appears in the cuticle approximately 20 μ from the anterior end. The appearance of the larvae at this stage is shown in Figure 3.1A. The outermost layer of the cuticle is swollen in the region of the ring and the innermost layer becomes thinner and eventually breaks. Finally the outer layer also breaks and a cap of cuticle is detached (Figure 3.2). While the sheath is breaking down, the larva moves its head continually and probably exerts some pressure on the sheath which helps to break it at the refractile ring. As soon as the cap is cast, the larva starts to emerge from the rest of the sheath. Frequently a second refractile ring appears in the cuticle about 63 μ from the anterior end and the sheath between the two rings becomes distended (Figure 3.1B) or the whole anterior end of the sheath, including the cap, is swollen (Figure 3.1C). The sheath, however, invariably breaks at the anterior refractile ring.

Figure 3.1 The formation of the refractile ring.

A. Typical refractile ring.

B & C. Larvae in which two refractile rings have formed and the cuticle between these is swollen.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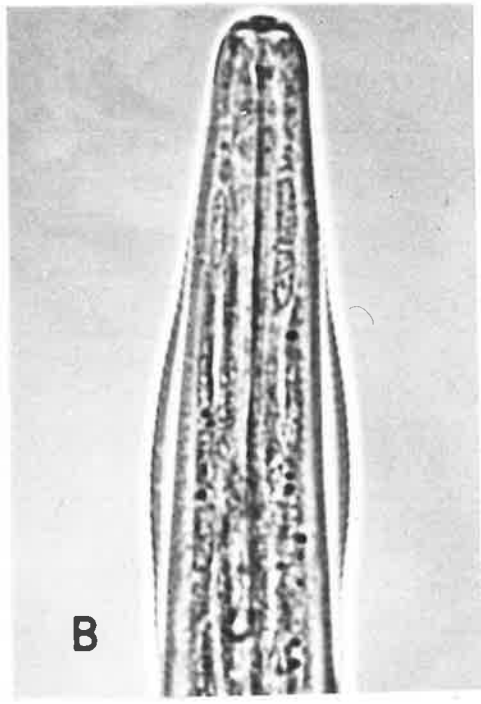
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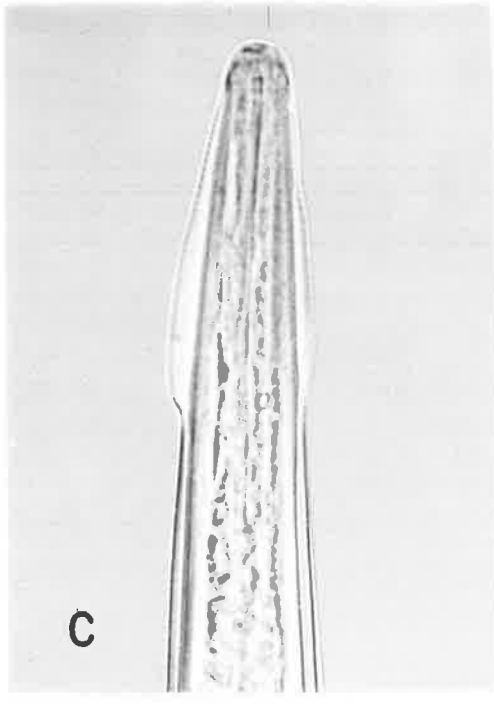
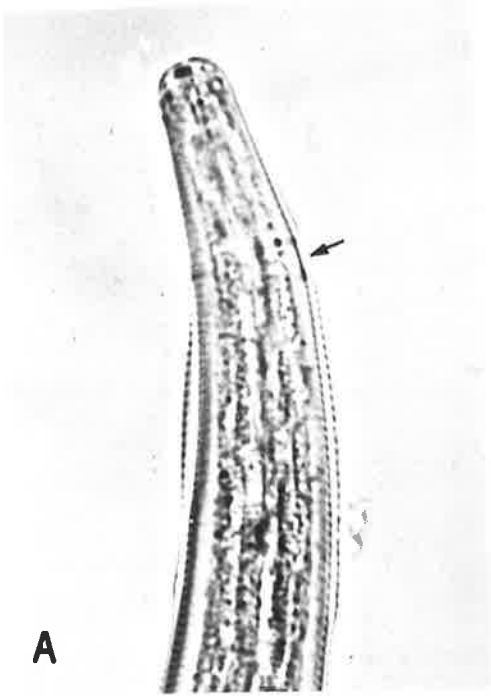
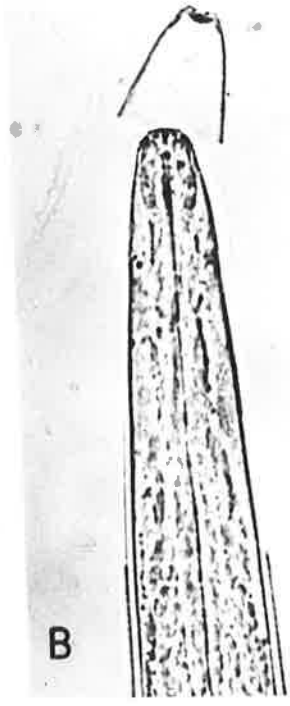

C

Figure 3.2 The fracture of the sheath of infective larvae

A. Cuticle starting to break at the refractile ring

B. Larvae starting to emerge from the sheath.


A


B

The third moult takes place differently. Instead of the cuticle splitting in a ring, a longitudinal split develops at the anterior end and the larva emerges through this, as described by Veglia (1915) and Lapage (1935d) in other species of the Trichostrongylidae. Before the moult, a second cuticle forms and a space appears between the two cuticles in the head and tail regions. By retracting its head and moving it from side to side, the larva sheds the lining of the oesophagus and amphidial sacs. Regular contractions also occur in the region of the rectum which free its old lining. Finally the third-stage cuticle splits. This moult occurs about 60 hours after the larva has been ingested by the host.

The final moult occurs after the larva has been in the host for about 5½ days. It is preceded by similar changes to those which occur before the third moult, but the split which appears in the cuticle is circular. As in exsheathment, a cap of cuticle is cast (Figure 3.10C page 35). The adult emerges slowly from the fourth-stage cuticle, and is probably not completely free of it until a few hours after the cap has been cast.

3.2 Change in the length of larvae

Table 3.1 gives the mean length and its standard deviation of larvae at different times after they had been ingested. When larvae that had been in the host for less than 96 hours were measured, a random sample was taken and the sex of individuals was ignored. However, once larvae had been in a rabbit for more than 96 hours, females were appreciably longer than males and the mean length of both males and

Table 3.1 The length of *Trichostrongylus retortaeformis* at different times after infection

No. of hours after ingestion	Stage of larvae	Mean length of larvae (mm)
0	3rd (exsheathed)	0.63 ± 0.02
24	3rd	0.65 ± 0.05
48	3rd	0.82 ± 0.08
54	3rd	0.85 ± 0.15
63	3rd	0.91 ± 0.06
63	4th	1.48 ± 0.22
72	3rd	0.88 ± 0.13
72	4th	1.78 ± 0.18
84	4th	2.03 ± 0.26
96	4th ♂	2.50 ± 0.21
96	4th ♀	2.70 ± 0.33
120	4th ♂	2.68 ± 0.32
120	4th ♀	2.79 ± 0.26
132	4th ♂	2.70 ± 0.33
132	4th ♀	3.28 ± 0.42
144	adult ♂	4.43 ± 0.36
144	adult ♀	5.24 ± 0.54

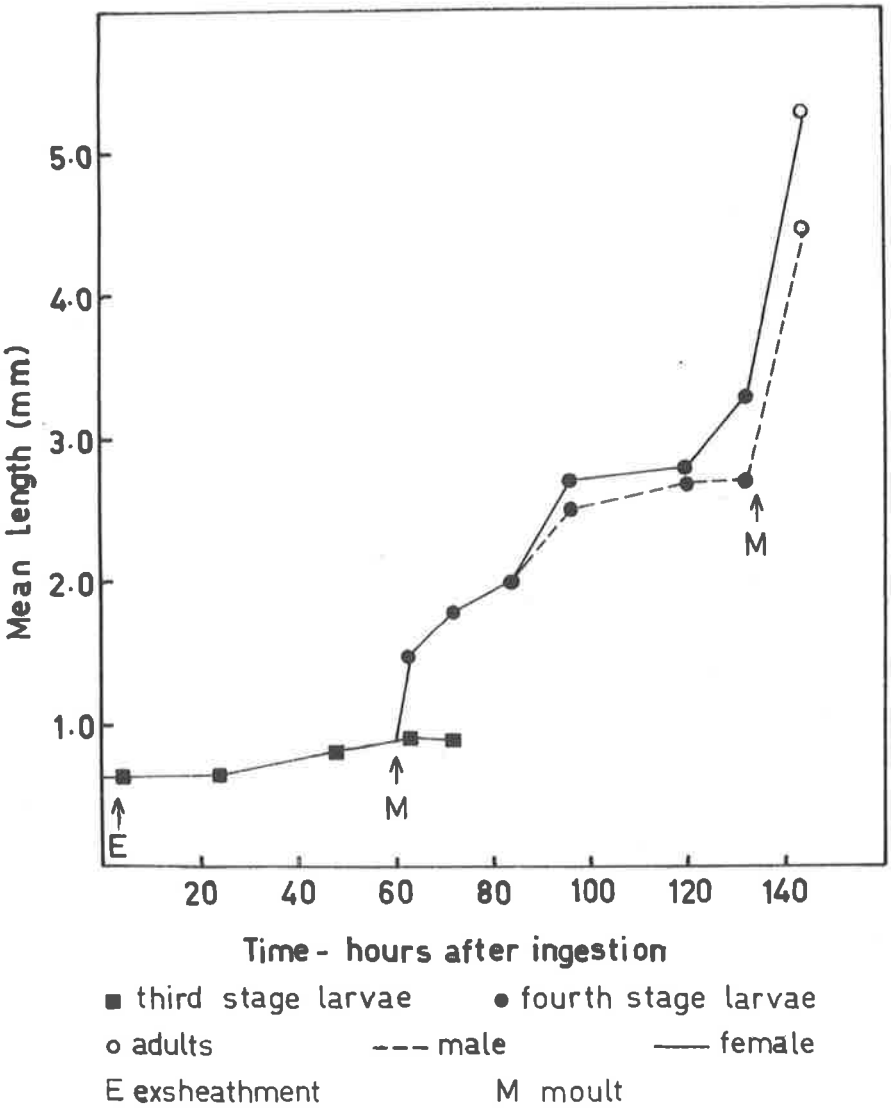


Figure 3.3 The mean length of Trichostrongylus retortaeformis at different times after ingestion

females was determined. Figure 3.3 shows the growth curve of larvae in the host.

It seems that larvae grow very little, if at all, for a few hours before the third and fourth moults, but immediately after the moults they increase enormously in length, fourth-stage larvae from a rabbit killed 63 hours after it was infected were almost twice as long as third-stage larvae from the same rabbit and recently moulted adults were almost twice as long as fourth-stage larvae about to moult. Exsheathment, which completes the second moult, differs from the later moults for it is not followed quickly by a period of growth; larvae begin to increase in length only after they have been in a rabbit for at least 24 hours and have almost certainly been free of their sheath for at least 20 hours. However third-stage larvae that are about to moult are approximately 0.3 mm longer than they were immediately after they exsheathed.

3.3 Changes in the shape of larvae

Figure 3.4 shows the structure of third-stage larvae.

Throughout development the shape of the parasite does not change markedly; after the third moult, the excretory pore is easier to detect as it lies in a depression in the cuticle and after the final moult the head is more rounded and the cuticle behind the buccal capsule may be inflated. The most noticeable changes are, however, those which occur in the tail region. These enable the different stages to be easily distinguished. Figure 3.5A shows the tail of a sheathed larva.

Figure 3.4 Third-stage larvae of Trichostrongylus retortaeformis

A. Immediately after exsheathment.

B. Male after 48 hours in a rabbit.

c = coelomocyte; e.p. = excretory pore; g.p. = genital primordium; h = hypodermis; n.r. = nerve ring; r.c. = renette cell; r.c.n. = renette cell nucleus; v.n.n. = ventral nerve nucleus.

THIRD STAGE LARVAE OF
TRICHOSTRONGYLUS RETORTAEFORMIS

A immediately after exsheathment
B male after 48 hours in a rabbit

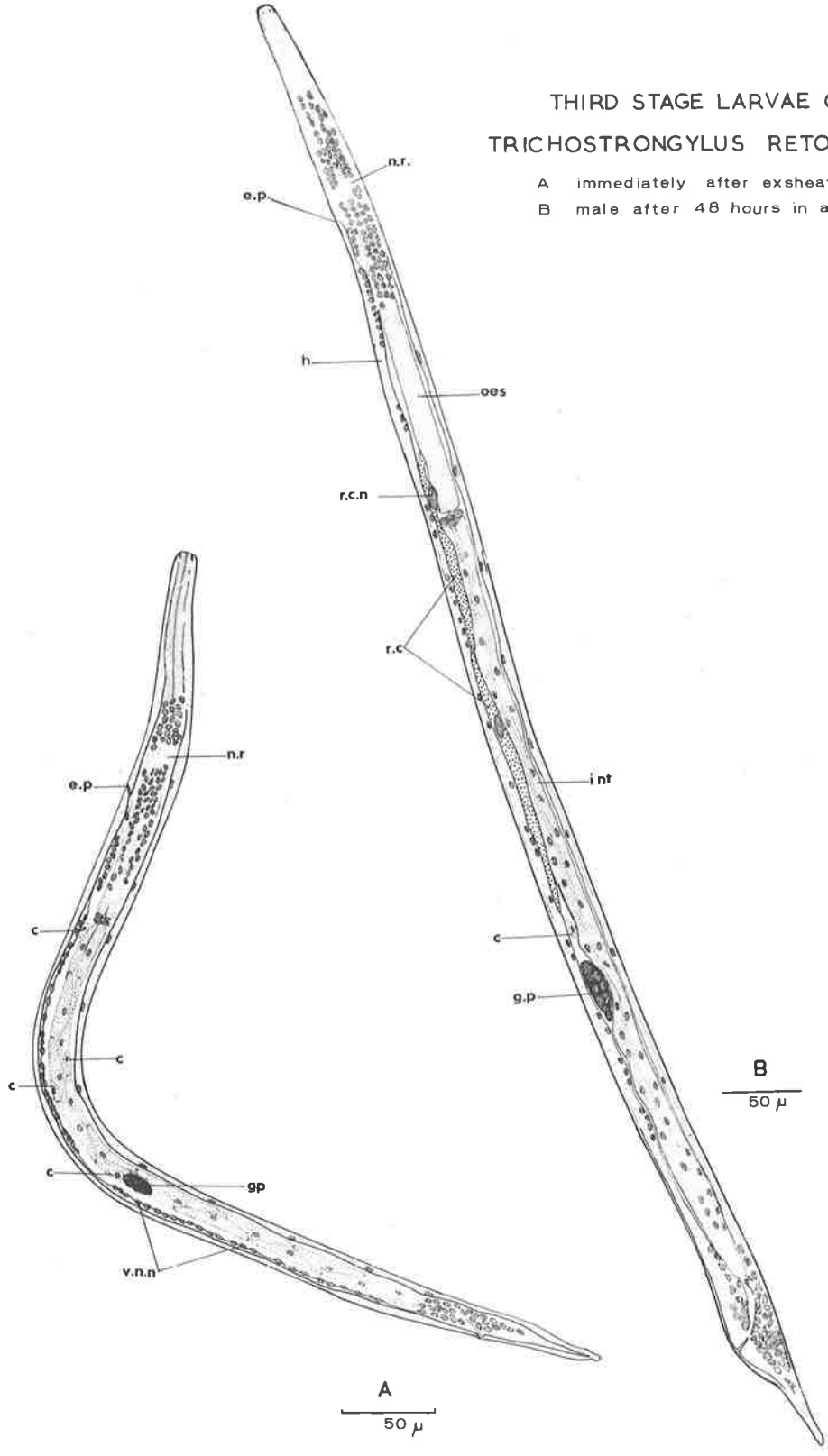


Figure 3.5 The posterior end of third-stage larvae.

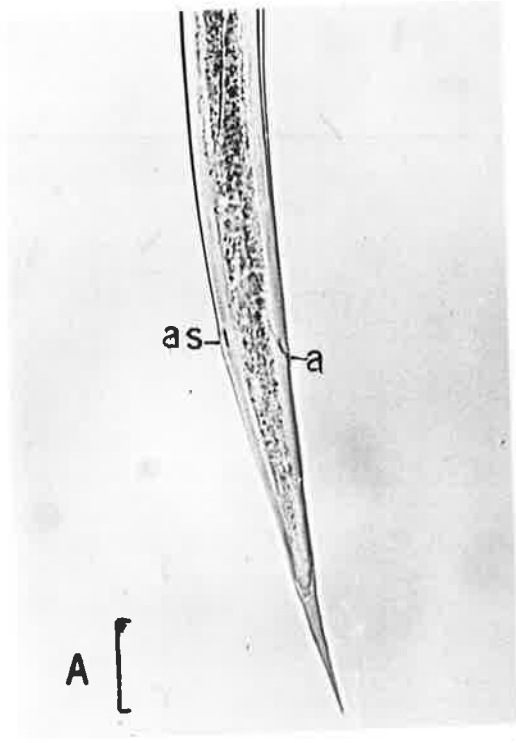
A. Sheathed larva, lateral view.

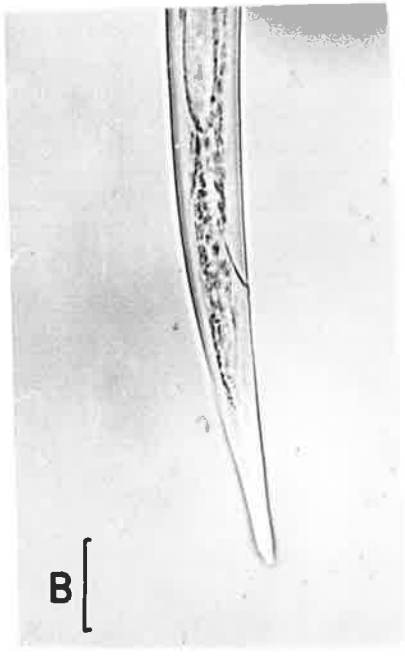
B and D Exsheathed larva, lateral view.

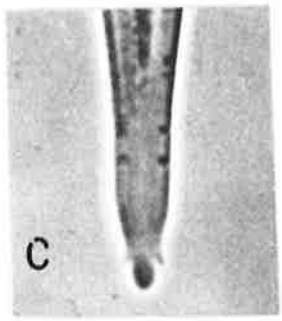
C and E Exsheathed larva, ventral view.

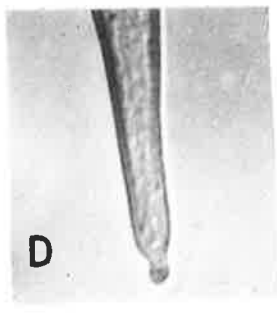
Scale represents 20 μ

a = anus; a.s. = anal aperture of sheath.


as
a
A


B


C


D

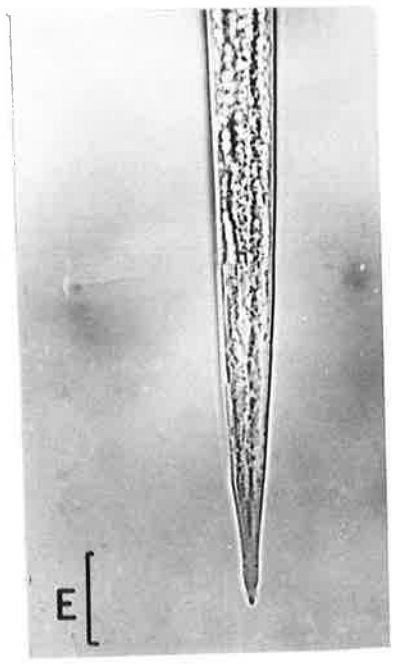

E

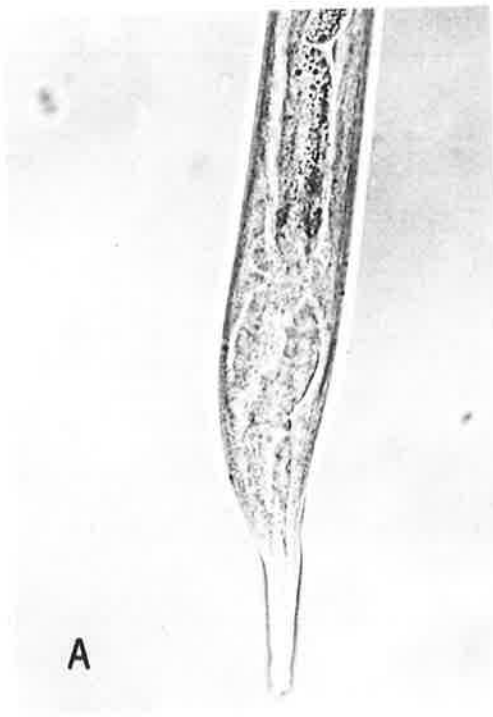
Figure 3.6 The posterior end of third and fourth-stage larvae.

A. Third-stage male, 48 hours after ingestion.

B. Third-stage female, 60 hours after ingestion.

C. Fourth-stage male, 66 hours after ingestion.

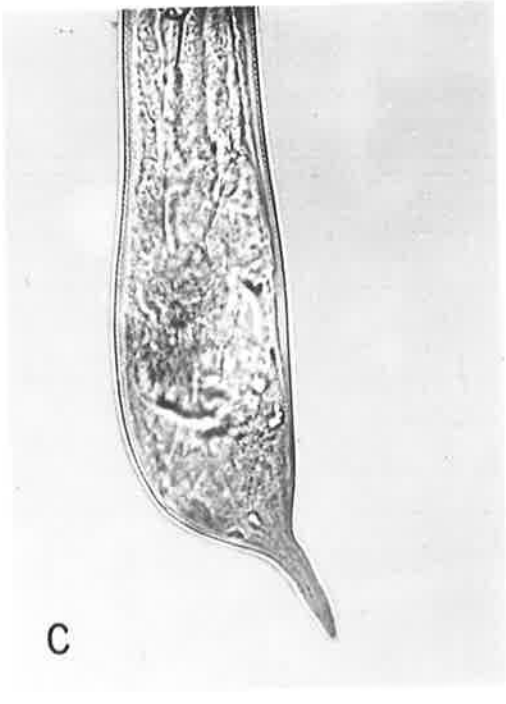
D. Fourth-stage female, 66 hours after ingestion.



A

B

20μ



C

D

Figure 3.7 The posterior end of females, 136 hours after ingestion.

A and B. Fourth-stage larvae

C. Adult

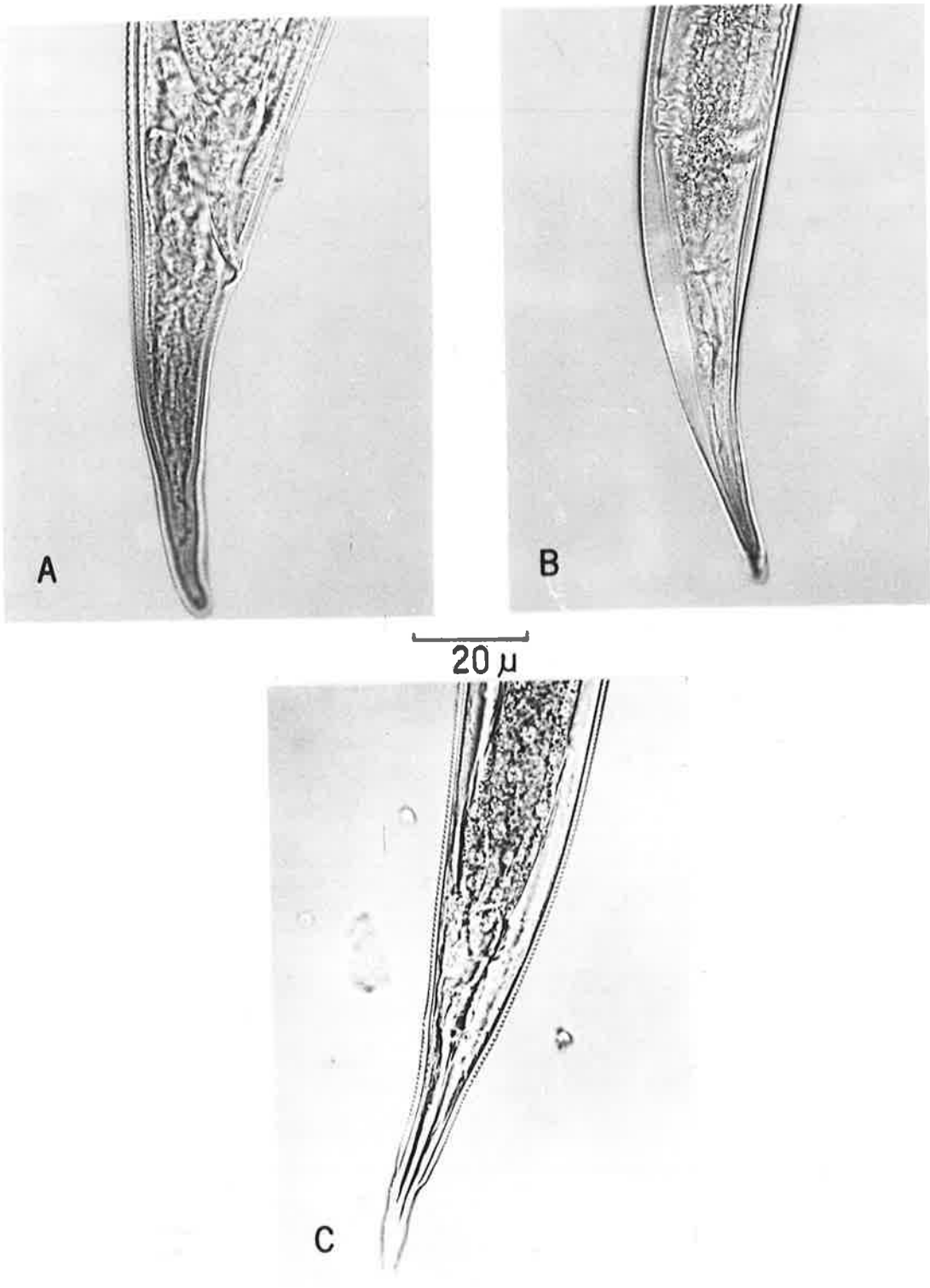
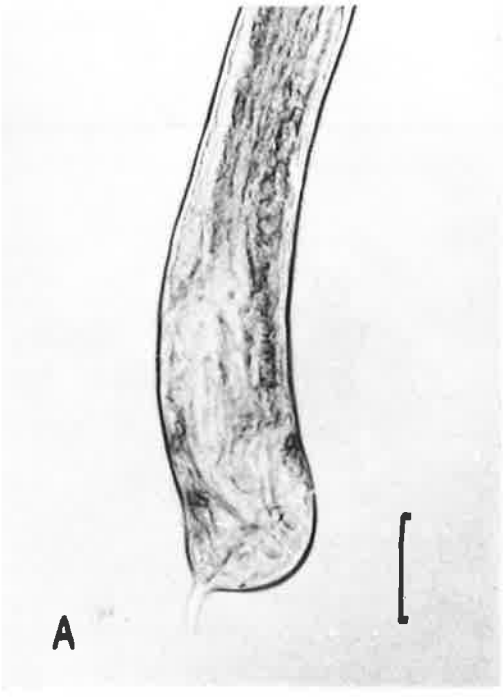

A
B
20 µ
C

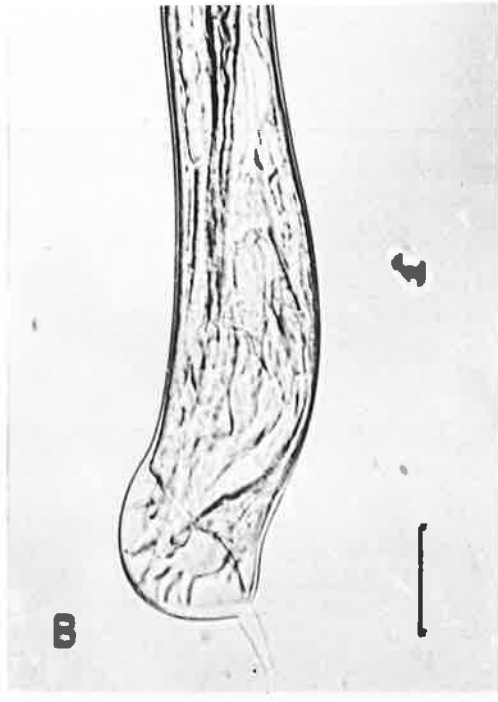
Figure 3.8 Formation of the bursa and spicules

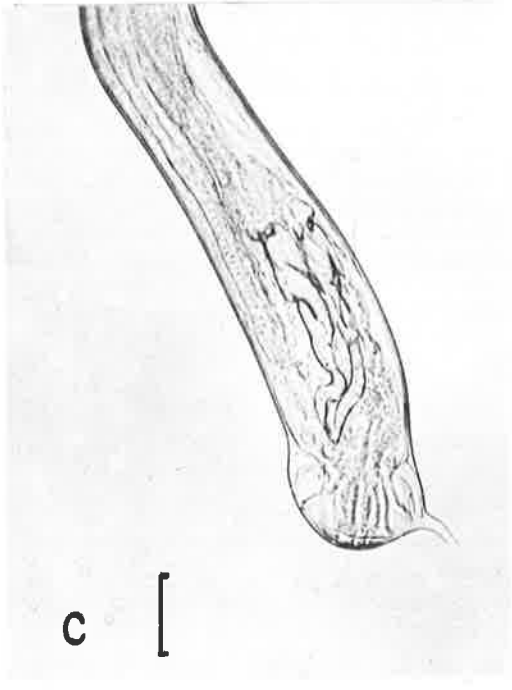
A, B and C. Development in fourth-stage larvae, 5-5½ days after ingestion.

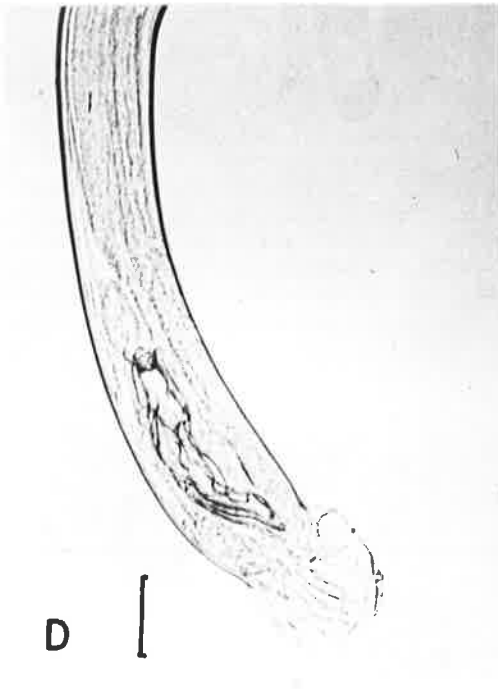
D. Young adult, 6 days after ingestion.

Scale represents 50 microns.


A


B


C


D

The sheath continues beyond the end of the third-stage larva and is very pointed. The tail of the third-stage larva ends in a small spherical process (Figure 3.5B-E) which is lost at the third moult (Figure 3.6). It is also possible to distinguish between the sexes before the third moult by the shape of the tail, that of males becoming swollen ventrally as spicules start to form (Figures 3.6A and B). After the third moult the tail of females changes little in structure (Figure 3.7). When male larvae have been in the host for 5 days bursal rays start to form and before the final moult the bursa is as it appears in adults. The fourth-stage cuticle projects beyond it as a short spike (Figure 3.8).

3.4 Development in the alimentary canal

(i) Buccal cavity and mouthparts

The buccal cavity of third-stage larvae is not well developed and is distinguished from the remainder of the oesophagus (more correctly termed the pharynx) only by the cheilorhabdions and protorhabdions which simulate stylets (Figure 3.9A). An obvious, though relatively unspecialised, buccal cavity first appears immediately before the final moult.

As larvae approach the third moult, vacuoles appear in the head region (Figure 3.9B and C). These enlarge and fuse and the mouth parts of the fourth-stage larva form. Fourth-stage larvae are characterised by two papillae, one dorsal and one ventral to the mouth. These are shed with the cuticle during the final moult and are not present in the adult (Figures 3.9 and 3.10).

Figure 3.9 Anterior end of third and fourth-stage larvae.

A. Recently moulted third-stage

B. Third-stage larva, 48 hours after ingestion

C. Third-stage larva, 60 hours after ingestion

D. Fourth-stage larva, 72 hours after ingestion

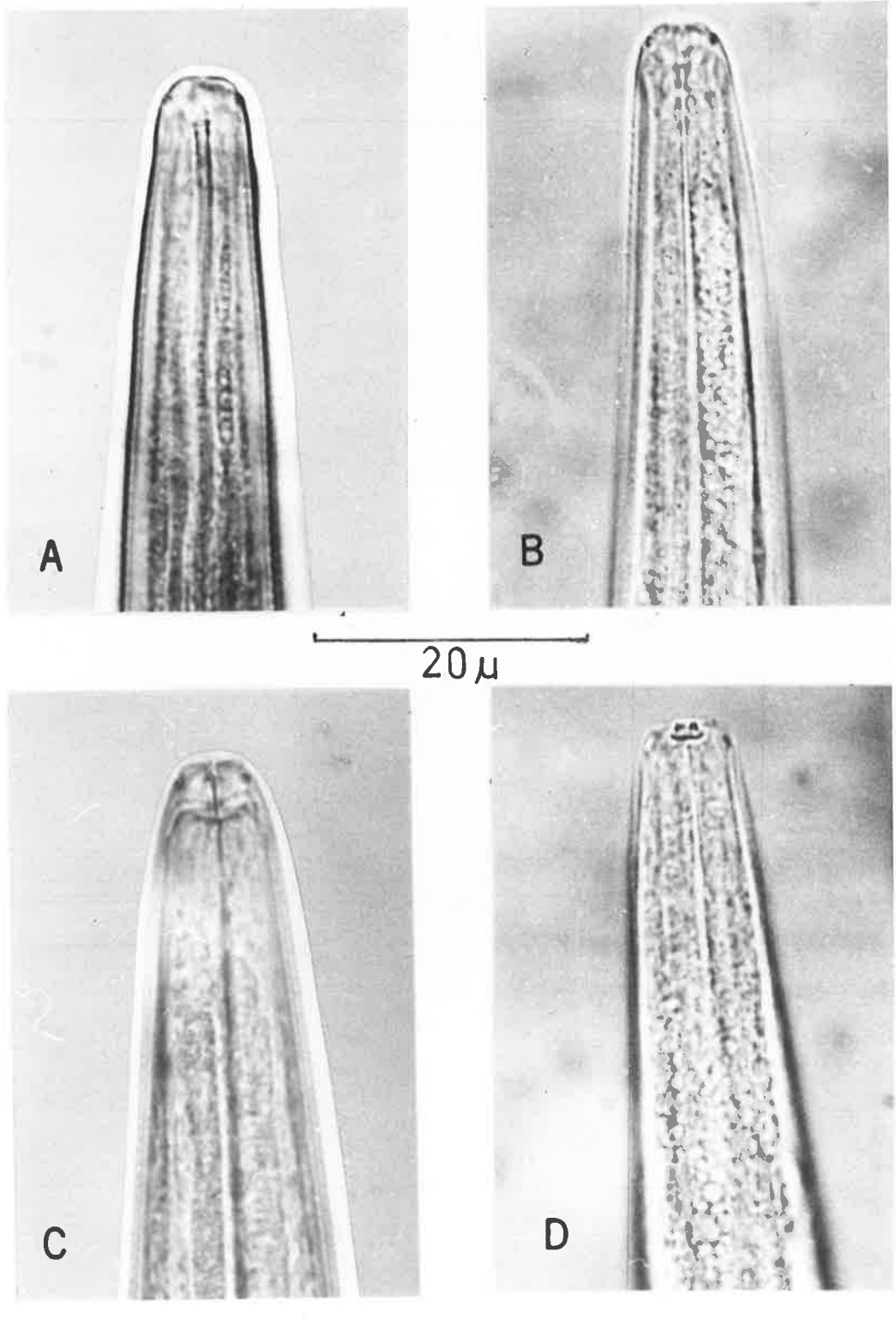

A
B
20 µ
C
D

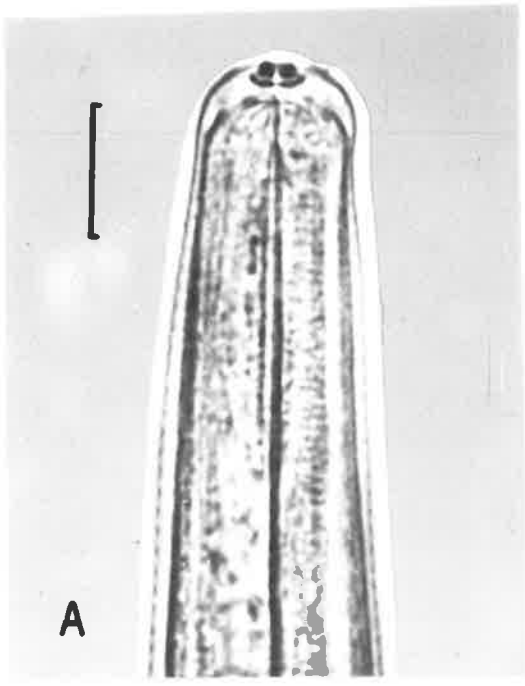
Figure 3.10 Anterior end of fourth-stage larvae and adult

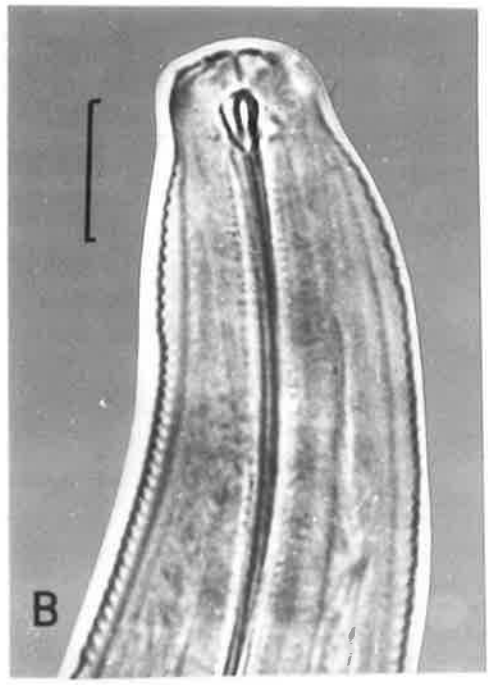
A. Fourth-stage larva, 123 hours after ingestion

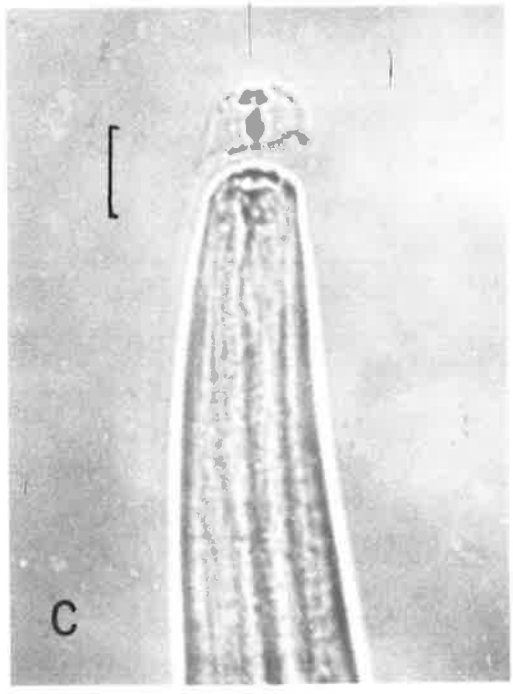
B. Young adult

C. Young adult casting cap of fourth-stage cuticle

Scale represents 10 μ


A


B


C

(ii) The intestine

The intestine of infective larvae consists of eight dorsal and eight ventral cells, as in other species of Trichostrongylus (Chitwood, 1950; Mönnig, 1926). The intestine appears unchanged in larvae which have been in a rabbit for 24 hours but after this the number of nuclei increases. When larvae have been in a rabbit for 56 hours, there are about 40 nuclei. I was unable to count the number of nuclei in later stages or determine whether the intestine was a syncitium.

(iii) Movement in the alimentary canal

Pulsations were observed in the oesophagus of third-stage larvae that had been in a rabbit for 56 hours or longer but not in larvae which had been in the host for 24 hours. The pulsations seemed to originate in two regions, one immediately anterior and one posterior to the excretory pore. The intestine also moved, propelling material in its lumen. Very little movement was observed in the alimentary canal of larvae which were about to moult.

(iv) Ingestion of food

It was predicted that, if larvae had been feeding in the host, they would ingest the fluorochrome Rhodamine B isothiocyanate conjugated with serum in vitro and its fluorescence could be detected in their alimentary tract. Larvae that had been in a rabbit for various times were incubated at 39°C for 3 hours in Kreb's bicarbonate Ringer containing either 5 or 15% of the conjugated serum. They were then washed with 0.9% sodium chloride and examined for fluorescence in ultraviolet-blue light. Exsheathed third-stage larvae which had been stimulated in vitro and mature adults were treated similarly and examined. Table 3.2 summarises the results.

Table 3.2 The ingestion of Rhodamine B isothiocyanate conjugated with sheep serum

No. of hours in the host	Stage of larvae	Concentration of conjugated serum (%)	Dye present (+) or absent (-)
-	3rd (exsheathed)	15 "	-
14	3rd	15 "	+
24	3rd	5 "	+
39	3rd	15 "	+
48	3rd	5 "	±
68	3rd	15 "	-
68	4th	15 "	+
96	4th	15 "	+
140	4th	15 "	-
140	adult	15 "	+
8 days +	mature	15 "	+

Larvae that had been in the host for 14 or 24 hours contained small amounts of the fluorochrome; it was present in the lumen of the oesophagus but seldom in that of the intestine and the fluorescence emitted was weak. Dye was present in the lumen of both oesophagus and intestine in larvae that had been in the host for more than 24 hours except for those about to moult. Presumably the reorganisation occurring in the mouthparts prevented ingestion of the dye; all the

late third-stage larvae which did not contain the fluorochrome had obvious vacuoles in the head, as shown in Figure 3.9C,(page 34). Similarly fourth-stage larvae which had formed the adult cuticle did not contain any fluorochrome. Both fourth-stage larvae and adults seemed to ingest the conjugated serum as soon as their head was free of the old cuticle. Mature adults all contained some Rhodamine B, but often it was not present throughout the alimentary canal or the fluorescence emitted was weak. This suggests that the worms ingested the conjugated serum sporadically.

The intestinal cells of all larvae and adults examined emitted strong green autofluorescence. This made it difficult to determine with certainty whether Rhodamine B had entered the cells, but it seemed to be confined to the lumen of the alimentary tract.

Rhodamine B entered the excretory sinus and renette cells as well as the alimentary canal of young adults. The amount which entered these excretory organs varied. No fluorescence was emitted from the lateral line canals. If the adults had moulted 2 or 3 hours before they were incubated with the conjugated serum, the fluorescence emitted from the excretory sinus and renette cells was often as bright as that emitted from the alimentary canal (see Figure 3.11). Very little of the fluorochrome entered the excretory sinus or renette cells during any other stage of the life cycle. Occasionally some entered these structures in fourth-stage larvae or mature adults, but the fluorescence emitted was very weak, even if the parasites were incubated in the conjugated serum for 12 hours.

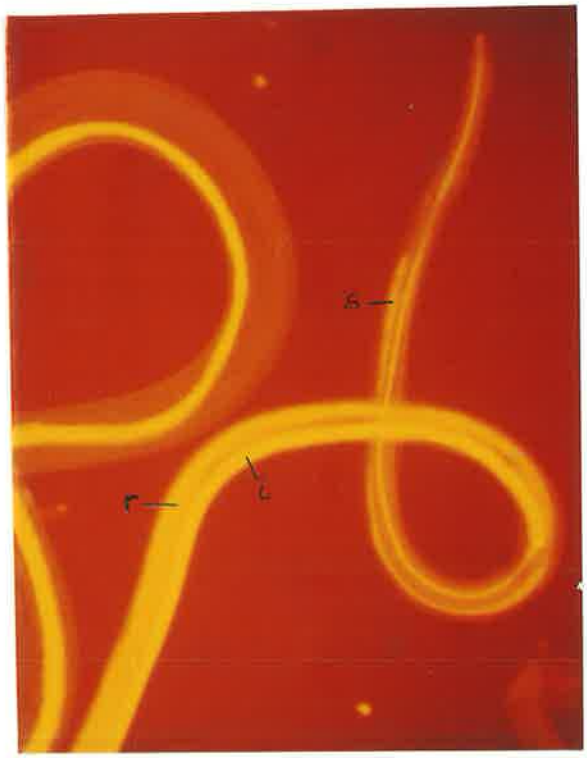


Figure 3.11 A young adult which was incubated with Rhodamine B isothiocyanate conjugated with serum. The fluorochrome has entered the alimentary canal, renette cells and excretory sinus.

i = intestine; r = renette cell;

s = excretory sinus.

Attempts were made to determine whether the fluorochrome entered the excretory sinus through the excretory pore or from the intestine. Recently moulted adults were incubated with the conjugated serum and examined at 15 minute intervals. The fluorochrome appeared in the excretory system only after it had entered the anterior portion of the intestine. This suggests that it might pass from this portion of the alimentary tract, but there was no indication of how it did this; no fluorescence was emitted from the body cavity and no processes like those which link the excretory system with the intestine in Panagrellus redivivus (Smith, 1965) were seen.

Ligatures were tied round some adults before they were incubated in the conjugated serum but, if these were tight enough to prevent the fluorochrome from getting past, they damaged the worms and all of the region anterior to the ligature became diffusely stained.

Other adults were incubated in the fluorescent medium until the fluorochrome had entered the intestine. They were then transferred to 0.9% sodium chloride and examined at intervals. It seemed as if the intensity of the fluorescence emitted from the excretory system increased while the parasites were in saline, but the increase was very slight. Most of the Rhodamine B passed out of the intestine through the anus.

It was thought that perhaps Rhodamine B passed into the renette cells after it had been split from the serum in the intestine. Fourth-stage larvae, recently moulted adults and mature adults were incubated in Kreb's bicarbonate Ringer containing Rhodamine B isothiocyanate not conjugated with serum. The concentration of the fluorochrome was

approximately the same as that in the medium containing 15% of the conjugated serum. The fluorochrome entered the alimentary tract of all the specimens examined but entered the renette cells and excretory sinus only in recently moulted adults.

3.5 The reproductive system

The genital primordium of exsheathed third-stage larvae lies at the junction of the fourth and fifth ventral cells of the intestine. It is made up of two germinal cells surrounded by about twelve epithelial cells. Alicata (1935) noted that the genital primordium at a similar stage of development in Hyostrongylus rubidus was always associated with a coelomocyte. This lay anterior to the genital primordium in male larvae, whereas in female larvae it was lateral or posterior. A coelomocyte was noted in several larvae of Trichostrongylus retortaeformis lying in the same position as that described by Alicata for male larvae. No coelomocyte was seen lateral or posterior to the genital primordium. Possibly when no coelomocyte was seen associated with the genital primordium, the larva was a female and the coelomocyte was in some other position where it was difficult to detect.

(i) Development of the male genitalia

No apparent changes take place in the genital primordium until the larva has been in the host for about 48 hours. After this the primordium elongates slightly and the germinal cells move anteriorly. Following the third moult, the genital primordium enlarges rapidly and

further differentiation occurs. The germinal cells which will form the testis become separated from the cells that form the gonoduct by three cells that represent the future seminal vesicle. When the larva begins to shed its cuticle in the final moult, the gonoduct connects with the rectum and the regions of the adult reproductive system are clearly differentiated. As mentioned previously, the bursa is fully formed and the spicules are also developed, though not yet brown. These changes are shown in Figure 3.12.

(ii) Development of the female genitalia

The genital primordium of a female does not differ obviously from that of a male during the first 48 hours in the host. After this it grows in length but, whereas in a male both germinal cells move anteriorly, in a female only one moves anteriorly and the other moves posteriorly. At the third moult the genital primordium lies very close to the hypodermis and a connection is beginning to form at the site of the future vulva. As in the male, the third moult is quickly followed by enlargement of the primordium. The epithelial and germinal cells multiply and cells in the hypodermis become attached to the primordium. After the larva has been in the host for about 5 days, the ovaries begin to reflex and immediately before the final moult all the structures of the adult reproductive system are clearly differentiated. Figure 3.13 illustrates these changes.

Figure 3.12 Development of the male reproductive system

A. Third-stage larva after exsheathment (male or female).

B. Third-stage larva, 48 hours after ingestion.

C. Fourth-stage larva, 66 hours after ingestion.

D. Fourth-stage larva, 84 hours after ingestion.

E. Fourth-stage larva, $5\frac{1}{2}$ days after ingestion.

c = coelomocyte; ed = ejaculatory duct; gc = germinal cel
int = intestine; t = testis; vs = vesicula seminalis.

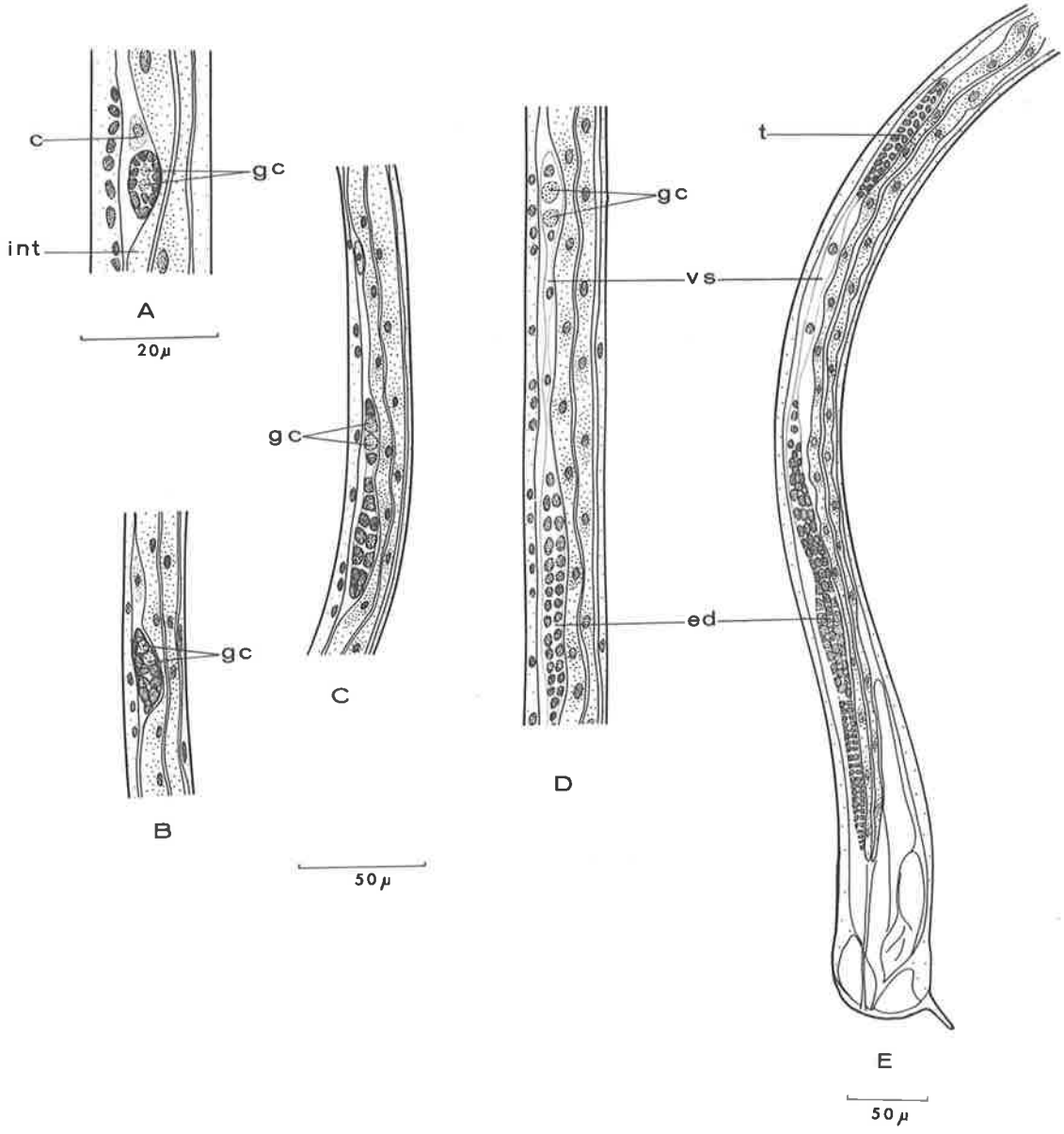

c
gc
int
A
20μ
gc
C
gc
B
50μ
gc
vs
ed
D
t
E
50μ

Figure 3.13 Development of the female reproductive system

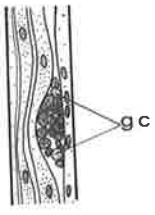
A. Third-stage larva, 56 hours after ingestion

B. Fourth-stage larva, 66 hours after ingestion.

C. Fourth-stage larva, 72 hours after ingestion.

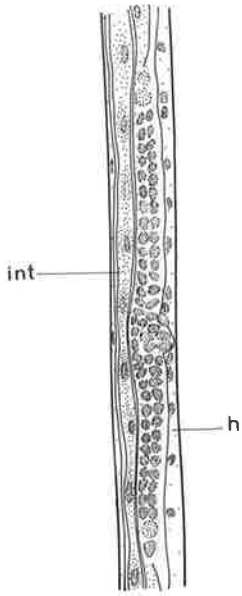
D. Fourth-stage larva, 96 hours after ingestion.

gc = germinal cell; h = hypodermis; int = intestine;
o = ovary; oj = ovojector; u = uterus; v = vagina.

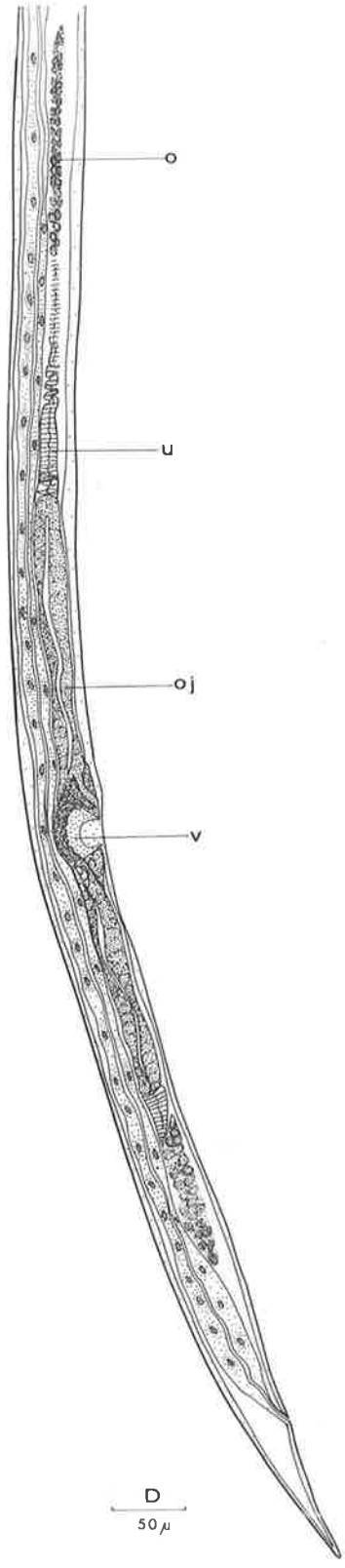


A
20 μ

B
50 μ



C
50 μ



D
50 μ

3.6 The excretory system

I was unable to interpret the exact relationship of parts of the excretory system, but some very obvious changes take place as the larvae mature. The excretory ampulla (Figure 3.14) pulsates in sheathed third-stage larvae and those which have been exsheathed *in vitro*. It is not apparent in larvae recovered from rabbits and was not seen pulsating in any of these.

The intestine fills the body cavity of sheathed third stage larvae and those which have been in the host for up to 24 hours. After this the renette cells appear near the base of the oesophagus and grow posteriorly. They lie ventrolateral to the intestine (Figure 3.4, page 28). Each of the cells has a very large nucleus. When stained with aceto-carmine these appear not only larger than the other nuclei in the larvae, but of a different composition. During the fourth-stage, the renette cells continue to lengthen and become coiled with the intestine. They always terminate anterior to the reproductive system.

The excretory sinus becomes full of dense material before both the third and fourth moults. This disappears within a few hours after the moult and probably passes out through the excretory pore; although this was never observed, droplets or granules were seen moving in the sinus, suggesting that once the excretory pore was open they would be voided. Figure 3.15 shows the appearance of the sinus before and after moults.

Figure 3.14 The excretory ampulla of third-stage larvae

a = expanded ampulla.

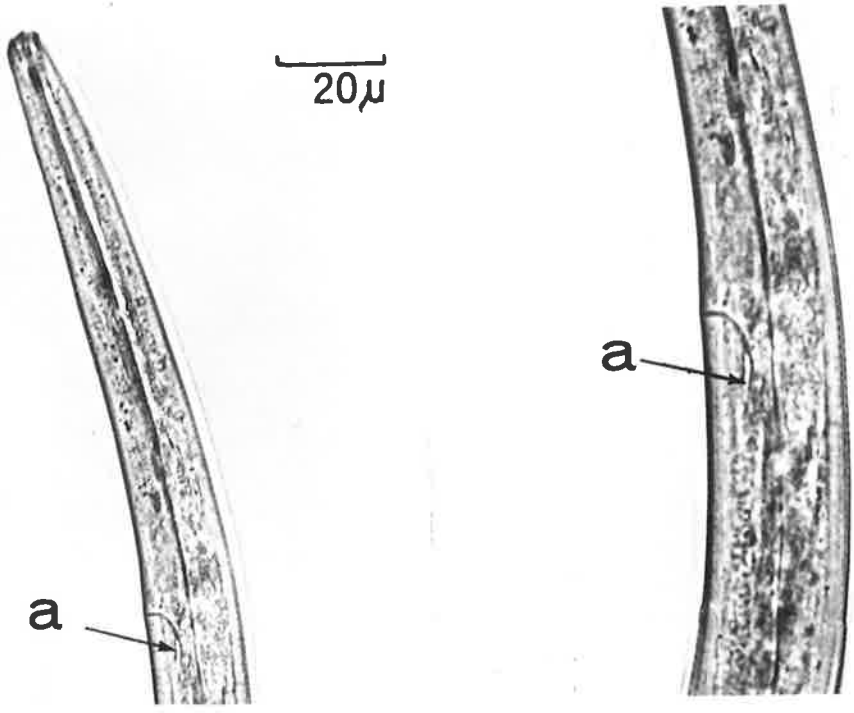

20μ
a
a

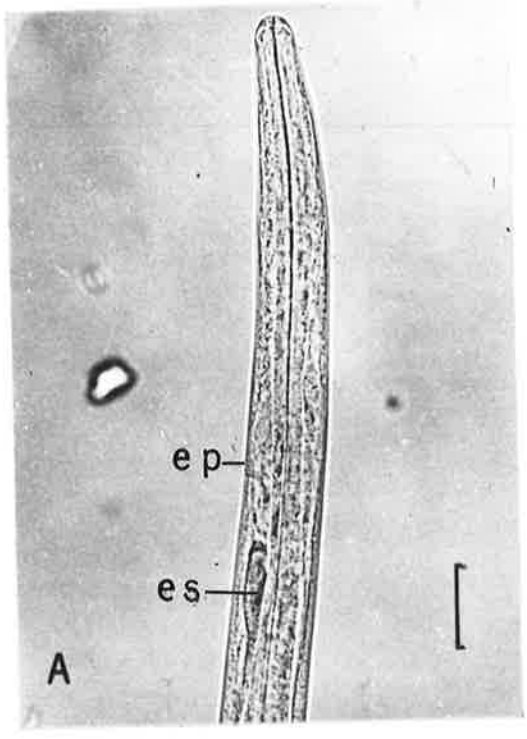
Figure 3.15 The excretory pore and excretory sinus.

A. Larva before the third moult, 66 hours after ingestion.

B. Larva before the fourth moult, 5½ days after ingestion

C. Young adult, 5½ days after ingestion.

Scale represents 20 μ; ep = excretory pore; es = excretory sinus. The excretory sinus appears much denser before a moult than after.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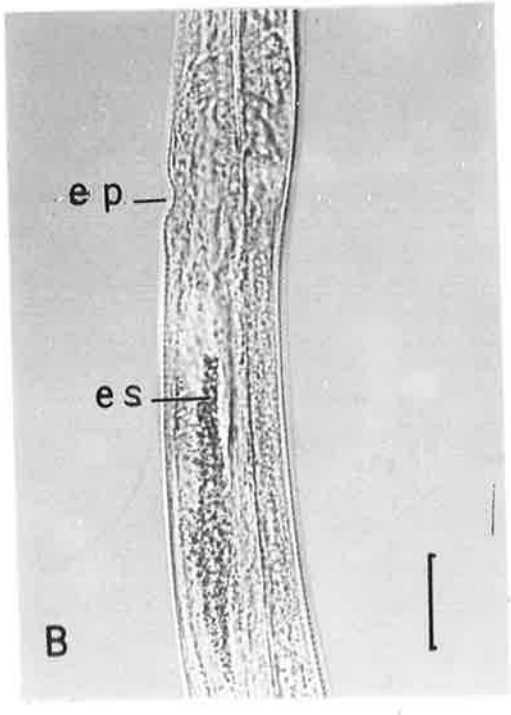
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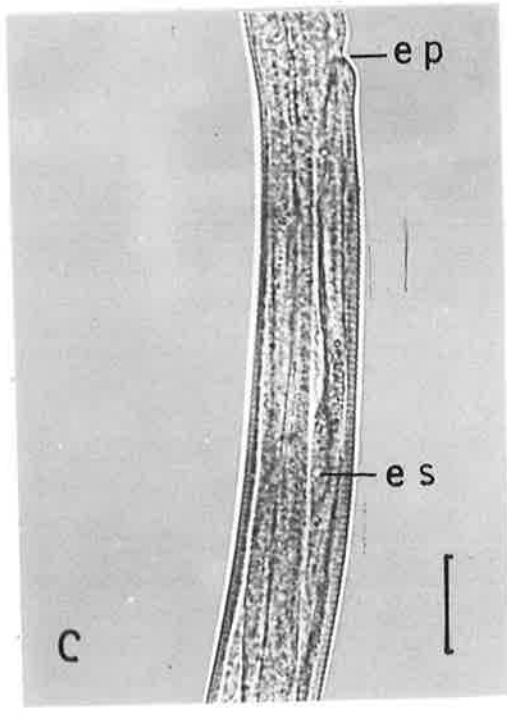
ep
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C

3.7 Discussion

The main aim of this study was to discover what changes took place in larvae after they had exsheathed, how quickly these changes occurred and whether development, once initiated, proceeded steadily.

(i) The initiation of development

Development is similar to that of Trichostrongylus colubriformis and Trichostrongylus rugatus, described by Mönnig, (1926). No apparent development occurs during the first 24 hours in the host. During this period, physiological changes may occur which are not reflected in morphological changes.

The development of third-stage larvae differs from that of Haemonchus contortus in some respects. In both species, renette cells develop and changes take place in the head region as the mouthparts and buccal capsule of the fourth-stage larvae are formed, but no other changes are apparent before the third moult in Haemonchus contortus (Veglia, 1915; Sommerville, 1966) whereas third-stage larvae of Trichostrongylus retortaeformis grow in length, the number of nuclei in the intestine increases and cells in the genital primordium are rearranged. If it is correct to assume that worms which ingest serum conjugated with a fluorochrome in vitro would feed if they were in the host, it seems that third-stage larvae of Haemonchus contortus do not feed (Sommerville, 1966) whereas those of Trichostrongylus retortaeformis start to feed when they have been in the host for only 14 hours. Possibly growth and development of the intestine and genital primordium take place only after larvae have ingested food and are no longer dependent on

reserves accumulated during the first and second stages in their life cycle.

(ii) The influence of moulting on development

When feeding ceases before the third and fourth moults, growth in length also ceases and the appearance of the excretory sinus suggests that excretory products are not ejected but accumulate until the cuticle has been shed. The very rapid increase in length which follows these moults may not involve much increase in the amount of tissue in the parasites, for the intestine of larvae before the third moult tends to be convoluted and its cells are thick; after the moult, the intestine is straighter and its cells are thinner. Alternatively, the increase in length may indicate a period of rapid metabolism.

The moults seem to interrupt development of the reproductive system relatively little. By the time that the third-stage cuticle is shed, the germinal and epithelial cells have altered in position but few, if any, cells have divided. Before the final moult, the reproductive system is essentially adult in form.

(iii) Excretion

Weinstein (1952) studied the pulsation of the excretory ampulla in third-stage larvae of Nippostrongylus brasiliensis and Ancylostoma caninum. There was an inverse relationship between the pulsation rate of the ampulla and the solute concentration of the environment. Weinstein concluded that the ampulla is concerned with osmoregulation and expells excess water from the larvae when they are

in a hypotonic environment. A brief study of the pulsation rate of the ampulla in third-stage larvae of Trichostrongylus retortaeformis suggested that the ampulla is concerned with osmoregulation in this species also; when the larvae were placed in various concentrations of sodium chloride, the rate of pulsation decreased as the concentration of sodium chloride increased. The ampulla pulsated most rapidly when the larvae were in distilled water.

After the infective larvae are ingested by the host, they are probably in a hypertonic environment. There is evidence that several nematode species, which live in the intestine, have body fluids slightly hypotonic to the intestinal fluids of their host (Duval and Courtois, 1928; Schopfer, 1932; Hobson, Stevenson and Beadle, 1952; Harpur and Popkin, 1965; Anya, 1966). In a hypertonic environment larvae need not pump out large volumes of water. This may explain why the ampulla was not observed pulsating in larvae and adults recovered from rabbits.

It is difficult to postulate why Rhodamine B entered the excretory sinus and renette cells only in adults that had moulted recently. For some reason, the excretory pore may remain open in these parasites and allow the fluorochrome to diffuse in, but it seems more likely that the fluorochrome enters from the intestine. Recently moulted adults increase in length rapidly and may have a greater metabolic rate than any other stage. Small amounts of fluorochrome may pass from the intestine into the excretory system in all larvae and adults, but its concentration in regions other than the alimentary canal may be too low for its fluorescence to be detected. The increased metabolic rate

in adults immediately after the final moult may increase the amount of fluorochrome which enters the excretory system and detectable amounts accumulate in the renette cells and excretory sinus. However, the fluorochrome entered the excretory system of young adults which were incubated in the conjugated serum at 15°C and were presumably metabolising more slowly. The fluorochrome took an hour longer to appear in the excretory system of these adults, but the fluorescence emitted was as strong as that emitted in adults which were incubated at 39°C.

Behrenz (1956) exposed nematodes to a variety of fluorescent dyes, incubating the worms in solutions of the fluorochromes and also injecting the fluorochromes into the host or into the body cavity of the worms. Seven species of nematodes were studied. Two of these were Strongyloids: a species of Oswaldocruzia, which is a member of the Trichostrongylidae and Uncinaria stenocephala of the family Ancylostomatidae. The remaining species were Oxyuroids and lacked renette cells.

None of the Strongyloids excreted any of the fluorochromes through the excretory pore. Some fluorochromes entered only the alimentary canal, others passed from this into the body cavity and were absorbed by the walls of the renette cells and lateral canals, but did not enter their lumina. The Oxyuroids excreted potassium salts of fluoresoein and erythrosin via the excretory system. These fluorochromes passed from the alimentary canal through the body cavity into the lateral canals and excretory sinus. Lissamine Rhodamine B however entered only the alimentary canal. These results suggest that

fluorochromes were excreted through the excretory pore only if they passed into the body cavity and were then able to pass through the walls of the excretory system.

Weinstein (1960) has reviewed the literature on excretion in nematodes and points out that the function of the so-called excretory system remains uncertain. Even if some substances are excreted by this system, others may be eliminated through the cuticle or anus; Savel (1955) has shown that Ascaris lumbricoides not only eliminates undigested and partially digested material through the anus, but also some, if not all, products of nitrogen metabolism.

The function of renette cells is unknown. Weinstein (1960) suggests that their very rapid development reflects a change in the physiology of larvae when they become parasitic. Enigk and Grittner (1952) suggest that they are concerned with the elimination of products of protein metabolism whereas the lateral lines are concerned with the products of carbohydrate and fat metabolism. There is as yet little evidence to support this hypothesis. It might explain why Rhodamine B entered the renette cells rather than the lateral canals of young adult Trichostrongylus retortaeformis; the fluorochrome was attached to protein and, if this was broken down in the intestine of the parasite, the Rhodamine B might be excreted by the same route as other unwanted substances. There is however, little point in further speculation until there is stronger evidence that Rhodamine B passed from the intestine into the excretory system of these worms and did not enter through the excretory pore.

IV THE ENVIRONMENT OF THE PARASITE IN VIVO

In the rest of this thesis, the term "small intestine" is used to refer to the jejunum and ileum.

4.1 Methods

i. Collection of samples

Rabbits were killed by a quick blow behind the head and their abdomens opened. Haemostats were placed at either end of the stomach duodenum and small intestine. These regions were excised and their contents expelled into tubes and centrifuged at 1,500 *g* until clear supernatants were obtained. The osmotic pressure, conductivity and content of sodium and potassium of these supernatants were measured. Schopfer (1932) observed that the osmotic pressure of material from the gut of horses changed on standing and Hollansbee (1945) showed that this could be accounted for by the activity of micro-organisms. Samples for analysis were therefore prepared within 30 minutes of the death of the rabbit and stored in a container of ice and salt or a refrigerator at 5°C when not being tested. Their osmotic pressure and conductivity were measured within 5 hours of the death of the rabbit. The samples were then diluted by one part in two hundred and stored at -5°C until the amount of sodium and potassium in them could be determined.

ii. Osmotic pressure determinations

Measurements of osmotic pressure were made with a Fiske model H osmometer. Samples of either 2 ml or 0.2 ml can be tested with this instrument, but whenever possible 2 ml samples were used as it is easier to obtain consistent results using samples of this size. Usually, the stomach and small intestine yielded plenty of fluid, but only small amounts were present in the duodenum and 0.2 ml samples had to be tested. Sometimes large amounts of mucus were present in the duodenum and small intestine and this remained in the supernatant, making results more erratic as the sample tended to freeze spontaneously.

iii. Total concentration of ions

A Phillips conductivity measuring bridge model PR 9500 was used to measure the conductivity of 0.5 ml samples in conjunction with a type PR 9513/00 immersion cell. The conductivity of standard solutions of sodium chloride was also measured and graphs were plotted of conductivity against concentration in milliosmols. The graphs were straight lines. From these, the conductivities of material from the alimentary canal could be read in terms of the equivalent concentration of sodium chloride. Care was taken that both the samples and standards were at the same temperature when their conductivities were measured. This method gave an estimate for each sample of the partial osmotic pressure that was due to electrolytes. Obviously the estimate will not be very accurate, for the non-electrolytes present in gut contents may modify the behaviour of electrolytes by altering the number of particles in the solution. Also, the estimate is based on the assumptions that most of the conductivity will be due to sodium chloride and that

relatively small errors will be introduced by ignoring the differences between the conductivity of this electrolyte and that of others present in small amounts.

To find out whether the use of the conductivity meter was justified, Tyrode's solution and other solutions with an electrolytic composition similar to that of gut fluids, were tested with the osmometer and conductivity meter. The concentration of electrolytes measured by the two instruments in terms of equivalent milliosmols of sodium chloride agreed to within 4%.

iv. Concentration of sodium and potassium

To measure this, an EEL flame photometer was used (Vogel, 1961).

4.2 Results

Table 4.1 shows the means of the results obtained and their standard deviations. Group A consisted of three rabbits that were given food and water until they were killed while the three rabbits in Group B were fasted for 1 hour before they were killed.

Table 4.1 The composition of fluid from the alimentary canal of rabbits

Group	Region	$\Delta f.^{\circ}C$	Total osmotic pressure m.osm	Total conc. of electrolytes equivalent m.osm	Na mmol./l.	K mmol./l.
A	Stomach	0.5554	299±23	475±126	33.62±11.25	20.64±9.69
	duodenum	0.6503	350±20	241±10	90.58±14.48	33.84±9.69
	small intestine	0.6187	333±15	239±13*	102.17±15.57	19.61±4.52
B	stomach	0.5630	303±22	745±81	18.84±3.70	15.78±4.01
	duodenum	0.5816	313±11	233±11*	86.96±19.22	30.44±5.42*
	small intestine	0.5797	312±66*	227±1*	89.71±10.47	25.58±0*

* results obtained from 2 rabbits only

To test whether there was any difference between the rabbits of group A and group B, a t test was carried out on comparisons of the mean measurements from the two groups. The mean total ionic concentration in the stomach was significantly greater at the 5% level of probability in the rabbits of group B, but there were no other significant differences between the two groups. The calculations are given in the Appendix, Tables 7.1, 7.2 and 7.3.

i. The influence of food

To investigate whether the environment of the parasites altered greatly when the host had not fed recently, one rabbit was killed after it had been without food for 12 hours. The results are given in Table 4.2.

Table 4.2 The composition of fluid in the alimentary canal of a rabbit which had been fasted for 12 hours

Region	Osmotic pressure m.osm.	Total conc. of electrolytes equivalent m.osm. Nacl	Conc. of sodium mmol./l.	Conc. of potassium mmol./l.
stomach	286	510	34.78	21.99
duodenum	315	300	142.61	31.20
small intestine	320	245	126.96	30.18

The only appreciable difference between this rabbit and the others examined was the higher concentration of sodium in the duodenal contents.

ii. The effect of a barbiturate anaesthetic

A rabbit was given a lethal dose of Nembutal (pentobarbital sodium). The measurements from this rabbit are given in Table 4.3. Although the conductivity of the material from the stomach was lower than in the other rabbits examined, the anaesthetic appeared to increase the concentration of sodium and potassium and the osmotic pressure in this region. The amount of sodium in the duodenal contents was also increased.

Table 4.3 The contents of the alimentary canal of a rabbit killed with Nembutal

Region	Osmotic pressure m.osm.	Total conc. of electrolytes equivalent m.osm. Nacl.	Conc. of Na mmol./l.	Conc. of K mmol./l.
Stomach	490	330	108.70	66.50
duodenum	306	240	165.22	30.69

iii. Comparison of the stomach, duodenum and small intestine

The mean measurements for material from the stomach and small intestine were compared with those for material from the duodenum. A t test was used to test the significance of the differences and Table 4.4 shows the calculated probabilities. The measurements for rabbits from both groups A and B were pooled for these comparisons. The concentrations of sodium and potassium were significantly lower in the stomach than in the duodenum but the measurements on material from the small intestine did not differ significantly from those for contents of the duodenum.

Table 4.4 Comparison of mean measurements on material from different regions of the alimentary canal

Measurement	Regions compared	
	Stomach and duodenum	small intestine and duodenum
osmotic pressure	$p > 0.10$	$p > 0.7$
sodium content	$p < 0.01^{***}$	$p > 0.4$
potassium content	$p < 0.05^{**}$	$p > 0.05$

4.3 Discussion

i. Osmotic pressure

Measurements made with the osmometer indicated that the osmotic pressure in the three regions studied remains fairly constant at about 300 milliosmols. The conductivity meter readings suggested that about 100 milliosmols of the total osmotic pressure is contributed by non-electrolytes in the duodenum and small intestine. The readings obtained for stomach contents using the conductivity meter suggested that many more ions were present than was indicated by the osmometer. This can probably be explained by the high concentration of hydrogen ions in the stomach. The equivalent conductivity of hydrogen and hydroxyl ions is very much greater than that of any other ions, the equivalent conductivity of hydrogen ions at 18°C is 314 mho. per cm whereas that of sodium ions is 43.4 mho. per cm (Hodgman, 1961). Alternatively the osmometer reading may be lower because of interionic attraction or adsorption of ions by protein molecules. When a current is passed through the fluid, some of these ions may be released. Probably more than one factor is involved, but the appearance of the discrepancy only in tests on stomach contents suggests that it is largely due to the presence of hydrochloric acid.

Altman (1961) quotes the freezing point depression of rabbit serum as 0.592°C. This suggests that the contents of the stomach, duodenum and small intestine were approximately isotonic with serum. Davey (1936) concluded that the contents of the stomach compartments of sheep were isotonic with blood. Similarly, Harpur and Popkin (1965) found that the intestinal contents of fasted guinea pigs were almost

isotonic with serum. They found however that in normally fed guinea pigs the intestinal contents were hypertonic to serum and that the intestinal contents of pigs were generally hypertonic to serum; although the osmolality of the contents may vary with age and diet, the osmotic pressure of duodenal contents was approximately 320 milliosmols, and of fluid from the remainder of the small intestine 380 milliosmols, whereas pig serum had an osmotic pressure of 311 milliosmols. Tables 4.5 and 4.6 give other results which indicate that intestinal contents are generally hypertonic to serum.

Table 4.5 The depression of the freezing point of gut contents

Animal	Δ °C	Author
sheep, abomasum	0.579	Davey, 1936
sheep, intestine	0.82	Schopfer, (1932)
pig intestine	1.02	Schopfer, (1932)
pig, intestine	0.869	Hobson, Stephenson and Beadle (1952)
horse, intestine	0.69	Duval and Courtois, (1928)
horse, intestine	0.75	Schopfer, (1932)

Table 4.6 Depression of the freezing point of serum (Altman, 1961)

Animal	Δ °C
sheep	0.619
pig	0.615
horse	0.564

Digestive secretions are usually isotonic with plasma and probably gut contents tend towards isotonicity. However, even if osmotic pressure is influenced by the composition of the food, the variation in osmotic pressure within the gut may be no greater than that which occurs between rabbits; the rabbit that was fasted for 12 hours did not differ significantly from the others examined. Probably Trichostrongylus retortaeformis is not obviously affected by changes in osmotic pressure between about 270 and 370 milliosmols.

ii. The concentration of sodium and potassium

Measurements with the flame photometer indicated that the concentrations of sodium and potassium were lower in stomach contents than in those from the duodenum and small intestine. This probably reflects the composition of the digestive secretions when the animal is digesting food. Table 4.7 quotes the concentrations of sodium and potassium measured by some workers in the digestive secretions of dogs and cats and those measured by Hobson, Stephenson and Eden (1952) in the contents of the small intestine of pigs. The concentration of potassium in gastric juice does not vary greatly (Gray and Bucher, 1941; Gamble and McIver, 1928a), but that of sodium varies with the rate of secretion and decreases as the hydrogen ion concentration of the juice increases (Grossman, 1963). Gamble and McIver (1928a) found that fasting increased the amount of sodium in gastric juice of a cat from 12.2 mmols per litr 2-4 hours after a meal to 55.7 mmol per litr 18 hours after feeding. The stomach contents of the rabbit which was fasted for 12 hours did not contain appreciably more sodium than those from the other rabbits. Either rabbits are like men and horses and secrete gastric

juice continuously (Dukes, 1955) or 12 hours was too short a period of fasting to cause a detectable change in sodium concentration.

It seems probable that the concentration of sodium in the duodenum and small intestine of a rabbit also fluctuates more than that of potassium; there was more variation between the rabbits examined in the concentration of sodium than that of potassium and fasting a rabbit for 12 hours appeared to influence the concentration of sodium but not potassium in the duodenum.

Table 4.7 The sodium and potassium content of fluid from the alimentary canal

Animal	Fluid	Mean conc. of Na mmol./l.	Mean conc. of K mmol./l.	Author
Cat	gastric juice	12.17	11.5	Gamble & McIver (1928a)
Dog	gastric juice	9.57	9.49	Rosemann, (1907)
Dog[■]	gastric juice	52.8-12.8	7.3	Gray & Bucher (1941)
"	pancreatic juice	148	7	Gamble & McIver (1928b)
"	jejunal secretion	142	6.5	De Beer, Johnston & Wilson (1935)
"	ileal secretion	149	6.3	" "
Pig	intestinal contents	124	26.7	Hobson, Stephenson & Eden (1952)
"[x]	intestinal contents	277	46.8	" "

■ concentration of sodium fell steadily during continuous secretion of gastric juice.

x exceptionally high values recorded from two animals.

Despite its incompleteness, this study of gut contents indicates that third-stage larvae of Trichostrongylus retortaeformis encounter sodium concentrations from about 15-150 mM and concentrations of potassium within the range 10-40 mM.

iii. General conclusions

More detailed information about the environment of Trichostrongylus retortaeformis could be obtained from rabbits with a permanent gastric fistula or partially exteriorised intestine. Samples of the gut contents could then be analysed at frequent intervals and the influence of food assessed. However, the variable nature of the gut contents is already apparent.

Tyrode's physiological saline is frequently used to simulate fluids in the alimentary canal. This has an osmotic pressure of about 240 milliosmols and contains 137 mmol. per litre of sodium and 2.69 mmol. per litre of potassium. This may be improved as a medium for Trichostrongylus retortaeformis if the amount of potassium in it is increased and the osmotic pressure not due to electrolytes is increased by adding rabbit serum.

The measurements obtained with Nembutal suggest that this anaesthetic alters the nature of the fluids in the stomach and duodenum appreciably.

V. PHYSIOLOGICAL ASPECTS OF EXSHEATHMENT

5.1 Methods

In preliminary experiments, larvae were placed in 10^{-4}M hydrochloric acid in an atmosphere of carbon dioxide and nitrogen for 3 hours. No exsheathment occurred when the partial pressure was 300 mm. of mercury or less and, although a few larvae exsheathed if they were in an atmosphere of pure carbon dioxide, the percentage did not increase even if the larvae were left in this for up to 18 hours. It was then discovered that high concentrations of carbonic acid inhibit the actual casting of the sheath; when the atmosphere of carbon dioxide and nitrogen was replaced by air after 1 hour and the larvae were incubated for a further 3 hours in 0.9% sodium chloride over 50% exsheathed.

i. Standard procedure

Approximately 2,500 larvae were placed in a centrifuge tube and 10^{-3}M hydrochloric acid and distilled water were added to give 2 ml. of suspension of pH 4. Carbon dioxide was bubbled through the suspension for 2 minutes and the tubes were stoppered and placed in a water bath at 37°C for 1 hour. At the end of the hour the larvae were washed with 0.9% sodium chloride, being spun at 400 *g* for 1 minute between washings. They were then incubated for a further 3 hours at 37°C.

ii. Estimation of the proportion of larvae exsheathed

Estimates of the proportion of larvae that had exsheathed were made by withdrawing samples with a pasteur pipette and counting the numbers sheathed and exsheathed. If the experiment was replicated two or three times, a hundred larvae were counted in a sample, but if the experiment was repeated four or five times, only fifty larvae were counted. A larva was considered exsheathed if the anterior cap of the sheath had split off, even if the larva had not yet emerged.

iii. Replication of experiments

All trials and replicates in experiments to compare the effects of particular treatments were done with larvae from the same stock and, because Lapage (1935c) found that the age of larvae influenced their capacity to exsheath, the replicates were done within three days. The different treatments in an experiment were tested in a random order. When a factor was tested for an effect on the casting of the sheath as opposed to an effect on the stimulus, the larvae for all the replicates of one treatment were stimulated in the same tube and five samples were withdrawn and treated in separate tubes with the saline that was being investigated. Differences between replicates due to differences in the stimulus were thus eliminated but variance between treatments due to differences in the saline was possibly confounded with variance caused by difference in the stimulus. However care was taken to stimulate all larvae in an experiment similarly and it is unlikely that significant differences between treatments can be explained in terms of differences in conditions in the stimulating medium.

5.2 In vivo experiments

A rabbit was anaesthetised and its stomach exposed. A cellophane sac attached to a piece of glass tubing 3.5 cm long and with a bore of 0.7 cm diameter was inserted into the stomach and secured with a purse string suture drawn tightly round the tubing (see Figure 5.1). Larvae were placed in the dialysis sac and the exposed end of the tubing was closed with a rubber bung. At intervals, samples of larvae were withdrawn and the numbers of sheathed and exsheathed larvae were counted. Figure 5.2 shows the results obtained from two rabbits. A probit analysis of the mean results was carried out as described by Finney (1947). In the second cycle of maximum likelihood calculations, the slope (b = 0.0122) differed by only 0.0001 from that calculated in the first cycle, so that a further approximation was unnecessary. Figure 5.3 shows the probit regression equation $y = 3.935 + 0.0122\,x$. The calculated time taken for 50% of the larvae to exsheath was 88 minutes; with a fiducial probability of 95%, the true value may be expected to lie between 77 and 99 minutes.

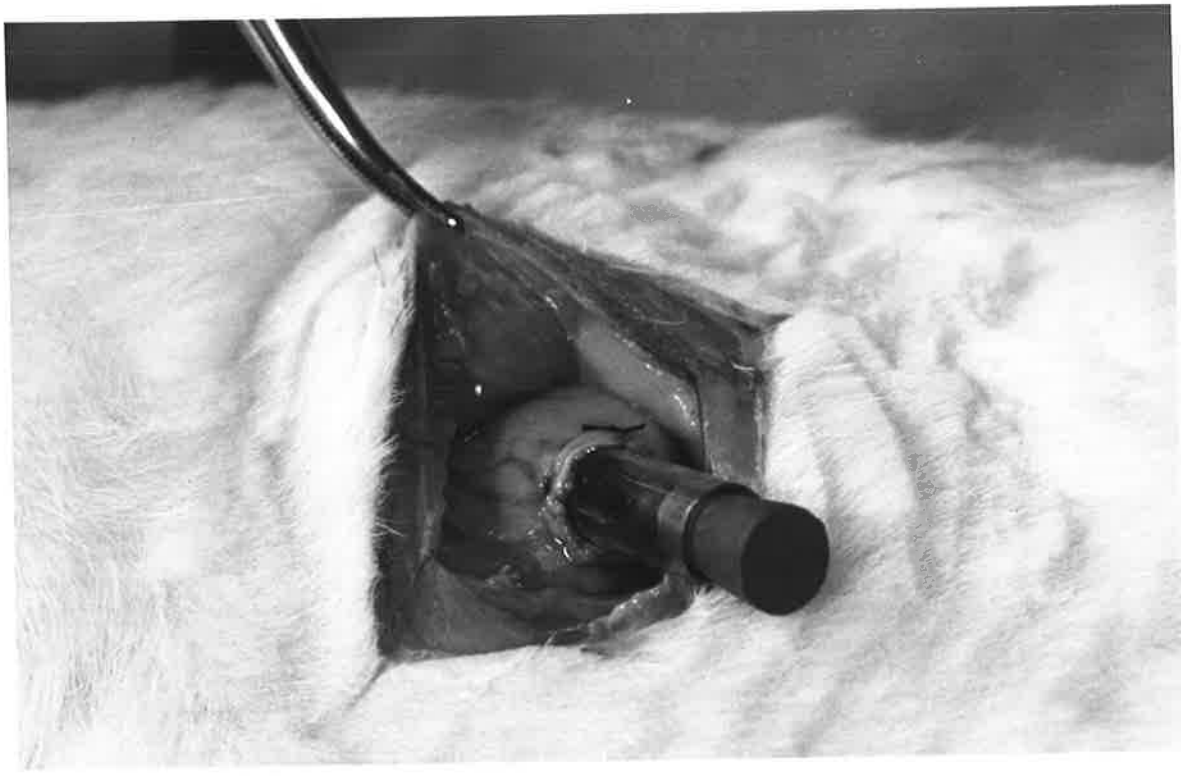
It was suspected that conditions in the stomach normally stimulated larvae to exsheath. To find out whether larvae must pass through the stomach if they are to establish themselves in the duodenum, four rabbits were anaesthetised and the body cavity opened. Distilled water containing about 2,500 infective larvae was injected into the lumen of the duodenum about 5 cm behind the pyloris. The site of injection was marked by sewing a short length of sterile cotton to the gut wall. The same operation was performed on three more rabbits. The larvae injected into these rabbits were stimulated beforehand in

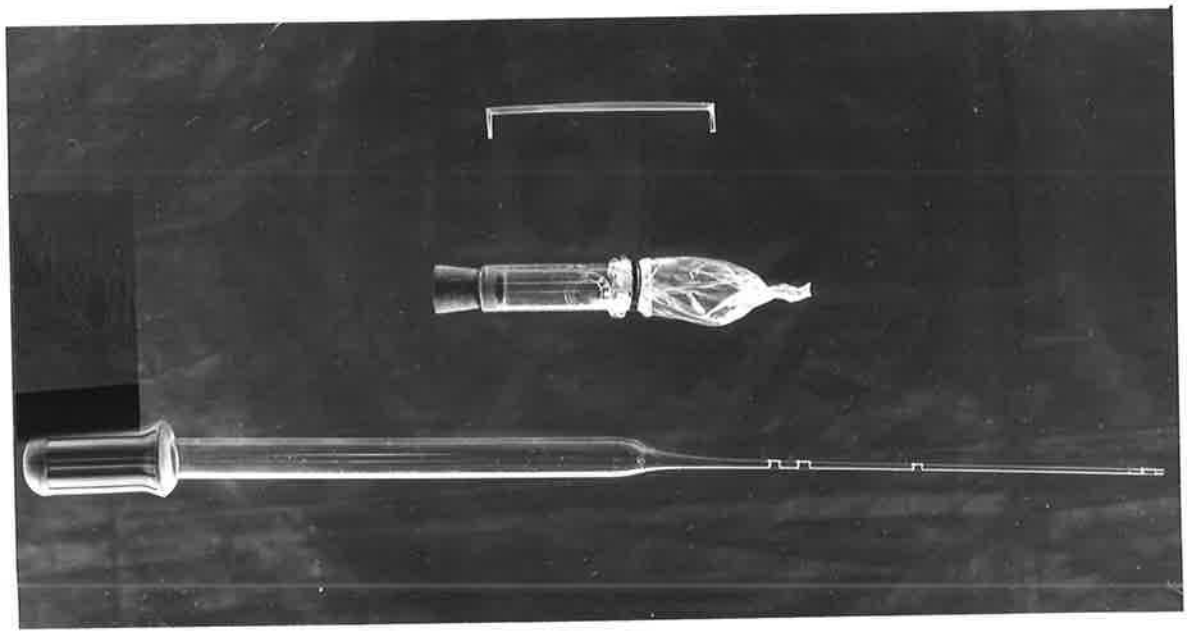
Figure 5.1

Upper photograph : dialysis sac inserted in the stomach of a rabbit.

Lower photograph: the dialysis sac and the pipette used to withdraw samples of larvae.

Scale represents 5 cm.





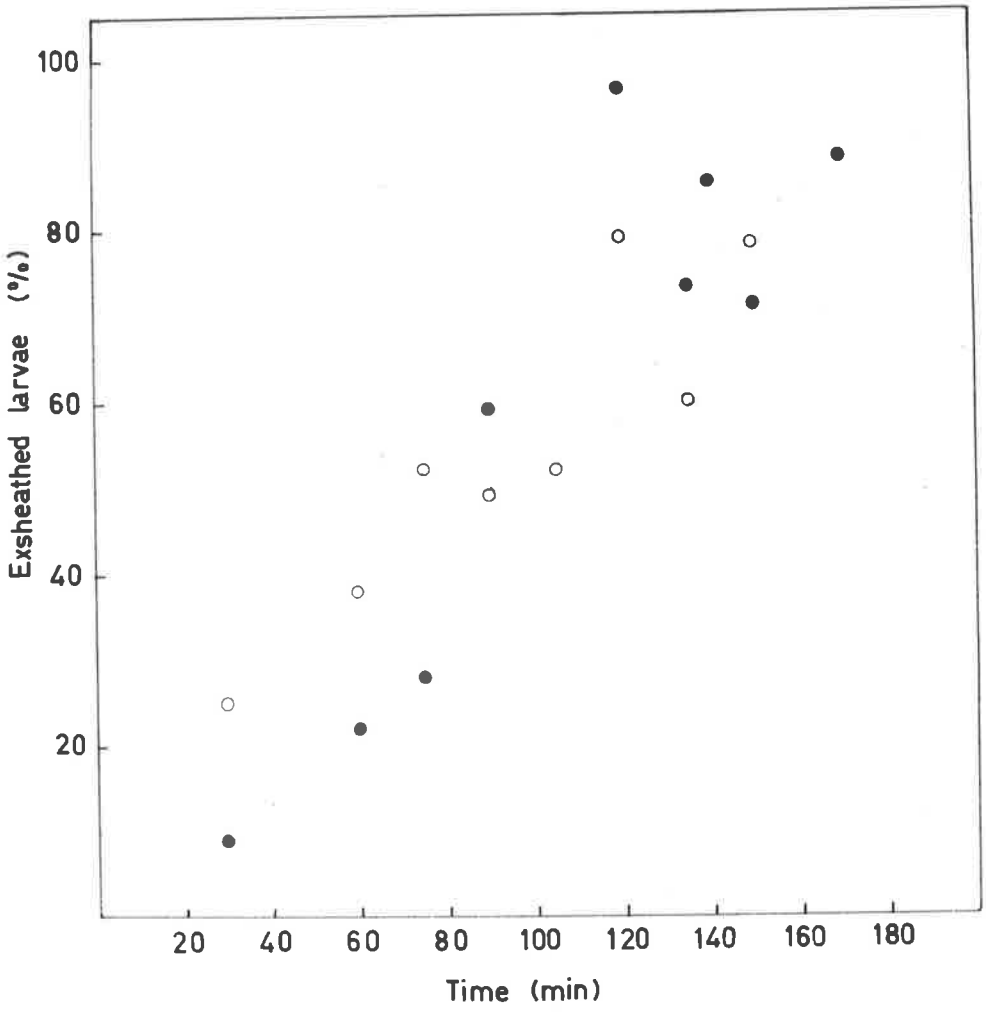


Figure 5.2 Exsheathment of infective larvae in a dialysis sac in the stomach of a rabbit

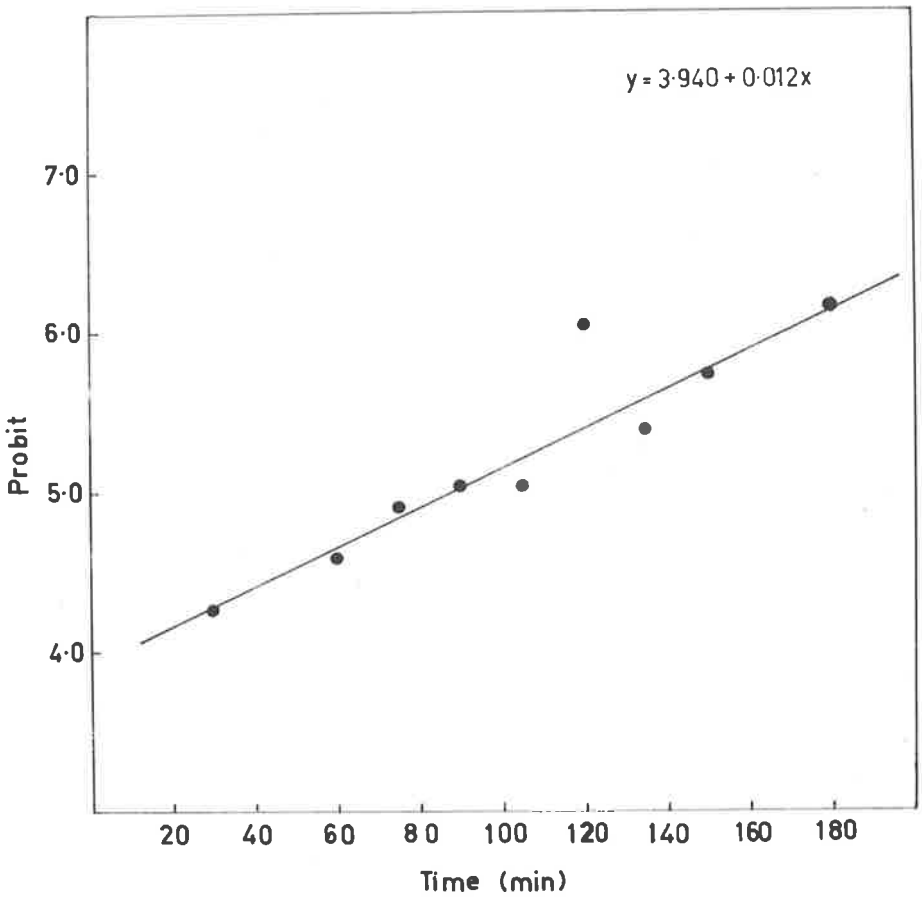


Figure 5.3 Probit regression line for the influence of the environment in the stomach of a rabbit on exsheathment

10^{-4}N hydrochloric acid under an atmosphere of carbon dioxide (see page 64). To check that the larvae used were infective, four rabbits were infected orally with 2,500 larvae from the same stock. All the rabbits became infected; the times taken for eggs to appear in the faeces are given in Table 5.1.

It seemed as if eggs appeared in the faeces a day or two earlier when the rabbit had been injected with stimulated larvae, but the differences were only slight.

Table 5.1 Duration of the prepatent period in rabbits infected by injecting infective larvae into the duodenum

Rabbits infected with exsheathed larvae	Rabbits infected with sheathed larvae
(days)	(days)
11	21
9	16
11	12
	12

This experiment suggested that conditions in the duodenum may also stimulate larvae, however it was possible that regurgitation occurred and that the larvae injected into the duodenum were actually stimulated in the stomach.

Two other experiments were done to test whether larvae could exsheath in the duodenum. In the first experiment, larvae in distilled water were placed in 3 cm of dialysis tubing with a diameter of approximately 1 cm. The ends of the tubing were tied and it was then folded

in two lengthwise and inserted into the duodenum of an anaesthetised rabbit 15 cm beyond the pyloris. After the tubing had been in position for 3 hours, it was withdrawn and the rabbit killed. Samples of larvae were withdrawn from the tubing and examined, but none had exsheathed.

In the second experiment, a rabbit was anaesthetised and haemostats were placed 4 cm apart on the posterior part of the duodenum. A concentrated suspension of infective larvae was injected into the region between the haemostats and at the same time 3 cm of dialysis tubing, closed at both ends and containing larvae from the same stock, was inserted into the stomach. The incision in the wall of the stomach was then closed with sutures. The haemostats were replaced by ligatures and the rabbit was left unaesthetised for 2 hours. At the end of this time samples of larvae were withdrawn from the duodenum and the dialysis tubing in the stomach. The rabbit was then killed. Although 58% of the larvae in the stomach had exsheathed, all those examined from the duodenum were still enclosed in sheaths. The region of the duodenum that contained the larvae became distended with blood and other fluid and this probably made the environment somewhat abnormal. However the results from both these experiments indicate that the factors inducing exsheathment are not present in the duodenum.

5.3 In vitro experiments

i. Exsheathment in contents of the alimentary tract

To obtain fluid from the stomach and duodenum, a rabbit was killed by a blow behind its head and the abdominal cavity was opened. Haemostats were placed at either end of the stomach and duodenum and these regions were excised. To avoid the contents of the two regions

coming into contact with the atmosphere, the haemostat at one end of the stomach or duodenum was placed under paraffin oil in a centrifuge tube before it was removed and the contents of the region were squeezed out. The suspensions were centrifuged at 1,500 g until clear supernatants were obtained and 2 ml volumes of these were withdrawn with a hypodermic syringe containing paraffin oil and added to larvae in about 0.1 ml of distilled water under paraffin oil. Gas mixtures containing different proportions of carbon dioxide, oxygen and nitrogen were bubbled gently through the fluids for 2 minutes and the tubes were then incubated at 39°C for 3 hours. Table 5.2 shows the percentage of larvae that exsheathed in fluid from the stomach after treatment with different mixtures of gasses.

Table 5.2 Exsheathment of infective larvae in fluid from the stomach

	Gas mixture used to saturate the fluid (vol/vol)				
	No gasses added	10% CO_2 90% N_2	10% CO_2 10% O_2 80% N_2	20% CO_2 80% O_2	20% CO_2 10% O_2 70% N_2
Larvae exsheathed (%)	59	39	69	53	60
	50	64	60	54	74

Larvae exsheathed in the fluid from the stomach in all the tubes and it appears that, if factors in the gaseous phase of the environment are important, these were preserved by the paraffin oil, for none of the gas mixtures studied increased the percentage of larvae that exsheathed.

Few larvae exsheathed in the fluid from the duodenum even if they were incubated in it for several hours. In only two tests did more than 10% exsheath; when the partial pressure of carbon dioxide in the gas mixture bubbled through the fluid was equivalent to 152 mm of mercury, 22% of the larvae exsheathed in one tube and 48% in another. This concentration of carbon dioxide probably lowered the pH of the fluid considerably. No larvae exsheathed in fluid from the duodenum that had not had any carbon dioxide bubbled through it.

ii. The relationship between the duration of the stimulus and exsheathment

Assuming that larvae are carried through the stomach with the food, it seems likely that conditions in the stomach must stimulate the larvae to exsheath within 3 hours.

To find the time of stimulation necessary for exsheathment to occur *in vitro*, larvae were incubated in 10^{-4}M hydrochloric acid under an atmosphere or carbon dioxide for 15, 30, 45, 60 or 90 minutes. They were then washed with saline and incubated in this as previously described (page 64). The percentage of larvae that had exsheathed was estimated 3 hours after they were placed in the stimulating medium and the mean results are shown in Figure 5.4.

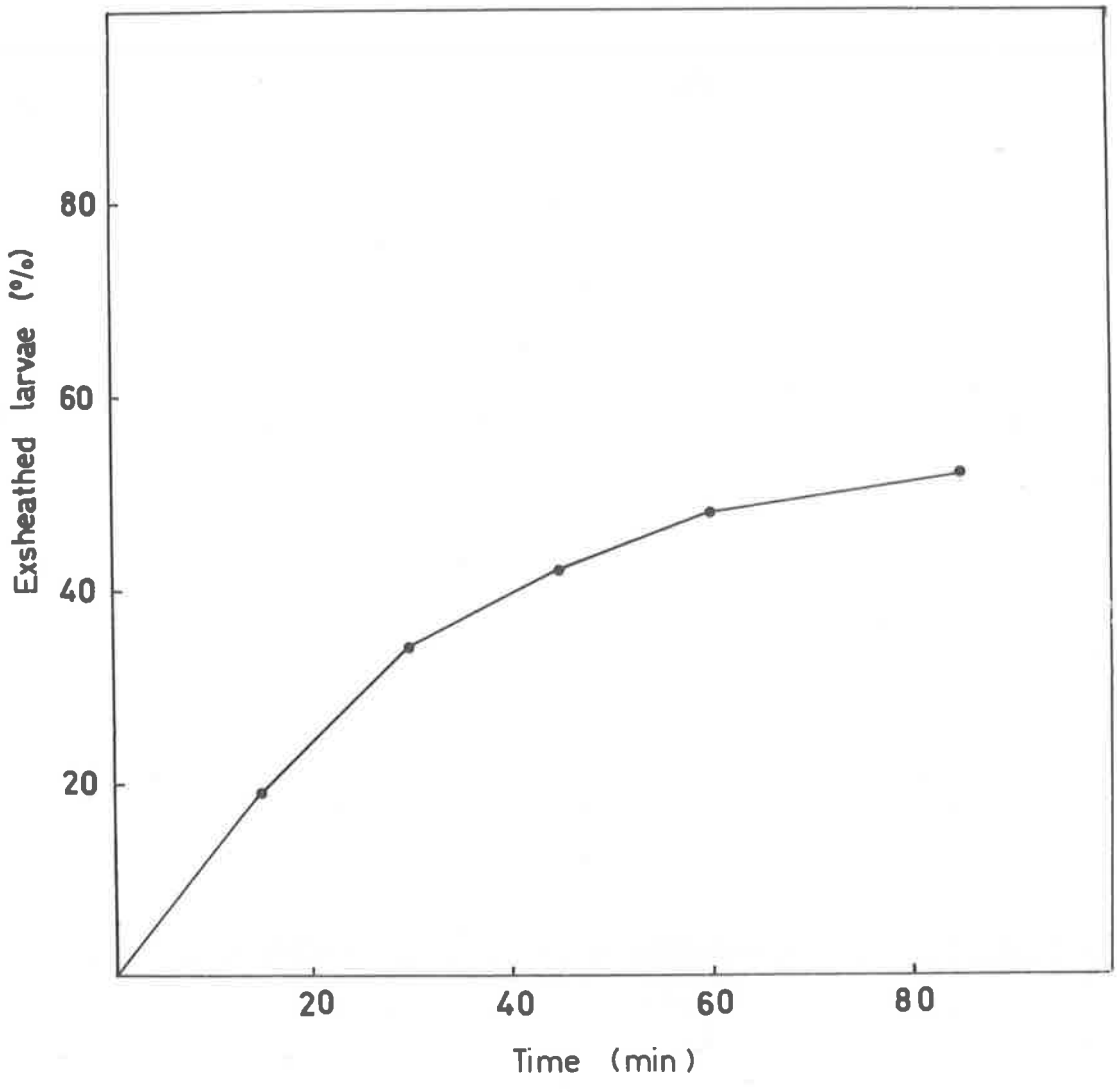


Figure 5.4 The relationship between the duration of the stimulus *in vitro* and exsheathment

An analysis of variance of the results showed that there was a significant difference between the treatments (see Table 5.3). A t test on the comparisons of the means showed that, at the 5% level of probability, stimuli of 45, 60 and 90 minutes did not differ significantly. A stimulus lasting for 30 minutes did not differ significantly from one of 45 minutes, but differed significantly from longer stimuli and also from a stimulus of 15 minutes. It seems therefore that some larvae exsheath when they are stimulated for less than 30 minutes, but that the stimulus must be applied for more than 45 minutes to stimulate exsheathment of the maximum number of larvae.

Table 5.3 Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
total	24	1867.7	77.8	
between treatments	4	1320.5	330.1	12.0
within treatments	20	547.2	27.4	

The duration of the stimulus did not seem to alter the time the larvae took subsequently to cast their sheaths (see Figure 5.5).

iii. The effect of carbon dioxide

Larvae were stimulated in 10^{-4}M hydrochloric acid under atmospheres containing carbon dioxide and nitrogen in various proportions. It was assumed that a solution with a pH of 4 would contain dissolved gaseous carbon dioxide and carbonic acid which would be largely undissociated. Consequently the gas mixtures used would not alter the

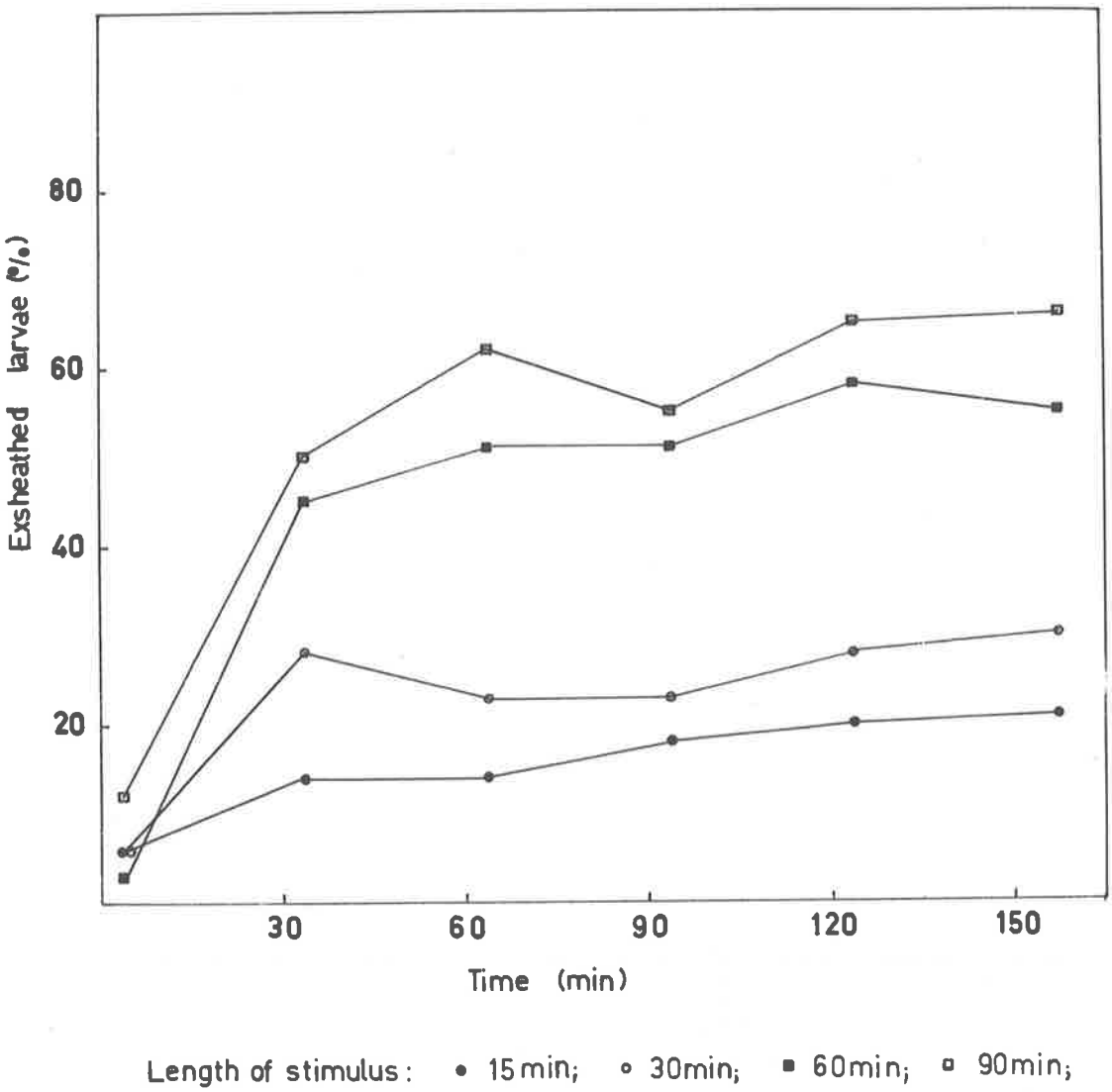


Figure 5.5 The time relations of the stimulus <u>in vitro</u>; the abscissae are the number of minutes larvae were incubated in 0.9% sodium chloride after they had been stimulated.

pH of the solution appreciably. For convenience, the term carbonic acid will be used in this section to include both the dissolved gaseous carbon dioxide and the undissociated acid, as the effects of these two components could not be separated.

Gas mixtures containing 5, 10, 25, 50 and 100% carbon dioxide (vol/vol) and nitrogen were studied and the mean results are shown in Figure 5.6.

An analysis of the results showed that the treatments differed significantly (see Table 5.4).

Table 5.4 Analysis of variance

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	4	4272.0	1068.0	75.74***
within treatments	5	70.7	14.1	
total	9	4342.7	482.5	

A t test on comparisons of the mean percentages of larvae that exsheathed with the different concentrations of carbonic acid showed that at the 5% level of probability the three greatest concentrations did not differ significantly in their effects. A carbonic acid concentration of 2.4×10^{-3}M stimulated significantly more larvae to exsheath than a concentration of 1.2×10^{-3}M but significantly fewer than a concentration of 6.0×10^{-3}M. This indicates that increase in

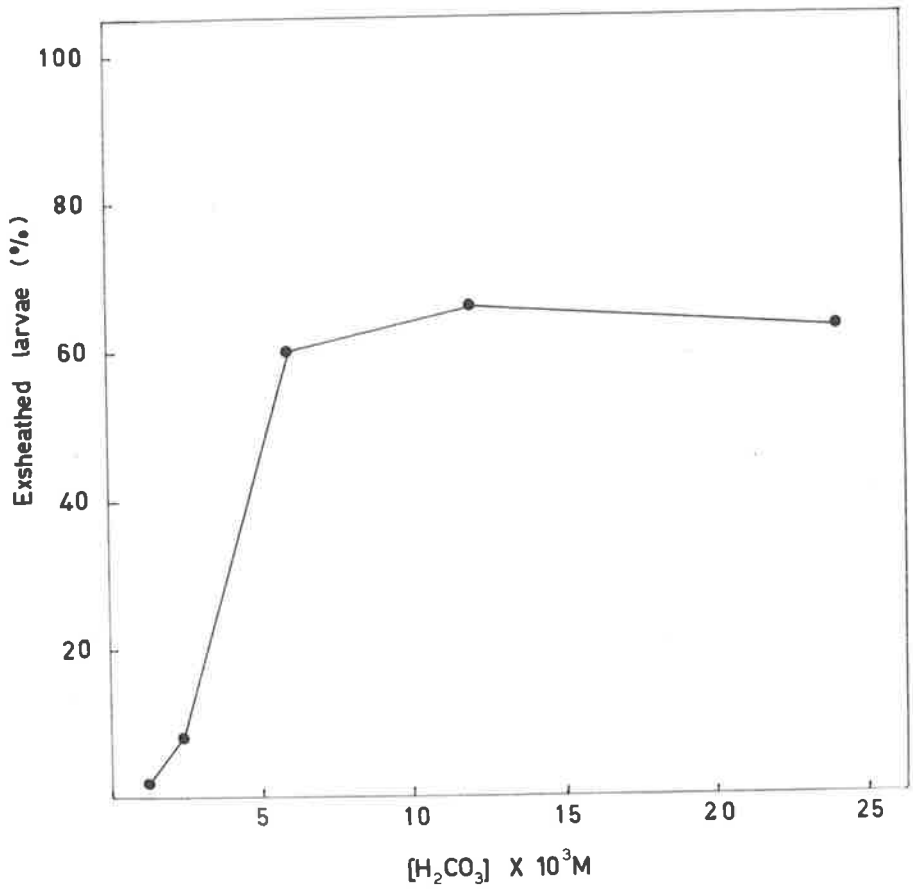


Figure 5.6 The influence of carbon dioxide or its derivatives on exsheathment.

the concentration of carbonic acid stimulates an increasing number of larvae to exsheath until a maximum number are stimulated at a concentration of approximately 6.0×10^{-3}M.

Gas mixtures containing carbon dioxide and oxygen were also used to stimulate larvae and the results were compared with those obtained using mixtures of carbon dioxide and nitrogen. It seems that a low oxygen tension is not an important part of the stimulus as the substitution of oxygen for nitrogen did not appreciably alter the percentage of larvae that exsheathed.

iv. The effect of temperature

The influence of temperature on both the stimulus for exsheathment and the casting of the sheath was studied. Larvae were stimulated at various temperatures by incubating them for an hour in 10^{-4}M hydrochloric acid under an atmosphere of carbon dioxide. The temperatures studied were 15, 25, 30, 37, 40 and 50°C. The larvae were subsequently washed and incubated in the standard way. Table 5.5 shows the percentage of larvae that had exsheathed after 3 hours incubation. Only larvae that had been stimulated at either 37°C or 40°C exsheathed, while all the larvae stimulated at 50°C died.

Table 5.5 The effect of temperature on the stimulus for exsheathment

	Temperature (°C)				
	15°C	25	30	37	40
larvae	0	0	0	78	68
exsheathed	0	0	0	50	90
(%)	0	0	0	64	70
	0	0	0	72	88
	0	0	0	80	74

To study the influence of temperature on the casting of the sheath, larvae were stimulated to exsheath at 37°C and then incubated for 3 hours at the temperatures studied in the previous experiment. It seems that temperature is less important during this phase of exsheathment because larvae cast their sheaths at all the temperatures studied except 50°C which was again lethal.

Figure 5.7 shows the mean results and Table 5.6 the analysis of variance.

Table 5.6 Analysis of variance

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	4	4880.7	1220.2	59.2***
within treatments	20	411.5	20.6	
total	24	5292.2	220.5	

A series of t tests on comparisons of the means showed that at the 5% level of probability temperatures of 37° and 40°C did not differ significantly in their effect but that the three lower temperatures led to significantly fewer larvae exsheathing; each increase in temperature significantly increased the percentage of larvae that exsheathed.

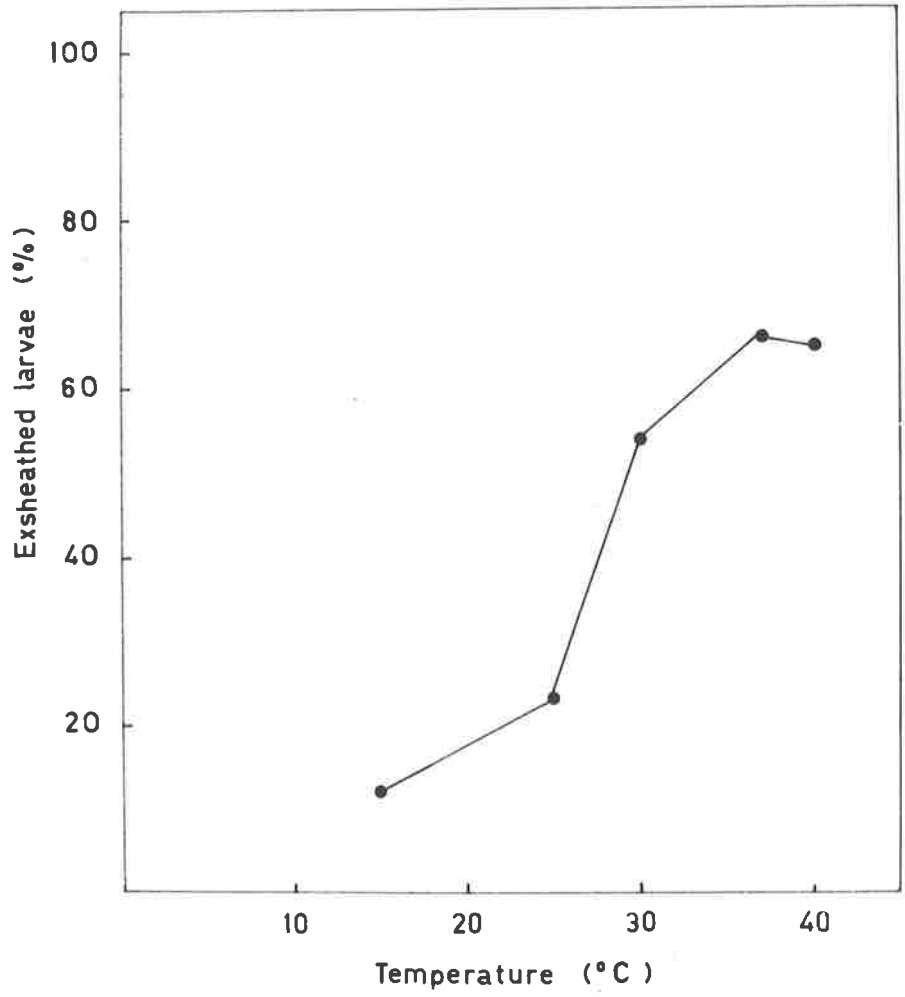


Figure 5.7 The relationship between exsheathment and the temperature of the saline in which larvae were incubated after they had been stimulated at 37°C for 1 hour.

v. The effect of pH

Larvae were stimulated in solutions with a pH of 2, 3, 4 or 6 under an atmosphere containing carbon dioxide and nitrogen. Some larvae were stimulated in a bicarbonate buffer of pH 6 under an atmosphere which contained 52% (vol/vol) carbon dioxide. This gas mixture was made up in the laboratory (see page 17). Other larvae were stimulated in 10^{-4}, 10^{-3}, or 10^{-2}M hydrochloric acid under an atmosphere containing 40% carbon dioxide. The bicarbonate buffer was made up according to Umbreit, Burris and Stauffer (1957) so that the concentration of undissociated carbonic acid would approximately equal that in the hydrochloric acid solutions. It was assumed that in the latter solutions all the carbonic acid present would be undissociated (Davis, 1961). Sodium chloride was also added to the bicarbonate buffer so that the concentration of chloride was approximately the same as that in 10^{-4}M hydrochloric acid.

The mean results and an analysis of their variance are given in Figure 5.8 and Table 5.7 respectively. The hydrogen ion concentration seemed to be an important part of the stimulus because when the pH was 6, very few larvae exsheathed. A series of t tests on comparisons of the means showed that at the 5% level of probability the other three concentrations of hydrogen ions differed significantly in their effects, the solution of pH 3 inducing the greatest percentage of exsheathed larvae.

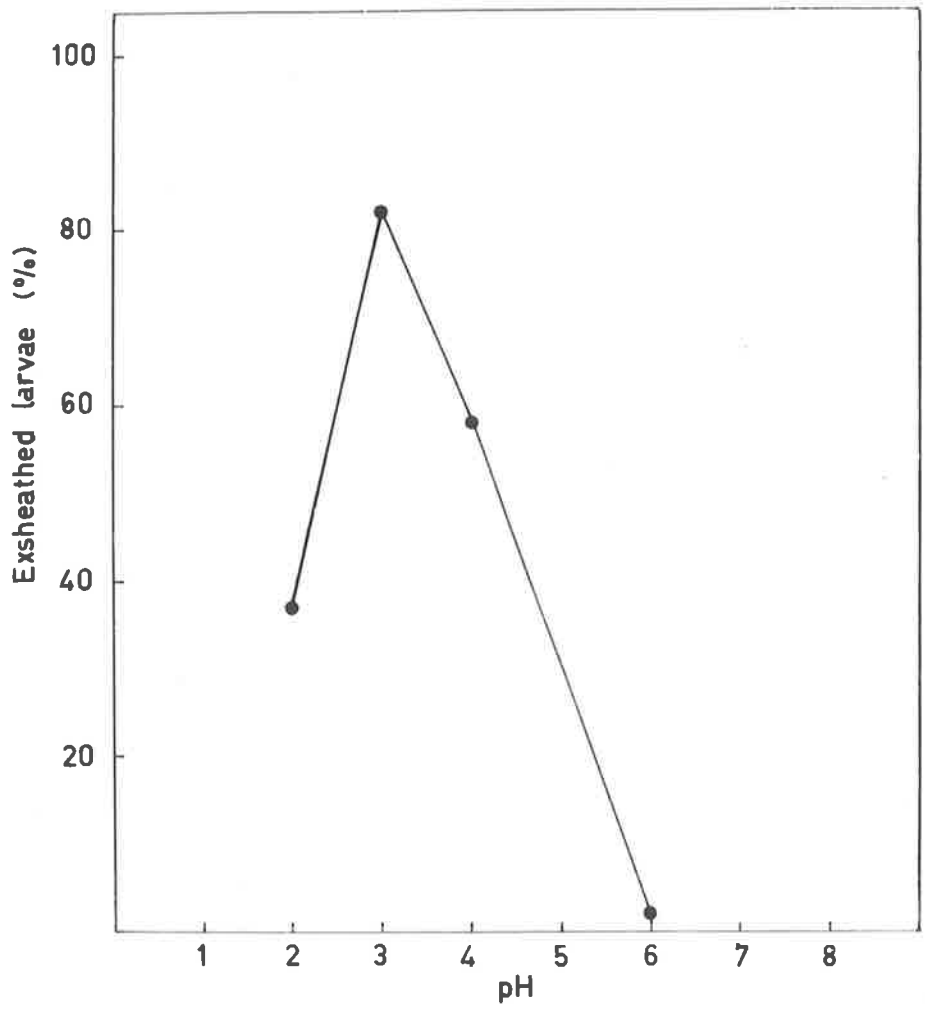


Figure 5.8 The influence of the pH of the stimulating medium on exsheathment.

Table 5.7 Analysis of Variance

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	3	9930.6	3310.2	112.2***
within treatments	16	472.3	29.5	
total	19	10402.9	547.5	

It was thought that hydrogen ion concentration might also influence the casting of the sheath. To investigate this, larvae were stimulated in the standard way and then washed and placed in saline that had been brought to a pH of 4 or 6 by adding normal hydrochloric acid or to a pH of 8 by adding normal sodium hydroxide. Neutral saline was also used. The larvae were incubated in saline for three hours and the percentage of larvae exsheathed in the different salines were estimated by the usual method.

The mean results are shown in Figure 5.9 and an analysis of their variance is given in Table 5.8. A t test on comparisons of the means showed that at the 5% level of probability the salines with a pH of 6 and 7 did not differ significantly. Significantly more larvae exsheathed when the pH was 4 and significantly fewer when the pH was 8.

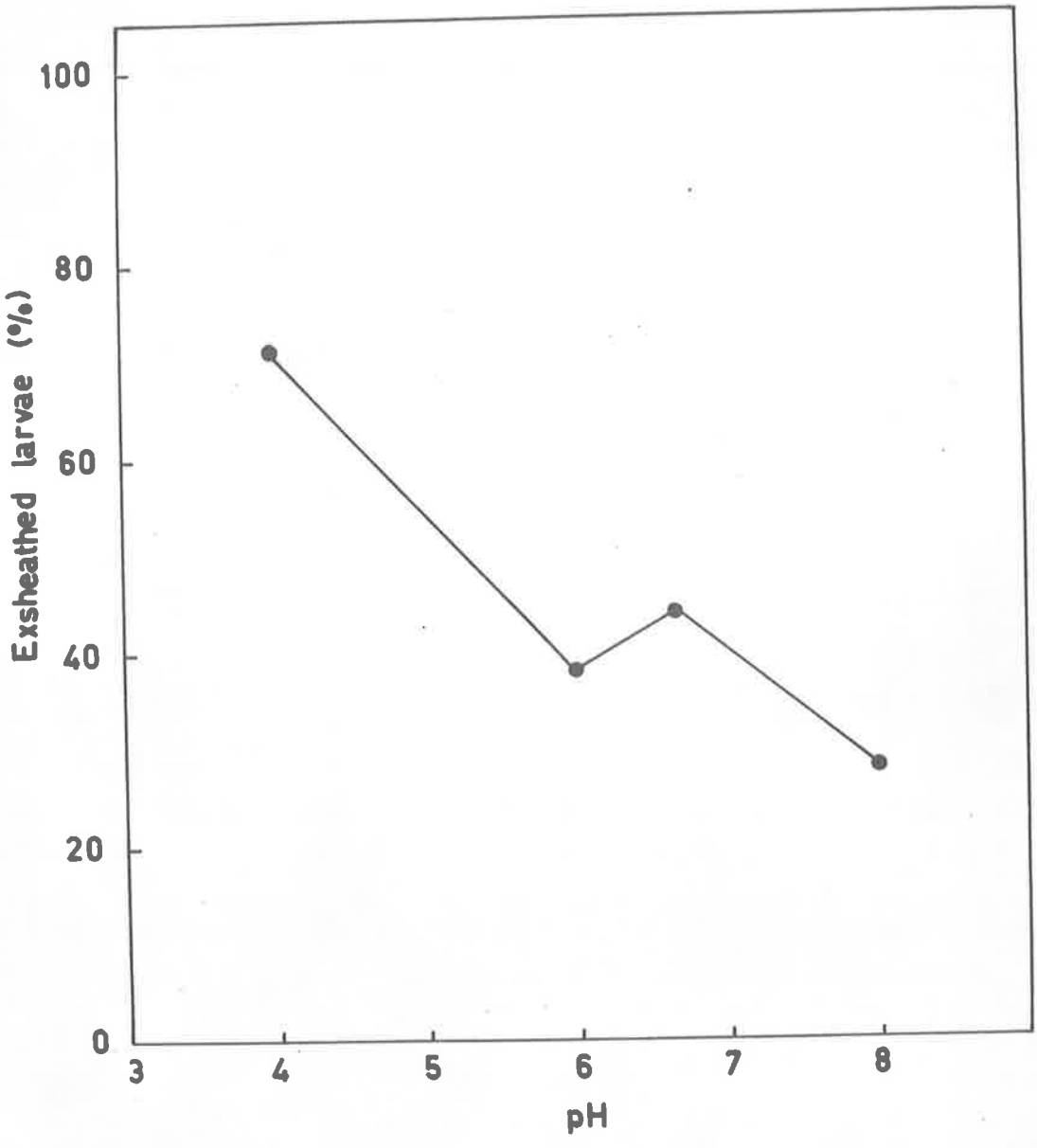


Figure 5.9 The influence of pH on the actual casting of the sheath; larvae were stimulated for 1 hour and then incubated for 3 hours in saline containing various concentrations of hydrogen ions.

Table 5.8 Analysis of variance

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	3	1768.6	589.5	32.6***
within treatments	16	290.0	18.1	
total	19	2058.6	108.3	

vi. The effect of reducing agents

To find out whether a low redox potential constituted part of the stimulus for exsheathment, larvae were stimulated in a medium of pH 4 which contained hydrochloric acid and 0.05 M cysteine. They were incubated at 37°C for an hour under an atmosphere of carbon dioxide and were treated subsequently in the standard way. However, comparison with a control in which larvae were stimulated in the absence of cysteine gave no evidence that cysteine enhanced or diminished the stimulus. The stronger reducing agent, sodium dithionite, could not be tested at the high concentration of hydrogen ions needed for these experiments.

In another experiment larvae were stimulated and then half were washed in saline to which cysteine and 1.0 N sodium hydroxide had been added so that the saline contained 0.05 M cysteine and had a pH of 6. The remainder of the larvae were incubated in saline which had been acidified to pH 6 with 1.0 N hydrochloric acid.

Table 5.9 shows the percentage of larvae that exsheathed in five replicates and these indicate that a low redox potential favours the casting of the sheath.

Table 5.9 The influence of cysteine on the casting of the sheath

	0.9% NaCl	0.9% NaCl + 0.05 M cysteine
Percentage of exsheathed larvae	44	72
	36	76
	44	74
	32	64
	54	84

vii. The effect of osmotic pressure

Solutions containing different amounts of sodium chloride were made up in 10^{-3}M hydrochloric acid and their osmolality checked. To test whether osmotic pressure exerted an influence on the stimulus, larvae were washed and incubated in these solutions at 37°C under an atmosphere of carbon dioxide. After an hour, they were washed with neutral 0.9% sodium chloride and incubated in this for a further 2 hours before the percentage of exsheathed larvae was estimated. Some larvae were also stimulated in a solution which contained 0. 02M potassium chloride in addition to sodium chloride and had an osmotic pressure of 100 milliosmols. It was expected that the concentration of potassium ions in this solution was approximately equal to that in the stomach (see page 63). Table 5.10 gives the results.

Table 5.10 The relationship between osmotic pressure and the stimulus for exsheathment

	Solution					
	NaCl + HCl 100 m.osm.	NaCl + HCl 300 m.osm.	NaCl + HCl 400 m.osm.	NaCl + HCl 750 m.osm.	NaCl + KCl + HCl 100 m.osm.	HCl
exsheathed larvae (%)	52	44	58	52	36	42
	55	54	60	46	44	44
	44	40	48	40		
mean	50	46	55	46	40	43

To test whether osmotic pressure influenced the casting of the sheath, larvae were stimulated then washed and incubated for a further 2 hours in sclutions containing various amounts of sodium chloride. The effect of a 100 milliosmolal solution containing potassium in addition to sodium was again compared with that of 100 milliosmolal sodium chloride. Table 5.11 shows the percentage of larvae that had exsheathed after they had been in saline for 2 hours.

Table 5.11 The effect of osmotic pressure on the casting of the sheath

	Solution				
	100mosm. NaCl	100 m.osm. NaCl + KCl	300 m.osm. NaCl	400 m.osm. NaCl	750 m.osm. NaCl
exsheathed	26	86	83	95	98
larvae	95	90	89	95	92
(%)	90	82	96	88	98
mean	70	86	89	93	96

The results do not indicate that osmotic pressure is important either in stimulating larvae or in encouraging them to cast their sheaths. Nor do they indicate that 0.02 M potassium chloride influences exsheathment.

viii. Other treatments investigated

Crofton (1947) exsheathed larvae of Trichostrongylus retortaeformis in hydrochloric acid containing 0.2% pepsin and Silverman and Podger (1964) found that this enzyme favoured exsheathment of Dictyocaulus viviparus and Trichostrongylus colubriformis; the influence of pepsin and trypsin on both the stimulus and the casting of the sheath was therefore investigated. Some larvae were incubated in 10^{-4}M hydrochloric acid containing 0.2% pepsin (wt/vol) under an atmosphere of carbon dioxide. They were treated subsequently in the standard manner. Others were stimulated normally and then incubated for 3 hours in 0.9% sodium chloride which contained 0.2% pepsin or trypsin. The saline which contained pepsin was brought to a pH of 3 by adding

1.0 N hydrochloric acid. The effect of trypsin on the stimulus could not be examined because its optimum pH is 7.8 and the larvae do not exsheath when stimulated at a pH greater than 6 (see page 82).

The results were compared with those from controls in which the larvae were treated similarly but the enzymes were either absent or had been inactivated by heat before they were added. Under the conditions of these experiments, there was no indication that either enzyme influenced exsheathment.

It was thought that the enzyme carbonic anhydrase might play a part in the response of larvae to carbon dioxide. Sodium acetazoleamide ("Diamox") which specifically inhibits the enzyme, was neutralised with 1.0 N hydrochloric acid and added to the stimulating medium so that this contained 0.5 mg per ml. However no inhibition of exsheathment was apparent when the results were compared with controls lacking sodium acetazoleamide.

5.4 Discussion

i. Exsheathment in vitro

The results obtained in this study are similar to those obtained with other species of the Trichostrongyloidea. Larvae exsheath after they have been exposed to high concentrations of undissociated carbonic acid and hydrogen ions at temperatures in the range 37° to 40°C, but these conditions need not persist throughout the process; when larvae are examined after they have been in such an environment for 45 minutes, few have a refractile ring in the cuticle but after they have been incubated for a further 2 hours in 0.9% sodium

chloride over 50% have cast their sheaths. Assuming that the process of exsheathment *in vitro* is essentially the same as that *in vivo*, this supports the hypothesis that the host provides a stimulus and the larvae produce the substance which attacks the sheath.

Most trichostrongyloids that have been exsheathed *in vitro* in physiological media have responded to carbon dioxide or its derivatives. The exceptions reported are *Dictyocaulus viviparus* (Silverman and Podger, 1964) and *Nematodirus battus* (Christie and Charleston, 1965). *Trichostrongylus retortaeformis* larvae required relatively high concentrations of undissociated carbonic acid, concentrations greater than 6×10^{-3}M stimulating significantly more larvae than lower concentrations. The relative importance of undissociated carbonic acid and dissolved gaseous carbon dioxide cannot at present be assessed, but dissociated carbonic acid is unlikely to be important in the stimulus; firstly, the pH of the solutions which stimulated larvae was such that very little, if any, carbonic acid would be dissociated; secondly, carbonic anhydrase does not seem to be involved in exsheathment and this enzyme catalyses the hydroxylation of carbon dioxide, forming bicarbonate (Davis, 1961).

Temperature seems to be an important part of the stimulus for no larvae exsheathed after they had been stimulated at 30°C or lower temperatures. It influenced the actual casting of the sheath less markedly, although a higher percentage of larvae exsheathed when the saline was at 37° or 40°C. Rogers and Sommerville (1960) also found that low temperatures had a more marked effect on the stimulus than on the subsequent casting of the sheath.

Larvae were sensitive to the concentration of hydrogen ions, very few exsheathing unless this was greater than 10^{-6}M. This was observed even when the concentration of undissociated carbonic acid was kept constant, indicating that pH influenced the larvae directly rather than indirectly by altering the concentration of undissociated carbonic acid. Although an increase in the concentration of hydrogen ions favoured exsheathment, many larvae died when the pH was as low as 2.

Reducing agents influence the exsheathment of larvae of some species, but their effect is more marked when the hydrogen ion concentration falls and the concentration of undissociated carbonic acid is relatively low. Rogers (1960) observed this with Trichostrongylus axei and Haemonchus contortus, while Trichostrongylus colubriformis larvae, which are stimulated in 10^{-3}M hydrochloric acid containing about 5×10^{-3}M undissociated carbonic acid, did not exsheath more readily if a reducing agent was added. Consequently it is not surprising that Trichostrongylus retortaeformis larvae, which have similar requirements to Trichostrongylus colubriformis, are not influenced by the addition of cysteine to the stimulating medium, although a higher percentage cast their sheaths when cysteine was added to the saline.

The experiments of Crofton (1947) indicate that pepsin induces exsheathment but neither pepsin nor trypsin seemed to be important in my experiments in vitro. Also, the larvae that exsheathed in cellophane sacs in the stomach should not have encountered pepsin as the mean pore size of the dialysis tubing used was 24 Å and its permeability is unlikely to have been altered during the 3 hours it was in the stomach.

Possibly in Crofton's experiments, the high concentration of hydrogen ions stimulated the production of small amounts of exsheathing fluid and this altered the composition of the sheath, making it susceptible to attack by pepsin. Alternatively, pepsin may be able to attack the sheath but does so more slowly than exsheathing fluid.

Taylor and Whitlock (1960) found that some salts were important in exsheathment of Haemonchus contortus. The effect of a salt seemed to depend on its nature rather than the osmotic pressure which it exerted. Oxy-acid salts influenced exsheathment more than neutral salts and it was suggested that they acted through their ability to catalyse the reaction

$$H_2O + CO_2 \rightleftharpoons H_2CO_3$$ (Roughton and Booth, 1938).

Rogers (1960) also observed that the addition of sodium chloride enhanced exsheathment, particularly that of Trichostrongylus colubriformis. However, Trichostrongylus retortaeformis did not seem to be sensitive to concentrations of sodium chloride between 100 and 750 milliosmols (approximately 0.05-0.4 Molal) and would exsheath even when the stimulating medium was replaced with distilled water rather than the customary 0.9% sodium chloride.

ii. Exsheathment in vivo

Sommerville (1957) suggested that larvae are stimulated in the region of the alimentary canal immediately anterior to that in which they develop to maturity. This is true of Trichostrongylus retortaeformis. About 80% of the larvae in cellophane sacs implanted in a rabbit's stomach exsheathed within 3 hours. This implies that most larvae

ingested by the host will have been stimulated when they reach the duodenum. However even if they pass through the stomach too rapidly to receive an adequate stimulus, they may later re-enter it; very few larvae exsheathed in fluid from the duodenum, yet when ensheathed larvae were injected direct into the duodenum, some developed to maturity, suggesting that they had been regurgitated into the stomach.

Little is known about the conditions in the stomach of a rabbit, but probably the temperature and high concentrations of hydrogen ions and undissociated carbonic acid that stimulate exsheathment *in vitro* also induce exsheathment *in vivo*. Thus, the body temperature of a rabbit fluctuates, but probably lies within the range 37°-40°C (Kanitz, 1925), which is necessary for exsheathment *in vitro*. The hydrogen ion concentration also varies but appears always to be greater than 10^{-6}M (Redman, Willemot and Wokes, 1927; Griffiths and Davies, 1963; Beauville and Raynaud, 1963). Consequently it could form part of the stimulus.

It seems less certain that the high concentrations of undissociated carbonic acid that stimulated the maximum number of larvae *in vitro* occur in the stomach of a rabbit; carbon dioxide tensions of only 40-60 mm of mercury were measured by Campbell (1933). Another indication that concentrations greater than 6×10^{-3}M are not encountered in the stomach is that, *in vitro*, these concentrations inhibit the actual casting of the sheath whereas the experiments *in vivo* indicate that larvae can exsheath within the stomach. This

may imply that some other factor not investigated, is important in the stimulus. Alternatively, if all other conditions are optimal, the majority of larvae may be stimulated by lower concentrations of carbonic acid.

Nevertheless, it is possible that the concentration of undissociated carbonic acid increases during coprophagy; Griffiths and Davies (1963) give evidence for the fermentation of carbohydrates by the bacteria in soft pellets and consider that the fundus may be analogous to the rumen of sheep and cattle.

VI. CONCLUSIONS

6.1 The life cycle of nematodes

In some respects, the life cycles of nematodes resemble those of arthropods. In both these groups of animals, development is interrupted by moults. Frequently the development of arthropods is suspended during a dormant period, or diapause. During this, metabolism is reduced and resistance to adverse climatic conditions, such as extremes of temperature and drought, increases. Similarly, development is suspended in the dauer larvae of some free-living nematodes and in the infective stage of parasitic species; dauer larvae and infective eggs or larvae have small energy requirements and are more resistant to the environment than earlier stages in the life cycle (Fairbairn, 1960; Rogers and Sommerville, 1963).

The main difference between the life cycles of nematodes and those of arthropods is that arthropods usually have a metamorphosis, when profound changes in their morphology occur, whereas all the postembryonic stages in the life cycle of nematodes are morphologically similar.

Metamorphosis frequently occurs when an organism leaves one environment and enters a different one. Thus, many free-living animals live in one type of environment as adults, but have a larval stage which is adapted to live in a different type of environment. The larvae may be aquatic and the adults terrestrial, or the larvae may live in freshwater and the adults in the sea. During metamorphosis,

larvae lose those features of the their anatomy which were adapted to life in the environment they are about to leave and attain the adult morphology, better adapted to life in the second environment.

The infective stage of nematodes might be expected to metamorphose when it infects the host, for it enters a completely different environment; as a free-living organism, it must be able to resist extremes of temperature and desiccation whereas, inside the host, these harmful factors will be replaced by others such as the antibodies, anti-enzymes and tissue responses of the host. Profound changes in the morphology of many parasites take place when they infect the intermediate or final host; for example, the free-living miracidia of trematodes lose their cilia and become immobile sporocysts when they enter the molluscan intermediate host. No such morphological changes take place in larvae of nematodes when they enter the host.

Although the most obvious changes in an animal during metamorphosis are morphological, profound biochemical and physiological changes probably also occur. Rogers and Sommerville (1963) have suggested that the biochemical changes which must take place in a nematode when it enters the host are comparable to those which take place during metamorphosis.

Metamorphosis in arthropods is always associated with a moult. The physiological metamorphosis of nematodes may also be associated with a moult, for it seems that the moult which precedes the formation of the infective stage is incomplete; in some species which infect the host as an egg, the larva is in the second stage but is still enclosed in the cuticle of the first-stage larva (Alicata, 1935); similarly the

infective third-stage larva is surrounded by the sheath, which represents the cuticle of the second-stage larva. The moult is completed after the nematode has entered the host.

6.2 The control of moulting and metamorphosis

Growth and metamorphosis seem generally to be controlled by an endocrine system. In amphibians and teleosts, a hormone is released from the anterior lobe of the pituitary. This stimulates the thyroid gland to produce another hormone, to which the various tissues involved in metamorphosis respond. Similarly, in insects, parts of the brain release a neurosecretion which stimulates the prothoracic glands to release the moulting hormone, ecdysone. Moulting in insects is also influenced by the juvenile hormone, secreted by the corpora allata. When juvenile hormone is present in the blood, metamorphosis is inhibited and another larval stage is formed. When the corpora allata stop producing juvenile hormone, larvae of hemimetabolous insects moult and become adults; those of holometabolous insects pupate and subsequently moult to become adults. Ametabolous insects do not have a marked metamorphosis and little is known about the process which controls moulting in them, but probably a similar system operates.

The control of growth and metamorphosis by an endocrine system in such widely separated groups of animals as the Arthropoda and Vertebrata suggests that development of nematodes may be influenced by such a system. Rogers (1962) has suggested that the mechanism which controls moulting may be analogous with that in insects. The development of free-living nematodes is influenced by such factors in the

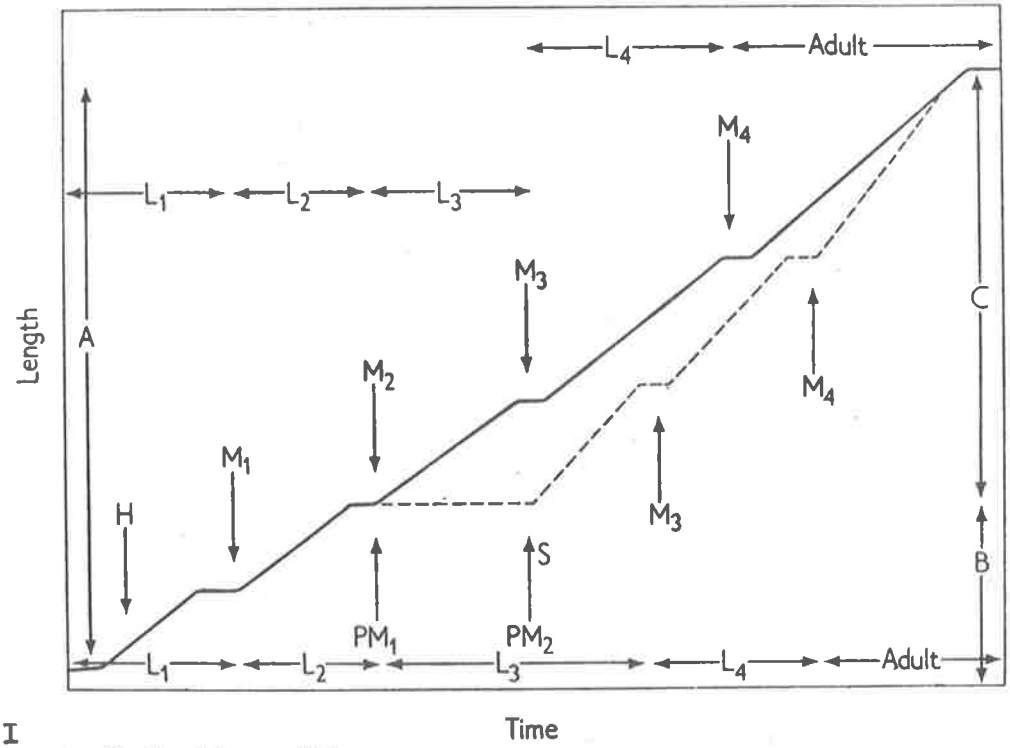


Fig.6.I An idealized form of the basic life cycle of nematodes (from Rogers, 1962). The life cycle of a free living nematode is represented by a solid line. Hatching (H) is "spontaneous" and there are four moults (M_1–M_4). The broken line represents a life cycle in which a change in environment is necessary to stimulate (S) the completion of the second moult (PM_2). A, B and C are different environments.

environment as temperature and food supply but it may also be controlled by internal secretions. Figure 6.1 shows the development of a hypothetical free-living nematode.

The development of each larval stage consists of a period of rapid growth, when some cell division and differentiation may also take place, and then a lethargus. During the lethargus, neurosecretory substances may be released into the tissues and induce the changes associated with a moult. The first three moults may also be influenced by a secretion analogous with the juvenile hormone of insects. This would delay the onset of maturity until after the final moult.

6.3 The influence of the host on development of parasitic nematodes

In parasitic nematodes, part of the mechanism which controls moulting may be lost when the infective stage is formed. The parasite may rely on the host to supply the missing part. Development will consequently be suspended until the infective stage has entered the host.

The host may induce exsheathment of infective larvae in one of several ways; (i) it may stimulate larvae to produce the internal secretion which induces moulting; (ii) it may supply some missing internal secretion or (iii) it may act on the tissues which respond to the internal secretion, supplying substrates for metabolic processes or enzymes which attack the sheath. In those species of nematodes which infect the host as an egg, the host may induce hatching in a similar way (see Figure 6.2).

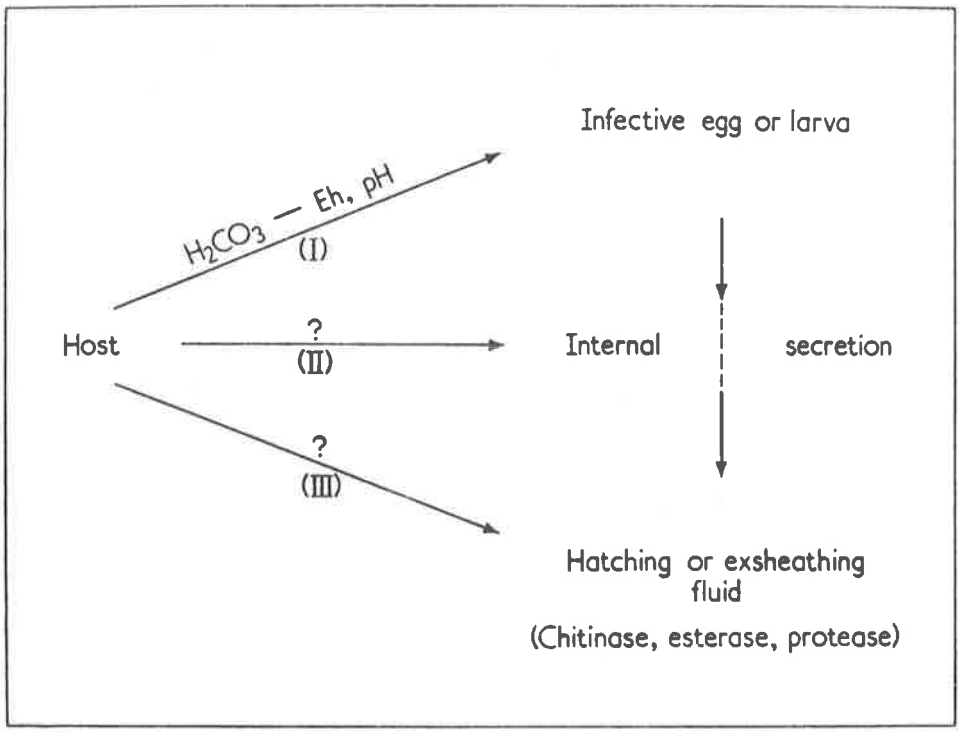


Fig. 6.2 Host-parasite relationships in the process of infection. The host may start infection by providing an environment (I) which stimulates the infective agent to produce, directly or indirectly, hatching or exsheathing fluids which contain certain enzymes. There is no evidence, as yet, to show that the host may function as in (II) or (III).

(from Rogers, 1960)

There is some evidence that the host influences hatching or exsheathment of the infective stage by providing a stimulus and that the infective stage releases the substances which attack the egg shell or sheath (Sommerville, 1954, 1957; Rogers and Sommerville, 1957, 1960; Rogers, 1958, 1960, 1961; Fairbairn, 1960; Taylor and Whitlock, 1960). The main components of the stimulus are dissolved carbon dioxide or its derivatives, hydrogen ions, the oxidation-reduction potential and temperature.

It is unlikely that vertebrate hosts induce exsheathment by replacing missing internal secretions; such substances would probably have large molecules, yet larvae exsheath if they are enclosed in dialysis tubing and incubated in fluid from the appropriate region of the gut. Rogers (1960) has suggested that chemical substances which are given off by the roots of plants may be related to the postulated internal secretions. These substances induce moulting of some larval stages of Paratylenchus species and hatching of Heterodera eggs. However, some species of these plant parasites respond more readily to substances from the roots of non-host plants than to those from host plants. Other species do not respond at all to root diffusates.

It is also unlikely that vertebrate hosts attack the egg membranes or sheath of the infective stage directly. Vertebrates seldom possess chitinases and these would be needed to break down the shell of infective eggs. There is evidence that pepsin aids exsheathment in some species (Crofton, 1947; Silverman and Podger, 1964), but it is not necessary for exsheathment in most of the species which have

been studied. Bird and Rogers (1956) studied the composition of sheaths of several trichostrongyles. No information about the composition of the small region of sheath which is attacked during exsheathment was obtained, but the remainder of the sheath was not hydrolysed by pepsin or trypsin and lacked the particular amino acids which would make it susceptible to attack by these enzymes.

Dependence of the infective stage on the host for a stimulus to hatch or exsheath would be an important adaptation to parasitism; it would ensure that adaptations which protect larvae in their environment as free-living animals are not lost until they are in the host. The stimulus may also induce physiological or behavioural changes which enable the larvae to become established in the host.

Rogers and Sommerville (1963) have discussed more comprehensively the way in which development of nematodes may be controlled. Figure 6.3 shows their model of the system which may operate in parasitic nematodes; it is applicable to nematodes which are infective as ensheathed, third-stage larvae.

The third and fourth moults may be controlled solely by intrinsic mechanisms, or the larvae may rely on a stimulus from the host, as they do for exsheathment.

Although complex substances need not be supplied by the host for larvae to hatch or exsheath, these may be necessary for other stages in development; growth and differentiation may be resumed only if the host supplies substrates necessary for metabolic processes in the nematode.

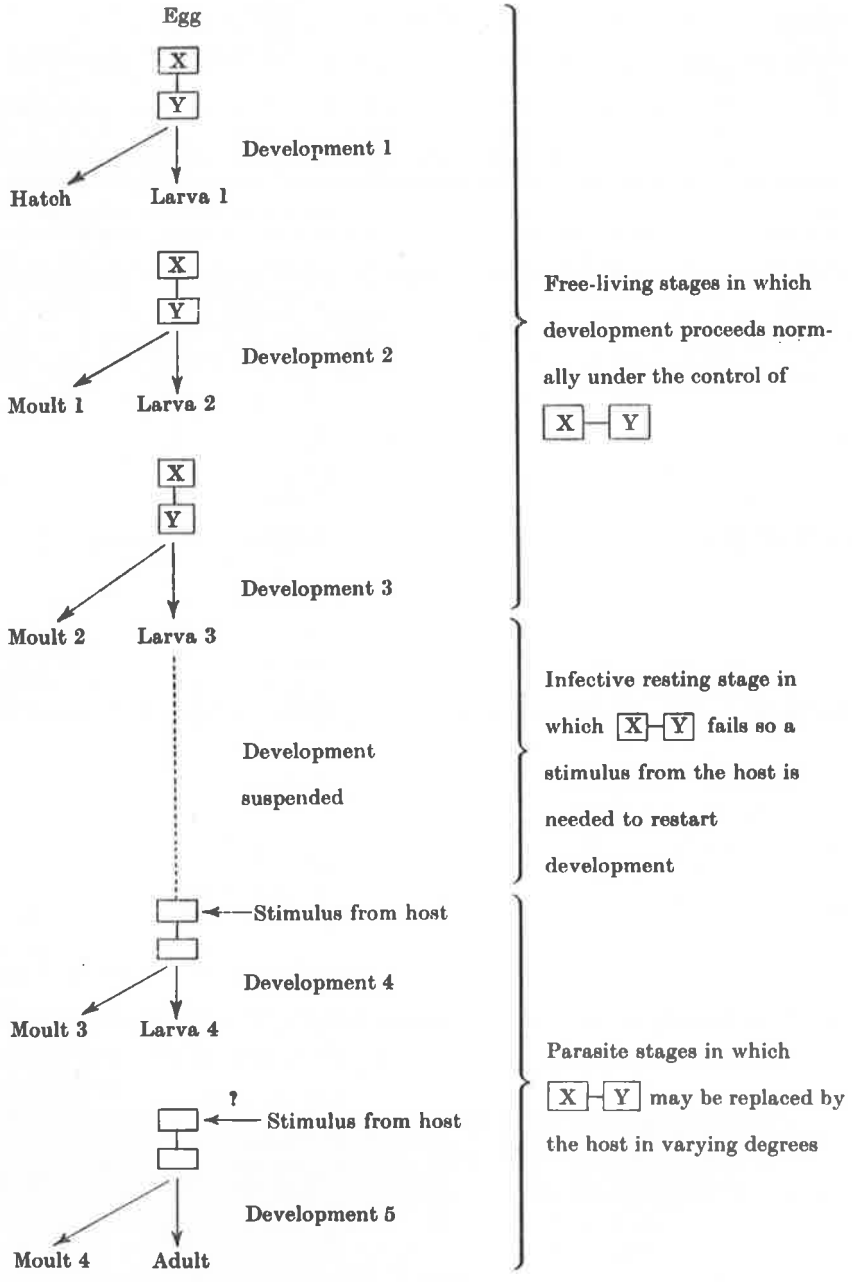


Fig. 6.3 A model of possible mechanisms controlling development of a parasite of which the infective stage is an ensheathed third-stage larva.

Hatch	Production of fluid containing the appropriate enzymes (and change in the activity of the embryo) for the rupture of the membranes of the egg shell.
Moult 1, 2, 3, 4	Production of fluid (and change in the behaviour of the larva) which causes the rupture of the outer cuticle and escape of the larva.
Development 1	Embryonic development culminating in the hatching of the egg.
Development 2, 3, 4	Developmental changes, under a "juvenile influence" in tissues and organs including the formation of a new cuticle underneath the old one.
Development 5	Developmental changes in the absence of a "juvenile influence" and without the formation of a new cuticle.
X–Y	Internal system governing the timing of moulting, the production of moulting fluid and the substances causing differentiation etc. Analagous to the neurosecretory cell-prothoracic gland-corpora allata system. Stimuli from the host may either replace X or Y or change one or both from an inactive to an active state.

(from Rogers and Sommerville, 1963)

6.4 The response to the stimulus from the host

Early experiments indicated that the infective stage releases hatching or exsheathing fluid in response to the stimulus from the host. The hatching fluid released by infective eggs of Ascaris lumbricoides contains an esterase, which alters the vitelline membrane of the egg, and a chitinase which hydrolyses the hard outer shell. A protease may also be present and aid the breakdown of the outer shell (Rogers, 1958).

Rogers and Sommerville (1960) studied exsheathing fluid from Haemonchus contortus and found that it contained a protein. A heat stable cofactor, replaceable by manganese or magnesium ions, was necessary for its activity. Exsheathing fluid from one species did not necessarily induce exsheathment in larvae of another species as readily as it did in larvae of its own species.

Rogers (1963, 1965) identified a leucine aminopeptidase in exsheathing fluid from Haemonchus contortus and Trichostrongylus colubriformis. This enzyme seems to be responsible for the breakdown of the sheath during exsheathment. Exsheathing fluid was fractionated on acrylamide columns and the activity of the various fractions was tested using sheaths, which had been dissected from infective larvae, as substrate. The only fraction which attacked sheaths was that which showed activity of leucine aminopeptidase. The activity of leucine aminopeptidase was influenced by the same factors which are important for the activity of exsheathing fluid; thus the enzyme was released by larvae which were stimulated to exsheath and manganese or magnesium ions were required as a cofactor. The enzyme from Haemonchus contortus did not attack sheaths from larvae of Trichostrongylus colubriformis and

vice versa, so it seems that there may be several leucine aminopeptidases which differ in the substrates which they attack.

Recent investigations by Davey (1966) support the hypothesis that neurosecretions are involved in moulting.

The final larval stage of Phocanema decipiens is found in the muscles of fish. If an infected fish is ingested by a seal, the larvae moult and become adults. Davey (1966) studied this moult in vitro. He found that the excretory sinus has a cycle of production and release of leucine aminopeptidase which is correlated with the moulting cycle. He also found two groups of neurosecretory cells which undergo a cycle of secretion during moulting. The secretion of these cells does not influence the deposition of the new cuticle but may influence the production and release of leucine aminopeptidase; preliminary experiments indicate that if larvae are ligated between the neurosecretory cells and the excretory sinus, leucine aminopeptidase does not accumulate in the excretory sinus.

It seems likely that in trichostrongyles as well as Phocanema decipiens, leucine aminopeptidase is released from the excretory sinus. Rogers and Sommerville (1960) found that precipitates formed at the excretory pore when infective larvae of Haemonchus contortus were stimulated to exsheath and then placed in antiserum to exsheathing fluid. This suggests that exsheathing fluid is released through the excretory pore. Experiments with larvae of Trichostrongylus axei which had been ligated or irradiated with a narrow beam of ultraviolet light indicate that exsheathing fluid is stored in the region between the base of the oesophagus and the excretory pore (Sommerville, 1957; Rogers and

Sommerville, 1960). This includes the excretory sinus.

At present there is no direct evidence that the same mechanism which controls exsheathment operates in later moults but indirect evidence suggests that it probably does. Soulsby, Sommerville and Stewart (1959) found that Haemonchus contortus released antigens at the time of the third moult which were similar to those released during exsheathment. This suggests that a moulting fluid, similar to exsheathing fluid and possibly also containing leucine aminopeptidase, is released. Preliminary experiments by Rogers (1965) indicate that the amount of leucine aminopeptidase present in tissues of parasitic nematodes varies during the life cycle. It is often present in high concentrations before moults other than exsheathment.

6.5 The receptor for the stimulus to exsheath

It seems that one of the most important components of the stimulus for exsheathment is carbon dioxide or undissociated carbonic acid. Presumably some receptor in the infective stage responds to these substances. Tissues which are sensitive to carbon dioxide may be scattered throughout the infective stage, but the experiments of Rogers and Sommerville (1960) suggest that there is a localised receptor; this is situated in the region between the base of the oesophagus and the excretory pore.

Rogers (1966) has tested a model of the way in which carbon dioxide might react with the receptor. The stimulus is enhanced *in vitro* if, in addition to carbon dioxide, reducing agents are present in the stimulating medium, but reducing agents are less important if the

medium contains high concentrations of hydrogen ions. This suggests that the receptor is more receptive to carbon dioxide in its reduced form and that it reacts less readily with reducing agents in the presence of high concentrations of hydrogen ions.

Carboxyl groups can react with sulphydryl groups :

$$-SH + C(=O)- \longrightarrow -S-C(-OH)- \qquad \text{(Rabin and Trown, 1964)}$$

Consequently, carbon dioxide might react with suitably placed sulphydryl groups in infective larvae to form an $-S-\underset{|}{C}-S$ link. This link might form between sulphur containing amino acids in two adjacent protein chains. In the presence of oxidising agents disulphide bonds would tend to form and the reaction with carbon dioxide would be inhibited. This inhibition would be reversed by reducing agents, which could restore the sulphydryl groups.

The cuticle of nematodes is impermeable to many of the substances commonly used as oxidising and reducing agents. Rogers used iodine as an oxidising agent and hydrogen sulphide as a reducing agent. Exsheathment of infective larvae of Haemonchus contortus and Trichostrongylus colubriformis was inhibited when larvae were incubated in solutions containing 10^{-4}M to 10^{-3}M iodine for 10 minutes before they were incubated in the stimulating medium. These concentrations of iodine were not apparently toxic to the larvae, which continued to move actively. Inhibition was decreased if larvae were placed in hydrogen sulphide—water for 10 minutes after they had been treated with iodine and before they were stimulated to exsheath. Infective larvae of Trichostrongylus colubriformis were more sensitive to hydrogen sulphide than larvae of Haemonchus contortus, and the inhibition could be completely reversed.

Inhibition of exsheathment in Haemonchus contortus was reduced further if the stimulating medium contained 0.01 M sodium dithionite. Both iodine and hydrogen sulphide could have acted on the larvae in a number of ways. It seems probable however that both substances acted at the same site, for hydrogen sulphide had little effect on larvae which had not been treated previously with iodine. Consequently, both substances may have influenced exsheathment by influencing the formation of -S-C-S groups.

6.6 The control of development of Trichostrongylus retortaeformis

There is as yet little information about the way in which moulting is controlled in Trichostrongylus retortaeformis. The results obtained in this study are, however, compatible with the model of development which has been discussed.

It seems that the host provides a stimulus to exsheath and does not attach the sheath directly. The components of the stimulus which initiates exsheathment in vitro are those which stimulate the release of exsheathing fluid and leucine aminopeptidase in other trichostrongyles. Consequently exsheathing fluid is probably released by infective larvae of Trichostrongylus retortaeformis, although no attempt to collect it was made.

The actual casting of the sheath took place most readily in acid solutions with a low redox potential. These conditions would not increase the activity of a leucine aminopeptidase. Leucine aminopeptidase has maximum activity when the pH is approximately 9.5 and is unstable at a pH of 7 or less; it does not require a reducing medium.

However, this enzyme attacks the inner layers of the sheath. If it is released by infective larvae of Trichostrongylus retortaeformis, it would probably be in a medium with quite different properties from those of the medium surrounding the larvae. Probably the high concentration of hydrogen ions and low redox potential, which were optimal for the casting of the sheath, stimulated the activity of larvae, encouraging fracture of the outer layers of the sheath rather than the breakdown of the inner layers by an enzyme.

Carbon dioxide, or a related compound, is an important component of the stimulus. Preliminary experiments indicate that exsheathment is inhibited by iodine and that this inhibition is reversed by hydrogen sulphide, as in Haemonchus contortus and Trichostrongylus colubriformis. Larvae are stimulated in media which contain high concentrations of hydrogen ions. These may favour the formation of sulphydryl groups which would be reactive with carbon dioxide and make a reducing agent in the stimulating medium unnecessary.

The occurrence of a lethargus before the third and fourth moults raises hopes that these moults can be studied in vitro; after larvae have entered a lethargus, the physico-chemical properties of the environment are probably more important than the nutrients it contains. It is hoped to study these later moults in vitro to find out whether the environment must contain appropriate concentrations of undissociated carbonic acid and hydrogen ions for larvae to release substances which will attack the cuticle. Preliminary experiments indicate that fourth stage larvae continue to develop in modified Tyrode's solution if they are removed from the host when they enter the lethargus, 4 days after

ingestion. When they are removed from the host, the cuticle adheres closely to the hypodermis and bursal rays are not apparent in male larvae. During the following 24 hours, the cuticle becomes loose around the larvae and bursal rays form. If the final moult is controlled by a mechanism similar to that which operates in exsheathment, the old cuticle may not split unless the appropriate stimulus is applied.

The substances which accumulate in the excretory sinus before the third and fourth moults and disappear when the moult occurs may represent excretory products. The investigations of Davey (1966) suggest however that the substance which accumulates may be a leucine aminopeptidase.

In addition to relying on the host to supply a stimulus for moulting, parasitic larvae may depend on the host to supply a stimulus or special substance for other stages of development. The stimulus for exsheathment does not appear to initiate growth or morphological changes directly in Trichostrongylus retortaeformis. It may initiate physiological or behavioural changes, or it may influence only exsheathment and some other factor may be necessary before growth and development resumes. Larvae apparently start to feed after they have been in the host for about 14 hours, but do not feed immediately after they exsheath. Probably, during the first few hours in the host, the larvae are passing through the stomach and burrowing into the mucosa. They may start to feed only after they are in contact with the tissues of the host; experiments using radioactive tracers indicate that small nematodes

which are gut parasites ingest the tissues of the host rather than material in the lumen of the gut (Rogers and Lazarus, 1949; Esserman and Sambell, 1951). The stimulus for exsheathment may also stimulate larvae to burrow into the mucosa when they have escaped from the sheath, or they may be stimulated to do this by the change in environment when they enter the duodenum. As larvae do not feed when they first enter the duodenum, any components of the environment which stimulate larvae must be able to pass through the cuticle. The environment in the duodenum differs from that in the stomach in the concentration of hydrogen ions (Redman, Willimott and Wokes, (1927) and content of sodium and potassium (see page 58). The concentration of other ions, the partial pressure of carbon dioxide and oxygen or components of the bile or pancreatic secretions might also form part of the stimulus.

No growth or morphological change was observed in larvae until after they had started to feed. The possibility that these changes begin when larvae no longer depend on energy reserves, accumulated when they were second-stage larvae, has already been discussed (see page 48).

Figure 6.4 summarises the ways in which development of Trichostrongylus retortaeformis may be influenced by the host. Although it is based on very slender evidence, it suggests investigations which might lead to a better understanding of this aspect of host-parasite relationships.

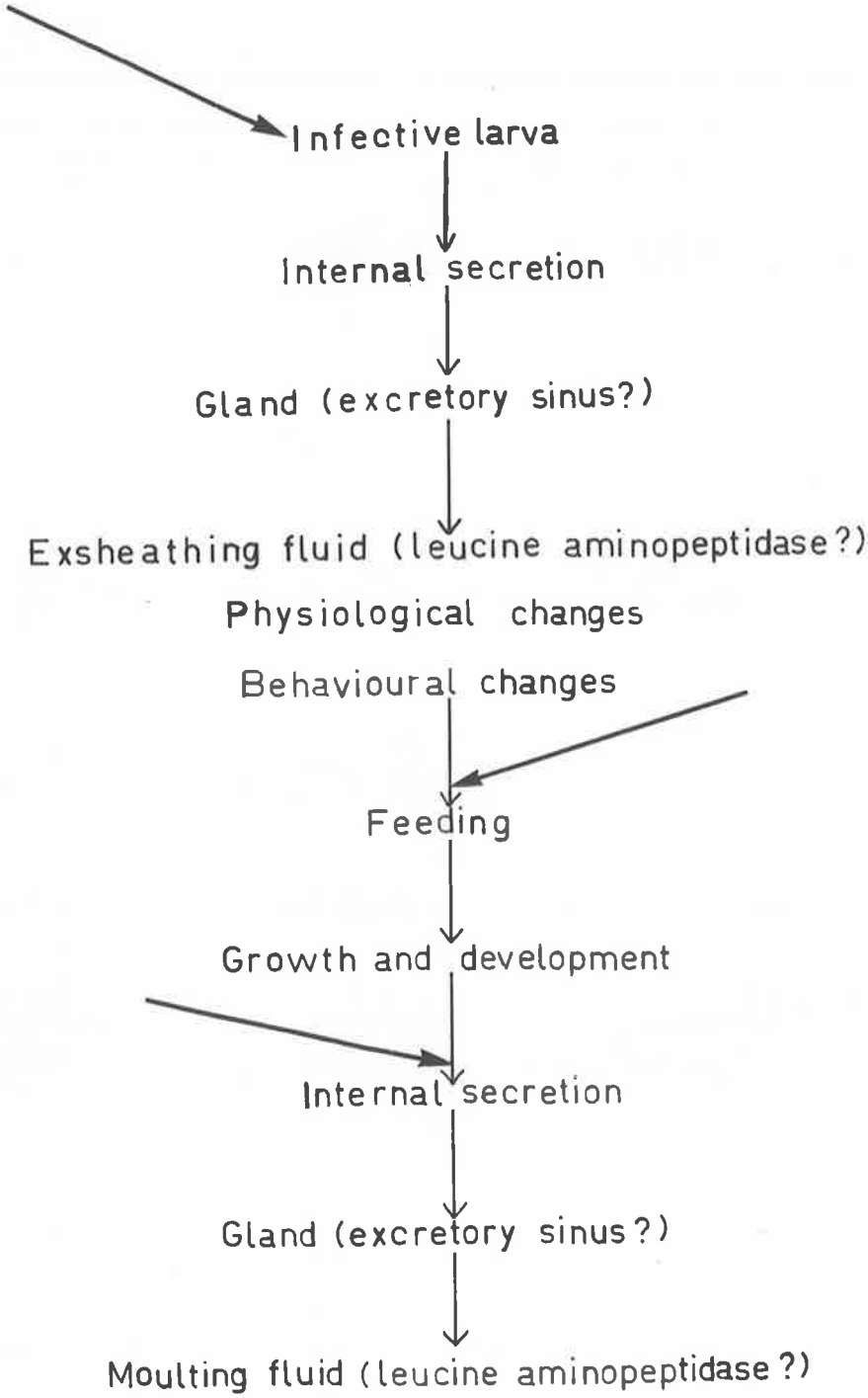


Figure 6.4 A possible sequence of events in the development of third-stage larvae of Trichostrongylus retortaeformis in a rabbit. → indicates a stimulus from the host.

VII APPENDIX

To find out whether the gut contents of rabbits in group A differed significantly from those of group B and whether they were any significant differences between the contents of the stomach, duodenum and small intestine a t test was used, based on the equation

$$t_{n+m-2} = \frac{\bar{x} - \bar{y}}{SEp}$$

where $$SEp = \sqrt{\frac{SSp}{1/n + 1/m}}$$

$$SSp = \frac{(x - \bar{x})^2 - (y - \bar{y})^2}{n + m - 2}$$

$\bar{x}$ = arithmetic mean of results in class x;

$\bar{y}$ = arithmetic mean of results in class y;

n = number of observations in class x

and m = number of observations in class y

Table 7.1 Comparison of stomach contents from rabbits of group A with those from group B

	Total osmotic pressure mOsm		Osmotic pressure due to electrolytes mOsm		Conc. of sodium mmol./l.		Conc. of pottassium mmol./.	
	Group B	Group A	Group B	Group A	Group A	Group B	Group A	Group B
	278	272	670	495	20.87	23.04	10.23	12.79
	320	316	734	590	37.83	17.39	22.28	20.46
	310	308	830	340	42.17	16.09	29.41	14.07
Σx	908	896	2234	1425	100.87	56.52	61.92	47.32
Σx^2	275784	268704	1676556	708725	3644.97	1092.14	1466.00	780.16
$\frac{(\Sigma x)^2}{n}$	274821	267605	1663585	676875	3391.59	1064.84	1278.03	746.71
$\Sigma(x-\bar{x})^2$	963	1099	12971	31850	253.38	27.30	187.97	33.45
Vx	481	549	6485	15925	126.69	13.65	93.99	16.72
Sx	21.92	23.44	80.53	125.80	11.25	3.70	9.69	4.01
$\bar{x}$	303	299	745	475	33.62	18.84	20.64	15.78
SSp	$\frac{136}{4} = 34$		$\frac{18879}{4} = 4720$		$\frac{226.08}{4} = 56.52$		$\frac{154.52}{4} = 38.63$	
SEp	$\sqrt{\frac{34x3}{2}} = 7.14$		$\sqrt{\frac{4720x3}{2}} = 84.14$		$\sqrt{\frac{56.52x3}{2}} = 9.21$		$\sqrt{\frac{38.63x3}{2}} = 7.61$	
$\bar{x}_1-\bar{x}_2$	4		270		14.78		4.86	
t_4	$\frac{4}{7.14} = 0.56$		$\frac{270}{84.14} = 3.21$		$\frac{14.78}{9.21} = 1.60$		$\frac{4.86}{7.61} = 0.64$	
p	N.S.		< 0.05		N.S.		N.S.	

Table 7.2 Comparison of duodenal contents from group A with those from group B

	Total osmotic pressure mOs		Osmotic pressure due to electrolytes mOs		Conc. of sodium mmol./l.		Conc. of pottassium mmol./l.	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
	350	300	230	225	86.96	69.57	25.06	34.27
	330	322	244	240	78.26	82.61	39.39	26.60
	370	316	248		106.52	108.70	37.08	
Σx	1050	938	722	465	271.74	260.88	101.53	60.87
Σx^2	368300	293540	173940	108225	25033.18	23480.09	3554.50	1881.99
$\frac{(\Sigma x)^2}{n}$	367500	293281	173761	108112	24614.20	22686.12	3436.11	1852.28
$\Sigma(x-\bar{x})^2$	800	259	179	113	418.98	793.97	118.39	29.41
Vx	400.0	129.3	89.3	113	209.49	396.98	59.19	29.41
Sx	20.0	11.4	9.5	10.6	14.48	19.22	7.69	5.42
$\bar{x}$	350	313	241	233	90.58	86.96	33.84	30.44
SSp	$\frac{541}{4} = 135.3$		$\frac{66}{3} = 22$		$\frac{374.99}{4} = 93.74$		$\frac{88.98}{3} = 29.66$	
SEp	$\sqrt{\frac{135.3x3}{2}} = 14.3$		$\sqrt{\frac{22x6}{5}} = 5.2$		$\sqrt{\frac{93.74x2}{3}} = 7.91$		$\sqrt{\frac{29.66x6}{5}} = 5.96$	
$\bar{x}_A - \bar{x}_B$	37		8		3.62		3.40	
t_4	$\frac{37}{14.3} = 2.59$		$t_3 = \frac{8}{5.2} = 1.54$		$t_4 = \frac{3.62}{7.91} = 0.46$		$t_3 = \frac{3.40}{5.96} = 0.57$	
p	N.S.		N.S.		N.S.		N.S.	

Table 7.3 Comparison of contents of the small intestine from rabbits of group A with those from group B

	Total osmotic pressure mOsm		Osmotic pressure due to electrolytes mOs		Conc. of sodium mmol./l.		Conc. of pottassium mmol./l.	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
	350	265	230	226	91.30	84.78	16.62	25.58
	325	358	248	228	120.00	82.61	24.81	25.58
	324				95.22	101.74	17.39	
Σx	999	623	478	454	306.52	269.13	58.82	51.16
Σx^2	333101	198389	114404	103060	31802.54	24363.09	1194.17	1308.67
$\frac{(\Sigma x)^2}{n}$	332667	194064	114242	103058	31318.17	24143.65	1153.26	1308.67
$\Sigma(x-\bar{x})^2$	434	4325	162	2	484.37	219.44	40.91	0
Vx	217	4325	162	2	242.18	108.72	20.45	0
Sx	14.7	65.8	12.7	1.4	15.57	10.47	4.52	0
$\bar{x}$	333	312	239	227	102.17	89.71	19.61	25.58
SSp	$\frac{3891}{4} = 972.7$		$\frac{160}{2} = 80$		$\frac{264.93}{4} = 66.23$		$\frac{40.91}{3} = 13.64$	
SEp	$\sqrt{\frac{972.7x6}{5}} = 34.2$		$\sqrt{\frac{80x2}{2}} = 8.9$		$\sqrt{\frac{66.23x3}{2}} = 9.97$		$\sqrt{\frac{13.64x6}{5}} = 3.37$	
$\bar{x}_A - \bar{x}_B$	21		21		12.46		5.97	
	$t_3 \frac{21}{34.2} = 0.61$		$t_2 \frac{12}{8.9} = 1.34$		$t \frac{12.46}{9.97} = 1.25$		$\frac{5.97}{3.37} = 1.77$	
p	N.S.		N.S.		N.S.		N.S.	

Table 7.4 Comparison of stomach contents with duodenal contents

	Total osmotic pressure mOs		Conc. of sodium mmol./l.		Conc. of pottassium mmol./l.	
	stomach	Duodenum	Stomach	Duo-denum	Stomach	Duo-denum
	280	300	23.04	69.57	12.79	34.27
	320	322	17.39	82.61	20.46	26.60
	310	316	16.09	108.70	14.07	25.06
	272	350	20.87	86.96	10.23	39.39
	316	330	37.83	78.26	22.28	37.08
	308	370	42.17	106.52	29.41	
Σx	1804	1988	157.39	532.62	109.24	162.40
Σx^2	544488	661840	4737.12	48513.27	2246.16	5436.50
$\frac{(\Sigma x)^2}{n}$	542402.6	658690.6	4128.60	47280.68	1988.90	5274.75
$\Sigma(x-\bar{x})^2$	2085.4	3149.4	608.52	1232.59	257.26	161.75
Vx	417.1	629.9	121.70	246.52	51.45	40.44
Sx	20.4	25.1	11.0	15.7	7.2	6.4
$\bar{x}$	301	331	26.23	88.77	18.21	32.48
SSp	$\frac{1064}{10} = 106.4$		$\frac{624.1}{10} = 62.4$		$\frac{95.5}{9} = 10.6$	
SEp	$\sqrt{\frac{106.4x5}{2}} = 17.9$		$\sqrt{\frac{62.4x6}{2}} = 13.7$		$\sqrt{\frac{10.6x30}{11}} = 5.4$	
$\bar{x}_1-\bar{x}_2$	30		62.54		14.27	
t_{10}	$\frac{30}{17.9} = 1.68$		$t_{10}\ \frac{62.54}{13.7} = 4.56$		$t_9\ \frac{14.27}{5.4} = 2.64$	
p	>0.10		<0.01		<0.05	

Table 7.5 Comparison of contents of the duodenum with those of the small intestine

	Total osmotic pressure mOs		Conc. of sodium mmol./l.		Conc. of pottassium	
	duodenum	small intestine	duodenum	small intestine	duodenum	small intestine
	300	265	69.57	84.78	34.27	25.58
	322	358	82.61	82.61	26.60	25.58
	316	350	108.70	101.74	25.06	16.62
	350	325	86.96	91.30	39.39	24.81
	330	324	78.26	120.00	37.08	17.39
	370		106.52	95.22		
Σx	1988	1622	532.62	575.65	162.40	109.98
Σx^2	661840	531490	48513.27	56165.63	5436.50	2502.85
$\frac{(\Sigma x)^2}{n}$	658690.6	526176.8	47280.68	55228.82	5274.75	2419.12
$\Sigma (x-\bar{x})^2$	3149.4	5313.2	1232.59	936.81	161.75	83.73
Vx	629.9	1328.3	246.52	187.36	40.44	20.93
Sx	25.1	36.4	15.7	13.68	6.4	4.58
$\bar{x}$	331	324	88.77	95.94	32.48	22.00
SSp	$\frac{2163.8}{9} = 240.4$		$\frac{295.8}{10} = 29.6$		$\frac{78.1}{8} = 9.8$	
SEp	$\frac{240.4 \times 30}{11} = 25.6$		$\frac{29.6 \times 6}{2} = 9.4$		$\frac{9.8 \times 5}{2} = 5.0$	
$\bar{x}_1 - \bar{x}_2$	7		7.17		10.48	
t_9	$\frac{7}{25.6} = 0.27$		t_{10} $\frac{7.17}{9.4} = 0.76$		t_8 $\frac{10.48}{5.0} = 2.10$	
p	> 0.70		> 0.40		> 0.05	

When an analysis of variance indicated that treatments differed significantly in their influence on exsheathment, the least significant difference between the mean results was calculated from the equation

$$\bar{x} - \bar{y} = t_{0.05}^{n+m-2} \sqrt{s^2\left(\frac{1}{n} + \frac{1}{m}\right)}$$

where $\bar{x}$ = arithmetic mean of results with treatment x

$\bar{y}$ = arithmetic mean of results with treatment y

s^2 = residual variance i.e. within classes mean square

and

$t_{0.05}^{n+m-2}$ = the value of t with n+m-2 degrees of freedom and a probability of 0.05.

Table 7.6 Analysis of variance of data from experiments to determine the time relations of the stimulus

	Time - minutes				
	15	30	45	60	90
percentage of larvae exsheathed	18	40	56	50	56
	26	56	42	48	46
	10	28	40	44	54
	16	28	34	52	52
	24	18	36	44	50

	15	30	45	60	90
percentage of larvae exsheathed transformed to angles	25.10	39.23	48.45	45.00	48.45
	30.66	48.45	40.40	43.85	42.71
	18.43	31.95	39.23	41.55	47.29
	23.58	31.95	35.67	46.15	46.15
	29.33	25.10	36.87	41.55	45.00
Σx	127.1	176.7	200.6	218.1	229.6
Σx^2	3326.0	6558.0	8150.3	9530.5	10652.7
$\bar{x}$	25.4	35.3	40.1	43.6	45.9

Correction Factor (C.F.) $= \frac{(\Sigma x)^2}{n}$

$= \frac{952.1^2}{25} = 36259.8$

Total sum of squares $= \Sigma x^2 - C.F.$

$= 38127.5 - 36259.8$

$= 1867.7 \quad . \quad . \quad . \quad . \quad (1)$

Sum of squares between treatments $= \Sigma \frac{(\Sigma x)^2}{n} - C.F.$

Table 7.6 (Contd.)

$$= \frac{127.1^2}{5} + \frac{176.7^2}{5} + \frac{229.6^2}{5} - C.F.$$

$$= 1320.5 \quad . \quad . \quad . \quad . \quad (2)$$

Sum of squares within treatments = (1) - (2)

= 547.2

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	4	1320.5	330.1	12.0 p 0.001
within treatments	20	547.2	27.4	
total	24	1867.7	77.8	

Least significant difference between means

$$\bar{x} - \bar{y} = t^{20}_{0.05} \sqrt{\frac{2 \times 27.4}{5}} = 2.09 \times 3.32$$

Time (mins)	90	60	45	30	15
$\bar{x}$	45.9	43.6	40.1	35.3	25.4

Difference between means

Time of stimulus	Time of stimulus			
	90 mins	60 mins	45 mins	30 mins
15 mins	20.5*	18.2*	14.7*	9.9*
30 mins	10.6*	8.3*	4.8	-
45 mins	5.8	3.5	-	-
60 mins	2.3	-	-	-

* difference significant at 5% level of probability

Table 7.7 Analysis of variance of data from experiments to determine the effect of carbon dioxide on exsheathment

	Concentration of carbon dioxide (vol/vol)					
Larvae exsheathed (%)	5%	10%	25%	50%	100%	
	0	7	55	67	62	
	3	10	64	64	64	
Larvae exsheathed	5%	10%	25%	50%	100%	
	0	15.34	47.87	54.94	51.94	
transformed to angles	9.97	18.43	53.13	53.13	53.13	
x	9.97	33.77	101.00	108.07	105.07	357.88
x^2	99.4	575.0	5114.3	5841.2	5520.6	17150.5
$\bar{x}$	4.99	16.89	50.50	54.04	52.54	

Correction factor (C.F.) $= \frac{(\Sigma x)^2}{n}$

$= \frac{(358.0)^2}{10}$

$= 12807.8$

Total sum of squares . . . . (1)

$= \Sigma x^2 - C.F.$

$= 17150.5 - 12807.8$

$= 4342.7$

Sum of squares between treatments . . (2)

$= \Sigma \frac{(\Sigma x)^2}{n} - C.F.$

$= \frac{9.97^2}{2} + \frac{33.77^2}{2} \ldots + \frac{105.07^2}{2} - C.F.$

$= 4272.0$

Table 7.7 (Contd.)

Sum of squares within treatments = (1) - (2)

= 70.7

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	4	4272.0	1068.0	75.74 p 0.001
within treatments	5	70.7	14.1	
total	9	4242.7	482.5	

least significant difference between means

$$\bar{x} - \bar{y} = t^{5}_{0.05} \quad \frac{2 \times 14.1}{2} = 2.57 \times 3.76 = 9.65$$

concentration of CO_2 vol/vol	100%	50%	25%	10%	5%
$\bar{x}$	52.54	54.04	50.50	16.89	4.99

Difference between means

concentration of CO_2 vol/vol	50%	100%	25%	10%
5%	49.05*	47.55*	45.51*	11.90*
10%	37.15*	35.65*	33.61*	
25%	3.54	2.04		
100%	1.50			

* Difference significant at 5% level of probability

Table 7.8 Analysis of variance of data from experiments to determine the effect of temperature on the casting of the sheath

	15°C	25°C	30°C	37°C	40°C
exsheathed larvae	20	30	52	64	68
(%)	8	22	54	70	76
	13	26	68	66	72
	7	20	54	68	51
	12	18	42	62	57

	15°C	25°C	30°C	37°C	40°C	
proportion of	26.57	33.21	46.15	53.13	55.55	
larvae exsheath-	16.43	27.97	47.29	56.79	60.67	
ed transformed	21.13	30.66	55.55	54.33	58.05	
to angles	15.34	26.57	47.29	55.55	45.57	
	20.27	25.10	40.40	51.94	49.02	
Σx	99.7	143.5	236.7	271.7	268.9	1020.5
Σx^2	3068.6	4161.2	11320.0	14783.2	14616.0	46949.0
$\bar{x}$	19.9	28.7	47.3	54.3	53.8	

Correction factor (C.F.) $= \frac{(\Sigma x)^2}{n}$

$= \frac{1020.5^2}{25}$

$= 4,656.8$

Total sum of squares . . . . . (1)

$= \Sigma x^2 - C.F.$

$= 46949.0 - C.F.$

$= 5292.2$

Table 7.8 (Contd.)

Sum of squares between treatments . . . . (2)

$$= \frac{(\Sigma x)^2}{n} - C.F.$$

$$= \frac{99.7^2}{5} + \frac{143.5^2}{5} \ldots + \frac{268.9^2}{5} - C.F.$$

$$= 4880.7$$

Sum of squares within treatments = (1) - (2)

$$= 411.5$$

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	4	4880.7	1220.2	59.2 p 0.001
within treatments	20	411.5	20.6	
total	24	5292.2	220.5	

Least significant difference between means

$$\bar{x} - \bar{y} = t^{20}_{0.05} \sqrt{\frac{2 \times 20.6}{5}} = 2.09 \times 2.88 = 6.0$$

Temp. °C	40	37	30	25	15
$\bar{x}$	53.8	54.3	47.3	28.7	19.9

Difference between means

Temperature	37°C	40°C	30°C	25°C
15°C	34.4*	33.9*	27.4*	8.8*
25°C	25.6*	25.1*	18.6*	
30°C	7.0*	6.5*		
40°C	0.5			

* Difference significant at the 5% level of probability

Table 7.9 Analysis of variance of data from experiments to determine the influence of pH on the stimulus for exsheathment

pH 2	pH 3	pH 4	pH 6
44	81	46	0
24	76	70	0
38	88	60	0
36	88	64	2
42	76	50	6

	pH 2	pH 3	pH 4	pH 6	
	41.55	64.16	42.71	0	
	29.33	60.67	56.79	0	
	38.06	69.73	50.77	0	
	36.87	69.73	53.13	8.13	
	40.40	60.67	45.00	14.18	
Σx	186.2	325.0	248.4	22.3	781.9
Σx^2	7026.8	21202.7	12474.6	267.2	40971.3
$\bar{x}$	37.2	65.0	49.7	4.5	

Correction factor (C.F.) $= \frac{(\Sigma x)^2}{n}$

$= \frac{781.9^2}{20}$

$= 30568.4$

Total sum of squares . . . . . (1)

$= \Sigma x^2 - C.F.$

$= 40971.3 - 30568.4$

$= 10402.9$

Sum of squares between treatments . . . (2)

Table 7.9 (Contd.)

$$= \frac{\Sigma(\Sigma x)^2}{n} - C.F.$$

$$= \frac{186.2^2}{5} + \frac{325.0}{5} \ldots\ldots \frac{22.3^2}{5} - 30568.4$$

$$= 9930.6$$

Sum of squares within treatments = (1) - (2)

= 472.3

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	3	9930.6	3310.2	112.2
within treatments	16	472.3	29.5	
total	19	10402.9	547.5	

Least significant difference

$$\bar{x} - \bar{y} = t^{16}_{0.05} \sqrt{\frac{2 \times 29.5}{5}} = 2.12 \times 3.44 = 7.3$$

pH	2	3	4	6
$\bar{x}$	37.2	65.0	49.7	4.5

Difference between means

pH	3	4	2
6	60.5*	45.2*	32.7*
2	27.8*	12.5*	
4	15.3		

* Difference significant

Table 7.10 Analysis of variance of data from experiments to determine the influence of pH on the casting of the sheath

	pH 4	pH 6	pH 7	pH 8
	76	44	50	24
larvae	58	36	38	28
exsheathed	66	44	34	26
(%)	78	32	56	28
	76	34	44	35

	pH 4	pH 6	pH 7	pH 8	
proportion of	60.67	41.55	45.00	29.33	
larvae ex-	49.60	36.87	38.06	31.95	
sheathed	54.33	41.55	35.67	30.66	
transformed	62.03	34.45	48.45	31.95	
to angles	60.67	35.67	41.55	36.27	
Σx	287.3	190.1	208.7	160.2	846.3
Σx^2	16621.3	7271.4	8819.7	5157.4	37869.8
$\bar{x}$	57.5	38.0	41.7	32.0	

Correction factor (C.F.) $= \frac{(\Sigma x)^2}{n}$

$= \frac{716223.7}{20}$

$= 35811.2$

Total sum of squares . . . . (1)

$= \Sigma x^2 - C.F.$

$= 37869.8 - 35811.2$

$= 2058.6$

Table 7.10 (Contd.)

sum of squares between treatments . . . . (2)

$$= \frac{\Sigma(\Sigma x)^2}{n} - \text{C.F.}$$

$$= \frac{287.3^2}{5} + \frac{190.1^2}{5} \ldots + \frac{160.2}{5} - 35811.2$$

$$= 1768.6$$

Sum of squares within treatments = (1) - (2)

$$= 290.0$$

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio
between treatments	3	1768.6	589.5	32.6
within treatments	16	290.0	18.1	
total	10	2058.6	108.3	

Least significant difference

$$\bar{x} - \bar{y} = t^{16}_{0.05} \sqrt{\frac{2 \times 18.1}{5}} = 2.12 \times 2.69 = 5.7$$

pH	4	6	7	8
$\bar{x}$	57.5	38.0	41.7	32.0

pH	4	7	6
8	25.5*	9.7*	6.0*
6	19.5*	3.7	
7	15.8*		

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