DOCTOR OF PHILOSOPHY

Invasion of host tissue by infective larvae of Ascaris suum (Goeze, 1782)

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INVASION OF HOST TISSUE BY INFECTIVE LARVAE OF ASCARIS SUUM (GOEZE, 1782)

A Thesis submitted to the

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by

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ABSTRACT

The strategy by which infective A. suum larvae gain entry into their hosts' intestinal tissue has been investigated. The infective larvae develop in the egg between the temperatures $16^{\circ} \pm 1^{\circ}C$ and $34^{\circ} \pm 1^{\circ}C$. Within this temperature range, increase in temperature increased the rate of development. The maximum rate of development of the eggs was attained at $31^{\circ} \pm 1^{\circ}C$. Eggs embryonated at $28^{\circ} \pm 1^{\circ}C$ and above gave rise to infective larvae which had a lesser ability to hatch *in vitro*, shorter longevity when aged in phosphate buffered saline (pH 7.2) at $37^{\circ}C$, and a limited ability to penetrate tissue membranes *in vitro*, compared with those embryonated at lower temperatures. Maximum larval viability was achieved when eggs were embryonated at $22^{\circ} \pm 1^{\circ}C$. These results suggest that the optional temperature for rate of development and larval viability or survivability are not the same.

In mice, given large oral infections, eggs hatched mainly in the small intestine. *In vitro* studies showed that the infective larvae (13 μm in diameter) actively emerged through a hole in the egg shell wall 9.7 μm in diameter. This hatching mechanism is considered to contract with the

situation in some hookworm eggs where the infective larva is liberated from the egg following splitting of the egg shell under high internal pressure. The egg shell retained its shape during and after hatching. There was no major configurational change in the structure of the egg shell. The inner lipid layer of the shell was broken down during hatching. The location of the hole on the egg shell through which the infective larva emerged varied and was not restricated to any one position. How the hole is made was not obvious. The presence of an opercular structure in the egg shell has been suggested but clear evidence for this was not found.

Ultrastructural study indicated that the infective larva possessed no specialised cuticular structures that might either assist its emergence from the egg shell or entry into the host tissue. Soon after hatching, the infective larvae invaded the host intestinal tissue mainly in the regions of the posterior third of the small intestine, the caecum and the colon. Penetration of the larvae into the host tissue was principally *via* the crypts of liberakun. Direct penetration *via* the villi appeared to be non-existent.

Entry into and subsequent passage of the infective larvae through the tissues was related to the production of larval secretions which caused

changes in the acellular materials of the host tissue. These made it easier for the larvae to separate the cells and fibres as they migrated. Passage of the infective larvae did not involve any extensive digestion of the host tissue. Enzymatic studies of the larvae revealed a correlation between the larval secretions and the ability to cause these changes.

Incubation with azocoll revealed that the larvae produced secretions which are capable of releasing dye from azocoll. These secretions were neither gelatinolytic nor histolytic.

The time course of penetration and subsequent migration to the liver and lungs of mice was followed and a possible mechanism for migration postulated.

The results are discussed in relation to the epidermiology of ascariasis and the known biology of the parasite.

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GENERAL INTRODUCTION

Penetration studies date as far back as the beginning of this century following pioneer works by investigators like Van Durme (1902), Looss (1911), Yokogawa (1922) and Goodey (1922, 1925). However, much of the early studies were preoccupied with determining the main conditions which influence penetration and little attempt was made to elucidate the actual mechanisms involved in the penetration process.

Lewert and Lee (1954) appear to have been the first to consider how parasites penetrate their host tissue. Since their studies on tissue penetration by the infective larvae of various helminths, interest in the mechanisms by which parasites invade has grown steadily over the years. In fact the British Society for Parasitology has recently devoted a complete symposium to the subject of parasite invasion (Taylor and Muller, 1977). Much of the work that has been done on helminths so far has been reviewed by Stirewalt (1963, 1966) and Matthews (1977). The majority of the work has concentrated on a few species; Schistosomes (Gordon and Griffiths, 1951; Griffiths, 1953; Standen, 1953; Lewert and Lee, 1954; Stirewalt and Hackney, 1956; Stirewalt, 1959; Stirewalt and Kruidenier, 1961;

Stirewalt and Fregeau, 1966; Stirewalt and Uy, 1969; Stirewalt, 1973; Stirewalt and Darsey, 1974); Fasciola (Dawes, 1959, 1960a and b; Wilson, Rullin and Denison, 1971); Ancylostoma (Lewert and Lee, 1954; 1956; Matthews, 1972; 1975); Strongyloides (Lewert and Lee, 1954; Barrett, 1969; Lee, 1972). Many of these species invade their host tissue via the skin. With the exception of the work by Lewert and Lee (1954), which included studies on T. spiralis, invasion studies involving nematode parasites which gain entry into their host via the oral route are meagre, even though a great number of these species have a tissue phase in their life-cycle during which they actively penetrate and extensively migrate through their host tissue.

Recent studies on invasion have revealed great diversity in the mode of penetration and subsequent passage of parasites through host tissues. Specialised mechanisms for effecting entrance into the body or tissues have been demonstrated in some species (Lewert and Lee, 1954; Silverman and Maneely, 1955; Erasmus, 1959; Sawada, 1961; Schell, 1961, 1962; Probert, 1965; Banerjee and Singh, 1969; Barker, 1970; Matthews, 1972, 1975; Doncaster and

Seymour, 1973).

Using a combination of *in vitro* and *in vivo* techniques which allowed them to follow the movements of the cercariae prior to their disappearance below the Stratum Corneum, Gordon and Griffiths (1951) first described the entry of *Schistosoma mansoni* into mammalian skin. However, a more comprehensive account of the route and the mechanisms involved was later provided by Stirewalt (1966). Cercariae are attracted to host skin mainly by temperature difference between the environment and the tissue (Stirewalt and Uy, 1969; Stirewalt, 1971). The invading cercariae feel-out the skin surface for varying lengths of time before attacking specific entry sites. "By such exploration, the potential invaders make contact with the deeply sculptured skin surface characterised by irregularities associated with hairs, bulbous outer ends of pilosebaceous follicles, loosened scafes, wrinkles, sulci, furrows, ridges and folds" (Stirewalt, 1966). The entry site may be at almost any point on the skin but usually wrinkles, furrows, desquamations of the keratinized cells as well as excavations

as a result of previous entry, are used (Stirewalt, 1959). During the exploration, the cercariae move across the skin surface by alternately attaching the oral and the ventral suckers. At each oral sucker attachment, they deposit minute droplets of secretions from the post-acetabular glands. These secretions are thought to be a carbohydrate-protein-lipid complex (Stirewalt and Evans, 1960) and have been considered by Stirewalt and Walters (1973) to be mucin. Once secreted, this mucus deposit swells in water becoming sticky and serves as adhesive attachment points for both the oral and the ventral suckers, the ventral being attached in it immediately after the oral is withdrawn from it and attached again at a new spot (Stirewalt, 1959b).

When a suitable entry site has been located, the cercariae orientate perpendicularly to the skin surface and exhibit vigorous muscular activity, lashing the tail and alternately contracting and elongating the body (Stirewalt, 1956). These body movements provide a means of enlarging the entry opening (Gordon and Griffiths, 1951) and have also been considered as a means of squeezing out secretions from the acetabular glands (Stirewalt, 1966). Simultaneously with this elongation and contraction of the body, the muscular oral sucker is alternately

everted and withdrawn thus hammering it into the crevice. It is assumed that in this way the Cuticular lips of the acetabular glands are brought into contact with the skin enabling the secretions to be deposited at the entry site. Although it has been considered that by this process the cercariae gradually penetrate the horney layer of the skin, this has been questioned (Dorsey and Stirewalt, 1971; Rifican, 1971). Stirewalt and Dorsey (1974) described passage between the keratinized cells rather than through them and suggested that the swelling of the post-acetabular gland mucus may be responsible for separating the strata seguames and breaking the close connection between the stratum corneum and the underlying tissue. When the body has just entered the stratum corneum and is relatively fixed by both the acetabular mucus and the enveloping skin tissue, the cercariae normally shed their tail. This is accomplished by mechanical rupture at the narrow body-tail junction as a result of the vigorous lashing movements that accompany entry (Gordon and Griffiths, 1951). The cercariae then orientate parallel to the skin surface and migrate along the line of the epidermis. During this migration, the secretions from the

pre-acetabular glands are continually released until they are depleted (Stirewalt and Kruidenier, 1961). The cercariae usually rest in the epidermis for varying lengths of time during which time it is assumed that the enzymatic secretions attack the intercellular matrix of the epidermis (Gordon and Griffiths, 1951). Passage through the epidermis by the cercariae is usually roughly parallel to the skin surface. The epidermal basement membrane presents a formendable obstacle to cercarial passage (Lewert, 1958) and it is assumed that cercariae normally avoid confronting it by migrating along the epidermis until it leads into a hair follicle or associated sebaceous gland. By following the follicles, cercariae are led directly into the dermis through which they migrate relatively rapidly to enter the veins and the lymphatic vessels.

"Although postulated there was little evidence of involvement of larval secretion in the penetration of the tissue by the cercariae until Lewert and Lee (1954) using histochemical techniques showed factors which affected the glycoprotein of the epidermal basement membrane and extracellular gland substance

of the dermis"(Matthews, 1977). Since then, however, a number of workers using various experimental techniques have confirmed their results. Bruce, Pezzlo, McCartney, Yajima (1970), using ultra-structural methods, reported cellular changes in the vicinity of the migrating cercariae indicative of enzymatic activity.

Lee (1972) studying the behaviour of the third-stage larvae of Nippostrongylus brasiliensis during penetration of mouse, rat and human skin at the electron microscope level described separation of host cells and dissolution of collagen during larval invasion which he suggested could be explained in terms of changes in pH or ionic composition of the bathing medium as an alternative to the possibility of direct larval enzyme activity.

Matthews (1975) advanced a mechanical rather than chemical concept of invasion of cat skin by Ancylostoma tubaeforme. He found no evidence for enzymatic secretion emanating from the worms under conditions that gave positive results for Necator americanus and Strongyloides fullebani species which have been considered to enlist larval enzymes in invasion of their host tissues. Exsheathment of the infective larvae of A. tubaeforme is not

essential prior to penetration of their host skin and entry can be at any point on the surface of the skin although hair follicles and desquamation of the Stratum Corneum are usually used (Matthews, 1972). The infective larvae show true thermopositive behaviour (Croll, 1973) and this is considered to be the most important factor in initiating penetration into the host tissue. As in the case of S. mansoni cercariae, initial contact is normally made with the larvae roughly perpendicular to the skin surface. The time spent in searching for an entry site may vary from 5 mins. to 4 hours. However, when a suitable site has been located, the larvae orientate parallel to the skin surface, following the line of least resistance into the skin. The stratum granulosum and the living layer of the epidermis present major barriers to penetration. However, once the epidermis has been passed, migration through the dermis is relatively rapid. Using a cinematographic technique, Matthews (1975) showed that passage through the dermis is accomplished by undulation activity, with the larvae travelling at an average speed of about $20 \mu m/sec^{-1}$. It is not possible to detect the path

of the larvae after they have passed. These observations coupled with the fact that larvae retained their sheath after penetration and lack of any direct evidence of enzymatic secretions emanating from the larvae formed the basis on which Matthews (1975) postulated a mechanical invasion mechanism for the infective larvae - a fact which has since received support by the work of Smith (1976) on the ultrastructure of the infective larvae.

The case of F.hepatica is uncertain. The migrating juveniles of F. hepatica have been considered to actively browse their way into their host tissue. Following a series of classical histological studies in mice, Dawes (1963) showed that penetration and subsequent migration through the gut wall by the fluke leaves a trail of damaged tissue and pyknotic nuclei in their wake. Recognisable fragments of the damaged host tissue are often seen in the fluke caecae during penetration (Dawes, 1962). However, the existence of enzymes in the juveniles of F. hepatica which may assist their passage through host tissues have been reported by Howell (1966).

Similar uncertainty surrounds entry of infective larvae of A. suum into their host

tissue. Although several studies have been conducted on the life-cycle of the parasite (Stewart, 1917-1921; Ransom and Foster, 1920; Ransom and Cram, 1921; Fulleborn, 1927; Roberts, 1934; Sprent, 1952, 1954; Jenkins, 1968), mechanisms by which the infective larvae gain entry into the host tissue remain enigmatic. Jenkins (1968) following his studies on early migration of A. suum in white mice, reported excavations in the host intestinal tissue which he suggested could be attributed to the action of larval enzymes. However, Chandler, Alicata and Chitwood (1974) in their recent description of the early life-cycle of the parasite presented a picture of a mechanical entry mechanism, with the infective larvae actively burrowing into their host tissue with the aid of a 'boring tooth'. Neither of these proposed entry mechanisms have so far been backed by any precise experimental evidence.

The present study is an attempt to elucidate the strategy by which the infective larvae of A. suum gain entry into their host tissue.

LITERATURE REVIEW

Ascaris suum, the common pig ascarid, occurs in the small intestine of pigs. Immature and even mature worms have occasionally been found in the intestine of sheep (Fallis, 1938; Todd, Pope and Mendlowski, 1954; Matoff and Wassileff, 1959; McDonald and Chevis, 1965). The species has also been reported from muskrat, Ondatra zibethica (Tiner and Chin, 1948; Gilford, 1954) and from dog (Berger, Wood and Willey, 1961). Although the parasite was previously considered as belonging to the same species as Ascaris lumbricoides - the human Ascarid (Tashiro, 1927; Matov, Vasilev, 1958; Barry and O'Rourke, 1967), recent studies have shown that the two species are indeed different and can be separated at the adult stage based on the characters of the head (Sprent, 1952; Abdulrachman and Lie, 1954; Lysek, 1963; Anser and Thibant, 1973; Matthews and Croll, 1974).

A. suum is common in pigs throughout the world and among other places has been reported in India (Sinha, 1968a); Canada (Martin, Gibbs and Pullin, 1974); Denmark (Jacobs, 1967); Greece (Himonas and Trintaphyllou, 1972), Papua, New Guinea (Talbot, 1972) and The Philippines (Tongson, Castillo, Arambulo and Sarmiento, 1971). Horak (1978) found

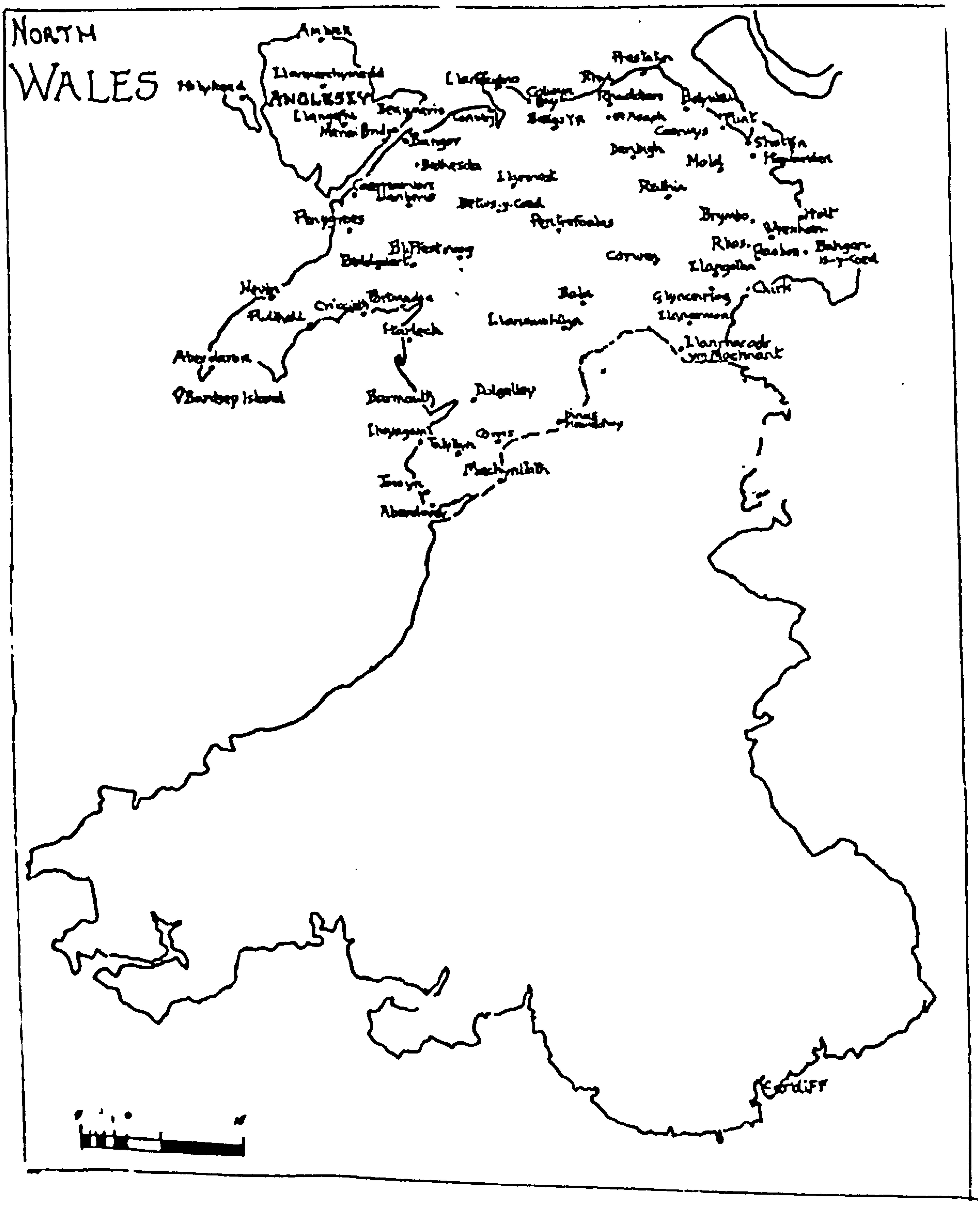
it in 30.8% of pigs slaughtered in the Pretoria Municipal abattoir in South Africa. Goldsby and Todd (1957a) found it in 65% of 101 pigs they examined in Wisconsin, U.S.A. In recent reports from Britain, Jenkins and Erasmus (1963) reported it in 41% of 27 slaughter pigs in South Wales. Gitter, Kidd and Davies (1965) found its eggs in 1% of 367 faecal samples from sows on 45 farms in England. Jacobs (1965) in a preliminary study of 75 pigs in abattoirs in South-West Scotland observed that 28% of the porkers (mean carcass weight, 90 lb.); 24% of the baconers (mean carcass weight, 160 lb.) and 4% of adults harboured A.suum. The present study necessitated regular visits to the local abattoir at Portdinorwic, North Wales to obtain fresh Ascaris. A one-year (1977/78) prevalence study of the infection amongst slaughter pigs (14-18 weeks of age) in this area of Wales was thus undertaken.

The pigs were reported to have come from various farms in North Wales as shown in the map (Figure 1). Details of individual farms were not available. Between 80-160 pigs are slaughtered at the abattoir weekly and the study was conducted on a fortnightly basis. On each occasion, 30 slaughtered pigs were randomly selected and their guts opened up and searched thoroughly for Ascaris.

A total of 750 pigs were examined in this way.

FIGURE 1

Map of Wales showing the sources of pigs examined for *Ascaris* infection.


NORTH
WALES
Amlwch
Holyhead
ANGLESEY
Llangefni
Menai Bridge
Beaumaris
Bangor
Bethesda
Caernarvon
Llandudno
Conwy
Colwyn Bay
Rhyl
Prestatyn
Rhuddlan
St Asaph
Holywell
Flint
Caerwys
Shotton
Hawarden
Denbigh
Mold
Llanrwst
Ruthin
Betws-y-Coed
Pentrefoelas
Brymbo
Holt
Wrexham
Rhos
Ruabon
Bangor-is-y-Coed
Penygroes
Bl. Ffestiniog
Beddgelert
Corwen
Llangollen
Nevin
Portmadoc
Criccieth
Pwllheli
Bala
Glynceiriog
Chirk
Llanarmon
Llanuwchllyn
Harlech
Llanrhaeadr ym Mochnant
Aberdaron
Bardsey Island
Barmouth
Dolgelley
Dinas Mawddwy
Corris
Tal-y-llyn
Machynlleth
Towyn
Aberdovey
Cardiff

The mean prevalence of the infection (mean percentage of pigs infected) was 32.92± 0.97. The prevalence of the infection did not vary significantly ($P > 0.05$) from one month to another (Figure 2) or between seasons of the year. The worm-burden (number of worms recovered from each infected pig) varied enormously from 1 to 90 worms throughout the survey period. However, on two occasions, an infected pig with over 200 worms was encountered. Plate 1 shows such a haul. Again this variation in worm-burden did not reflect any seasonal pattern (Figure 3). The sex ratio of the male to female worms recovered from the infected pigs were fairly constant averaging about 1 male to 3 females throughout the survey.

A. suum is important helminth parasite of pig and is most commonly found in pigs raised under less sanitary conditions. The life-cycle is direct. The female worms lay eggs in the host intestine and these pass out with the faeces. The egg production of a mature female is tremendous. Olsen, Kelly and Sen (1958) estimated that each female in a naturally infected pig produced nearly 2 million eggs per day. Seamster (1950), following

FIGURE 2*

Percentage of 30 fourteen-eighteen week old pigs carrying natural A. suum infections. The pigs came from various areas in North Wales.

* This figure and other subsequent figures are also reported in tabular form in the appendix.

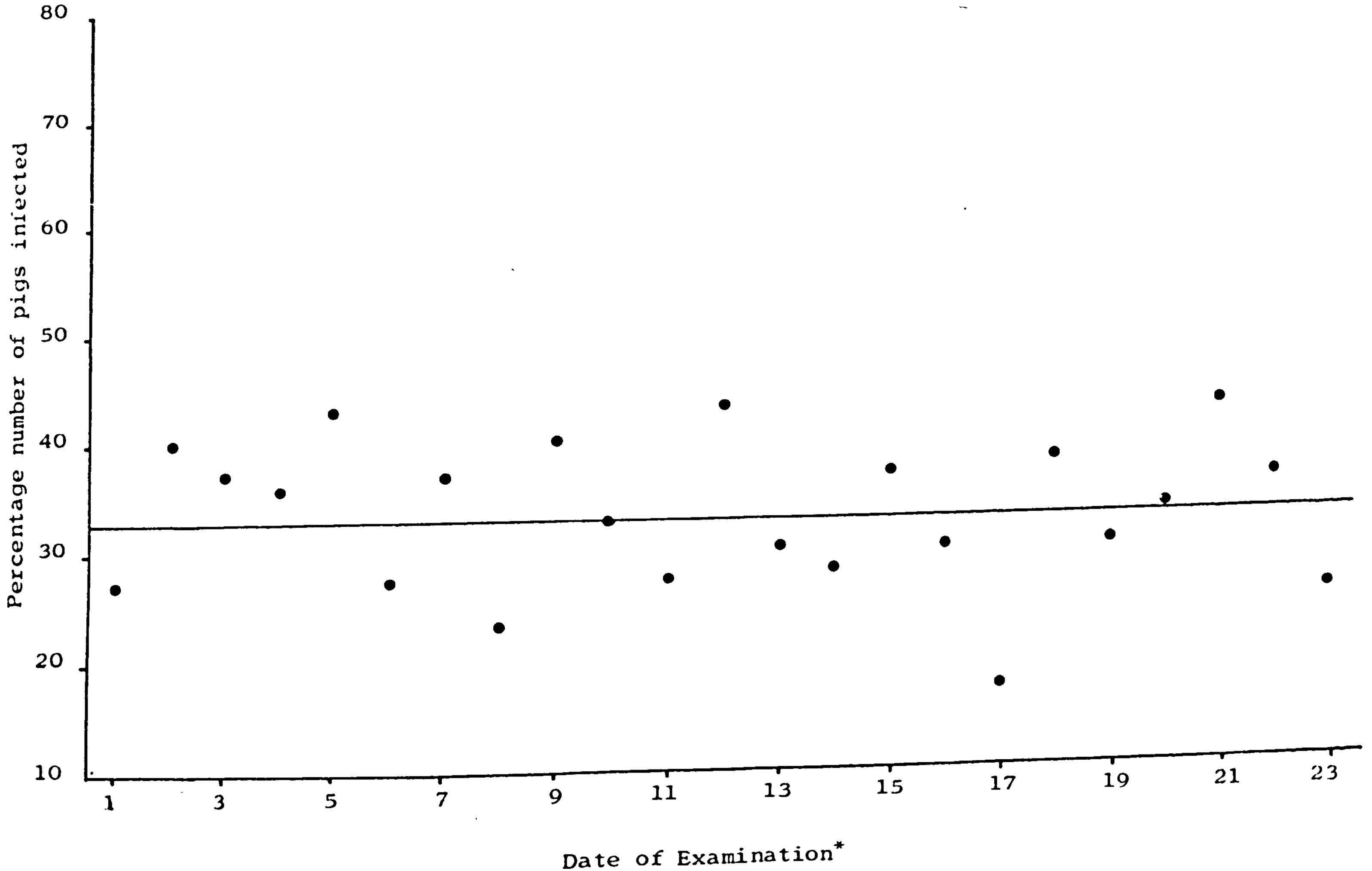
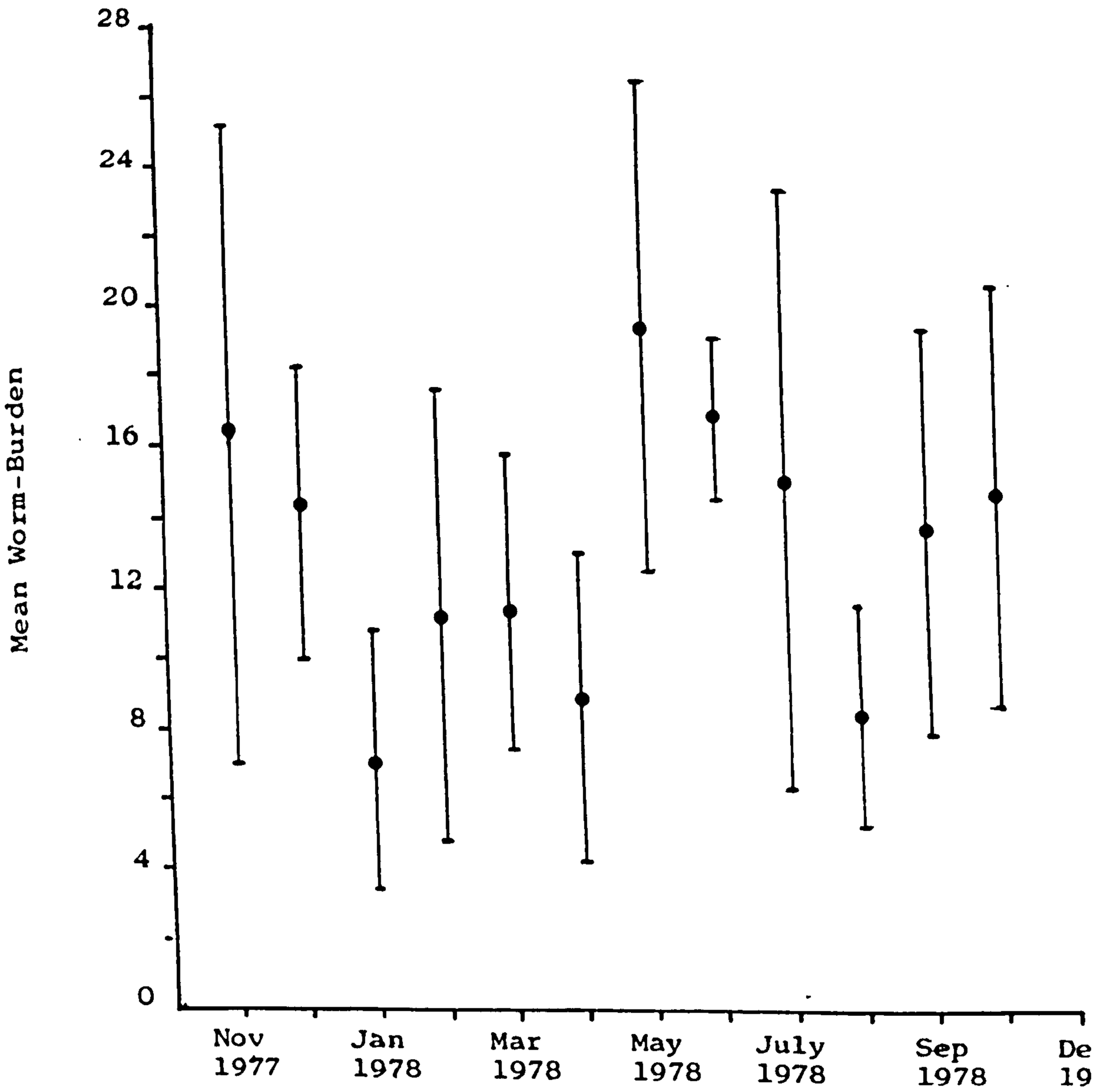

Percentage number of pigs infected
80
70
60
50
40
30
20
10
1
3
5
7
9
11
13
15
17
19
21
23
Date of Examination*

PLATE 1

Adult A. suum collected from the small intestine of a single naturally infected pig in North Wales. The pig was between fourteen-eighteen weeks old.

FIGURE 3

Mean number of adult A. suum recovered from slaughter pigs over a one-year survey period. The pigs were naturally infected and came from various farms in North Wales. The ages vary between fourteen-eighteen weeks. The points represent the mean and the vertical lines the standard errors.


Mean Worm-Burden
28
24
20
16
12
8
4
0
Nov 1977
Jan 1978
Mar 1978
May 1978
July 1978
Sep 1978
Dec 1978
Month of Examination

<u>in vitro</u> incubation of 16 adult female worms reported that each worm produced between 19,400 to 475,000 eggs per day. Eggs removed from the uterus are colourless whereas those found in faeces are golden-brown to yellowish. The eggs passed in the faeces may be fertilised or unfertilised. Fertilised eggs measure 70-80 /um by 50-60 /um and are slightly ovoid, thick-shelled with a mammillated outer layer. Unfertilised eggs are usually longer (108-124 /um by 58-75 /um) with a thin shell and irregular shape. The fertilised eggs are extremely resistant to drying, freezing, other adverse weather conditions and chemicals (Yoshida, 1919, 1920a,b; Yoshida and Hotta, 1919; Kobayashi, 1922; Asada, 1923; Oba, 1923; Ogata, 1924a, 1925a; Aoki, 1936; Fushimi, 1950; Fukui, Kitamura, Ogawa, 1952; Tobizawa, 1954; Yamazaki, Tomita and Yamashita, 1954a,b; Mitobe, 1954a; Antonova, 1959). Bizyulyavishyus (1955) found that they would survive for up to 3 months at -20°C and -30°C. They may live for several years under favourable conditions. The embryonated eggs are generally more resistant than unembryonated ones. Spindler (1940) found that undeveloped eggs were less resistant to sunlight than embryonated ones.

Munnich (1965) kept the embryonated eggs in 1% formalin for 4 years at room temperature. However, during this period, he observed that the number surviving and containing active larvae was greatly reduced.

Although as few as 2 and as many as 5 layers have been suggested (Leuckart, 1886; Biedermann, 1912; Schmidt, 1936; Woltge, 1937; Chitwood, 1938; Christensen, 1940; Rogers, 1956), the egg-shell is generally considered to be composed of four layers - an inner lipid layer often referred to as Ascaroside layer, a thick chitinous layer, a thin uterine layer and an outer protein coat - the uterine layer (Bird, 1971). The remarkable resistance of the egg to chemicals is attributed to the lipid inner layer of the egg-shell. This lipid layer is considered to be semi-permeable (Fairbairn, 1957); allowing easy diffusion of gases and organic chemicals. However, recent evidence suggests that it is also permeable to water (Clarke and Perry, et alia).

The eggs are unsegmented when they are laid. Their rate of development depends on temperature. Seamster (1950) found that the eggs developed to the motile embryo stage in 27 days at 18.9^{o}C and

in 13 days at 34.5°C. The infective larval stage is generally believed to be the 2nd stage larva enclosed within the cast skin of the first stage larva (Alicata, 1936). However, a number of workers including Araujo (1972) have reported two moults inside the egg.

Hosts become infected by ingesting eggs enclosing the infective larvae. The eggs hatch in the intestine. The physiological process of hatching has been investigated by a number of workers (McRae, 1935; Fenwick, 1939; Pitts, 1948; O'Connor, 1951; Rogers, 1958; Fairbairn, 1961; Hass and Todd, 1962; Jaskoski, 1964). In vitro they require 4 stimuli to hatch (1) a temperature of about 37°C, (2) a Carbon-dioxide level of about 5 volumes per litre, (3) a pH of near neutrality, and (4) non-specific reducing conditions such as those produced by cysteine, sodium metabisulphite and Sulphur dioxide (Fairbairn, 1961). In vivo, the conditions are less well understood although it has also been suggested that leucine aminopeptidase, which is present in the adult worm (Lee, 1962a), may also play a role in hatching (Arean and Crandall, 1962).

The hatched larvae penetrate the intestinal wall and migrate in the tissues. Ascaris was first shown to undergo a preliminary migration through the body of the host by Stewert (1914), contrary to earlier belief that the larva had a direct development in the intestine. Shortly afterwards, Ransom and Foster (1917), as well as Chandler (1918), called attention to the probability that the migration through the host body was part of the parasite's normal development. Experimental proof of this hypothesis was later supplied by Ransom and Foster (1920). Since then many others including Sprent (1954) have shown that adult stages of the parasite are unable to develop unless the larvae have completed a defined migration within the host. This migration is of the tracheal type; the larvae re-entering the small intestine after migrating through the liver, lungs and trachea.

Although the gross life-cycle of the parasite may no longer be in doubt, various detailed aspects of the tissue migration phase have remained controversial. Both the body cavity (Yashidia, 1919; Burus, 1963) and the circulatory system (Ransom and Cram, 1921; Jenkins, 1968) have been

considered as the main route of early larval migration to the liver. Similarly, although it is generally considered that the infective larvae moult to the 3rd stage in the liver, and to the 4th stage in the lungs before returning to the intestine via the trachea (Sprent, 1952; 1954), some workers have considered the 3rd and the 4th moults to occur in the lungs (Roberts, 1934).

A large number of 4th stage larvae have been reported in the small intestine of pigs 14-21 days after infection (Roberts, 1934). The moult to the adult stage occurs about 9 months after infection (Roberts, 1934; Schwartz, 1959). The prepatent period is about 49-60 days and very few adults live for more than one year. Olsen, Kelley and Sen (1958) found that most of the adult worms in a naturally infected young pig were expelled before the 23rd week of infection.

The larvae may migrate in the tissues and organs of many animals, including man, but they do not normally become mature in man although a few isolated cases have been reported (Koino, 1922a,b,c, 1923; Payne, Ackert and Harkman, 1925; Takada, 1950, 1951; Tanigawa, Jitsukawa and

Kikuchi, 1958; Jaskoski, 1961). Much research has been done on larval migration in "foreign" hosts including guinea pig, mouse, rat and rabbit (Roberts, 1934; Graham, 1937; Kozer, 1948a; Fallis, 1948; Nichols, 1956a; Kelley, Olsen and Hoerlein, 1957; Olsen and Kelley, 1960; Bhowmick, 1964; Lamina, 1964; Jenkins, 1968). Hoeppli, Feng and Li (1949) studied larval migration in mice. Olsen and Kelley (1960) reported good migration to the lungs in rats and mice, as did Nayak and Kelley (1968) in mice.

The larvae in pigs are generally larger than in laboratory animals (Kelley, Olsen and Hoerlein, 1957), but their route of migration is the same. The speed varies, however, being faster in mice than in rats, rabbit or pig (Olsen and Kelley, 1960; Dourves, Tromba and Malakatis, 1969). In the "foreign" hosts, the 4th stage larvae are unable to develop in the intestine and are expelled in the faeces. The same is true of a varying percentage of the 4th stage larvae in pigs (Schwartz, 1959).

A. suum infection is a major cause of economic loss in the pig industry. Spindler

(1947, 1951) estimated that loss due to condemnation of edible portions of pig carcasses amounted to about 50 cents per 100 pounds body-weight in U.S.A. in 1947. Bindseil (1967) reported that the total annual loss in Denmark was about £300,000. Any part of a liver showing parasitic damage is considered unfit for human consumption and depending upon the degree of damage, affected organs are either partially or totally condemned. This inevitably results in economic losses, but unfortunately a precise figure for such losses in the U.K. is not readily available. During the course of the prevalence study of Ascaris infection in pigs in North Wales reported earlier, records were obtained of the number of pig livers condemned. The condemnation was attributed mainly to Ascaris infection. Out of a total number of 882 pigs slaughtered at the abattoir between January 3, 1978 and February 28, 1978, a total of 81938.38g of liver was condemned - more than 7.48% of the total weight of liver obtain for the two-month period (Table 1). Though pig liver is relatively low priced compared to other portions of the carcass, at the slaughter house price of 34p. per lb. of pig liver, this

represented an economic loss to the slaughter house of about £61.42 over the 2 month period. There is little doubt that when projected on a national scale that this level of loss will run into millions of pounds. However, the true loss to the country is much more difficult to assess accurately. Infected pigs often have a low feed conversion rate - producing less pork per kg of feed consumed. This inevitably leads to higher costs of production.

Both the infective larvae and the adult worms have varying effects on the host; the effect of each depending upon its location and activities. The adults do not suck blood or graze on the intestinal mucosa as was previously considered (Gavin, 1913). Davey (1964) found that a large part of their ingesta consisted of host intestinal epithelial cells which have been sloughed into the lumen during the normal replacement cycle. They do, however, compete with the host for the feed it has digested. Large numbers cause emaciation, and slow growth and may occlude the intestine and thus interfere with the normal passage of material down the gut. In addition, some worms may enter the bile ducts,

occluding them.

For their part, the migrating larvae damage the liver while migrating through it, causing small haemorrhagic foci which are then replaced by connective tissue becoming the white spots (milk spots) and filaments of fibrosis that are characteristic of the infection (Oldham and Ostertag, 1922; Oldham and White, 1944; Jorgensen, 1973). In the lungs the larvae caused verminous pneumonia which may result in the death of the animal if a large number of larvae is present (Green and Oldham, 1964). Stray larvae have also been located in other tissues and organs of the host including the brain (Sprent, 1955, 1955a). In addition to causing disease by themselves, the infective larvae may carry or exacerbate virus diseases (Underdahl and Kelley, 1957; Underdahl, 1958; Shope, 1958; Nayak and Kelley, 1968).

Over the years, a number of anthelminthics have been developed which are active in eradicating the adult worms resident in the host's small intestine. By invading the host tissue, the infective larvae are able to evade the action of such anthelminthics. It is therefore of some considerable importance to try to understand the mechanism by which the infective larvae invade and penetrate the host tissue. It is hoped that

such knowledge may be of some help in the possible development of measures to counter or inhibit larval entry into the host tissue and thus prevent the establishment of the infection. Adult A. suum cannot develop unless the infective larvae have completed the tissue migratory phase of the life cycle (Sprent, 1954).

TABLE 1

Estimate of economic loss due to liver condemnation as a result of A. suum infection of pigs. The pigs were fourteen to eighteen weeks old and naturally infected they came from various farms in North Wales.

Date of Slaughter	Number of Pigs Slaughtered	Average wt. of pig liver	Number of whole liver condemned	Total wt. of liver trimmings	Total wt. of liver condemned
3/1/78	77	1204.14g.	2	7183.20g.	9591.48
10/1/78	82	1250.46g.	3	9928.60g.	13679.98
17/1/78	81	1284.02g.	-	9699.40g.	10983.42
21/1/78	140	1205.40g.	1	8210.00g.	9415.40
31/1/78	171	1243.22g.	2	4774.50g.	7260.94
7/2/78	84	1287.74g.	4	2683.30g.	7834.26
14/2/78	79	1240.82g.	5	1910.40g.	8114.50
21/2/78	61	1267.03g.	4	3161.20g.	7984.32
28/2/78	107	1242.57g.	4	2098.50g.	7069.08

Total weight of liver obtained over the two month period (January 3rd - February 28, 1978) = 1094209.20g

Total weight of liver condemned over the same period (January 3rd - February 28, 1978) = 81938.38g.

% of liver condemned over the given period = 7.49%

Slaughter house price of pig liver = 34p/1b. (1b. = 453.592g.)

Economic loss due to condemned liver over the two month period 6141.873p. = £61.42p.

PART ONE

DEVELOPMENT AND HATCHING OF

A. SUUM EGGS

GENERAL MATERIALS AND METHODS

Collection and Incubation of the Eggs

Adult female Ascaris suum were obtained from the intestines of pigs slaughtered at a local abattoir at Portdinorwic. These worms were dissected and the pro imal third of their bifurcate uteri removed and the eggs extracted and prepared for embryonation as outlined by Fairbairn (1970). The eggs were washed three times in 0.5m NaOH by gentle stirring using a magnetic stirrer. This treatment was necessary to remove the outer protein coat (the uterine layer) which otherwise makes the eggs very sticky and less easy to handle experimentally. After this initial washing, the eggs were rewashed with distilled water and placed in a 0.1m solution of H_2SO_4 allowing 10 ml of the acid for each gram of uteri. 5 ml of the egg suspension were then pipetted in a 50 ml conical flask stoppered loosely with cotton wool and incubated at the required temperatures.

The incubation medium was changed every 5 days to keep fungal growth in check.

Method of assessment of rate of development of the Incubated Eggs

The rate of development of the incubated eggs was checked every two days and the percentage

development estimated. 200 eggs were pipetted onto a clean microscope slide and the number of eggs which had developed to pre-determined stages of development counted Six stages were recognised - 1-celled stage (no development); 2-celled stage; 4-celled stage; 8-celled stage; morula stage; the first larval stage; as well as "degenerates". These stages of embryonation were chosen because of their relative ease of observation (Figure 4). Index values of 0; 1; 2; 3; 4; 5; and 0 respectively were assigned to each of these developmental stages. The rate of embryonation was expressed as a total index of development (S) which is given by the formula

$$S = \Sigma(X.Y)$$

where X = % of a given developmental stage in the egg sample

Y = the index value of the given developmental stage.

e.g.

When the % of the various development stages in a given egg sample are

a%; b%; c%; d%; e%; f%; g%

Total index value of development (S) is given by

S = a.0+b.1+c.2+d.3+e.4+f.5+g.0

In vitro hatching of the Embryonated Eggs

The fully embryonated eggs were hatched *in vitro* using a slight modification of Fairbairn's (1961) method. Samples of the embryonated eggs were neutralized with 10 ml of 0.5m phosphate buffer (pH 7.0) in a centrifuge tube; washed three times with distilled water and incubated in a McCartney bottle containing 5 ml of 0.25m sodium chloride pregassed with N_2-CO_2 (95%/5%) for 10 minutes in an ice-bath. 16 mg of solid sodium metabisulphite were added to the suspension which was agitated for a few seconds before 5 mls of 0.1m sodium bicarbonate solution also pregassed with N_2-CO_2 for 10 minutes in an ice-bath were added. The final mixture was then treated with the same gas mixture for 10 minutes in an ice-bath before incubation in a shaking water bath at 37°C.

Observation of the *in vitro* Hatching Process

At periodic intervals during hatching of the embryonated eggs, samples were removed and the hatching process terminated by freezing in an ice-bath. The mechanics of larval emergence with particular reference to the structure of the egg-shell, were studied directly under the light microscope.

Later, empty egg shells together with the

embryonated eggs were prepared for electron microscopy. The specimens were fixed in 3% glutaraldehyde in 0.1m cacodylate buffer for 4 hours, followed by 1 hour in cacodylate buffered 1% osmium tehoxide (pH 7.2). The specimens were then dehydrated through an ascending series of ethyl alcohol, transferred to acetone and finally placed in miniature stainless wire baskets and transferred to a critical point-drier. The critical point drying was accomplished with liquid CO_2. The dried specimens were then mounted on standard specimen stubs, coated with a layer gold-palladium (150-200^{o}A) and examined using an SEM 151 M-7 microscope operating at 30 kv.

For transmission electron microscopy, the specimens were fixed in 3% glutaraldehyde in 0.1m sodium cacodylate buffer (pH 7.2) at 4^{o}C overnight. After washing three times in the same buffer at 4^{o}C, the specimens were post-fixed in 1% osmium tetroxide for 4 hours at 4^{o}C and then quickly dehydrated in an ascending series of ethyl alcohol and embedded in araldite after the procedure of Luft (1961). 700^{o}A thick sections were cut with a glass knife on an LKB ultratome III ultramicro-tome. Sections were picked up in copper grids coated

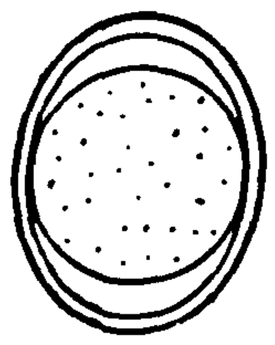
with celloidin (2% nitro cellulose) stained with lead citrate for 15 minutes (Reynold's, 1963), washed and counter stained either with 2% aquous Uranyl acetate or pottassium permangenate (Lawn, 1960) and examined on an AEI EM6 electron microscope operating at 80 kv.

Isolation of Hatched Infective Larvae

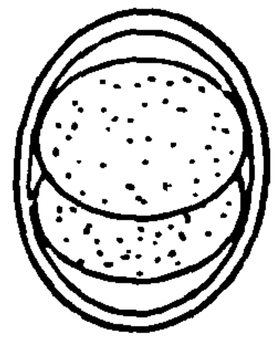
Hatched larvae were isolated from unhatched eggs, empty egg-shells and debris using a variation of the Baermann technique. A Baermann funnel was set up with a tap on the lower end. The funnel was filled with warm isotonic saline at 37°C. A nylon sieve (pore diameter 75 µm), was placed at the top of the funnel with the bottom of the sieve just touching the surface of the liquid; care being taken to remove all air that might have been trapped. The hatching medium containing the hatched larvae as well as the unhatched eggs and debris was then poured onto a piece of filter paper (Whatman No.1) so that the fluid soaked through but the larvae and other materials remained trapped. The filter paper was then inverted over the sieve. Larvae migrated to and concentrated at the bottom of the funnel from where they were run off in small volumes of saline.

FIGURE 4

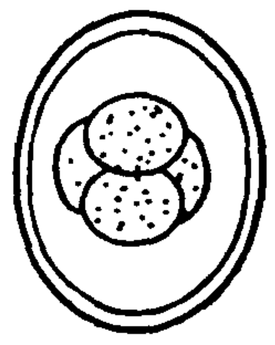
Stages of development of A. Suum ova used for assessment of the rate of development.



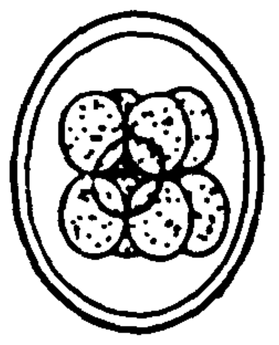
1-cell stage



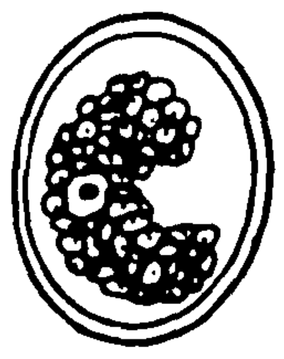
2-cell stage



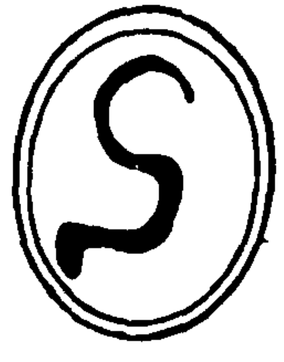
4-cell stage



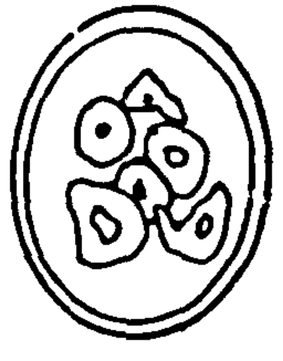
8-cell stage



morula stage



first larvae stage



Degenerate

Estimation of Larval Longevity

Isolated larvae were washed three times by centrifuging in distilled water and twice in two changes of 1.5% saline in 0.05m phosphate buffer (pH 7.2). After the final washing, the larvae were then resuspended in 10 ml of the fresh buffered saline in McCartney bottles and incubated at 37°C in a shaking water bath for up to 180 hours. The percentage of larvae alive after given time intervals was determined by observing 5 replicates of 30 larvae from each sample pipetted into small petri-dishes for signs of activity. The larvae were pipetted into the dishes after brief agitation of the incubation bottles. Larvae which showed no discernible movement after five minutes of observation were regarded as dead.

In vitro Penetration Assay

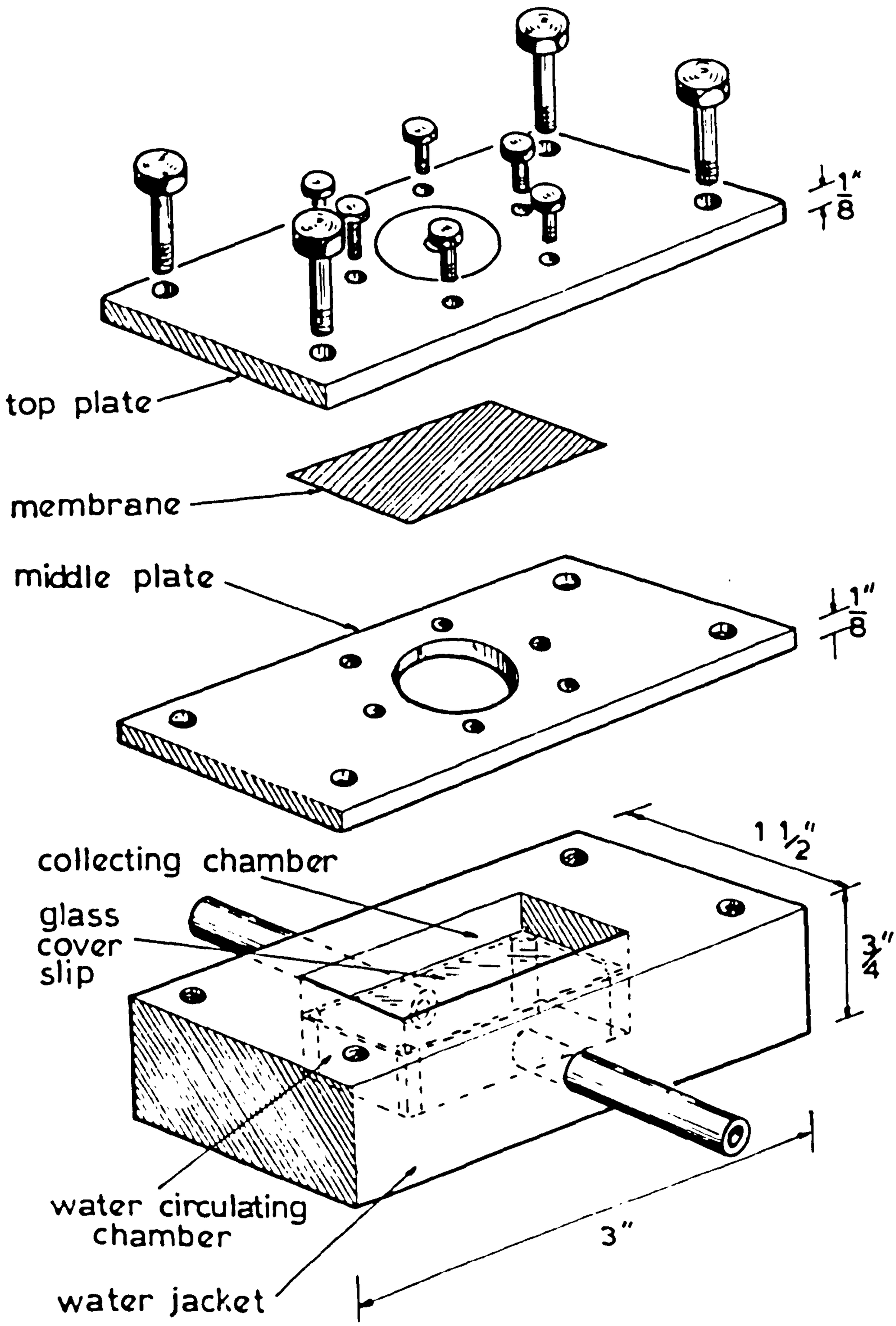
The ability of hatched infective larvae to penetrate various membranes was assayed using an in vitro penetration apparatus similar to that of Matthews (1972). A diagram of the apparatus used in the present study is shown in Figure 4.

The apparatus was constructed of perspex, it consists of a top plate (A), a middle plate (B) and a water jacket (C). The water jacket is divided into two chambers: an upper collecting chamber and a lower warm water circulating chamber. The two chambers are separated by a 22 x 44 mm

FIGURE 5

IN VITRO PENETRATION APPARATUS

In Vitro Penetration Apparatus



microscope glass cover slip which is cemented down to prevent passage of fluid between the chambers. On assembling the upper collecting chamber is filled with 0.005m phosphate buffered saline (pH 7.2) so that the surface of the solution is in contact with the underside of the membrane tightly screwed between the top and the middle plates. Particular care was taken during assembling the apparatus to avoid air bubbles. A current of warm water at 37°C was pumped through the lower water-circulating chamber so that a temperature gradient was established between the membrane and the bottom of the collecting chamber.

After rapid counting under a dissecting microscope, 30 larvae were concentrated in a small volume of buffered saline in the wells of micro-titre plates. These larvae were then transferred onto the surface of the membrane in the penetration apparatus with as little saline as possible. Constant checks were made to ensure that the membranes did not dry out during the duration of the experiments; a few drops of the saline being added, if and when necessary to replace losses due to evaporation.

Larvae which succeeded in penetrating the

membranes sank through the saline to the bottom of the collecting chamber. At the end of the assay, larvae in the saline above the membrane were pipetted into a watch glass and counted. The apparatus was then dismantled and the "penetrants" counted. Gelatin membranes were then either teased apart or stained with 0.05% methylene blue, orange G or mallory triple stain and observed under an inverted microscope. Other membranes were fixed in 70% alcohol, embedded in wax and sections 10 μm thick cut and stained with mallory triple stain. The number of larvae present (if any) were recorded.

Preparation of Penetration Membranes

The technique used to prepare gelatin membranes was essentially that of Clegg (1969). A 10% (w/v) solution of sheet gelatin was prepared in hot glass distilled water. The solution was brought to 100^{o}C for a few seconds to ensure maximum rupture of the hydrogen bonds between the protein molecules. The hot gelatin solution was then poured into a 500 ml beaker and maintained at 60^{o}C on a hot plate. Surgical cotton gauze, as well as various grades of filter paper were stretched across pieces of polystyrene sheet (8 cm by 7 cm) and secured with either office pins

or adhesive tape. A sheet of celophane material was placed between the tissues and the polystyrene sheet to prevent the penetration membranes sticking to the polystyrene after preparation. The polystyrene sheets were then held with the tissues facing the surface of the gelatin solution and dipped slowly into it, evenly impregnating the tissues with gelatin. After 30 seconds, the polystyrene sheet was lifted away from the gelatin, by raising one edge first, and then held perpendicular to allow excess gelatin to drain away leaving a thin film clinging to the tissues. The prepared membranes were then allowed to stand on the bench for 10 minutes after which they were dried in an incubator at 40°C for 1 hour to eliminate surface moisture. The tissues were later chrome tanned to decrease the solubility of the gelatin.

A number of natural membranes were also used. These consisted of either whole or scraped intestinal tissue from mice or pigs and were used fresh, dried in air, fixed in 70% alcohol for 15 min. to 24 hrs. or tanned.

Membranes were tanned in 0.006m chrome potassium sulphate in 0.2m sodium acetate/acetic acid buffer at pH 5.0 for 45 mins. at 22°C, and then thoroughly washed in cold glass distilled water.

Both the duration of tanning and the pH of the chrome solution were carefully checked as Clegg (1969) reported that these could be important in preventing excessive hardening of the membranes.

The concentration of the chrome salt, the period of tanning and the pH of the tanning solution were varied empirically in later trials in an attempt to obtain a membrane sufficiently stable under the conditions of the *in vitro* penetration experiments and that could at the same time allow larval penetration, but to no avail.

Passage of larvae through natural and artificial membranes *in vitro*

The ability of larvae hatched, collected and aged as described earlier to penetrate various tissue membranes *in vitro* was assessed. The membranes tried and the percentage penetration achieved are shown in Table II.

Staining of larvae for lipid level determination

Active larvae, hatched and isolated as described, were hand picked without bias. These were aged in McCartney bottles in phosphate buffered saline either at 37°C in a shaking water bath, which meant that the larvae were continuously agitated; or, at various temperatures in standing incubators to avoid the

inherent variation between cultures, which has been reported in similar studies (Croll, 1972), each experiment was conducted using larvae from the same culture.

Larvae were stained in saturated oil Red O (Croll, 1972). The larvae were transferred to solid watch glasses and the saline withdrawn, they were immediately flooded with 70% alcohol saturated with oil Red O at 60°C and kept at 60°C for 20 mins. Larvae were then washed in three changes of distilled water and transferred to a water-glycerol (50 : 50, v/v) mixture in a loosely covered watch glass for 24 hours. The water slowly evaporated, permitting gradual dehydration and clearing to about 95% glycerol (Seinhorst, 1959, Croll, 1972). The larvae were finally mounted in glycerine jelly. This technique provided permanent preparations of larvae in which the unbound neutral lipid was stained pinkish red (Pearse, 1960). Care was taken to mount larvae flatly, on spotless slides in spotless glycerine. Glass-wool was used to support the cover-slips to ensure that they did not rest on the larvae.

The density of oil Red O in the larvae was measured using a Vickers M85 scanning microdensitometer at 520 nM, the peak absorbance of the stain (Figure 6).

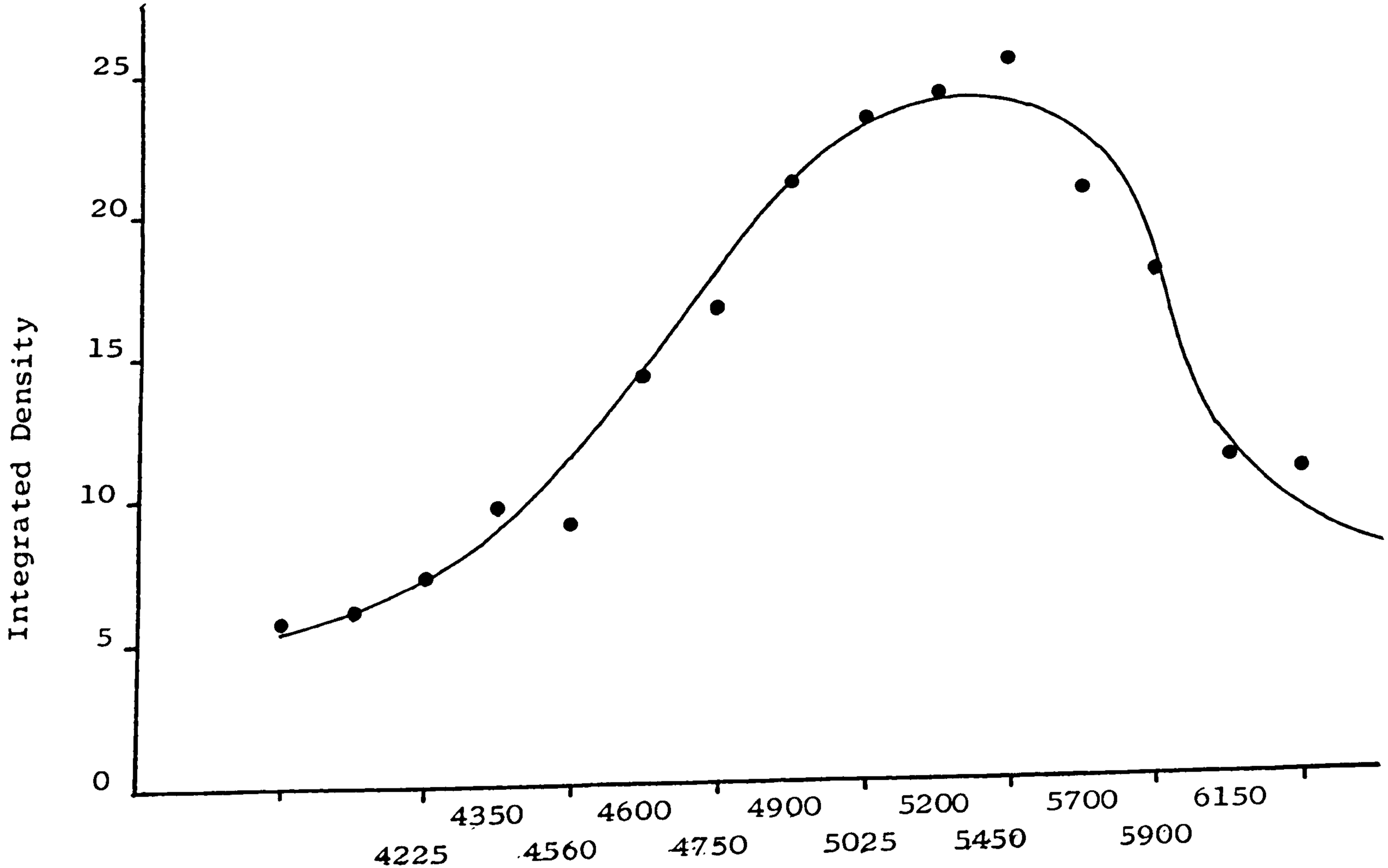
TABLE II

PERCENTAGE OF INFECTIVE LARVAE OF A. SUUM ABLE TO PENETRATE VARIOUS TISSUE MEMBRANES IN VITRO

Membrane	Duration of Assay	% Penetration
Fresh pig small intestine	15 min- 16 hr.	ø
Fixed " " "	"	ø
Tanned whole pig small intestine	"	ø
Tanned scraped " " "	"	ø
Fresh mouse small intestine	"	ø
Fixed " " "	"	ø
Tanned whole mouse small intestine	"	ø
Tanned scraped " " "	"	ø
Filter Paper (Whatman Grades 42, 44, 542)	3h.	100%
Tanned thin gelatin film	15 min- 6 hr.	ø
Tanned filter paper impregnated with gelatin (Whatman Grades 42, 44, 542)	"	ø

FIGURE 6

Graph of integrated density of Oil Red O stained Ascaris suum larvae.


Integrated Density
25
20
15
10
5
0
4350
4600
4900
5200
5700
6150
4225
4560
4750
5025
5450
5900
- Instrument Setting

SECTION A

HATCHING OF A. SUUM EGGS

INTRODUCTION

Relatively little information is available on the mechanics by which infective A. suum larvae escape from their egg-shells. Much more is known about physiological aspects of the process (Rogers, 1958; Fairbairn, 1961; Hass and Todd, 1962; Jaskoski and Colucci, 1964; Perry and Clarke et alia). As invasion of the host tissue by the parasite initially depends on the availability of infective larvae, a consideration of the mechanism of hatching of infective ova seemed an appropriate starting point in investigations into the strategy by which the infective larvae gain entry to, and migrate through, host tissues. This was considered particularly necessary as it might provide some insight into the way in which infective larvae overcome barriers during their migration.

Hatching has been investigated in a number of nematode species. In most of these studies attention has been focussed on the physiological aspects. With few exceptions (Doncaster and Shepherd, 1967; Wallace, 1968; Bird, 1968; Croll, 1974), little attempt has been made to elucidate the mechanics of the hatching process. In the few species that so far have been studied, larvae emergence has been associated with a number of factors. These include mechanical

breakdown of the shell due to the activity of the larvae (Doncaster and Shepherd, 1967; Silverman and Campbell, 1959); osmotic factors (Croll, 1974); and the activity of larval enzymes or various combinations of these factors (Bird, 1971).

Doncaster and Shepherd (1967) considered that the process of hatching in Heterodera rostochiensis is largely mechanical. Using time lapse cinephotomicrography, they showed that the nematode cuts a site in the egg-shell with its stylet and emerges through it. Bird (1968) suggested that larval movement prior to hatching may occur in association with enzymatic distortion of the egg-shell. Rogers (1958) and Fairbairn (1961) in a series of in vitro experiments have shown that during hatching, the infective larvae of A. suum inside the egg are stimulated to produce a hatching fluid, containing an esterase, a chitinase and possibly a protease. The esterase is thought to alter the permeability of the previously otherwise semi-permeable ascaroside membrane (Fairbairn, 1957, 1961; Rogers, 1958), permitting the Chitinase and protease to act upon the chitinous layer (Rogers, 1958). During this change in the permeability of the ascaroside membrane, the membrane has been considered to remain intact with no chemical or conformational changes

involved (Barrett, 1976). However, emulsification of this layer in a number of other nematodes eggs during hatching has been reported (Wilson, 1958; Wallace, 1968a, b; Bird, 1968). Bird (1968), as a result of studies on the ultrastructure of the egg of Meloidogyne javanica during hatching showed that prior to larval emergence from the egg-shell, the ascaroside membrane becomes hydrolyzed.

In the present study, an attempt has been made to look at the structural changes that take place in the egg during the process of hatching of the infective larvae of A. suum. No attempt has been made to get involved with the physiology or the biochemistry of the hatching process as these have been excellently treated by a number of workers including Rogers, 1958, 1960; Fairbairn, 1961, and Clarke and Perry et alia.

OBSERVATIONS

Hatching of Infective Eggs

Fertilized eggs were incubated at 22° ± 1°C and 30° ± 1°C for 60 and 22 days respectively to attain full embryonation. The eggs were divided into 36 separate McCartney bottles and the percentage of eggs containing motile larvae was recorded. A few of the eggs were fixed and prepared for electron microscopy. Each sample was then stimulated to hatch.

After stimulation, small samples of eggs were removed at 15 minute intervals and the activity of the contained larvae checked by counting the number of eggs in which the larvae were motile. The nature of the larval movement inside the egg was also noted. The percentage of the eggs that hatched at each time interval was recorded, as well as the nature of the hatching process. The count at each time interval was in triplicate and each sample was determined five times.

Figure 7 shows the relationship between the percentage of eggs, in which the enclosed larvae were active, and time after stimulation. Before stimulation, in less than 15% of the eggs were the enclosed larvae observed to be motile. However, soon after stimulation, the percentage containing motile larvae rose sharply, reaching a peak (97.68 $\pm$ 1.95%) about 30 minutes post-stimulation, after which there was a gradual decline. Hatched larvae were not observed in the various egg samples until about 45 minutes after stimulation of the eggs. The percentage hatch

is shown in Figure 8.

The nature of the hatching process was examined with both normal transmitted light and under phase contrast. The eggs were mounted in phosphate buffered saline (pH 7.2) on microscope slides with glass-wool placed between the cover slip and the slide to prevent any distortion of the eggs. Plate 2a, b, c, d, and e show the sequence of the hatching process. The larvae exhibited repeated patterns of movement inside the egg before emergence. This is shown diagramatically in Figure 9. Within 15 minutes of stimulation the majority of the larvae inside the eggs tended to move inside in a figure of eight. This type of movement involved the whole of the body in a gliding wave which gradually extended from the head to the tail. After the next 15 minutes or so, much of the movement then appeared to be restricted to the anterior $\frac{3}{4}$ of the body, with the head moving round the egg-shell as if it were "feeling-out" the inner surface of the shell. After about 45-90 minutes post-stimulation, much of the movement ceased and the majority of the larvae at this time seemed to settle down in a "resting" position with much of the posterior end coiled and the head pressing against

FIGURE 7

Graph of percentage of A. suum eggs embryonated at 30° ± 1°C in 0.1N H_2SO_4 for 26 days, in which the enclosed larvae were observed to be motile at various time intervals after being stimulated to hatch in vitro at 37°C.. The points represent the mean of 15 determinants and the vertical lines indicate the standard error.

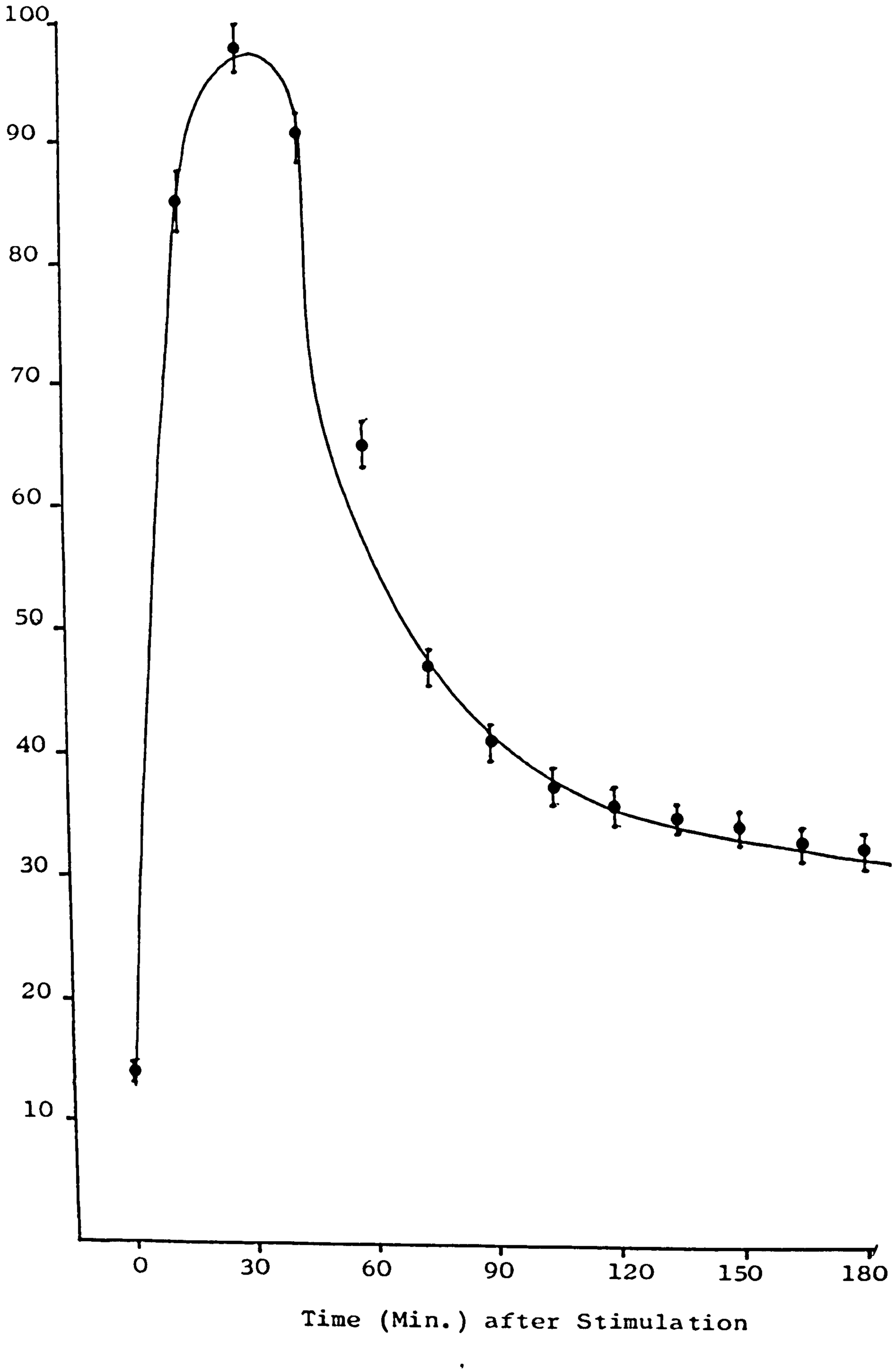
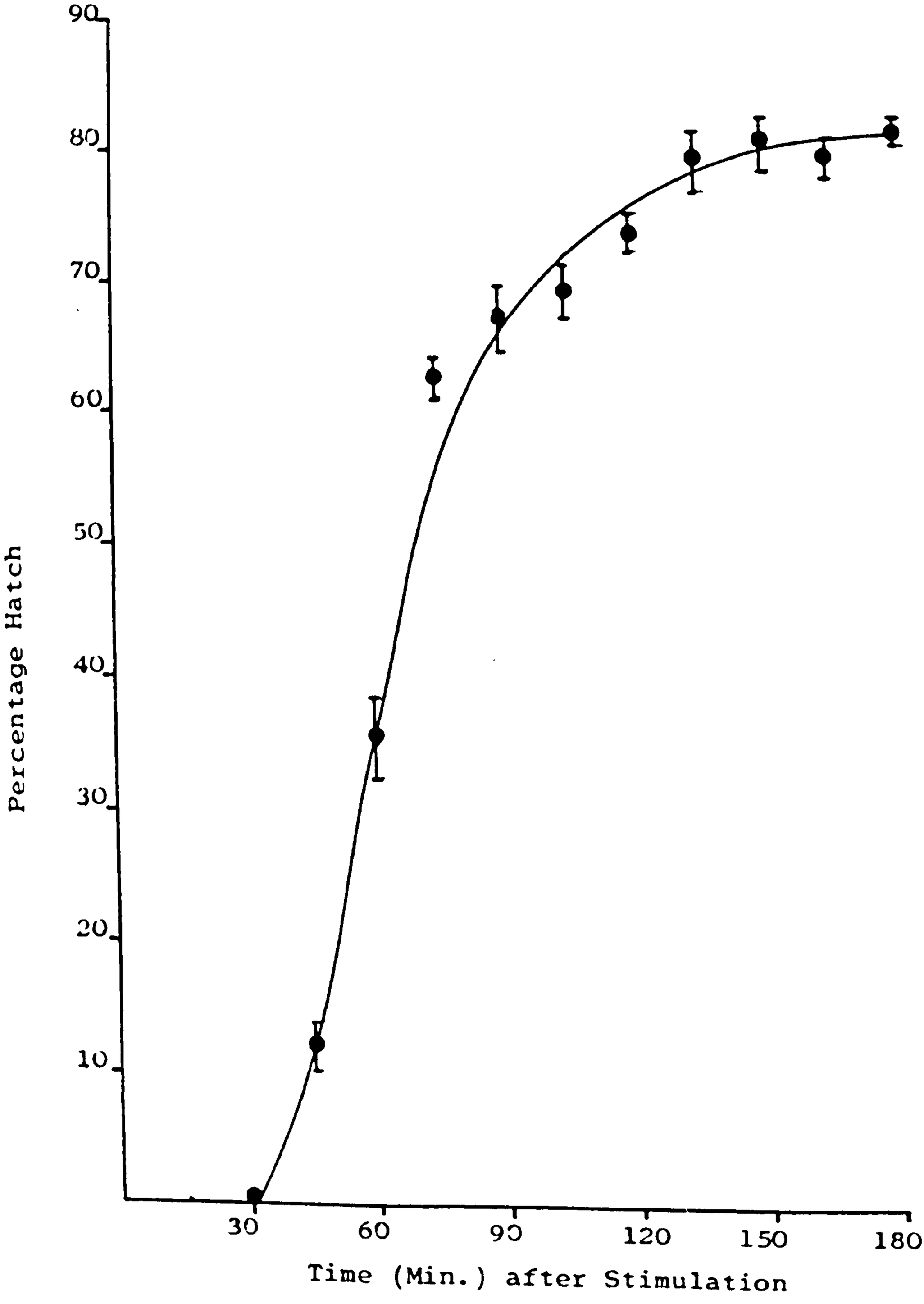

100
90
80
70
60
50
40
30
20
10
Percentage number of eggs containing Motile larva
0
30
60
90
120
150
180
Time (Min.) after Stimulation

FIGURE 8

Graph of percentage hatch of A. suum infective eggs embryonated at 30°± 1°C in 0.1N H_2SO_4 for 26 days and subjected to hatching stimulus in vitro at 37°C. The points represent the mean of 15 determinants and the vertical lines indicate the standard error.

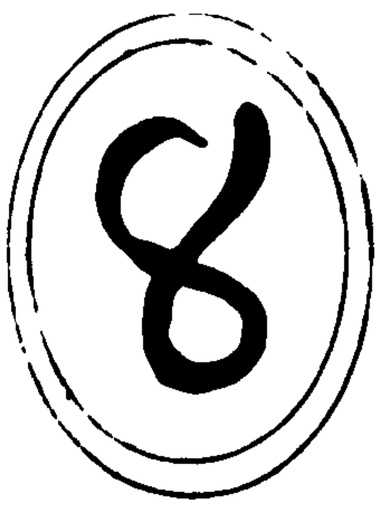

90
80
70
60
50
40
30
20
10
Percentage Hatch
30
60
90
120
150
180
Time (Min.) after Stimulation

the shell, Plate 2a. Although in some of the eggs recovered after 90 minutes of stimulation the larvae still maintained this "resting" position, the majority of them were by this time loosely coiled, Figure 9. Movement was much reduced and consisted of an occasional reversal of the coil.

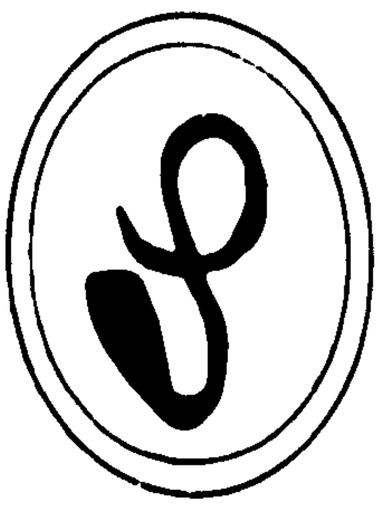
Close observation of the larvae inside the egg at high magnification did not reveal any oesophageal pulsation of the type which has been described in some nematode species (Bird, 1968; Croll, 1972). About 45 minutes after stimulation of the eggs some of the larvae (about 11%) were observed to protrude from a minute hole in the egg-shell. The position of the hole on the egg-shell varied and was not restricted to any one region. Early descriptions (Lee and Atkinson, 1976) referred to the inner shell membranes forming a small bleb or vesicle around the emerging larva, this was not apparent during the present study. Although either end of the larvae may emerge first, the anterior end emerged first in more than 73% of observations.

FIGURE 9

Diagrams illustrating patterns of repeated movement of the infective larva of A. suum at various time intervals after being stimulated to hatch in vitro at 37°C.

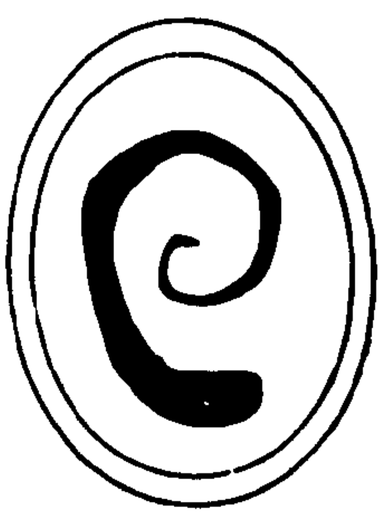


15 minutes after stimulation



30 minutes after stimulation

45 minutes after stimulation.
At this position much of the
larval movement has ceased
and larva appear to be "resting"
with the Head pressed on the egg-
shell.



90-180 minutes after stimulation

Once initiated emergence took between 90 and 135 seconds. Considerable effort appeared to be involved in emergence as the larvae were often constricted by the egg-shell at the place of emergence and were seen twisting and pulling in their efforts to emerge (see Plates 2c and d). The egg-shell retained its shape during and after the hatching process, and there was no indication of the egg-shell going plastic at any time (Plates 2 and 3).

Fine structure of the egg-shell

Ultrastructural examination of the egg-shell before and after hatching revealed interesting changes in the inner shell layer after hatching. The eggs varied considerably in size. However, the majority of them measured 52.87± 2.55 µm in length by 42.00± 3.68 µm in width (i.e. without the uterine layer).

In the thin sections (Plate 4), the shell was observed to consist of 3 layers - an outer vitelline layer (vl), a middle chitinous layer (cl) and an inner lipid layer (ll). The lipid inner layer was made up of two bands of spongy wavy membranes which in the unstimulated

egg (Plate 4) appeared very much intact. However, after hatching, this lipid inner layer was disrupted (Plate 4c) although not completely digested. No other layer of the egg-shell appeared to be extensively affected in this way except at the point of larval emergence where a hole was bored through the three layers (Plate 4c). How this hole was made is not obvious. The hole measures about 9.7 $\pm$ 1.20 μm in diameter against the larval body diameter of about 13.00 $\pm$ 1.64 μm.

DISCUSSION

Bird (1971) suggested that the mechanism of hatching amongst nematode eggs may be generally similar. He considered the basic mechanics to involve a behavioural response in the form of increased activity by the larvae to a hatching stimulus; breakdown of the lipid inner layer of the egg-shell and an increase in the plasticity of the egg-shell leading to its distortion. Increased movement of the infective larvae inside the egg prior to hatching has been reported in a number of species (Rogers, 1958; Wilson, 1958; Fairbairn, 1960; Flegg, 1968; Bird 1968; Doncaster and Shepherd, 1968). However, the time for initiation of such movement has varied from one species to another. In Heterodera rostochiensis larval movement in stimulated eggs did not start for at least a day after stimulation and stylet movement for at least another day (Doncaster and Shepherd, 1968; Bird, 1971). The present study has shown that in the case of A. suum, active movement of the infective larvae inside the eggs started soo n after application of the hatching stimulus, with over 90% of the larvae moving actively 30 minutes after stimulation (Figure7.). However, this number began to drop 15 minutes

later by which time emergence had started (Figure 8).

Wallace (1968) suggested that pre-hatch movement of infective larvae inside the egg may be necessary to bring their muscles to a high level of efficiency, particularly in those nematodes whose eggs hatch outside the host and therefore may need to "actively" invade their host. A. suum gain entry into their host via the oral route. The infective eggs hatch in the small intestine and the hatched infective larvae penetrate the host intestinal tissue en route to the deeper tissues and organs of the host.

Exercise alone, of course, may not be a sufficient explanation for pre-hatch movement (Croll, 1974). In a number of species, such pre-hatch movements have been associated with the production of larval secretions which bring about changes in the structure of the egg shell leading to its distortion and rupture (Flegg, 1968; Bird, 1968). The work of Rogers (1958, 1960) and Fairbairn (1961) on hatching of the eggs of Ascaris clearly indicated that the infective larvae produce a chitinase, a lipase and possibly a proteinase in response to host stimuli. The lipase was considered to alter the

permeability of the inner lipid layer allowing the chitinase and possibly the proteinase to attack the outer layers of the egg-shell (Rogers, 1958). The present study showed that the action of the larval enzymes does not render the egg-shell plastic (Plate 2a, b,c,d) as has been described in the case of Meloidogyne javanica (Bird, 1968) and Necator americanus (Croll, 1974). Nor did it involve extensive digestion of the shell layers (Plate 4). However, the lipid inner layer of the egg-shell was broken down following hatching (Plate 4). How far this breakdown of the lipid layer of the egg-shell was due to the action of the larval enzymes or to active movement of the larvae inside the egg before hatching is not clear. However, it is interesting to note that the lipid layer, although much broken down was not completely digested. So if the breakdown was due to the action of the larval secretions, it would appear that the secretions do not perhaps play much of a digestive role or at least are incapable of digesting the entire lipid layer. Wilson (1958) considered that movement might help to disrupt the lipid layer, this idea appears compatible with the present results.

At the point of larval emergence a hole was made through the 3 layers of the egg-shell (Plate 4). As reported earlier, it is not obvious how this hole was made. There are many recorded instances of mechanical force being used by hatching larvae to pierce the egg-shell prior to emergence. These have included widely separated genera such as Paratylenchus (Di. Edwardo, 1960); Paratylenchus (Rhoades and Linford, 1961); Haemonchus (Silverman and Campbell, 1959); Trichostrongylus (Wilson, 1958); Aphelenchus avenae (Taylor, 1962); Nacobbus (Clark, 1967) and Meloidogyne (Dropkin, Martain and Johnson, 1958; Bird, 1968; Wallace, 1968b). Croll (1974) attributed the split in the egg-shell of N. americanus during hatching to the action of the head (or tail) of the infective larva on the shell. This caused a sudden release of high internal pressure within the egg which carried the larva from the shell. The circular nature of the exit point in the case of A. suum (Plate 3) and the relatively slow rate of emergence, seem to rule out the possibility that the hole could have been made as a result of such a split and sudden release of internal pressure.

In many plant parasitic forms, the stylet is used to cut a hole in the egg-shell through which the larva escapes (Wallace, 1968; Doncaster and Shepherd, 1967). The infective larva of A. suum does not possess any such cuticular armature that might be utilised in this way.

Fairbairn (1960) in his description of the emergence of the infective larva of A. lumbricoides of porcine origin, diagrammatically illustrated polar emergence of the infective larva. He reported seeing no evidence of any structural modifications of the polar region of the infective eggs. Recently, Ubelaker and Allison (1975) have described a specilization of one pole of the egg of A. lumbricoides and A. suum. Based on scanning electron microscope examination of the morphology of fresh "undecoated" eggs they suggested the presence of an opercular region through which the larvae emerge. The presence of an operculum, although a common feature of trematode eggs, has been confirmed on only a few nematode eggs (Porrocaecum ratti, Sprent, 1973; Ascaridia galli, Cruthers, Weise and Hanson, 1974; S. muris, Van der Gulden and Van Aspert-van Evp, 1976; Porrocaecum ensicaudatum, Wharton, 1979; Aspiculuris tetraptera, Wharton, 1979; S. obvelata, Wharton, 1979b).

Wharton (1979) in his studies on the structure of the egg-shell of Aspiculuris tetraptera showed that both the uterine and the chitinous layers of the egg-shell were modified over the whole of the opercular region. The present study has failed to confirm the presence of an operculum on the egg-shell of A. suum. No modification of the chitinous shell of the egg of A. suum as clearly demonstrated in the case of Aspiculuris by Wharton (1979) was identified in the present study, and larval emergence from the egg-shell was not always polar (Plate 2).

Nevertheless, it is still possible that the egg-shell might be endowed with an area of chemical weakness which, although it may not be obvious as a physical structure beyond the uterine outer coat of the egg-shell (Ubelaker and Allison, 1975), could be susceptible to the action of the larval secretions. This might account for the rather localised digestion of the egg-shell at the point of larval emergence (Plates 3, 4c). The larva inside the egg is surrounded by a fluid filled cavity, and whatever secretions are produced by the larva might be expected to be dispersed in the fluid medium

reducing to a minimum the chances of localised action, unless such an area of chemical weakness exists on the egg-shell. Alternatively, it could be that by its reduced movement and fashion of "rest" with the head pressed on the egg-shell (Plate 2a and Figure 9) the action of the larval secretions is concentrated on a limited area causing the localised dissolution.

PLATE 2

Hatching of A. suum eggs. Photomicrographs illustrating sequence of the hatching process of the infective larva (of A. suum) under phase contrast. The infective eggs were embryonated at 22°C ± 1°C and hatched in vitro by a slight modification of the Fairbairn's (1961) technique at 37°C.

A. Fully developed egg containing the infective larva removed from the hatching incubator just before larval emergence begins

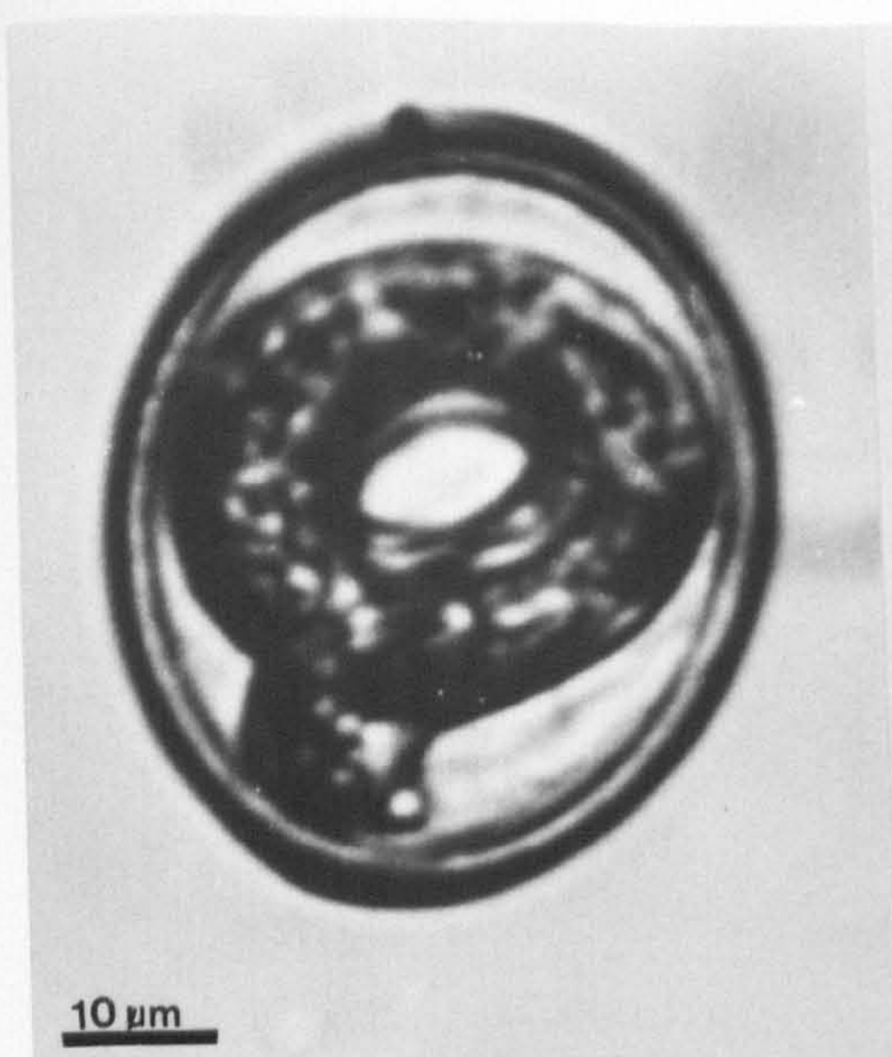
B.)

C.) Stages of larval emergence from the egg-shell

D.)

Note: the shape of the egg-shell did not become distorted during the process of larval emergence.
The eggs were from different egg samples collected at various times during the hatching process.

E. Second stage larva of A. Suum photographed alive under phase contrast soon after emergence from the egg-shell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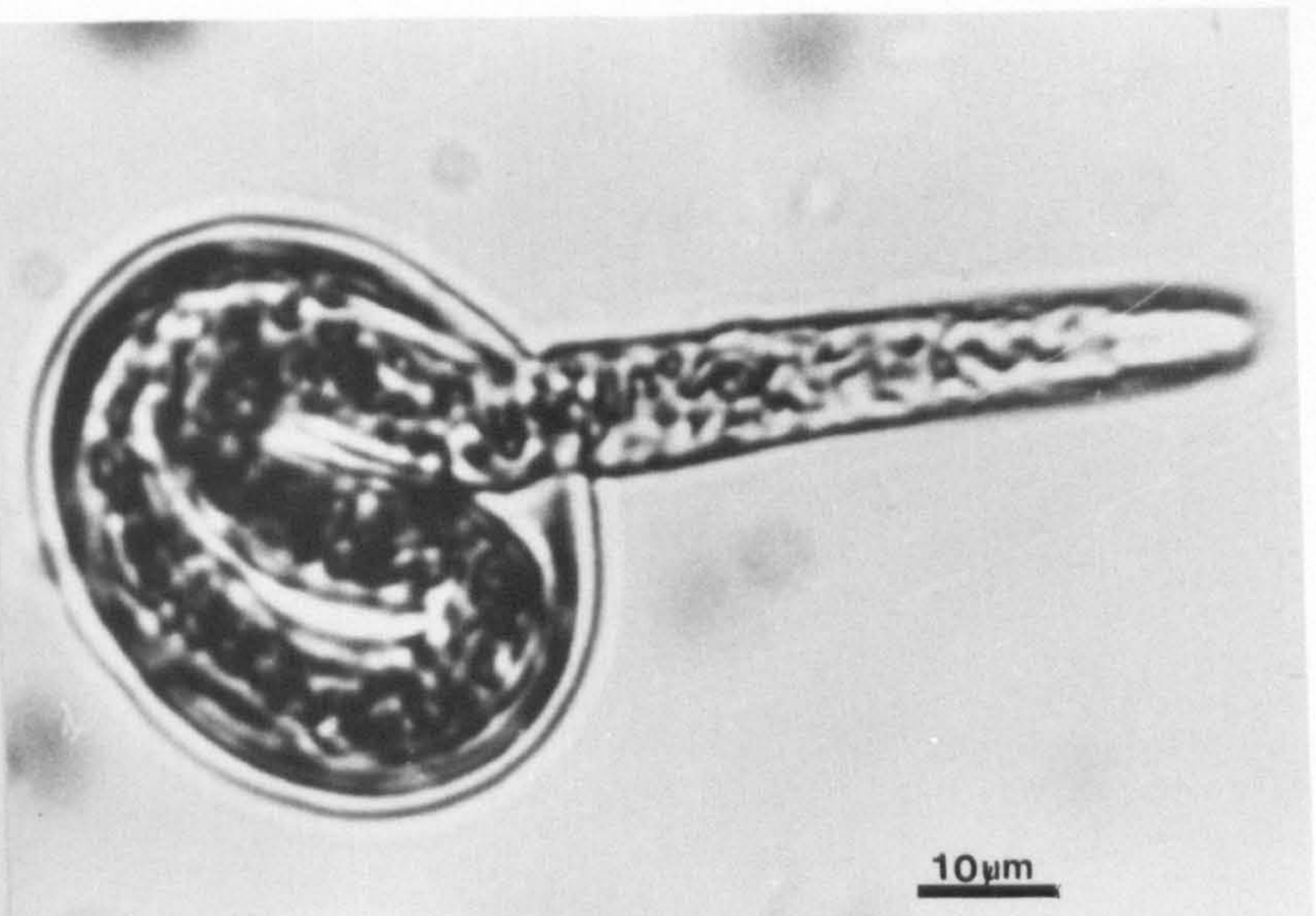
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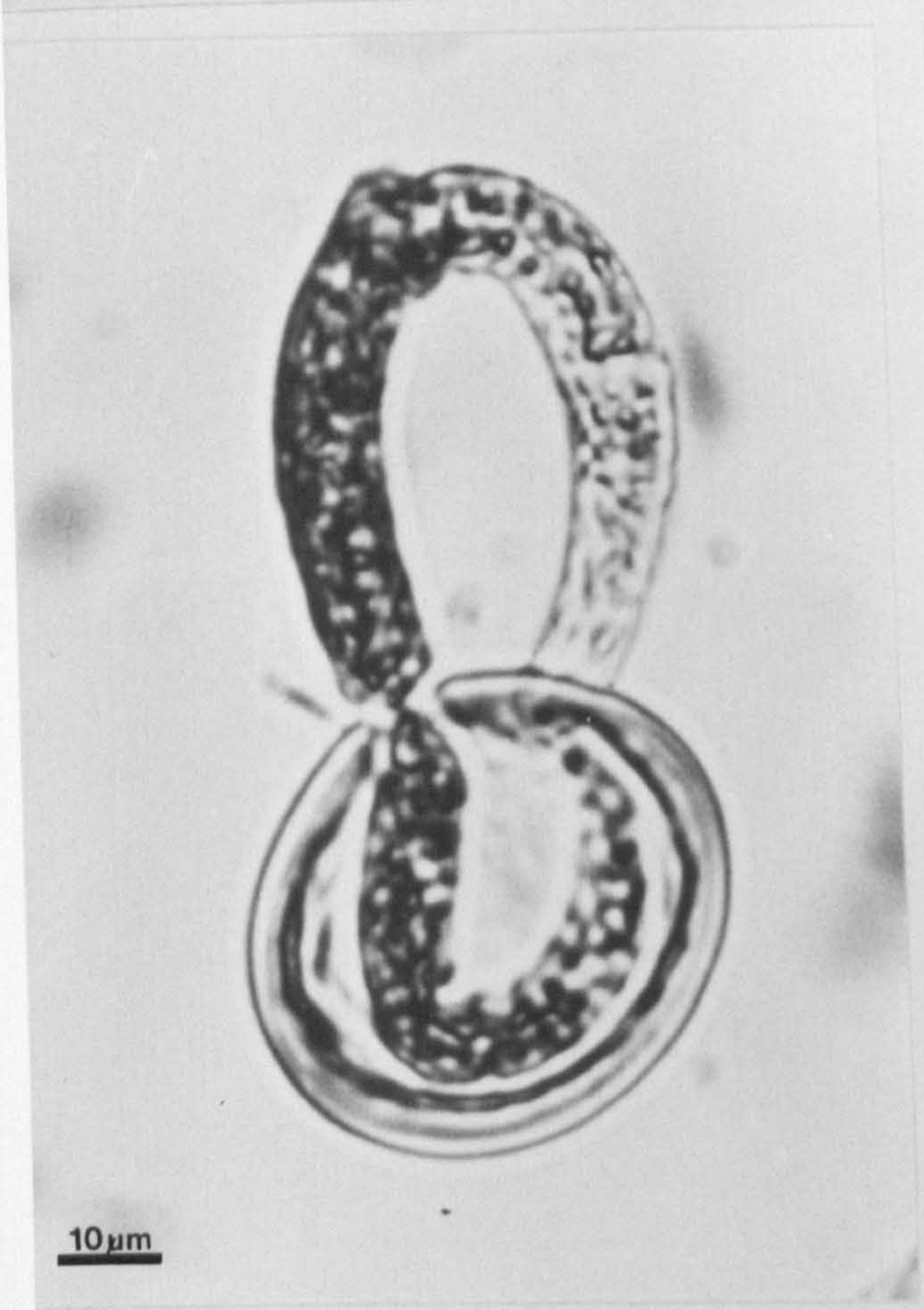
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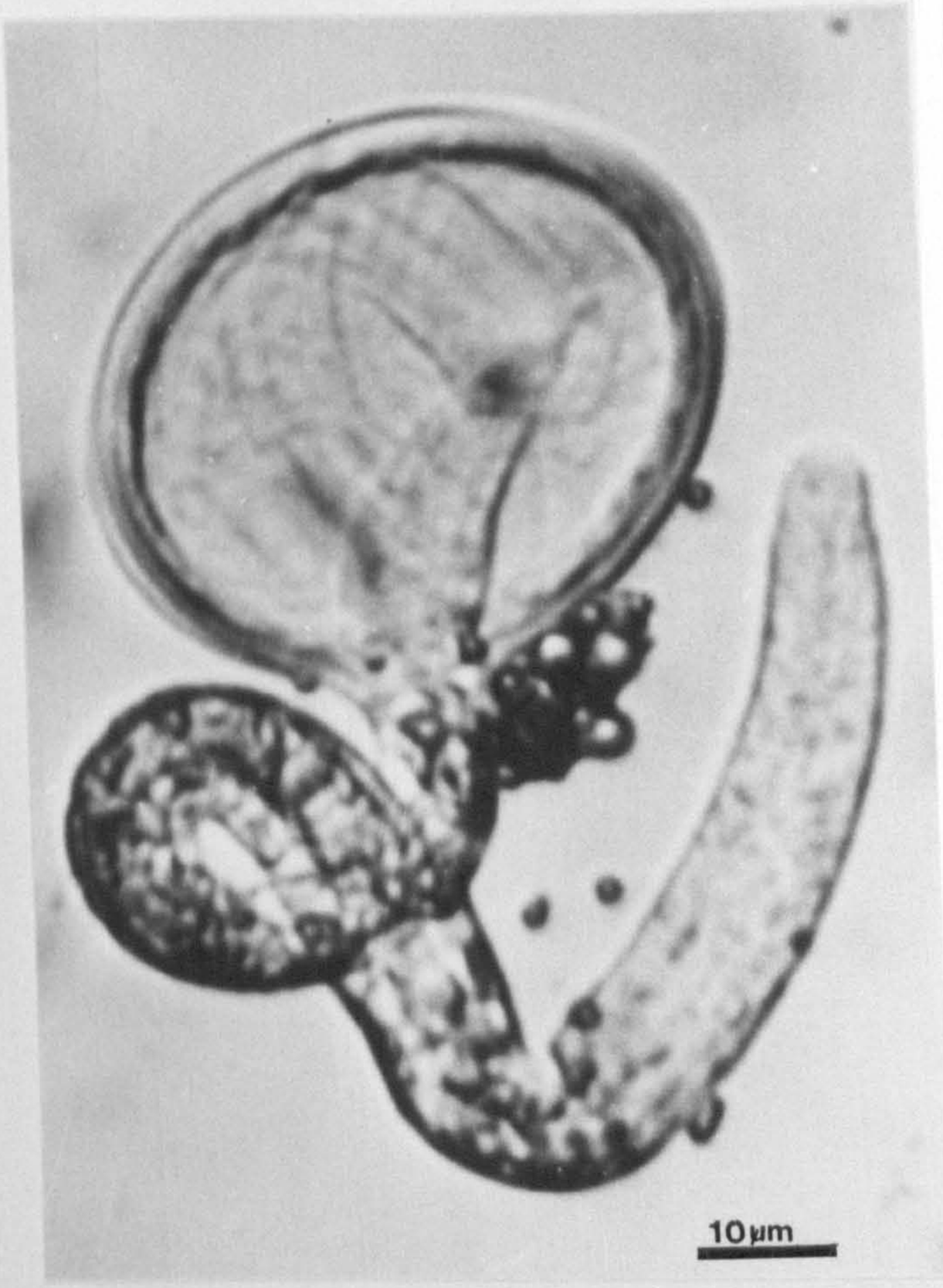

10µm


10µm

PLATE 3

Scanning electron microscope of an empty egg shell. Note the single circular hole on the egg-shell through which the infective larva has emerged. The shell retained its shape after hatching.

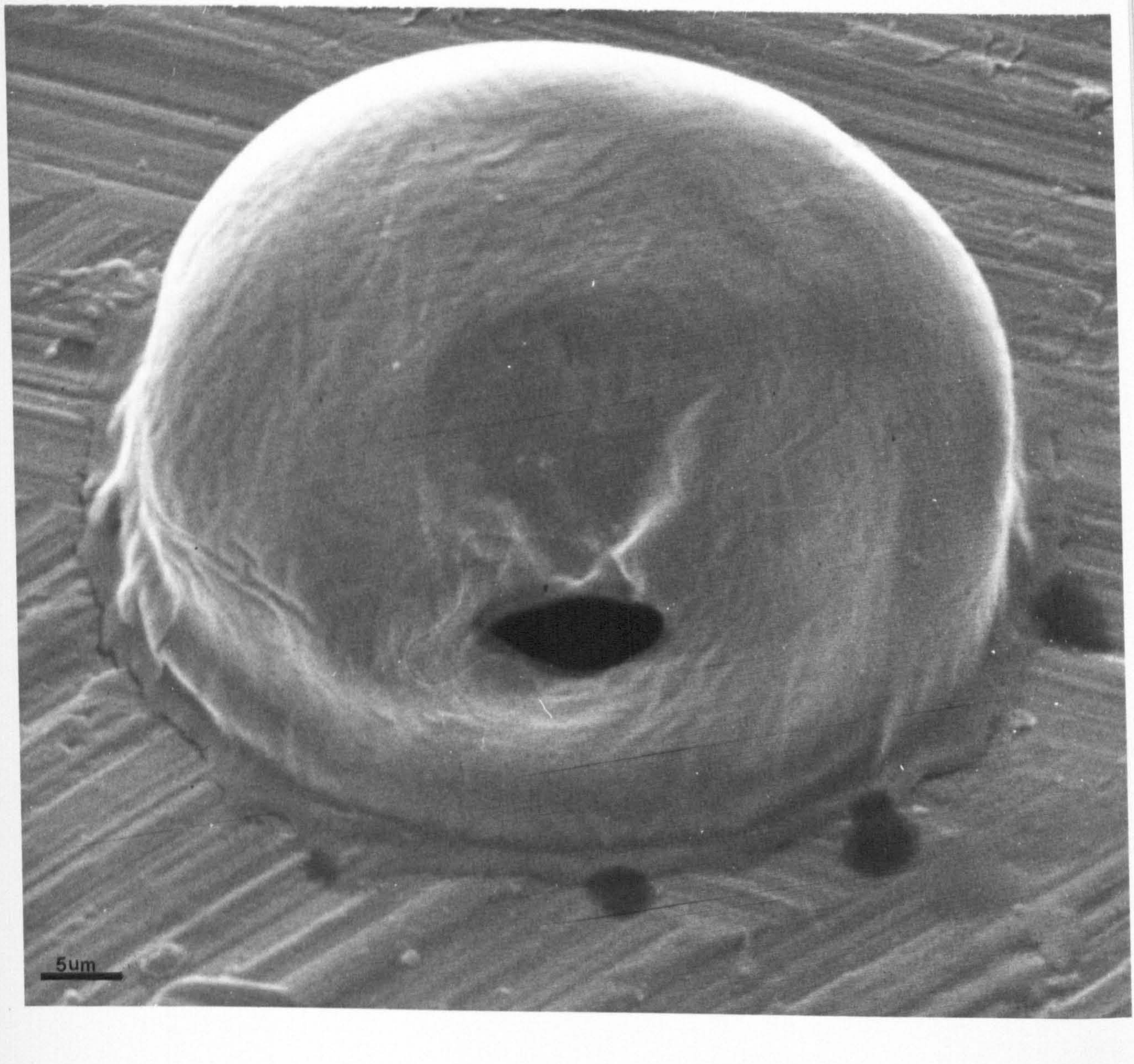

5um

PLATE 4

Transmission electron micrograph of section of *A. suum* egg:

a. Unstimulated egg containing infective larva

b. Stimulated egg containing infective larva

c. Hatched empty egg shell. Note the wavy unbroken nature of the lipid inner layer of the egg shell in the prehatched eggs, which in the hatched empty egg shell is much broken down.

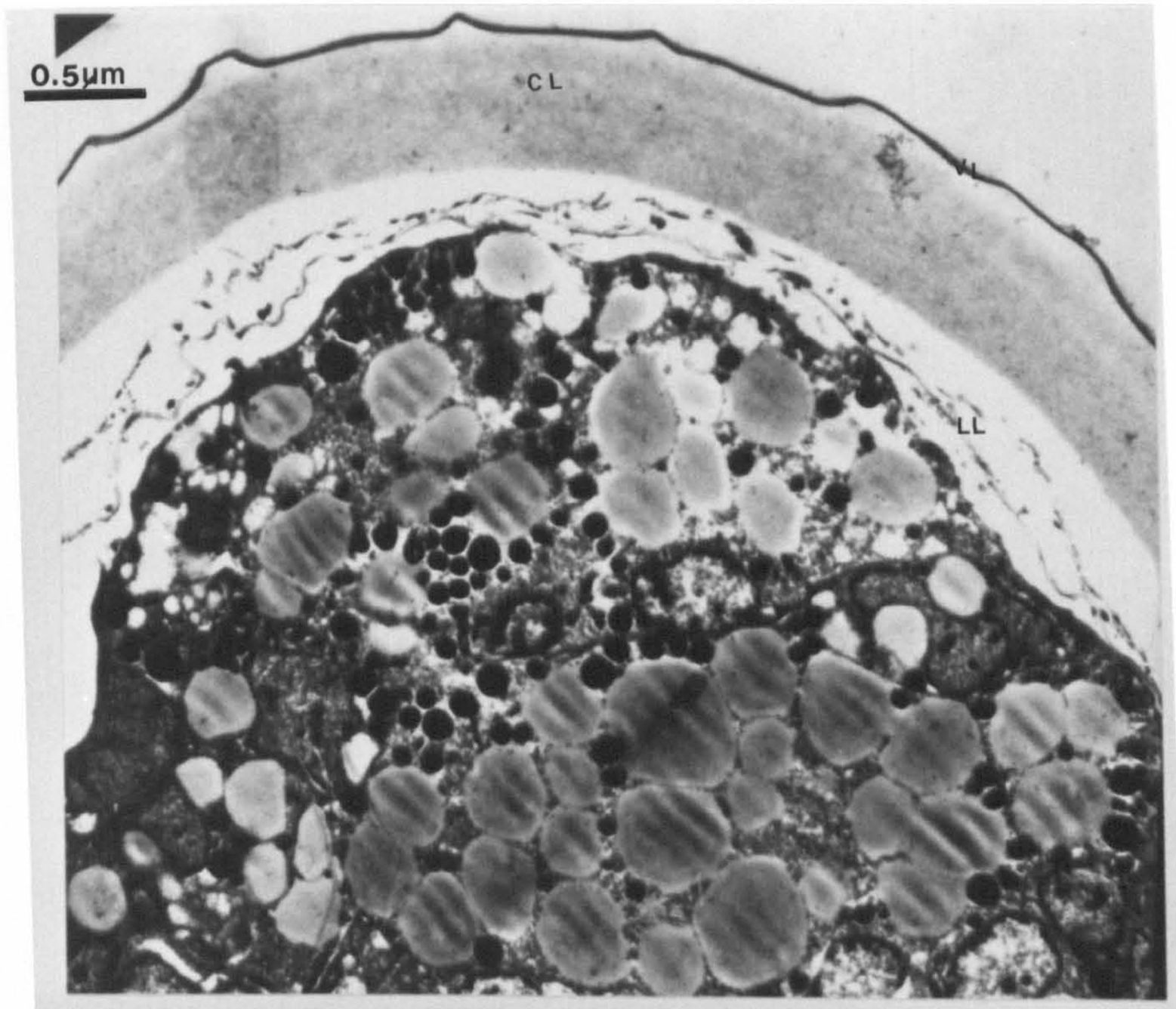
KEY

ll Lipid Layer

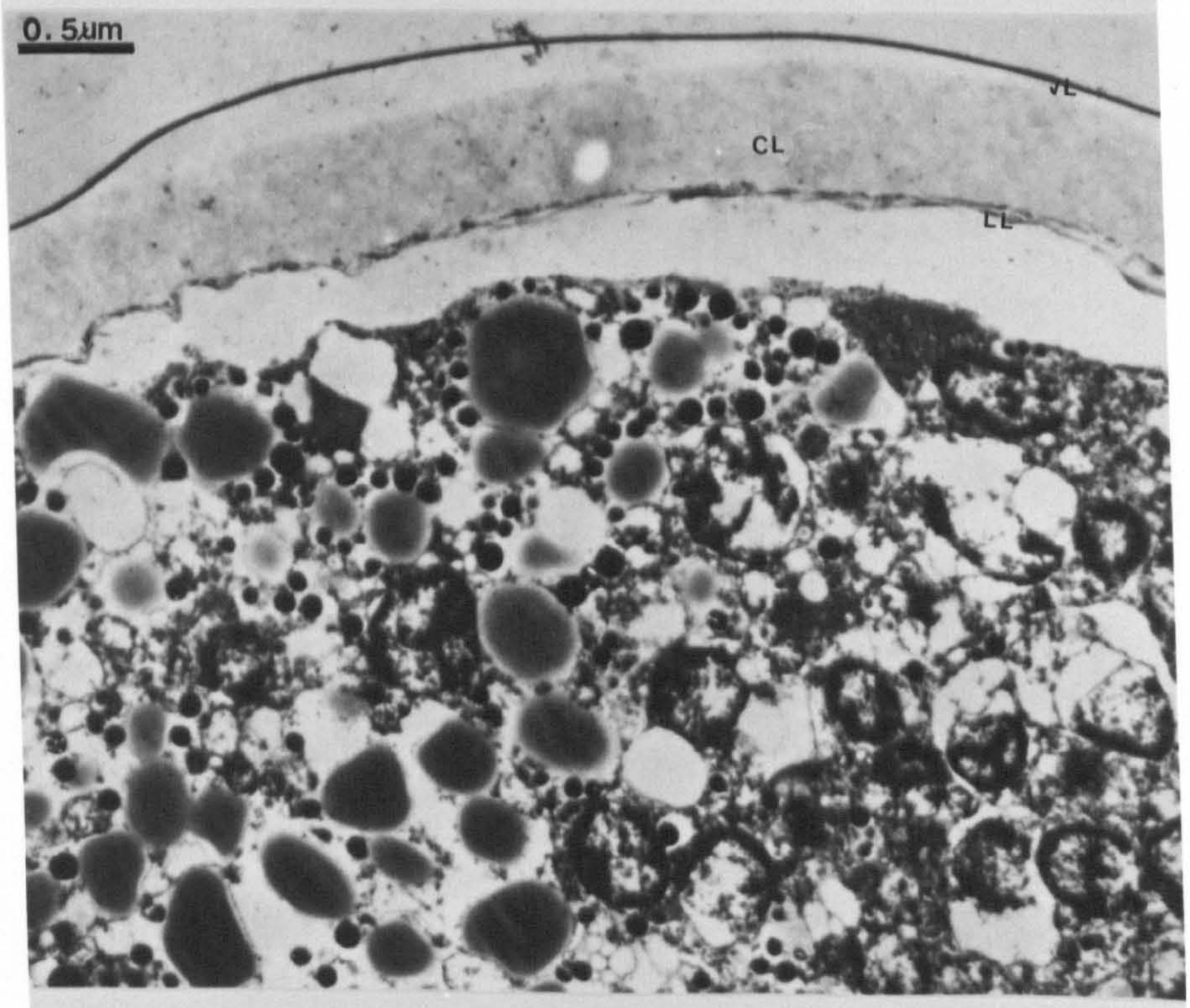
cl Chitinous layer

vl Vitelline layer

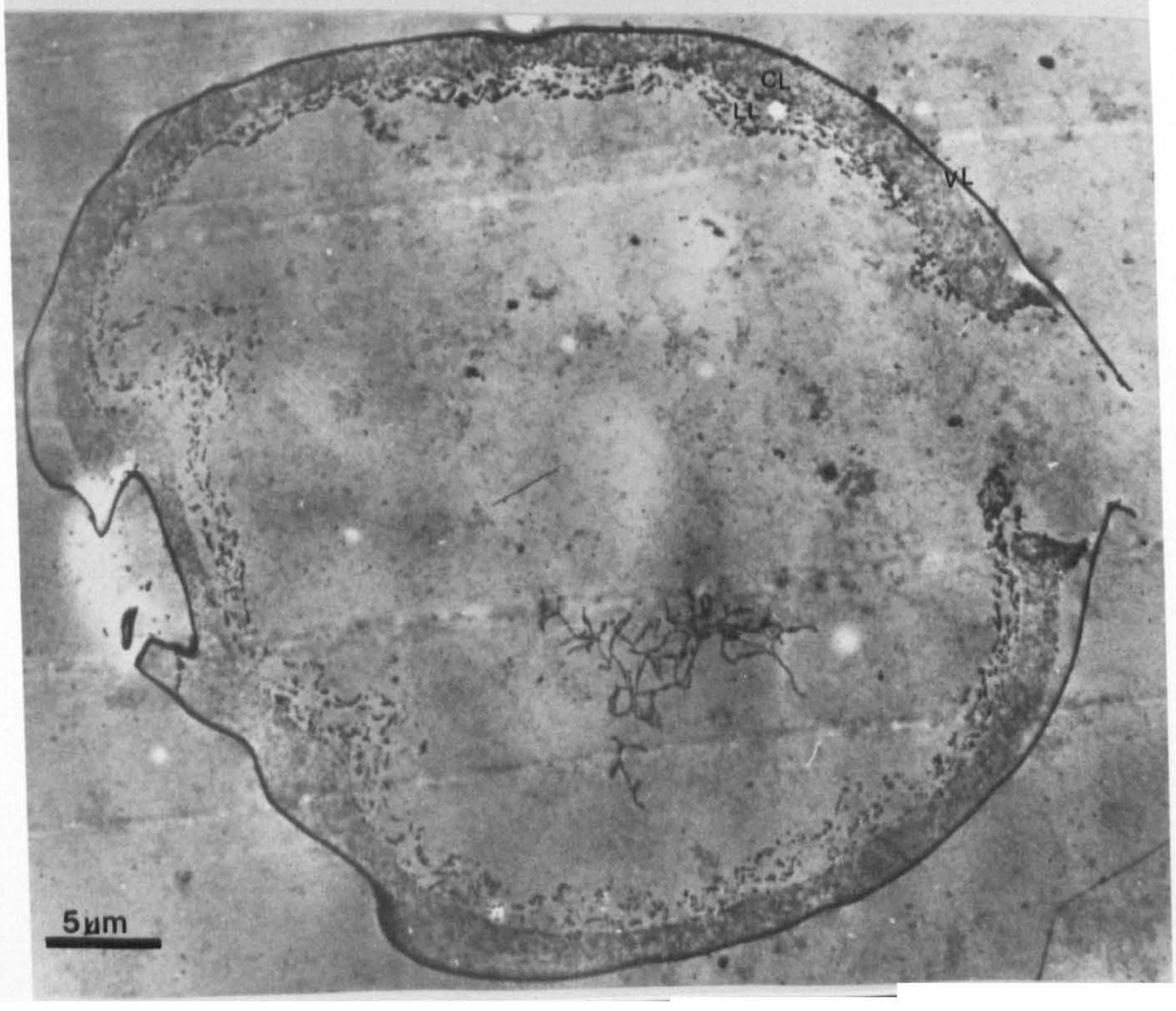
a



b



c



SECTION B

THE INFLUENCE OF EMBRYONATION TEMPERATURE OF A. SUUM EGGS ON THE VIABILITY OF THE DEVELOPED INFECTIVE LARVAE

INTRODUCTION

The development of viable infective larvae of A. suum is basic to the elucidation of the invasive mechanisms of the parasite as well as host tissue reactions following such larval activities. Although there is some literature on the *in vitro* cultivation of the infective larva of *A. suum* (Feng and Chu, 1938; Fenwick, 1939; Pitts and Ball, 1953, 1955; Pitts, 1948, 1960, 1962, 1963; Cleeland and Lawrence, 1962; Cleeland, 1963; Silverman, 1965; Levine and Silverman, 1969; Dourves and Tromba, 1970), early attempts to culture the larvae for use in the present study was hampered by difficulties in obtaining larvae which remained viable after hatching. Several methods for hatching *Ascaris* eggs *in vitro* have been described (McRae, 1935; Fenwick, 1939; Pitts, 1948; O'Connor, 1951; Haskins and Weinskein, 1957; Rogers, 1958; Cleeland and Lawrence, 1962; Todd, 1962; Fairbairn, 1961; Jaskoski and Colucci, 1964). Many of these methods were, however, either detrimental to the larvae or difficult to reproduce when large quantities of the freshly hatched infective larvae were desired. Although the technique of Fairbairn (1961) gave a consistently good hatch, it soon became obvious that for full utilisation of the technique more information was required.

The influence of temperature on the development, survival, size and infectivity of free-living stages of a number of animal parasitic nematodes is well documented (Splindler, 1938; Rogers, 1940; Premvati, 1958, 1961; Narain, 1965; Agrawal, 1966; Barrett, 1968). Narain (1965) found that not only is the size of free-living female Bunostomum trigonocephalum dependent upon the temperature at which it was cultured, but that they were better adapted to lower temperatures than higher temperatures. Barrett (1968) showed in the case of Strongyloides ratti that worms grown at 20°C lived longer than worms grown at any other temperature. Various embryonation temperatures have been quoted by a number of workers as the optimum temperature at which rapid and uniform development of Ascaris eggs is accomplished (30°C by Fairbairn, 1961; 31.1°C by Seamster, 1950). The effect that such a rapid rate of embryonation might have on the viability of the infective larvae has not been reported. Information for other animal parasitic nematodes whose eggs require a long period of development outside the host before becoming infective is scanty.

This section reports the results of a series of experiments designed to ascertain the effect of

embryonation temperature on the viability of the resultant infective larvae. The study was prompted by the rather accidental discovery that eggs embryonated at about 18-20°C on the bench in the laboratory performed better than those embryonated at the "optimum" temperatures. Moreover, attempts to establish a high level of infection in mice using such eggs gave consistently good results under conditions where similar eggs embryonated at the "optimum" temperatures proved less successful.

MATERIALS AND METHODS

Freshly extracted eggs in 0.1m H_2SO_4 were incubated at various temperatures ranging from $6^{\circ}C$ - $45^{\circ}C$ for up to 60 days. Temperatures of 6° - $20^{\circ}C$ were provided by incubators and the higher temperatures by shaking water baths. In the case of eggs in incubators the cotton wool stoppers were removed and the contents shaken several times a day to ensure that the eggs were in suspension and adequately oxygenated (Fairbairn, 1957, 1961). The incubation medium was changed every 5 days to avoid fungal growth. Several batches of eggs were used and 5 replicates were prepared at each of the embryonation temperatures.

Samples of eggs were removed at periodic intervals and the stage of development and percentage of eggs at each stage determined. From these data an index of development (S) was calculated, such that $S = \Sigma (X.Y)$

where X = % of the given developmental stage in the sample

Y = Index value of developmental stage (See p. 32).

Additionally, eggs were hatched *in vitro* for 2 hours using the modification of Fairbairn's (1961) method described earlier. After 2 hours, hatching was terminated by freezing and the percentage hatch determined from a randomly selected sample of 300 eggs. A hatched larva was considered to be one that was completely free from the egg shell. Preliminary experiments (Figure 10) had shown that there was little additional hatching after 2 hours.

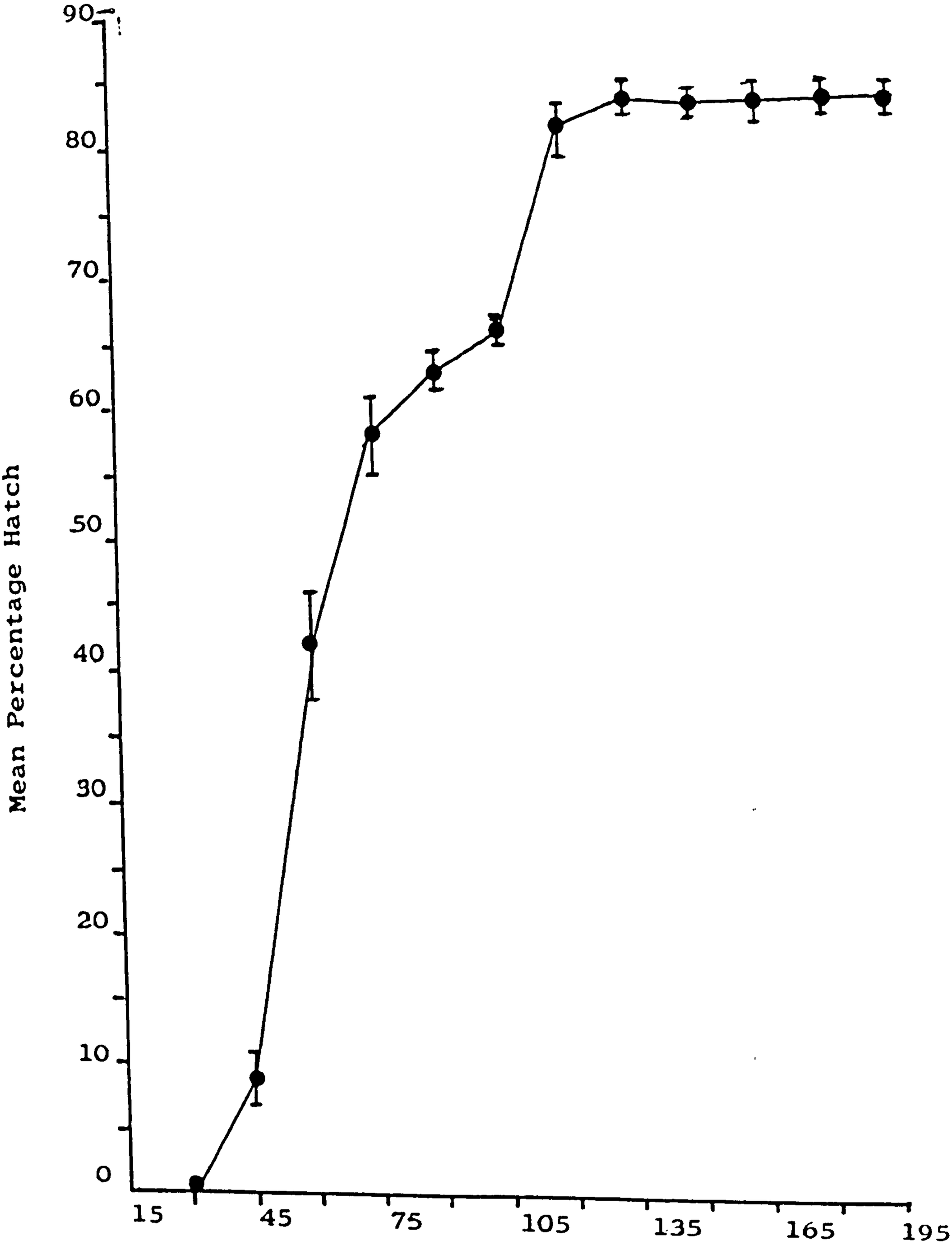
The hatched larvae were washed in PBS by centrifugation, resuspended in fresh PBS in McCartney bottles and stored at 37°C. Samples of larvae were removed at times up to 64 hours and the percentage mortality and activity recorded. Larval activity was assessed by their ability to penetrate Whatman No. 42 Filter Paper *in vitro*. This paper is graded as slow (filtration speed 240-280) with particle size 2.5 - 3 μm. This meant that larvae had to exert some activity to penetrate.

Larvae embryonated for 60 days at 22°C were

hatched for 2 hours at 37°C, washed in PBS and incubated for up to 26 hours at 37°C in fresh PBS. Samples were removed at 2 hour intervals and the contained neutral lipid stained with saturated Oil Red O at 60°C for 20 mins. A second group of larvae was incubated for 24 hours at a range of temperatures before staining. Neutral unbound lipid levels were measured using a Vickers M85 microdensitometer at 520 nM.

FIGURE 10

Percentage hatch of A. suum eggs embryonated for 60 days in 0.1N H_2SO_4 at 22° ± 1°C and hatched at 37°C. Five samples were taken from each of 5 replicate cultures and each point represents the mean of these 25 values. The vertical lines indicate standard error.


Mean Percentage Hatch
90
80
70
60
50
40
30
20
10
0
15
45
75
105
135
165
195
Time (Min.)

RESULTS

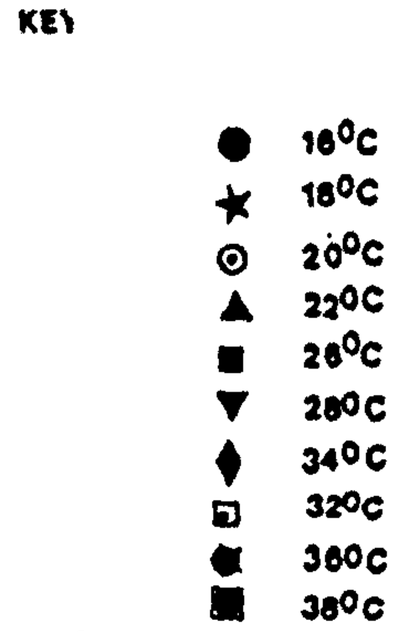
The Effect of Embryonation Temperature on the rate of Egg Development

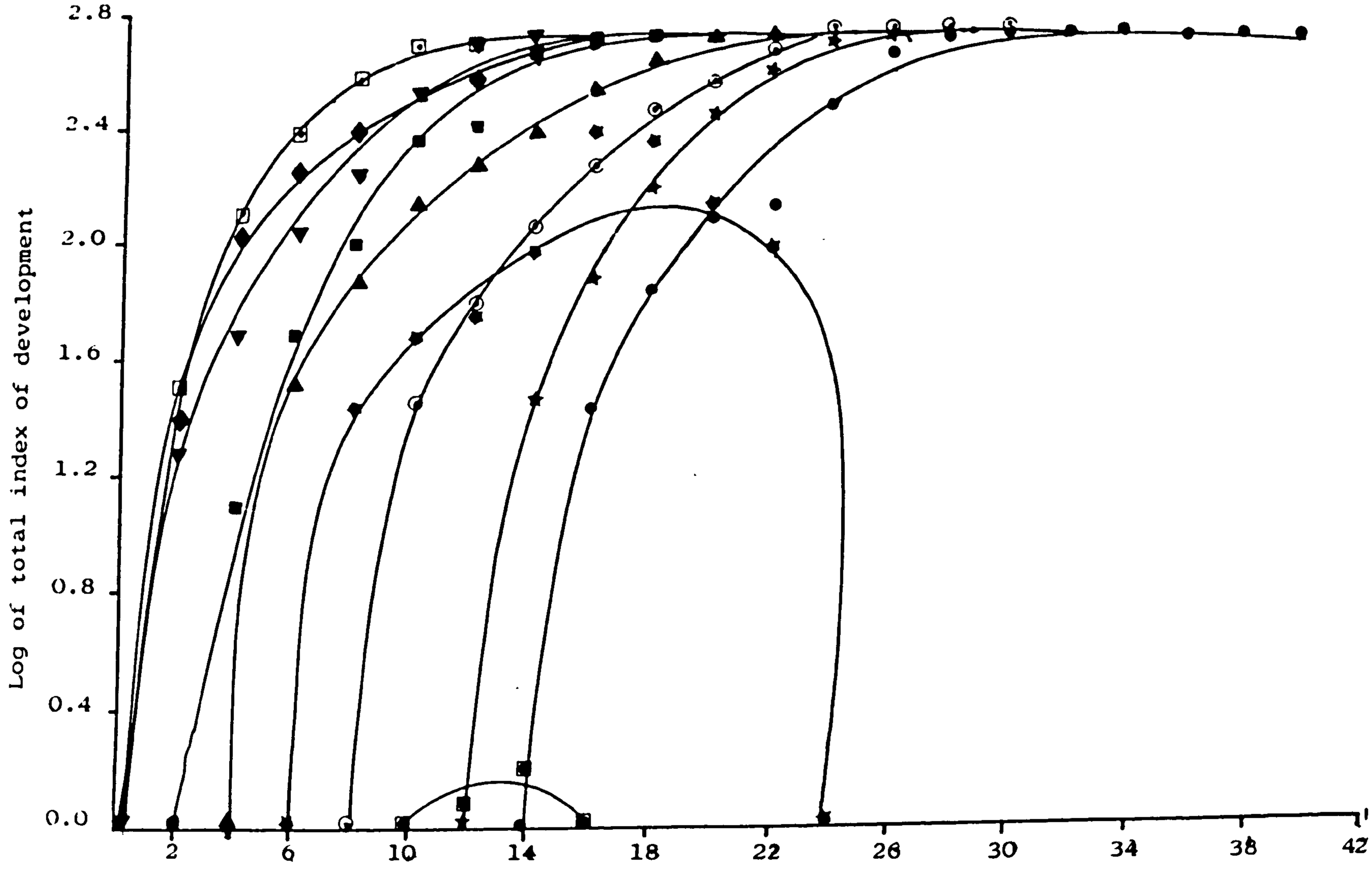
Development of the eggs only occurred between $16^{o} \pm 1^{o}C$ and $38^{o} \pm 1^{o}C$. However, full embryonation (i.e. development up to the larval stage) was possible only between $16^{o} \pm 1^{o}C$ and $34^{o} \pm 1^{o}C$. The effect of the various embryonation temperatures on the rate of development of the eggs is shown in Figure 11. The figure shows the relationship between the index of development (S) of the eggs at various incubation temperatures and incubation time. Between $16^{o} \pm 1^{o}C$ and $32^{o} \pm 1^{o}C$, increase in incubation temperature increased the rate of development of the eggs. Above $32^{o} \pm 1^{o}C$ development became irregular. At $36^{o} \pm 1^{o}C$, initiation of development of the eggs was delayed until about 6 days after incubation commenced. The rate of development reached its peak about ten days later (day 16); after which there was a decline, with the entire development coming to a halt 8 days later (day 24). By this time only about $14.3 \pm 2.51\%$ of the eggs had reached the morula stage.

Similarly, development of the eggs at $38^{o}C$ was delayed until about the 12th day after

FIGURE 11

Relation between rate of development of eggs embryonated at various temperatures and incubation time.




Log of total index of development
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
2
6
10
14
18
22
26
30
34
38
42
Duration of Incubation (Days)

incubation when only 1.2 ± 0.32% of the eggs had developed to the 2-celled stage. At this temperature, the rate of development reached its peak 2 days later (day 14) with only 1.6 ± 0.14% of the eggs reaching the 2-celled stage. Thereafter, rapid degeneration of the eggs occurred.

There was no development of eggs at temperatures of 40° ± 1°C and greater. All the eggs at these temperatures degenerated by the second day of incubation.

Figure 12 shows the mean time taken for 60% of the eggs embryonated at the various incubation temperatures to develop to the first larval stage. The results are based on 5 separate determinations.

Effect of Embryonation Temperature on Percentage Hatch of Infective Eggs

The temperature at which the infective eggs were embryonated affected hatching of the infective eggs in two ways. Firstly, eggs embryonated at 32° ± 1°C and greater had significantly lower mean percentage hatch ($P < 0.05$), than those embryonated at lower temperatures (Figure 13). Although there were slight differences in the percentage hatch between eggs embryonated at these lower temperatures, the differences were not

FIGURE 12

Graph of time to achieve 60% larval development in eggs incubated at various temperatures. The circles represent mean of 5 determinations and the vertical lines indicate standard error.

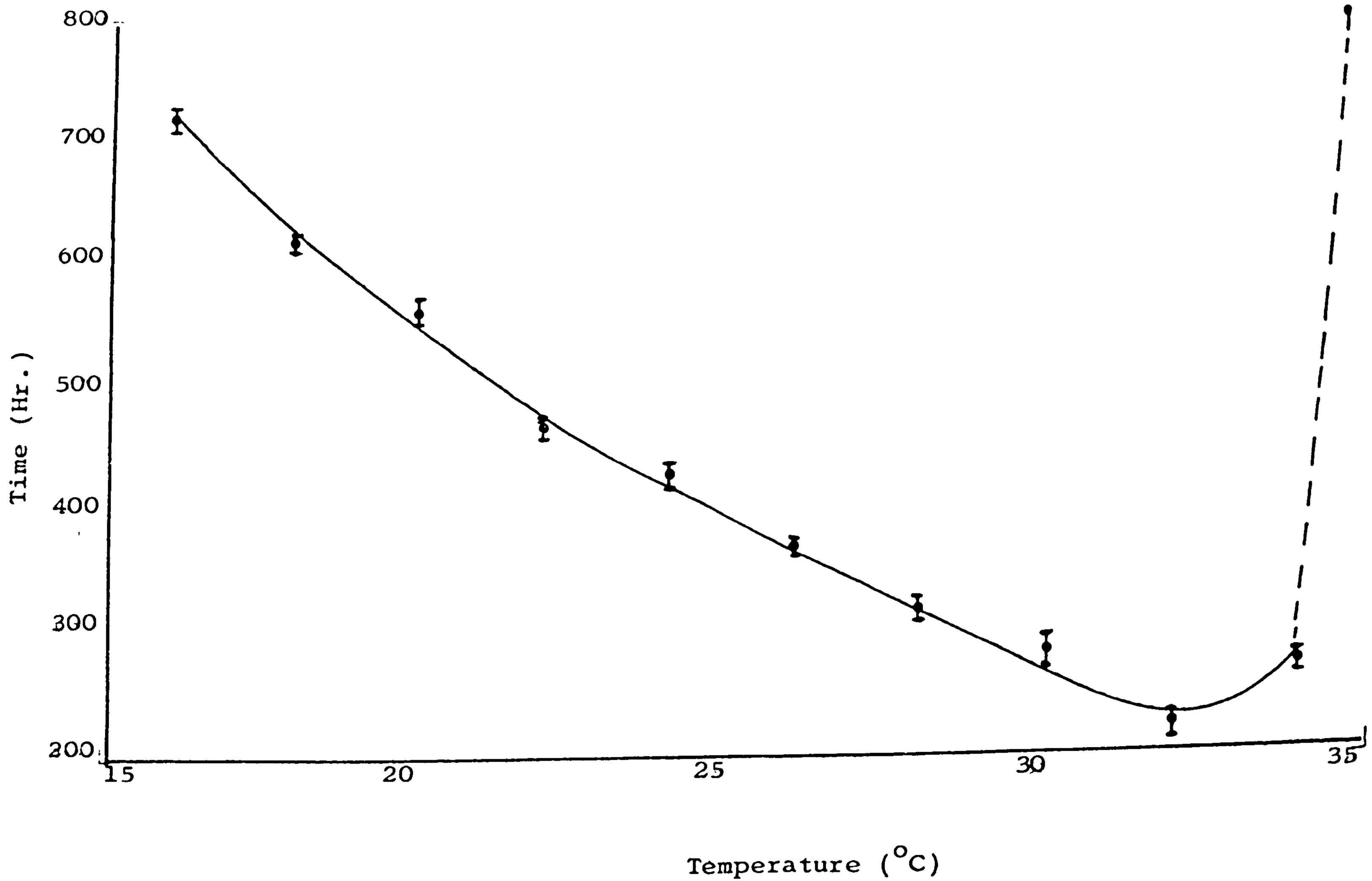
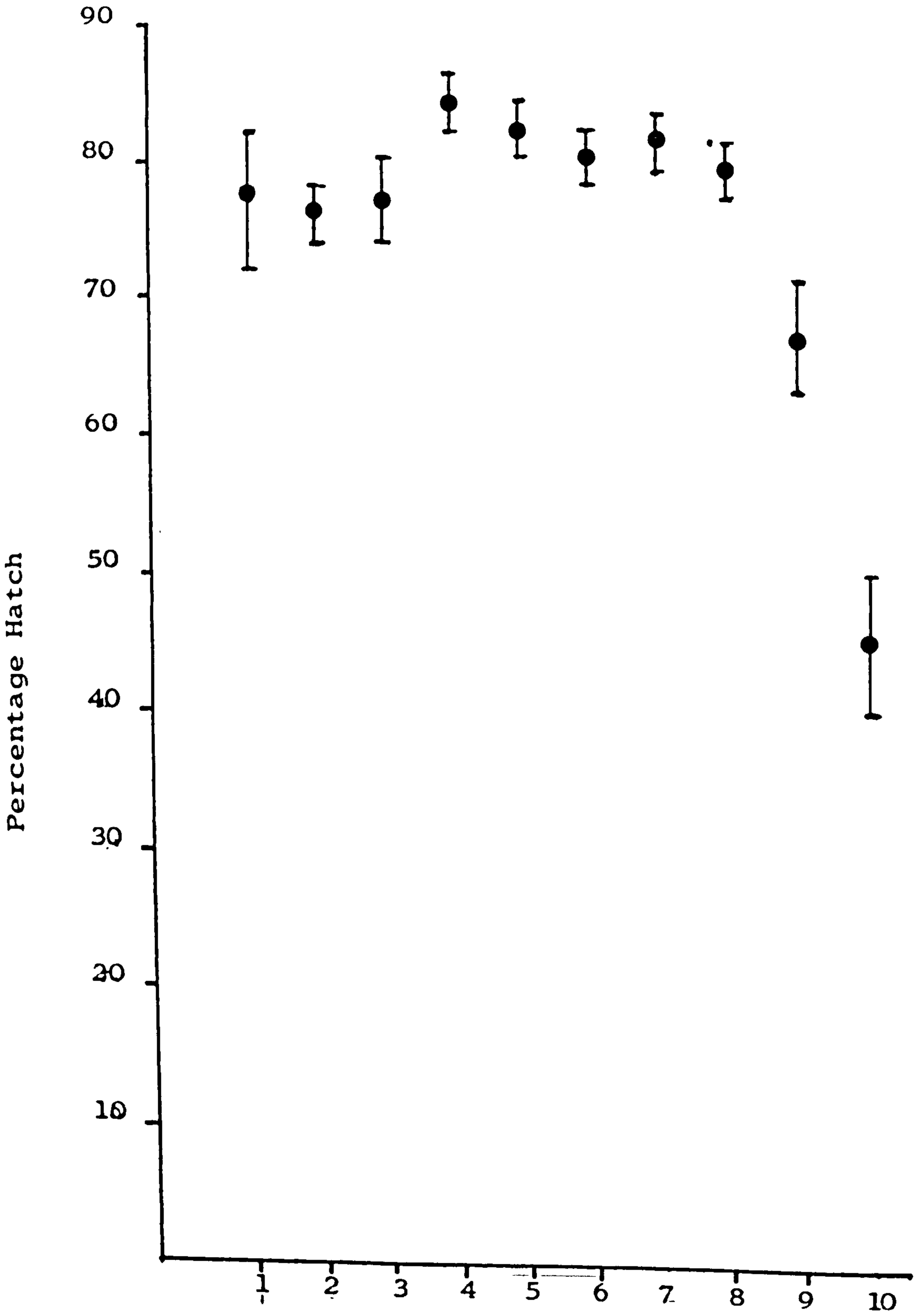

800
700
600
500
400
300
200
Time (Hr.)
15
20
25
30
35
Temperature (°C)

FIGURE 13

Mean percentage hatch of Ascaris suum eggs embryonated at various incubation temperatures for 60 days in 0.1N H_2SO_4 and hatched in vitro at 37°C for 2 hours. 5 samples were taken from each of 5 replicate cultures and each point represents the mean of these 25 values. The vertical lines indicate standard error.

KEY:

1	16° ± 1°C
2	18° ± 1°C
3	20° ± 1°C
4	22° ± 1°C
5	24° ± 1°C
6	26° ± 1°C
7	28° ± 1°C
8	30° ± 1°C
9	32° ± 1°C
10	34° ± 1°C


90
80
70
60
50
40
30
20
10
Percentage Hatch
1
2
3
4
5
6
7
8
9
10
Embryonation temperature (°C)

significant ($P > 0.05$). The maximum percentage hatch was obtained from eggs embryonated at $22^{o} \pm 1^{o}C$.

Secondly, the length of incubation required before maximum hatch could be achieved was temperature dependent (Table 11).

Influence of Embryonation Temperature on Longevity of the Infective Larva in PBS (pH 7.2).

Figure 14 shows the effect of embryonation temperature on the length of time the hatched infective larvae remained alive in phosphate buffered saline (pH 7.2), at $37^{o}C$. The longevity of the infective larvae was found to drop as the temperature at which the infective eggs were embryonated increased. Longevity in a given culture was assessed as the time to 90% mortality. To distinguish between dead and quiescent larvae viability was determined by a number of parameters, firstly motility after exposure of the larvae to light for 5 mins., secondly dead larvae as opposed to living or quiescent larvae tended to straighten out while living or quiescent larvae tended to retain a smooth curved shape. Larvae which were judged dead on the above criteria were confirmed dead by staining in 0.05% methylene blue for 5 mins.

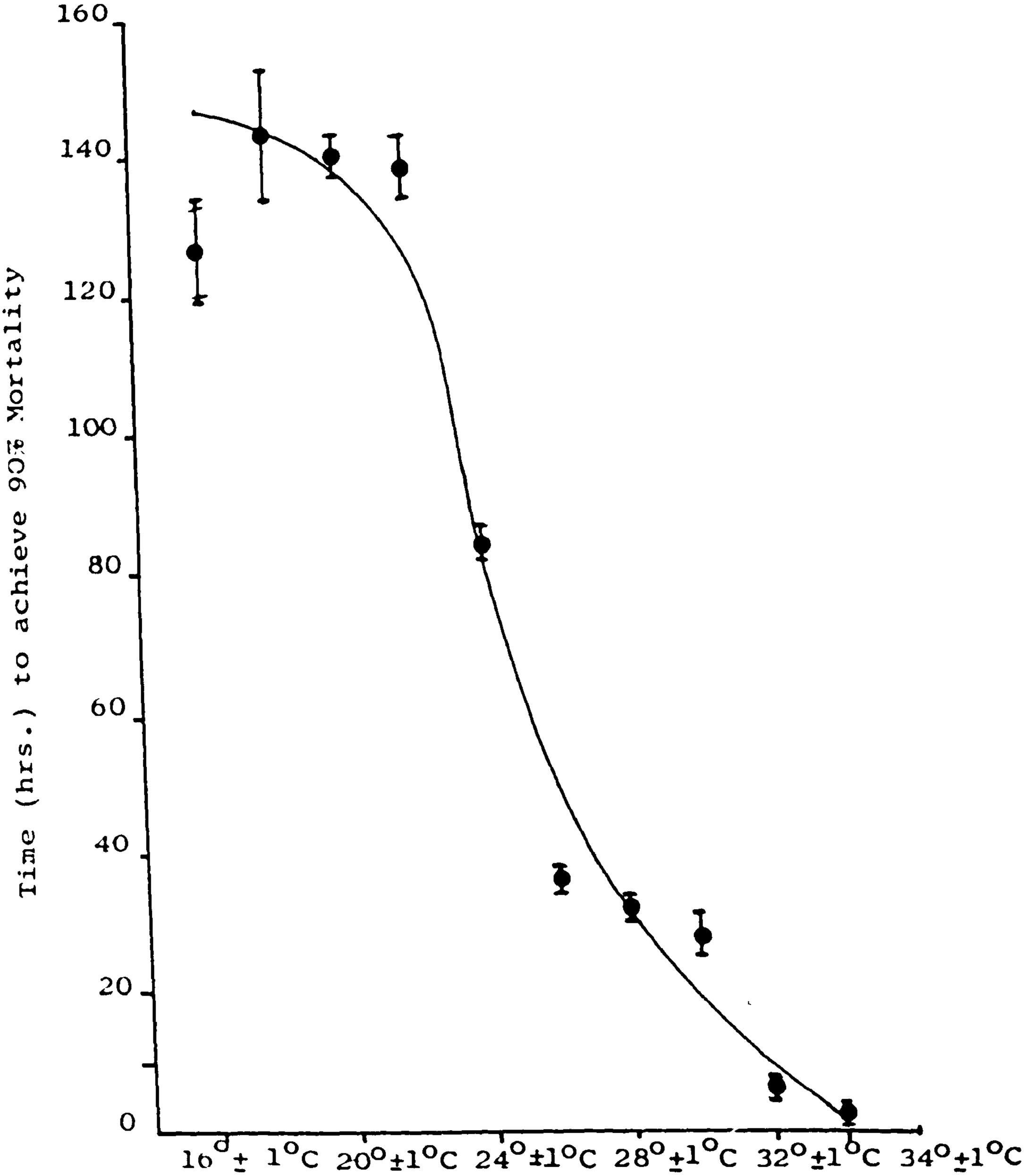
TABLE 11

RANGE OF INCUBATION TIME AT VARIOUS TEMPERATURES REQUIRED TO ACHIEVE MAXIMUM HATCH

Temperature of Embryonation	Time/Days
16 ± 1°C	53-58
18 ± 1°C	47-52
20 ± 1°C	46-48
22 ± 1°C	39-44
24 ± 1°C	34-36
26 ± 1°C	28-32
28 ± 1°C	22-26
30 ± 1°C	21-24
32 ± 1°C	21-24
34 ± 1°C	16-20

FIGURE 14

Effect of Embryonation temperature on *in vitro* survival of the hatched larvae in phosphate buffered saline (pH 7.2) at 37°C (the eggs were embryonated for 60 days and hatched by a slight modification of the Fairbairn technique).


160
140
120
100
80
60
40
20
0
Time (hrs.) to achieve 90% Mortality
16° ± 1°C
20° ± 1°C
24° ± 1°C
28° ± 1°C
32° ± 1°C
34° ± 1°C
Temperature of Embryonation (°C)

Living larvae remained unstained, while dead larvae stained deep blue and retained the blue colouration even after two washings by centrifugation in distilled water.

The highest mean survival time was recorded amongst larvae obtained from eggs embryonated at $18^{o} \pm 1^{o}C$ - 144.20 ± 8.14 hours. However, this value was not significantly different ($P \geq 0.05$) from larvae obtained from eggs embryonated at $22^{o} \pm 1^{o}C$; $20^{o} \pm 1^{o}C$ and $16^{o} \pm 1^{o}C$. Larvae obtained from eggs embryonated at $32^{o} \pm 1^{o}C$ survived for 6.00 ± 1.49; while those obtained from eggs embryonated at $34^{o} \pm 1^{o}C$ survived for only 2.01 ± 0.82 hours.

Influence of Embryonation temperature on the Activity of the Hatched Larvae

Freshly hatched larvae were generally active. But as they aged, they became increasingly lethargic and finally died. The influence of age on the activity of the infective larvae obtained from eggs embryonated at the various incubation temperatures is shown in Figure 15.

FIGURE 15

Passage of A. suum larvae through Number 42 Filter paper following 30 mins. exposure to larvae obtained from eggs embryonated at various incubation temperatures and aged for various lengths of time from 0 hour to 64 hours in phosphate buffered saline (pH 7.2) at 37°C. The points represent mean of 5 replicates and the vertical lines represent the standard errors.

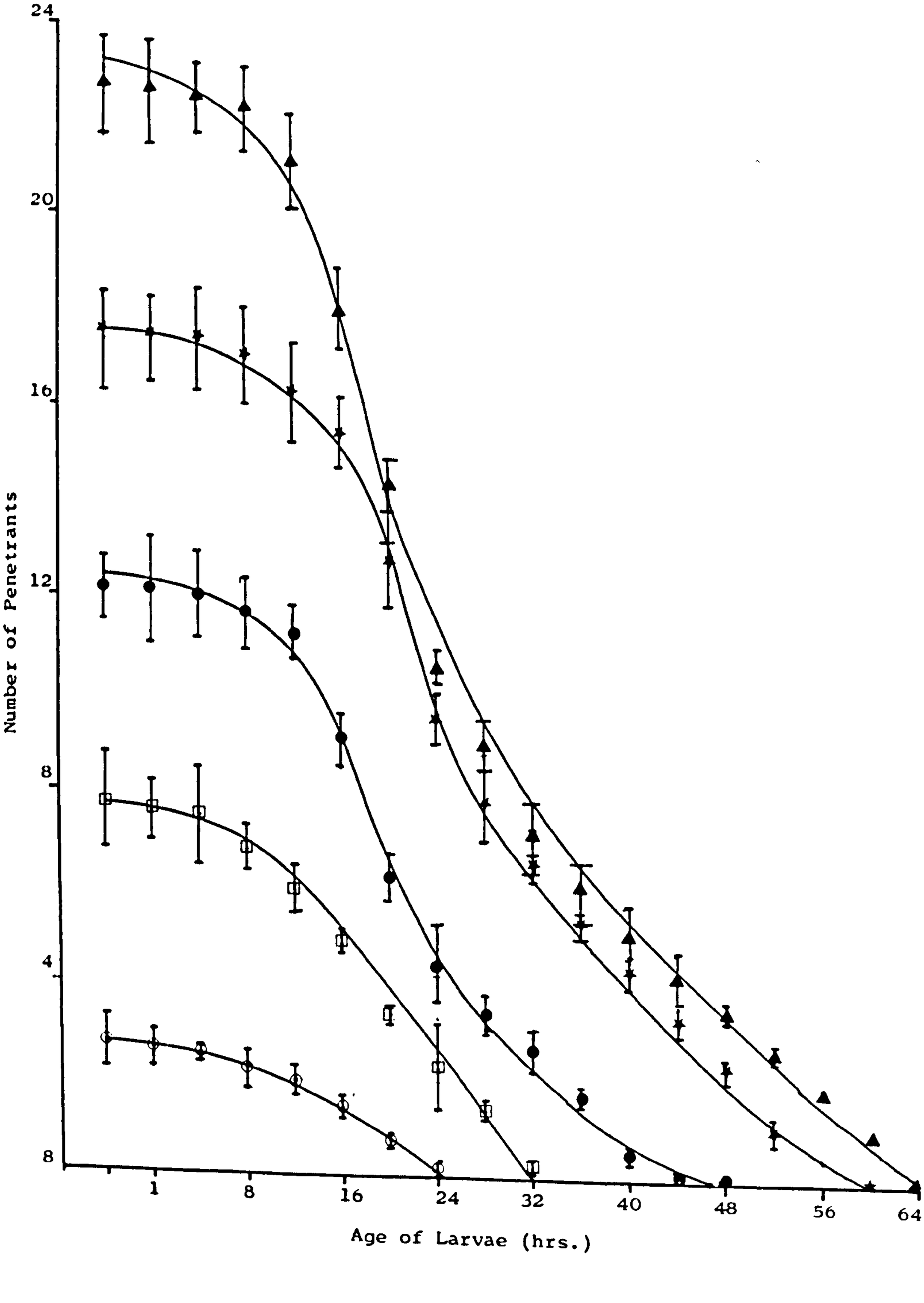
▲ 22° ± 1°C

✱ 20° ± 1°C

● 26° ± 1°C

□ 30° ± 1°C

○ 34° ± 1°C


24
20
16
12
8
4
8
Number of Penetrants
1
8
16
24
32
40
48
56
64
Age of Larvae (hrs.)

Most of the active larvae went through the filter paper within 3 hours and over 50% of them within 90 mins. of exposure (Table III.). Figure 16 shows the rate of passage of the larvae through the membrane. Larvae obtained from eggs embryonated at higher temperatures ($30^{o} \pm 1^{o}C$ and greater) were less active than those embryonated at lower temperatures, and smaller numbers of the former went through the membrane in 90 mins. (Table IV). All the larvae used in the experiment were of the same age (> 1 hr. after hatching) and were treated and handled alike.

The larvae were not able to penetrate tanned thin gelatin film, nor were they able to penetrate into or go through fixed or tanned pig or mouse intestinal tissue. Fresh tissues autolyzed too quickly under in vitro conditions but none of the larvae were able to either enter or pass through the tissues.

Lipid Loss by the Infective Larvae (following ageing in vitro).

Figure 17 shows the relationship between lipid loss and age of the infective larvae. The larvae were embryonated at $22^{o}C$ and aged in phosphate

TABLE III

Mean (± SE) % Passage through Filter Paper No. 42 of Larvae obtained from Infective Eggs Embryonated at various Incubation Temperatures

Time (mins.)	Temperature of Embryonation									
	16° ± 1°C	18° ± 1°C	20° ± 1°C	22° ± 1°C	24° ± 1°C	26° ± 1°C	28° ± 1°C	30° ± 1°C	32° ± 1°C	34° ± 1°C
30	6.25 ± 2.10	3.19 ± 1.77	11.00 ± 2.18	4.75 ± 1.86	8.32 ± 1.45	3.65 ± 1.00	5.27 ± 1.48	0	0	0
60	24.72 ± 3.99	28.46 ± 2.81	32.43 ± 3.69	39.26 ± 3.47	26.00 ± 2.57	13.37 ± 2.15	11.53 ± 2.40	8.67 ± 2.55	0	0
90	56.17 ± 2.45	61.08 ± 4.26	65.18 ± 3.00	61.43 ± 3.21	66.73 ± 4.26	37.00 ± 2.10	31.83 ± 3.11	26.47 ± 2.18	8.36 ± 2.14	7.26 ± 4.68
120	88.29 ± 1.63	83.74 ± 3.95	79.00 ± 2.97	84.10 ± 2.90	89.32 ± 3.44	65.71 ± 3.59	54.28 ± 2.19	31.10 ± 3.62	17.00 ± 2.18	13.00 ± 6.14
150	93.65 ± 3.21	100	100	100	100	86.51 ± 2.26	79.94 ± 3.48	62.33 ± 2.99	18.32 ± 1.96	21.15 ± 2.63
180	100	100	100	100	100	100	100	78.20 ± 2.15	24.00 ± 2.45	22.80 ± 3.15
210	100	100	100	100	100	100	100	100	26.16 ± 2.13	23.73 ± 2.49

FIGURE 16

Graph of percentage penetration through filter paper by larvae obtained from infective eggs embryonated at various incubation temperatures ranging from $16^{\circ} \pm 1^{\circ}C$ - $34^{\circ} \pm 1^{\circ}C$. The larvae were assayed immediately after hatching (1 hour) and the penetration membrane was maintained at $37^{\circ}C$.

KEY:

- ✱ $34^{\circ} \pm 1^{\circ}C$
- ● $32^{\circ} \pm 1^{\circ}C$
- O $30^{\circ} \pm 1^{\circ}C$
- ■ $28^{\circ} \pm 1^{\circ}C$
- □ $18^{\circ} \pm 1^{\circ}C$
- ▲ $22^{\circ} \pm 1^{\circ}C$

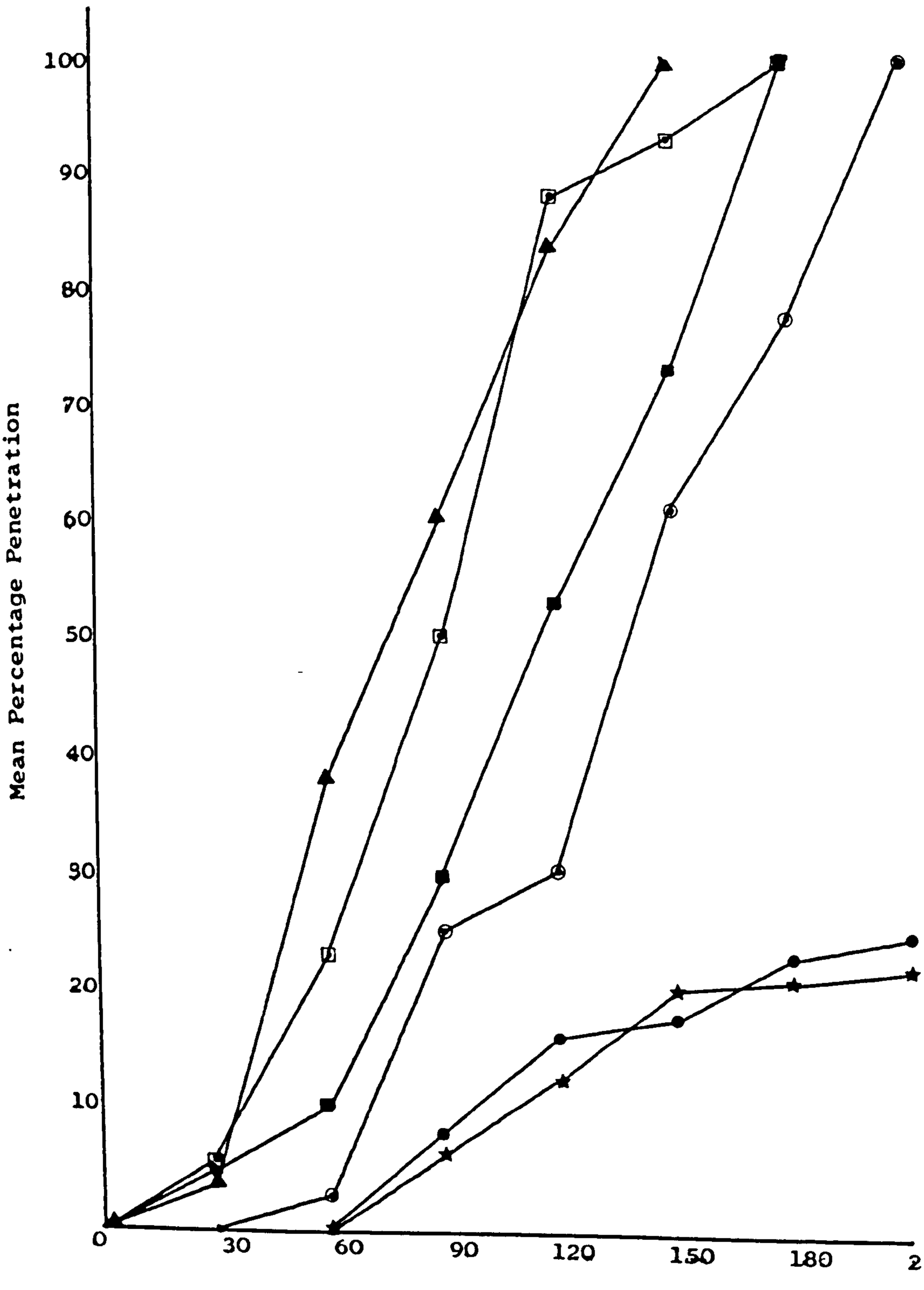

Mean Percentage Penetration
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
210
Time (Min.)

TABLE IV

Mean number of Infective Larvae passing through filter paper (Whitman No. 42) after 90 minutes of exposure.
Age of larvae — 1 hr. after hatching

Temperature at which the ova was embryonated	Number of Larvae in each sample	Mean number of Penetrants (5 replicates)
16 ± 1°C	30	16.20 ± 3.70
18 ± 1°C	30	17.40 ± 3.36
20 ± 1°C	30	18.40 ± 4.39
22 ± 1°C	30	21.60 ± 4.28
24 ± 1°C	30	18.20 ± 2.77
26 ± 1°C	30	12.00 ± 5.57
28 ± 1°C	30	10.80 ± 4.55
30 ± 1°C	30	7.60 ± 4.77
32 ± 1°C	30	2.60 ± 2.61
34 ± 1°C	30	2.60 ± 2.19

Means not significantly different ($P > 0.05$) are joined by a line.

buffered saline (pH 7.2) at 37°C in a shaking water bath for various lengths of time up to 26 hours after hatching. There was little decrease in lipid level during the first four hours but over the next 10 hours the level fell relatively rapidly to a more or less steady plateau.

Storage temperature up to 28°C had little effect on the loss of lipid but at higher temperatures the loss was much more rapid (Figure 18).

FIGURE 17

Relationship between lipid loss and age of infective A. suum larvae. The larvae were embryonated at 22° ± 1°C and aged in phosphate buffered saline (pH 7.2) in a shaking water bath at 37°C. for various lengths of time. The points represent the mean and the vertical lines the standard errors of at least 20 larvae.

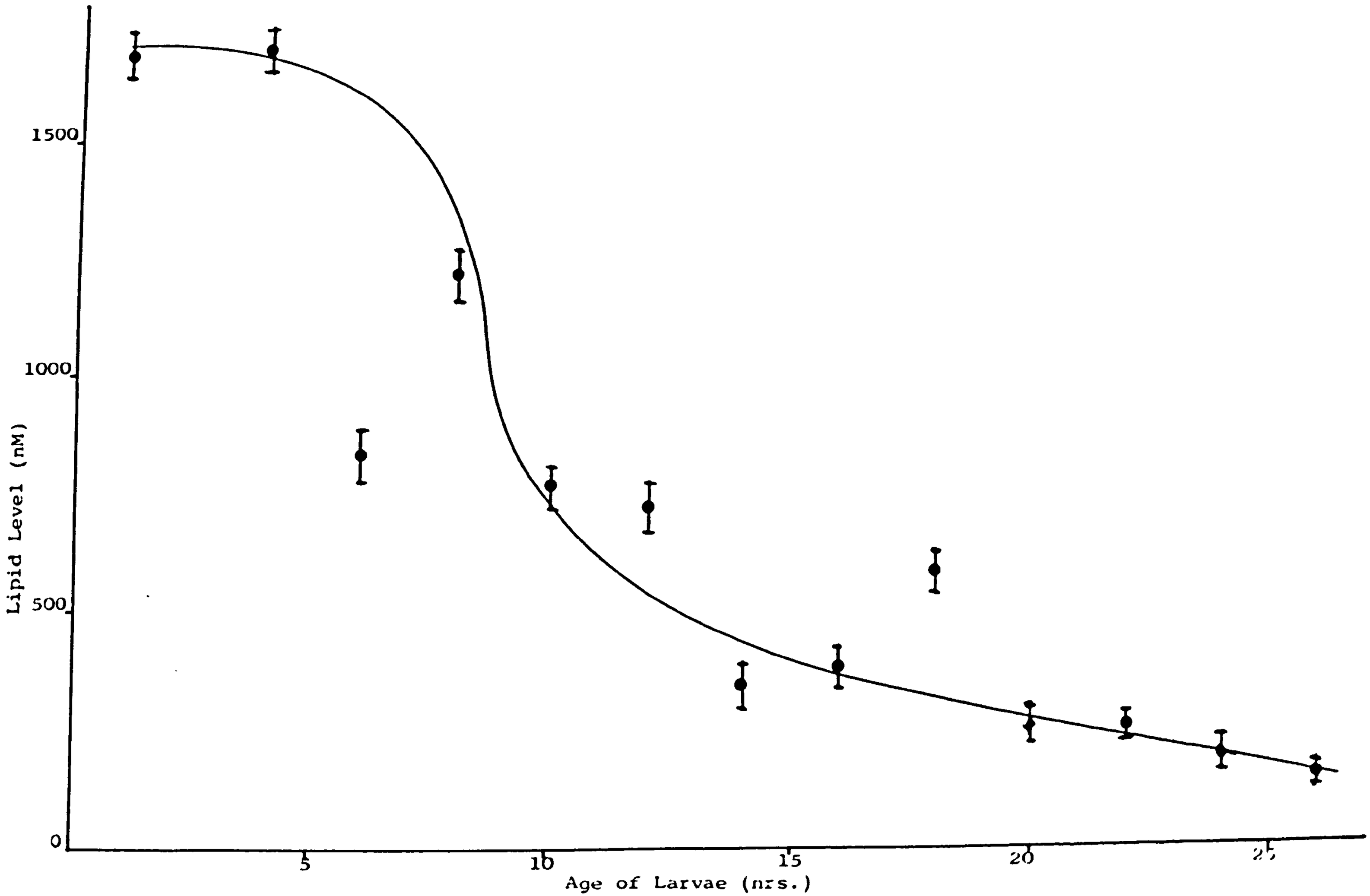
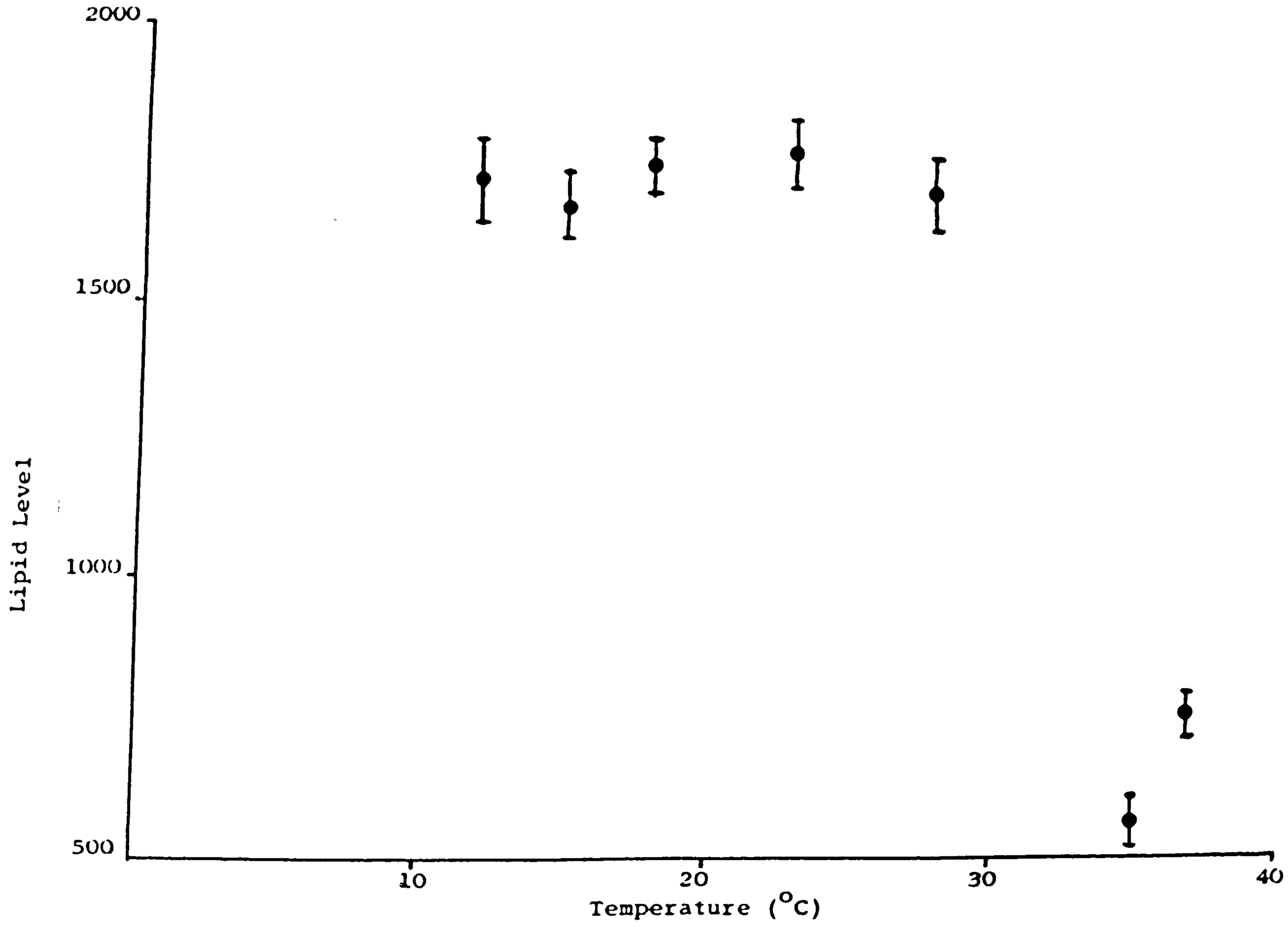

1500
1000
500
0
Lipid Level (nM)
5
10
15
20
25
Age of Larvae (nrs.)

FIGURE 18

Lipid levels of infective A. suum larvae aged at various temperatures in phosphate buffered saline (pH 7.2) for 24 hours. The points represent means and the vertical lines the standard errors of at least 20 larvae.


2000
1500
1000
500
Lipid Level
10
20
30
40
Temperature (°C)

DISCUSSION

The epidemiology of Ascaris infection depends to a large extent upon the eggs since they must embryonate outside the body of the host. Previous investigators (Spindler, 1940; Cram and Hicks, 1944; Parlov, 1958; Reyes, Kruse and Batson, 1963) have shown that under optimum conditions of aeration and moisture, the rate of embryonation of the eggs increases with increase in temperature. Seamster (1950) found that eggs develop to the Embryo (L_1) state in 27 days at 18.9°C at 100% relative humidity; in 8.3 days at 31.1°C and in 13 days at 34.4°C. Timoshin (1967) reported that development of Ascaris ova occurs at temperatures between 13°C and 36°C with optimal temperatures between 17°C and 30°C. Between these temperatures, the rate of development of the eggs was directly proportional to temperature. The infective larval stage within the egg is the second stage larva ensheathed within the first stage larval cuticle. Both Rogers (1958) and Fairbairn (1961) have reported that before the eggs can be hatched with optimum success in vitro an additional period of incubation is required following the appearance of the motile larvae. These results have been largely

confirmed in the present study. Full embryonation of the eggs was accomplished within the temperature range of $16^{o} \pm 1^{o}C$, and $34^{o} \pm 1^{o}C$. Within this range, increase in temperature had the effect of shortening the time required to reach the motile larval stage (Figure 12). At $16^{o} \pm 1^{o}C$, the first larval stage was reached in 29 to 31 days; at $32^{o} \pm 1^{o}C$, which was the temperature at which the index of development of the eggs was at its highest, the first larval stage was reached in 8 - 10 days. Beyond $32^{o} \pm 1^{o}C$, however, the index of development began to drop and at $34^{o} \pm 1^{o}C$ the first larval stage is reached in 13-14 days. The times for development of the second stage larvae and maximum hatch also varied with temperature (Table II). At $16^{o}C$ the minimum time was 53-58 days and at $30^{o}C$ 21-24 days.

Nitzulescu, Sorescu and Panaitescu (1956) following their laboratory experiments on the effect of temperature on development of various helminth eggs, concluded that eggs of Ascaris lumbricoides and Trichuris trichiura developed better at $20^{o}C$ than at $27^{o}C$ and $37^{o}C$, and in 2% agar than in 1% agar, although they did not elaborate. The present data indicate that the

temperature at which A. suum eggs are embryonated has a marked effect on the viability of the resultant infective larvae. Based on the criteria employed in these studies, it would seem right to conclude that an excessive rate of embryonation has the adverse effect of reducing the viability of the developed infective larvae. The finding that embryonation at high temperatures gave rise to infective eggs with a lower percentage hatch (Figure 13) and resultant infective larvae with reduced longivity (Figure 14), and a poor ability to penetrate filter paper membranes (Figure 15) when compared to those obtained from eggs embryonated at lower temperatures, illustrate the ill-effects of high embryonation temperature.

The practical significance of these results is clear and may help to provide an answer to some of the practical problems often experienced in experimental work with Ascaris.

The optimal temperatures for rate of development and larval survivability are not the same, a point which has not previously been emphasised. This phenomenon may well go a long way to explain why it has often proved difficult to obtain heavy experimental infections in pigs

while heavy natural infections occur commonly (Kelley, Olsen and Hoerlein, 1958; Schwartz, 1959; Soulsby, 1965).

The reason for this reduction in larval viability following embryonation of the eggs at higher temperatures is not obvious. Details of the nature of the biochemical changes which occur in the eggs during the course of their development have not yet been fully elucidated (Fairbairn, 1957; Castello and Smith, 1964). Large quantities of lipid are synthesised by the infective larva of A. suum during embryonation (Fairbairn, 1957). These are stored as food reserve mainly in the gut region. The infective larvae at this early stage of development have no functional gut lumen (Jenkins and Erasmus, 1971) and it is presumed that the infective larvae at this early stage depend to a great extent on their stored food for their activity (Munnich, 1965). Developing A. suum eggs are known to contain apart from carbohydrate, three major lipids. These include ascaryl alcohol, phospholipids and a triglyceride fraction (Fairbairn, 1955). The triglyceride which is normally abundant at the

beginning of embryonation of the eggs is extensively used throughout the period of development (Fairbairn and Passey, 1955). It has been suggested (Passey and Fairbairn, 1955) that once fully embryonated (L_2 stage) the metabolism of the larvae within the eggs is extremely low and although this may be increased at elevated temperatures there is no evidence that there is any dramatic effect. It is possible that at high temperatures the rate of utilisation of the developmental lipids may be accelerated or the metabolic pathways otherwise modified resulting in less viable larvae.

The activity of the infective larvae of a number of parasitic nematodes has been correlated with the rate of utilisation of their food or lipid reserve (Payne, 1922, 1923; Corts, 1925; Rogers, 1934; Giovannola 1936; Rogers, 1939, 1940; Elliot, 1954; Hailey and Clifford, 1958, 1960; Croll, 1972; Croll and Matthews, 1973). Among the Ascarids Elliot (1954) reported the existence of a relationship between diminution of larval fat reserves and loss of infectivity in aged Ascaridia galli eggs; Todd et al (1952) observed a reduced

infectivity when chicken ascarid eggs were allowed to age; Jaskoski (1960) related age and fat reserves in eggs of A. suum to infectivity in the hamster. Von Gundy et al (1967) associated a decrease in infectivity with a corresponding decrease in motility and body fat of second stage larvae of Meloidogyne javanica and Tylenchulus semipenetrans when aged and stored in 27°C in soil and water. Payne (1922,1923), Cort (1925) and Giovannola (1936) reported a decrease in the lipid content of hookworm larvae with age and suggested that the physiological age of the infective larvae can be determined by examination of the fat content.

The present study showed a similar trend of decrease in the lipid content with increase in physiological age of the infective larva of A. suum (Figure 17). Figure 15 also showed that there was a general decline in the number of the infective larvae that penetrated the tissue membrane as the infective larvae aged. These observations would seem to suggest the existence of some relationship between physiological age, lipid reserve and ability of the infective larvae

to penetrate the tissue membrane. However, although it may be tempting to assume that the level of lipid reserve in the infective larvae may be indicative of the capacity of the larvae to penetrate tissue membrane, work by Matthews and Croll (1973) on activity, ageing and penetration of hookworm larvae showed that such assumption may not be strictly correct. They showed that the ability of the larvae to penetrate tissue membranes depended largely on larval activity which was itself not directly related to lipid level.

It may be worthwhile to point out that the results of the present study are in keeping with the epidemiology of the parasite. A. suum is a common parasite of pigs and even in the U.K. where the temperature hardly exceeds 24°C throughout the year, the incidence of the parasite apart from being common (Jenkins and Erasmus, 1963; Gitter, Kidd and Davies, 1965; Gitter et al, 1966; Davidson and Taffs, 1965) does not vary from one season of the year to another.

PART TWO

OBSERVATIONS ON THE SITE, ROUTE AND STRATEGY OF PENETRATION AND SUBSEQUENT PASSAGE OF THE INFECTIVE LARVAE THROUGH HOST TISSUES.

INTRODUCTION

The gross early migration of infective *A. suum* larva from their site of hatching in the intestinal lumen to their developmental sites in the liver and lungs has been studied by Stewert (1917); Fulleborn (1920, 1927); Ransom and Cram (1921); Roberts (1934), Sprent (1952, 1954), Kelley, Olsen and Hoerlein (1957), Olsen and Kelley (1960) and Jenkins (1968). These studies have concentrated on the general course and route of invasion and there is little information available on the early host/parasite relationship with reference to the mechanism by which the infective larvae invade the host tissue.

Accounts in various textbooks have been vague referring to the infective larvae as boring their way through the host tissue. How this boring activity is accomplished has remained speculative. Both mechanical and enzymatic invasion mechanisms have been suggested (Jenkins, 1968; Chandler *et al*, 1974). However, neither of the suggested entry mechanisms has been backed by precise experimental evidence.

Ransom and Cram (1921a and b) in an extensive

study of the early migration of Ascaris in mice found a number of migrating larvae in the blood and concluded that early migration of the larvae from the intestine to the liver was preferentially *via* the blood stream. Similar conclusions have also been drawn by subsequent investigators including Roberts (1934) who studied the early migration in pigs. However, these conclusions have not been unanimous.

Although Stewert (1917) rather erroneously considered the bile duct as a probable route of larval migration to the liver, others including Yoshidia (1919) and Bhowmick (1964) have presented the view of a more active migration with the larvae penetrating the intestine and actively moving to the liver and lungs *via* the abdominal and thoracic cavities. Jenkins (1968) in his studies of the early migration of the infective larvae in white mice reported seeing no evidence that the larvae traversed the muscle layer of the small intestine and described the caecum as the region of maximal larval penetration into the host tissue.

The need for further study of this early phase of infection is accentuated by these disagreements which remain largely unresolved. Therefore experiments were designed to determine the precise site, route and course of larval penetration and

subsequent migration through the host intestinal tissue, as well as the nature of any host tissue alterations accompanying larval passage.

The second part of the following study is concerned with the in vitro assessment of the histolytic potential of larval extracts. Various tests have been utilised by a number of workers in an attempt to elucidate the type of enzymatic activity produced by tissue penetrating helminths. Stirewalt (1966) in her review on the skin penetrating mechanisms of helminths has drawn up a catalogue of such studies. Preparations of Schistosome - infected snail tissue have been shown to exert lytic effects on skin sections by Gordon and Griffiths (1951). A spreading factor has been described in Cercariae ocellata and Cercariae psendaumata (Kuntz, 1953; Ginetsinkaya, 1950); adult Ancylostoma duodenale (Bruni, 1939); Strongyloides ratti, S. simiae and S. mansoni (Lewert and Lee, 1954). Hyaluronidase-like activity has also been described in extracts of S. mansoni (Levine, Garzoli, Kuntz, Killough, 1948; Stirewalt and Evans, 1952; Evans, 1953) and in Cercariae psendaumata (Ginetsinkaya, 1950). It has also been suggested that the lipolytic activity of larvae

of Nippostrongylus muris is related to their ability to penetrate the skin (Thorsøn, 1953).

In order to complement the histochemical aspect of the present work, a comparative study of the activity of larval extracts of infective A. suum larvae was also undertaken using Azocoll, thin gelatin film and frozen sections of mouse small intestine as test substrates.

MATERIALS AND METHODS

One of the major difficulties in any invasion study is locating the larvae in a known region of tissue. A number of *in vitro* and *in vivo* techniques were employed in an attempt to obtain a limited area of host tissue containing migrating larvae. *In vitro*, a modification of the "sausage" technique of Silverman and Maneely (1955) was used. Mice were killed by cervical dislocation and following laparotomy the gut was exposed and ligatured at 2.5 cm intervals into a number of "sausages" down its length. Inocula consisting of either 5,000 infective eggs or 1,000 active larvae, hatched *in vitro*, were introduced into the lumen through a hypodermic needle. The gut was then either removed from the animal and incubated in phosphate buffered saline or mammalian Ringers solution at 37°C, or retained attached to the animal carcass which was kept moist with Ringers solution at 37°C.

The tissues were incubated for various lengths of time after which they were fixed in either 70% alcohol for 24 hours, or quenched in liquid Nitrogen, and prepared for histological and histochemical studies.

This technique was not very successful and yielded few useful results. There were two main reasons for this; firstly, infective eggs did not hatch within the "sausages" and secondly, autolysis set in so rapidly that tissue degeneration resulted and invalidated any histological studies.

To overcome these problems a series of *in vivo* tests were conducted. Mice were infected orally by stomach tube with a large number (20,000) of infective eggs and then sacrificed at periodic intervals. The gut was removed and used for histological and histochemical analysis. This technique made it necessary to precisely identify the region of the gut where larval penetration takes place. To do this, the following experimental procedure was adopted. Mice 2-4 months old, of both sexes from an inbreeding colony were each infected *via* stomach tubing with 10,000-20,000 infective eggs embryonated at $22^{o} \pm 1^{o}C$. The infective dose was calculated using the modified McMaster technique with saturated sodium chloride as the floation medium. The hosts were sacrificed at various intervals from 30 minutes to 144 hours.

Immediately after sacrifice, the gut was

removed and divided into stomach, small intestine, caecum, colon and rectum, with the small intestine further sub-divided into three equal parts which represented the anterior, middle and posterior regions. The various portions were either fixed in 70% alcohol and prepared for histological examination or opened along their length; and their luminal contents flushed into a petri-dish using 10 mls. of physiological saline. The number of larvae found in each portion was recorded.

The gut walls as well as other internal organs were removed and subjected to peptic digestion for 18 hours at 37°C (Hill, 1957). This treatment did not kill the larvae and they were recovered from digests by repeated centrifugation and later counted. The body cavity was also washed with saline and searched for larvae.

30 randomly selected larvae recovered from the luminal contents, the various organs and tissue digests at various intervals after infection were measured with an eye piece micrometer to determine their body size.

Histological Examination

Portions of the infected tissue were fixed in either 70% alcohol or 10% neutral buffered formalin

(Pearse, 1960) for 24 hours and embedded in paraffin wax. Serial sections were cut at 8-10 µm using a rotary microtome and sections were stained using Haematoxylin and Eosin, Alcian blue and Mallory's triple stain, the latter being especially useful for demonstrating the deposition of connective tissue. Regand's iron haematoxylin was also used, as this stain gave improved nuclear differentiation and better visualization of the larvae. This was especially valuable for photography. Staining techniques were used as given by Humason (1962).

Histochemical Studies

Histochemical tests were largely based upon the work of Lewert and Lee (1954) in their study of migration of various helminth larvae. It was hoped that changes in the host tissues caused by migrating A. suum larvae could be detected by looking at the changes in the ground substance and basement membrane and that these would give an indication of the mechanisms involved. The methods are based on the freeze-dry technique of tissue fixation, embedding and section preparation described by Gersh (1948).

Portions of the posterior $^{1}/_{3}$ of the small intestine, the caecum and colon of the infected mice were rapidly removed soon after death, quenched

in 150-pentane at -120°C and dehydrated under vacuum at -30°C. Following dehydration, the tissues were impregnated with molten paraffin wax at 62°C under vacuum at a pressure of 400 to 500 mm for 48 hours and then embedded in paraffin wax. Serial sections of 8-12 µm were cut and the paraffin removed from the sections by immersion in three changes of anhydrous petroleum ether. This technique has the advantage of preserving the cause of any tissue changes if caused by other than mechanical disruption. Normal histological techniques of fixation and section preparation might mask or destroy any evidence especially of water soluble products (Lewert and Lee, 1954).

Sections were transferred to absolute alcohol for six hours prior to staining. The staining method was based mainly on the Hotchkiss periodic acid leucofuchin technique (Hotchkiss, 1948).

Sections were rinsed in 70% alcohol and immersed in periodic acid solution for 5 minutes. The periodic acid solution was prepared by dissolving 0.4 gm of periodic acid (HIO_4 $2H_2O$) in 35 ml of ethyl alcohol to which was added 5 ml

of 0.2M - sodium acetate (27.2g of the hydrated salt in 1,000 ml) and 10 ml of distilled water. The sections were rinsed in 70% ethyl alcohol once removed from the periodic acid solution and immersed in a reducing bath for 1 minute. The reducing bath was prepared by dissolving 1 gm potassium iodide and 1 gm sodium thiosulphite in 30 ml of ethyl alcohol and adding 20 ml of distilled water and 0.5 ml of 2N-HCl (20% conc. HCl). On removal from the reducing bath, the sections were again rinsed in 70% alcohol and immersed in Schiff's solution (Berge and Delamater) for 20 minutues; washed in running water for 10 minutes and stained with celestin blue for 2-3 minutes, followed by Mayer's haemalum 2-3 minutes. The sections were then differentiated in 1% acid alcohol, washed in running water for 10 minutes, dehydrated in ethyl alcohol, cleaned in xylene and mounted in DPX.

Control sections to check colour reactions were prepared from uninfected mouse gut obtained from similar regions as the experimental samples. Sections were all processed at the same time in exactly the same way. To test the adequacy and specificity of the staining reactions, some sections were taken through Schiff's reagent without

previous oxidation by periodic acid. Finally portions of infected and uninfected mouse gut were processed using conventional methods of fixation and histological preparation and stained at the same time as the test sections. The sections were counter stained either with orange G or Harris Haematoxylin.

Other staining methods employed included Alcian Blue - Chlorantine fast red; Mallory's triple stain and H & E.

To complement the above histochemical methods, Evans' blue technique was also adopted. The technique has a diagnostic affinity for areas of formation of water soluble alcohol-insoluble carbohydrate-containing protein (Gersh, 1952). Evans blue was administered intravenously at the rate of 1 ml of a 1% solution of the dye in 0.85% saline per 100 gm body weight via the tail vein of anaesthetised infected mice. Prior to the injection of the dye, the animals were warmed in an incubator at 37°C for a few minutes. This treatment helped to expose the tail vein. Six mice were used each infected with 10,000 infective eggs. The dye was administered 15-20 minutes before the animals were sacrificed. The first mouse was sacrificed 30 minutes after infection and the rest at 1 hour intervals. Immediately after

death, the intestines were removed and quenched in liquid nitrogen at -135°C to prevent any shift in the site of the Evans blue. The intestines were then cut into 2.5 cm portions and opened along their lengths for examination.

Concurrent with the above histochemical studies, a histological study was undertaken to assess the rate of larval passage through the layers of the intestinal tissue. Twelve mice were killed at 30 minute intervals post-infection. The intestines were removed and cut into 20, 1.5 cm segments beginning at the duodenal end. The first eight segments were discarded and each of the five subsequent segments were placed in a McCartney bottle labelled with the intestinal region and duration of infection. The luminal contents of each of the segments were flushed out and the number of larvae present counted. The tissues were then fixed in 70% alcohol for 24 hours, dehydrated in alcohol, embedded in wax and later sectioned at 10 µm. Sections were taken 120 µm apart and affixed to microscope slides. Twelve slides were made from each block.

The tissues were then stained with Alcian blue and the slides scanned at X 80 for larvae either in the lumen or the various layers of the

gut wall and counts taken of the number of larval sections found.

0.25 Cm^3 portions of the liver were removed and digested and the number of larvae in the digest counted.

Assays for Enzymatic Activity

Living larvae and larval secretion(s) were assayed for the presence of enzymatic activity capable of digesting host tissue during larval invasion. Three assay methods were used; dye release from azocoll; thin gelatin film digestion, and the effect of the test materials on the frozen sections of host tissues.

PREPARATION OF TEST MATERIALS

(a) Infective larvae:

30,000 infective larvae hatched *in vitro* and isolated as described earlier, were washed three times by centrifuging in distilled water, and then twice in 1.5% saline in 0.05m phosphate buffer (pH 7.2). After the final washing, the larvae were resuspended in either 1 ml of fresh buffered saline or 1 ml of buffered saline containing antibiotics (Penicillin - 0.0627 gm/100 ml; Streptomycin - 0.01 gm/100 ml; Nystatin - 0.002 gm/100 ml).

(b) Larval Secretion(s):

30,000 larvae obtained and treated as described above were concentrated in 0.05m phosphate buffered saline (pH 7.2) or in 1 ml of saline containing antibiotics and incubated at 37°C in a shaking water bath for 4 hours. The larvae were centrifuged at 1000 rpm for 2 minutes and the supernatent retained as the test material.

EXPERIMENTAL PROCEDURES

Dye release from Azocoll

The procedure followed was a biochemical assay based on the liberation of dye from azocoll (Calibiochem) - a dye-linked general proteolytic substrate. Each test sample was obtained from approximately 30,000 freshly hatched larvae concentrated in 1 ml of the incubation solution. Each sample was made up to 5 ml with the appropriate incubation solution in a 10-ml centrifuge tube and the tubes incubated in a shaking water bath at 37°C. At times of 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours and 6 hours the tubes were centrifuged, the supernatants carefully removed and their absorbance measured spectrophotometrically at 520 nM. After each reading, the supernatents were returned for further incubation. Two separate

samples were used, one with and the other without, antibiotics (Penicillin-0.0627 gm/100 ml; Streptomycin-0.01 gm/100 ml; Nystatin-0.002 gm/100 ml). Each batch was replicated four times.

Controls consisted of samples heated to 100°C for 15 minutes to kill the larvae, and denature any of the enzymes which might have been present. 10 mg/ml pepsin (sigma) inthe appropriate incubation solutions was used as a positive control, and samples containing all the appropriate components but lacking larvae were included to eliminate spurious readings which might have been obtained from bacterial contamination of the incubation media.

GELATINOLYTIC ACTIVITY

The histolytic potential of the larval secretions was assessed using thin gelatin films (Lewert and Lee, 1954). The gelatin films were prepared by dipping clean microscope slides into a 5% solution of Eastman purified gelatin at 38°C and allowing the slides to drain and dry in a vertical position.

0.01 ml of the test samples was placed on the prepared slides and incubated at 37°C in a constant humidity chamber at 95% RH. Four sets of

slides were removed after ½ hour, 1 hour, 1½ hours, 2 hours, 3 hours, 4 hours, 5 hours and 6 hours. The slides were immediately fixed in 4% formalin for 10 minutes, washed with distilled water and stained with Delafield's haematoxylin for 5 minutes.

Boiled test samples; 10 mg/ml pepsin in appropriate incubation media, as well as incubation media lacking the larvae were used as controls.

HISTOLYTIC ACTIVITY

Concentrates of (a) living larvae, (b) larval secretions in buffered saline solutions (with and without antibiotics) were tested against frozen sections of intestinal tissue for their ability to digest host tissue *in vitro*.

Frozen sections, 8-10 μ thick, of uninfected mouse small intestine were cut on a cryostat, mounted on glass cover slips, fixed in absolute alcohol for 6 hours and later hydrated in buffered saline solutions. After hydration, the tissue sections were covered with 0.02 ml of saline containing 8,000 larvae, or the larval secretion(s). The tissue sections were then incubated in a moist chamber at 37°C for various lengths of time ranging

from 30 minutes to 6 hours. At the end of the incubation period, the sections were stained by the Hotchkiss periodic acid technique; the Alcian blue-chlorantine fast red technique or the Mallory's triple staining technique.

Controls included sections fixed and stained soon after cutting, sections treated with pepsin (10 mg/ml in the buffered saline solutions); sections treated with boiled test material and sections incubated with buffered saline solutions lacking the test samples.

RESULTS

There were some differences between individual mice but the general pattern of larval invasion was clear. Figure 19 illustrates the recovery of larvae from the gut lumen and reflects the normal passage of material through the mouse intestinal tract. Within an hour larvae had spread through the length of the intestine (with the exception of the rectum) although the build up in the more posterior regions was not complete until about 5 hr. post-infection. By this time the numbers recovered from the rectum were increasing, these reached a peak after about 10 hrs. before falling off. Most of the larvae recovered during the first 10 hours were alive and active but later many were non-motile and probably dead.

Although the larvae were spread throughout the length of the intestine, recovery from the intestinal wall was much less even (Figure 20). Many more larvae were recovered in the digests from the walls of the posterior third of the small intestine, the caecum and the colon than from the anterior two thirds suggesting some preferential invasion site. No larvae were

recovered from the stomach wall. Very few from the rectal wall and these only in the later stages of the infection. Initial penetration was rapid with larvae within the tissues within an hour of infection. There was no additional build up of larvae and the numbers remained relatively constant for the first 24 hours and then slowly declined.

No larvae were recovered from the body cavity, spleen, kidney, brain or carcass during the course of the infection. Larvae were first recovered from the liver within two hours of infection (Figure 21), the number at this time was small (3) but increased steadily, reaching a peak 2 days post infection. Larval build up in the lungs did not begin until 4 hours after infection but continued to increase throughout the 120 hours of the experimental infection. The decline in numbers in the liver was matched by an increase in the rate of entry into the lungs.

There was no significant size increase in larvae recovered from the intestinal tissues or lumen but considerable growth occurred during migration through the liver and lungs. The range of size of larvae recovered from the lungs was frequently

FIGURE 19

Distribution of "Free" infective A. suum larvae with the gut lumen of mice infected with 10,000 infective eggs, and sacrificed at various times post-infection.

★ Anterior 1/3 of the Small Intestine

○ Mid 1/3 of the Small Intestine

♦ Posterior 1/3 of the Small Intestine

■ Caecum

□ Colon

● Rectum

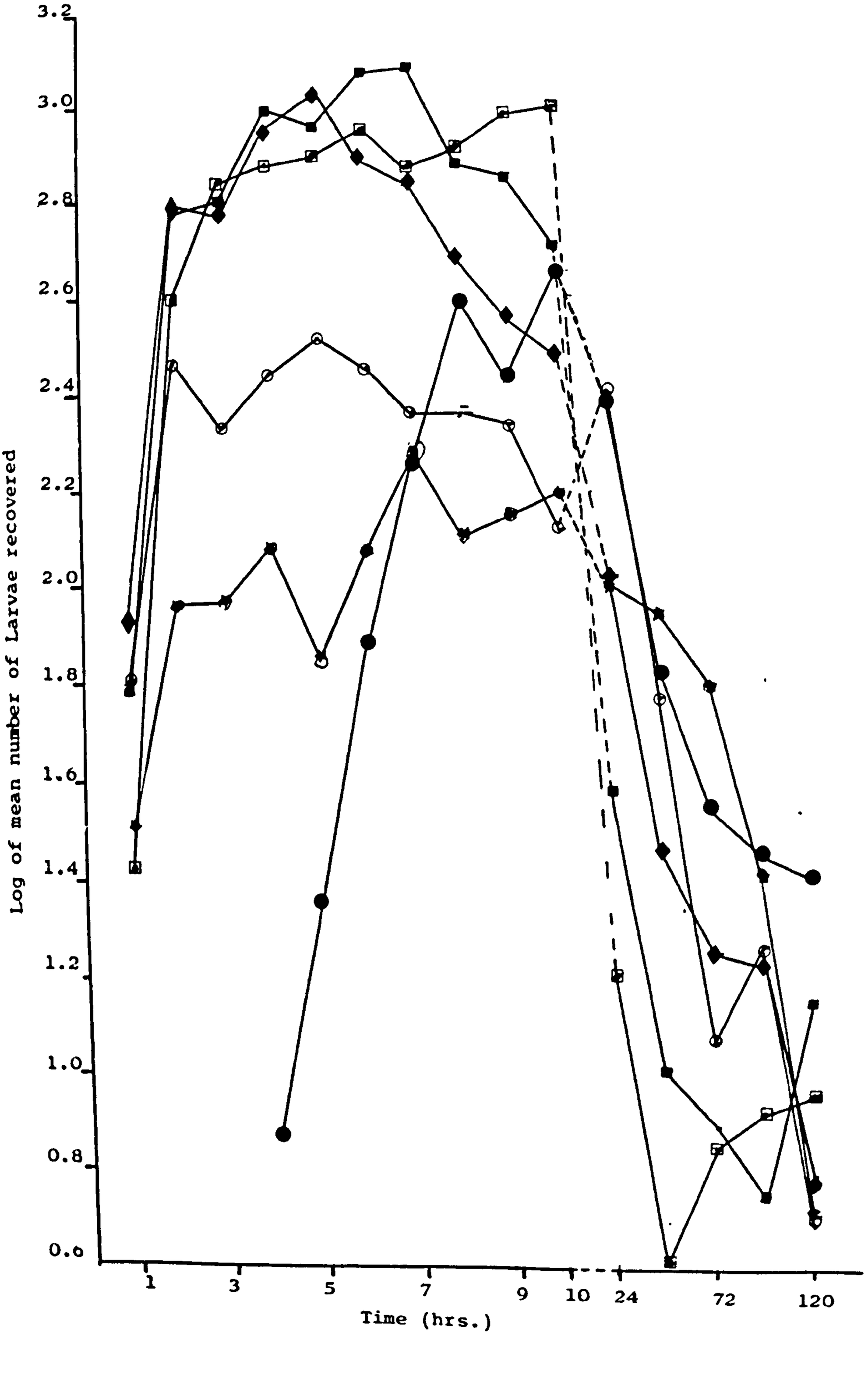
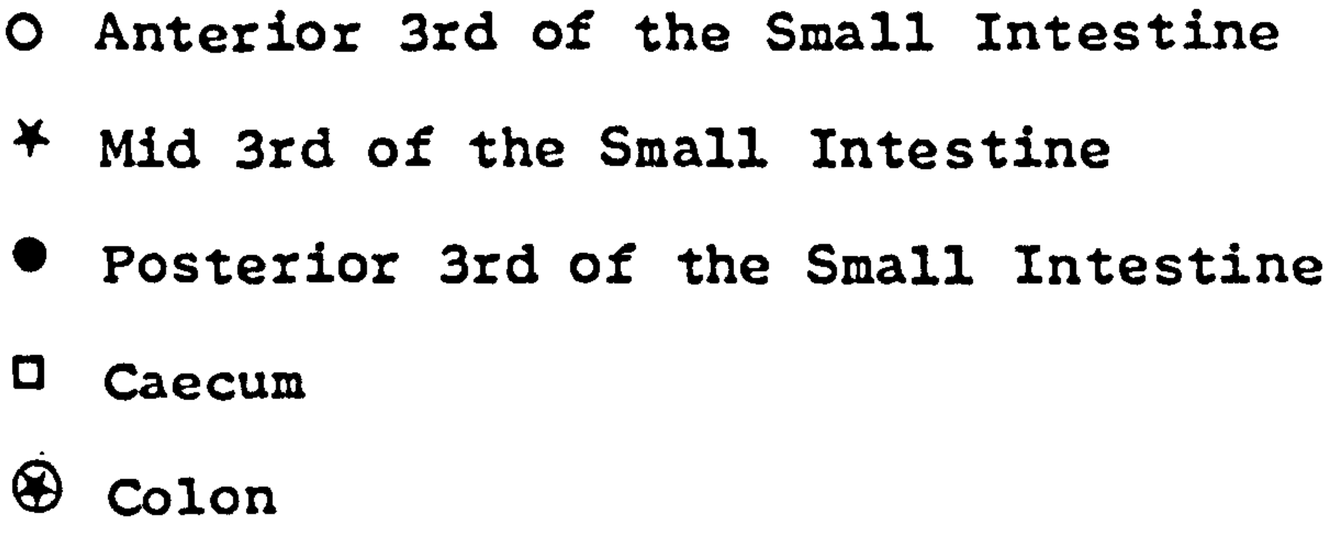

3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
Log of mean number of Larvae recovered
1
3
5
7
9
10
24
72
120
Time (hrs.)

FIGURE 20

Distribution of infective A. suum larvae in the gut wall of mice infected with 10,000 infective eggs, and sacrificed at various times post-infection.



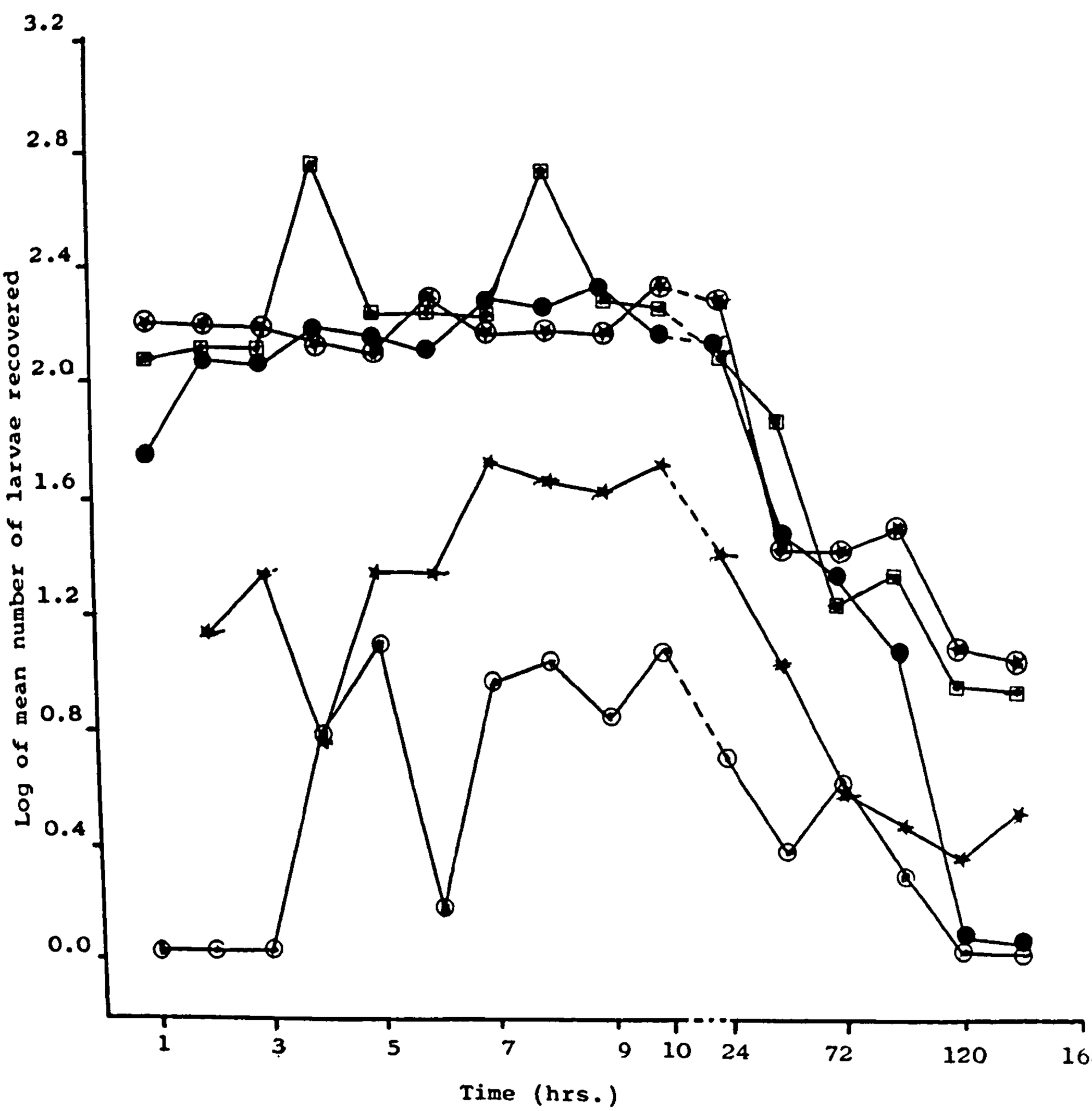
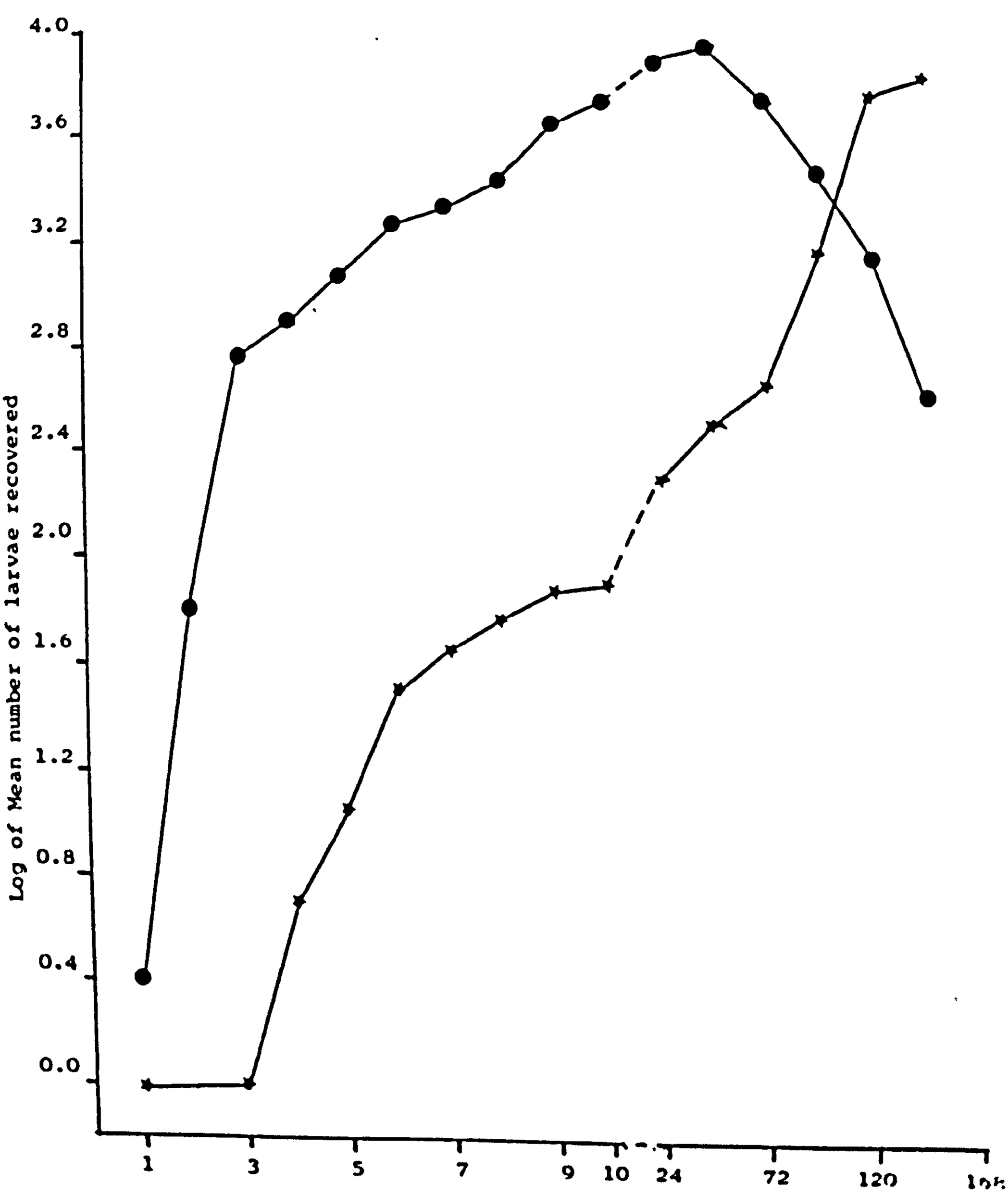

3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Log of mean number of larvae recovered
1
3
5
7
9
10
24
72
120
168
Time (hrs.)

FIGURE 21

Number of infective A. suum larvae recovered from the liver and lungs of mice infected with 10,000 infective eggs at various time intervals after infection.

● Liver

✱ Lungs


4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Log of Mean number of larvae recovered
1
3
5
7
9
10
24
72
120
Time (hours)

considerably greater than that of larvae in the liver at the same time (Table V a & b Figure 22) indicating that some larvae may also be reaching the lungs by-passing the liver.

Larval penetration was invariably through the crypts of Lieberkuhn. On no occasion was larvae found to penetrate the epithelium of the villi directly suggesting that if this does occur it is a rare event. During the process of initial entry the larvae orientated perpendicularly to the epithelial and paneth cells at the base of the crypts (Plate 5). On several occasions, when the larvae were caught in this act of initial penetration, the epithelial cells at the point of larval penetration were dislodged; there were no signs of any extensive lysis of the cells.

In a few cases, more than one larva was seen to penetrate the same crypt although they did not appear to follow exactly the same track (Plate 6).

Passage through the epithelial layer appeared to involve some physical effort on the part of the larvae. Their body was often constricted at the entry-point with the rest of the body thrown into sinusoidal waves.

TABLE Va

Body measurements of Larvae recovered from intestinal contents; intestinal wall; liver and lungs at intervals after infection (measurement in μm; number in bracket is the mean of the range of the measurements).

Time Post-Infection	Source of Larvae	Length	μ^3	Width
1 hr.	Intestinal Contents Intestinal Wall Larvae Hatched in vitro	204.6 - 267.2 (249.62)	29,228	11.3 - 15.7 (12.21)
2 hrs.	Intestinal Contents Intestinal Wall Liver	206.4 - 262.5 (248.81)	30,047	11.4 - 15.6 (12.40)
10 hrs.	Intestinal Contents Intestinal Wall Liver Lungs	207.8 - 263.9 (247.90)	29,841	(11.4 - 15.6) (12.38)
24 hrs.	Intestinal Contents	204.4 - 275.3 (247.33)	31,976	11.2 - 15.7 (12.83)
	Intestinal Wall	205.2 - 273.7 (248.24)	29,593	11.8 - 15.7 (12.32)
	Liver	213.8 - 272.2 (247.26)	31,027	11.4 - 15.6 (12.64)
	Lungs	203.6 - 247.4 (241.22)	27.738	11.2 - 15.5 (12.10)

TABLE V$_q$ continued

Time Post-Infection	Source of Larvae	Length	μ^3	Width
2 days	Intestinal Contents	204.4 - 281.6 (239.36)	30,369	11.2 - 15.6 (12.71)
	Intestinal Wall	209.2 - 274.5 (248.61)	32,191	11.8 - 15.6 (12.84)
	Liver	286.4 - 307.6 (293.21)	48,151	12.8 - 18.2 (14.46)
	Lungs	208.6 - 274.4 (248.71)	37,362	12.2 - 16.6 (13.83)
3 days	Intestinal Contents	206.2 - 279.8 (246.4)	30,335	11.2 - 16.5 (12.52)
	Intestinal Wall	203.4 - 276.2 (241.26)	33,821	11.6 - 15.4 (13.36)
	Liver	295.8 - 349.5 (321.11)	77,324	17.1 - 19.8 (17.51)
	Lungs	212.4 - 347.6 (302.68)	70,656	16.5 - 19.4 (17.24)
4 days	Intestinal Contents	208.2 - 299.4 (254.27)	48,475	12.6 - 17.8 (15.58)
	Intestinal Wall	212.6 - 289.8 (243.19)	50,685	12.1 - 18.5 (16.29)
	Liver	493.4 - 589.6 (538.68)	298,004	24.6 - 28.2 (26.54)
	Lungs	301.2 - 603.1 (492.52)	210,183	23.6 - 24.2 (23.31)
5 days	Intestinal Contents	206.4 - 296.2 (256.26)	41,503	12.1 - 17.6 (14.36)
	Intestinal Wall	212.2 - 302.1 (242.59)	50,064	12.5 - 18.8 (16.21)
	Liver	593.4 - 624.4 (573.34)	473,583	29.2 - 36.4 (32.43)
	Lungs	422.4 - 846.5 (702.48)	726,604	33.2 - 39.1 (36.29)

TABLE Va continued

Time Post-Infection	Source of Larvae	Length	μ^3	Width
6 days	Intestinal Content	206.5 - 302.6 (253.26)	47,912	12.6 - 17.8 (15.52)
	Intestinal Wall	210.4 - 302.2 (246.32)	41,748	12.2 - 17.2 (14.69)
	Liver	826.2 - 881.8 (843.41)	1,127,578	39.4 - 43.2 (41.48)
	Lungs	544.8 - 983.4 (896.44)	1,321,259	38.6 - 46.4 (43.32)

TABLE V6 Body measurements of Larvae recovered from liver and lungs at various intervals from mice infected with 10,000 infective Ascaris suum eggs (measurement in/um, number in bracket is the mean of the range of measurements).

Time Post-Infection	Source of Larvae	Length	/u^3	Width
24 hrs. (1 day)	Liver	213.8 - 272.2 (247.26)	31,027	11.4 - 15.6 (12.64)
	Lungs	203.6 - 247.4 (241.22)	27,738	11.2 - 15.5 (12.10)
2 days	Liver	286.4 - 307.6 (293.21)	48,151	12.8 - 18.2 (14.46)
	Lungs	208.6 - 274.4 (248.71)	37,362	12.2 - 16.6 (13.83)
3 days	Liver	295.8 - 349.5 (321.11)	77,324	17.1 - 19.8 (17.51)
	Lungs	212.4 - 347.6 (302.68)	70,656	16.5 - 19.4 (17.24)
4 days	Liver	493.4 - 589.6 (538.68)	298,004	24.6 - 28.2 (26.54)
	Lungs	301.2 - 603.1 (492.52)	210,183	23.6 - 24.2 (23.31)
5 days	Liver	593.4 - 624.4 (573.34)	473,583	29.2 - 36.4 (32.43)
	Lungs	422.4 - 846.5 (702.48)	726,604	33.2 - 39.1 (36.29)
6 days	Liver	826.2 - 881.8 (834.41)	1,127,578	39.4 - 43.2 (41.48)
	Lungs	544.8 - 983.4 (896.44)	1,321,259	38.6 - 46.4 (43.32)

FIGURE 22

Graph of length of larvae recovered from various locations in mice at various intervals after infection with 10,000 infective A. suum eggs.

△ Luminal content

□ Intestinal Wall

■ Liver

● Lungs

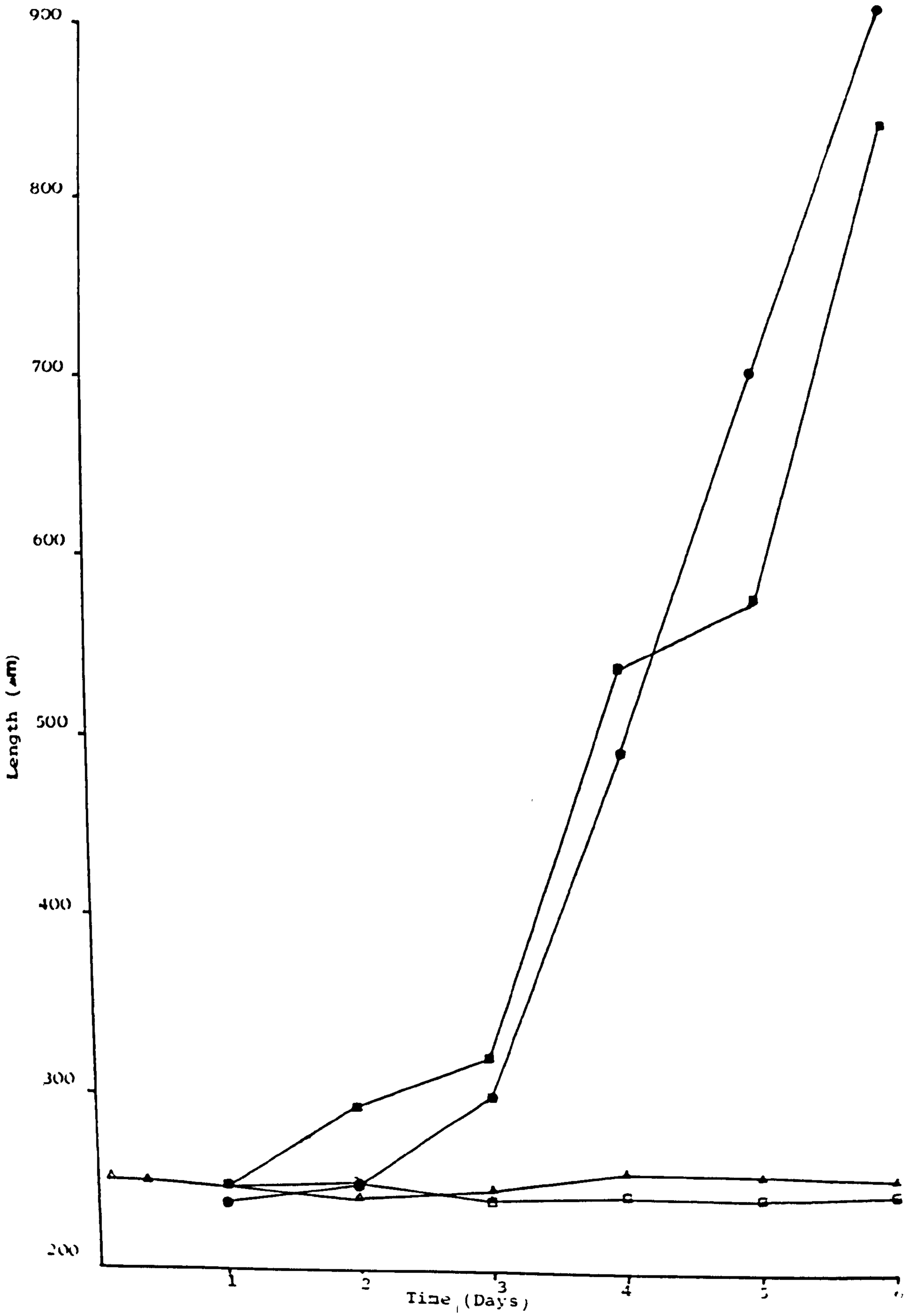

900
800
700
600
500
400
300
200
Length (μm)
1
2
3
4
5
Time (Days)

TABLE VI

Numbers of Infective Larvae in each location of host tissue sections at increasing time intervals after infection with 10,000 infective eggs.

Time Post-Infection	Location in Host Tissue Section			
	Intestinal * Lumen	Submucosa	Muscle Layer	Liver **
30 min.	2	Ø	Ø	Ø
1 hr.	13	9	Ø	8
1 hr. 30 min.	32	12	Ø	10
2 hr.	51	17	Ø	15
2 hr. 30 min.	48	23	Ø	28
3 hr.	53	27	Ø	26
3 hr. 30 min.	64	18	Ø	31
4 hr.	83	22	4	39
4 hr. 30 min.	91	16	8	56
5 hr.	86	24	12	71
5 hr. 30 min.	98	11	6	34
6 hr.	131	23	4	49

* Intestinal Lumenal Content

** Tissue digest

PLATE 5

Section of the small intestine of mouse infected with 20,000 infective A. suum eggs. The animal was sacrificed 2 hrs. after infection. Stained with Alcian blue. Chlorantine fast red. Mag. X640. Note the perpendicular nature of the entry of the infective larva into the submucosa.

PLATE 6

Section of the small intestine of mouse infected with 20,000 infective A. suum eggs. The animal was sacrificed 4 hrs. after infection; section was stained with Alcian Blue-Chlorantine fast red. Mag. X640. Note the tendency for 2 larvae to use a limited area of entry at the same time.

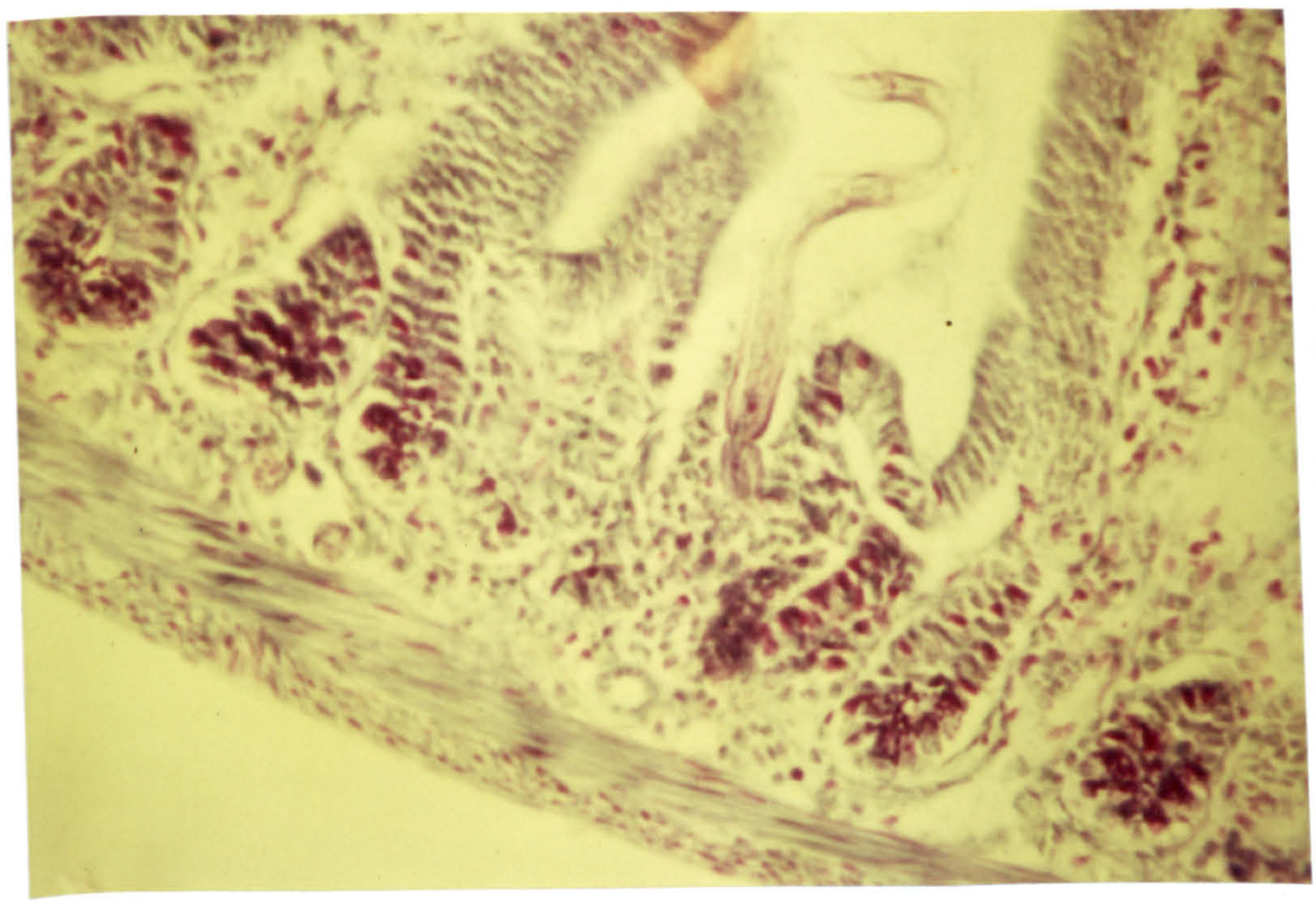


PLATE 5

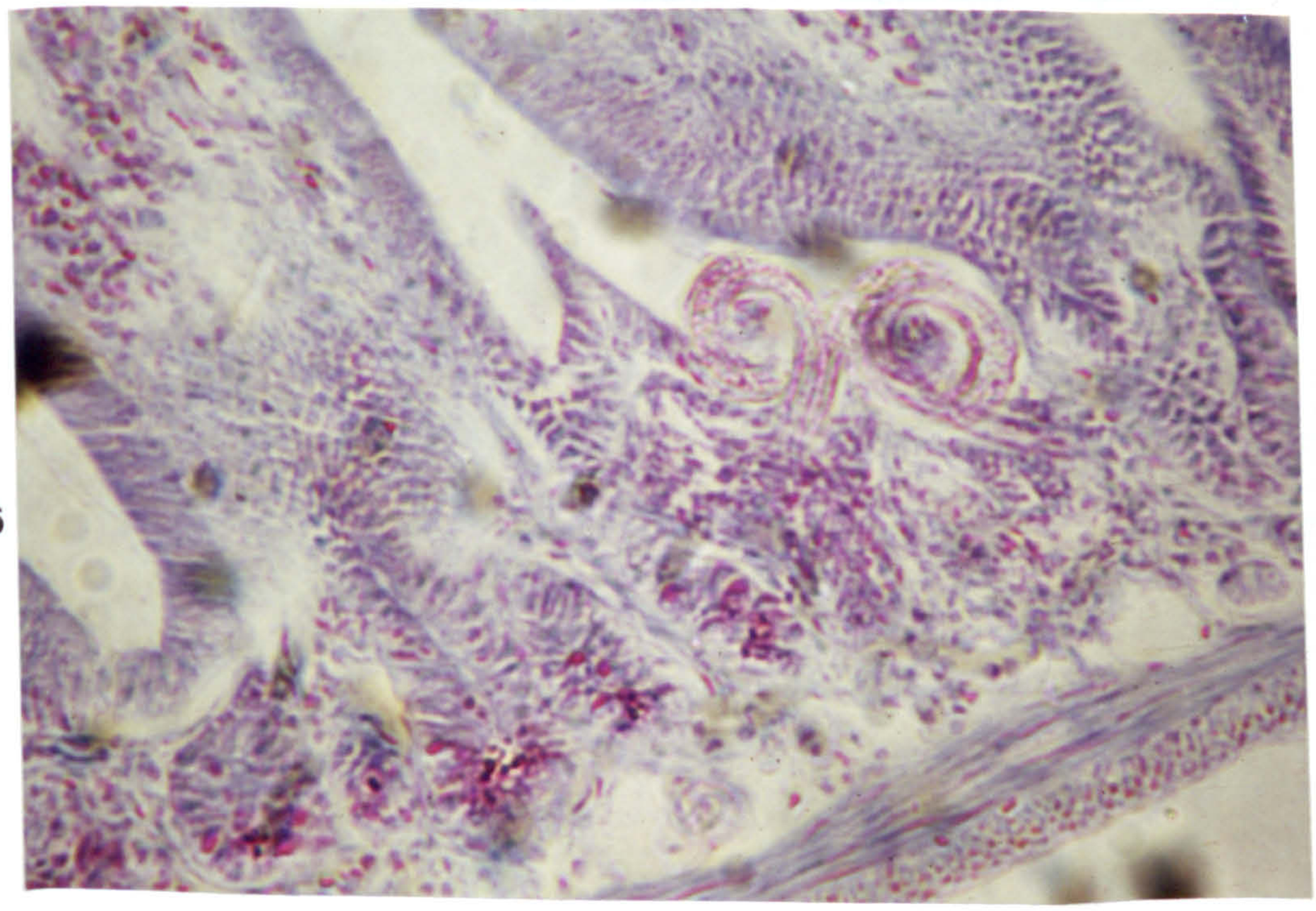


PLATE 6

It was not possible to determine the precise time required for larvae to cross the epitherial layer into the submucosa.

No larvae were found in the intestinal walls of mice sacrificed 30 minutes after infection but by an hour the eggs had hatched and larvae had entered the submucosa (Table VI) suggesting that the invasion process is relatively rapid.

Penetrating larvae caused Evans blue reaction in the gut mucosa. However, larval entry into the submucosa was never accomplished by the formation of permanent tunnels or excavation in the tissue. Instead, the penetrating larvae frequently caused changes in the ground substance which led to changes in the staining affinity of the tissue along the path of the larval passage. The ground substance was often extensively altered in a way which suggested that the glycoprotein materials were affected (Lewert and Lee, 1954). Within moments of larval passage, the ground substance appeared to be somewhat thickened and more diffusely stained (Plates 7 and 8). The normal ground substance stained a relatively even diffused pink but in the path of larval passage was lightly

coloured. The diffused area varied in extent from a relatively small locus approximately the diameter of the larvae to an area roughly two to three times the larval diameter (Plates 7 and 8).

Details of these tissue changes were not preserved by the usual methods of tissue fixation and section preparation, and in such sections, the larvae were often observed to be surrounded by a clear area formed largely by tissue shrinkage (Plate 9).

In sections of the tissue stained by the Hotchkiss method, the larvae were readily seen due to the presence of relatively large amounts of Hotchkiss-positive materials. The most prominent feature of the larvae was the intensively stained oesophagus (Plate 10). The intensity of stain decreased with time as the larvae remained within the tissue (Plate 11). The anterior of the worm and the tissue immediately surrounding it were often coated with PAS - positive material.

There was no evidence of extensive oesophageal glands in the infective larva (Plate 12) but granular bodies which Jenkins (1971) described as secretory bodies were present in larvae during the early stages of infection (Plate 13). These disappeared after penetration of the host tissue.

PLATE 7

Section of the small intestine of mouse infected with 10,000 infective A. suum eggs and sacrificed 4 hrs. after infection. Section was stained with PAS counter stained with Orange G. Mag. X640. Note the alteration of the host tissue along the route of larval entry. The section was prepared by the freeze-dry technique according to Gersh (1948).

PLATE 8

Section of the small intestine of mouse infected with 10,000 infective A. suum eggs and sacrificed 3 hrs. after infection. Section was prepared by the freeze-dry method of Gersh (1948) and stained with PAS counter stained with Orange G. Mag. X480. Note again the alteration of the host tissue along the route of larval passage.

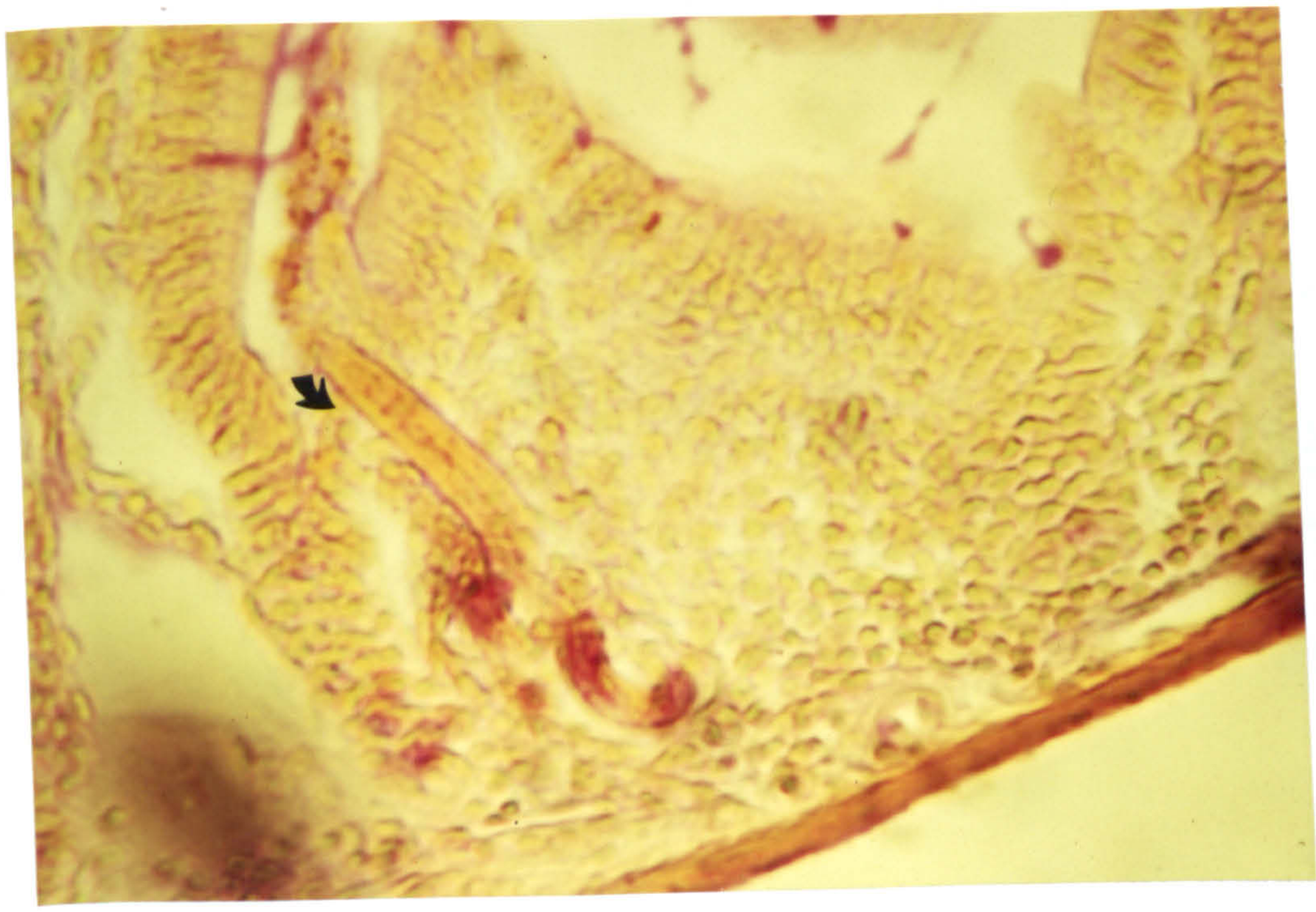


PLATE 7

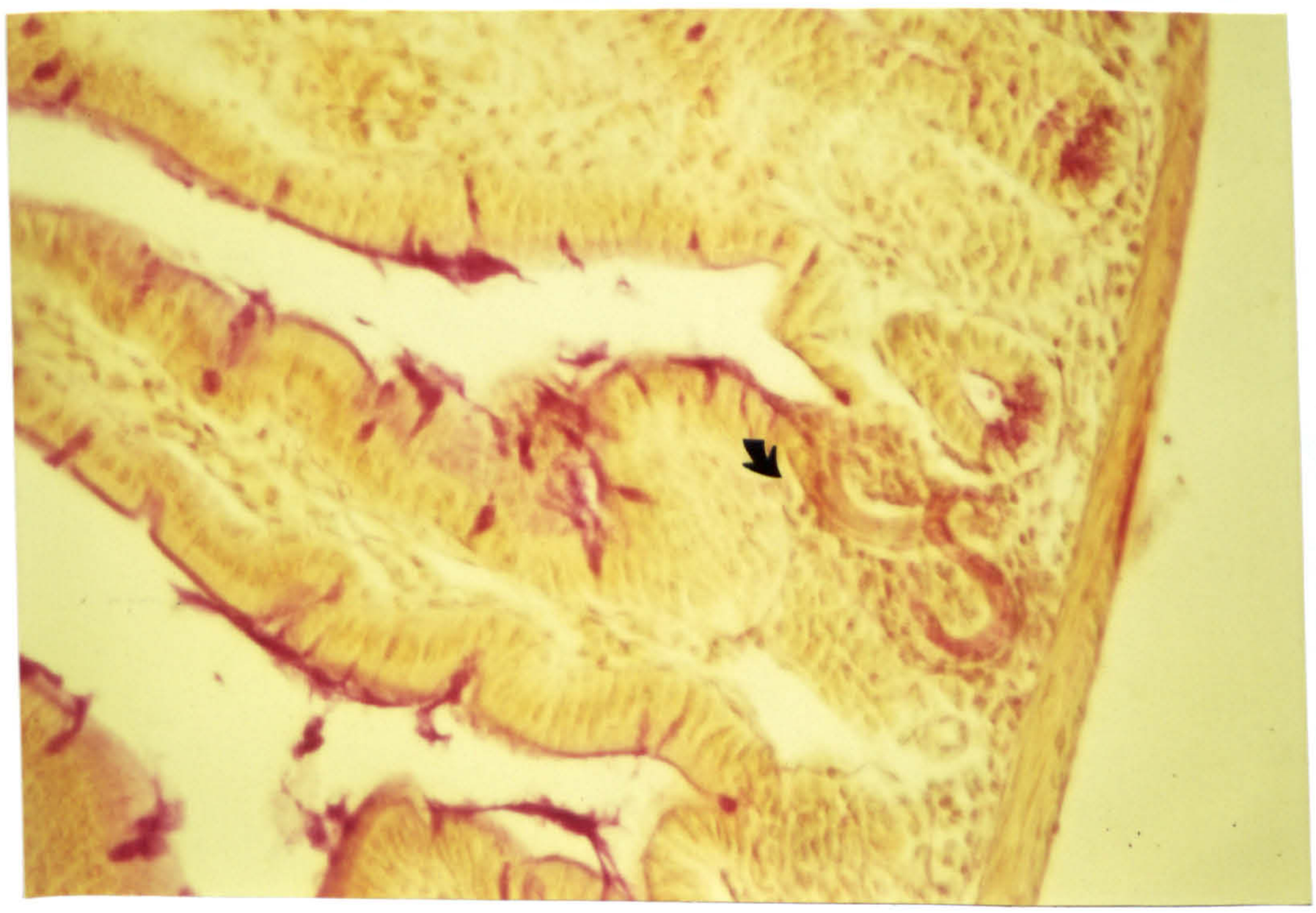


PLATE 8

PLATE 9

Section of mouse small intestine infected with 10,000 infective A. suum eggs and sacrificed 4 hours post-infection. The section was prepared by the usual method of tissue fixation and section preparation and stained with PAS counter stained with Orange G. Mag. X640. Note the clear area surrounding the larvae due to tissue shrinkage.

PLATE 10

Section of mouse small intestine infected with 10,000 infective A. suum eggs, and sacrificed 6 hrs. post-infection. Stained with PAS counter stained with Orange G. Mag. X640. Note the brilliant colouration of the anterior end.

PLATE 9

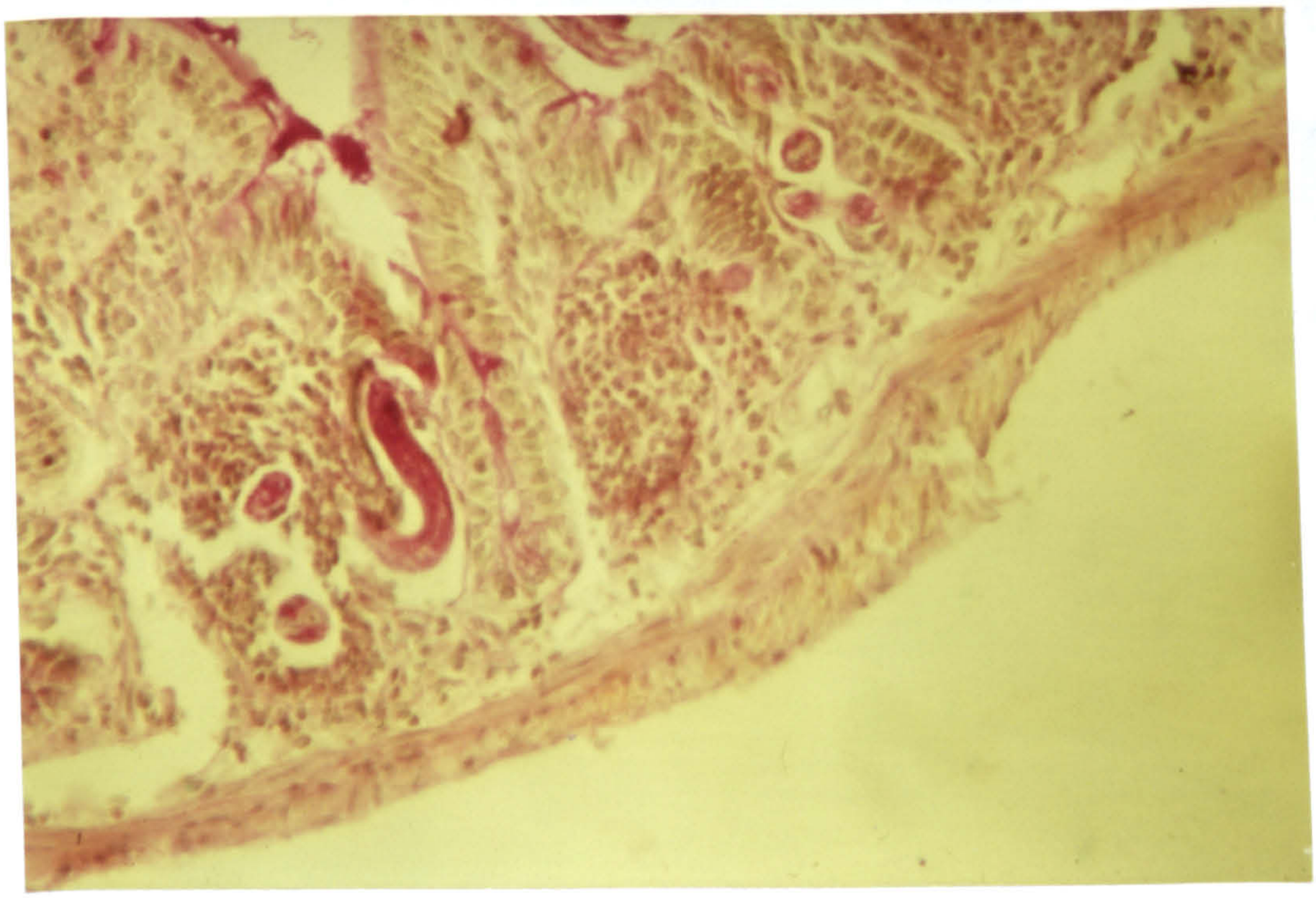


PLATE 10

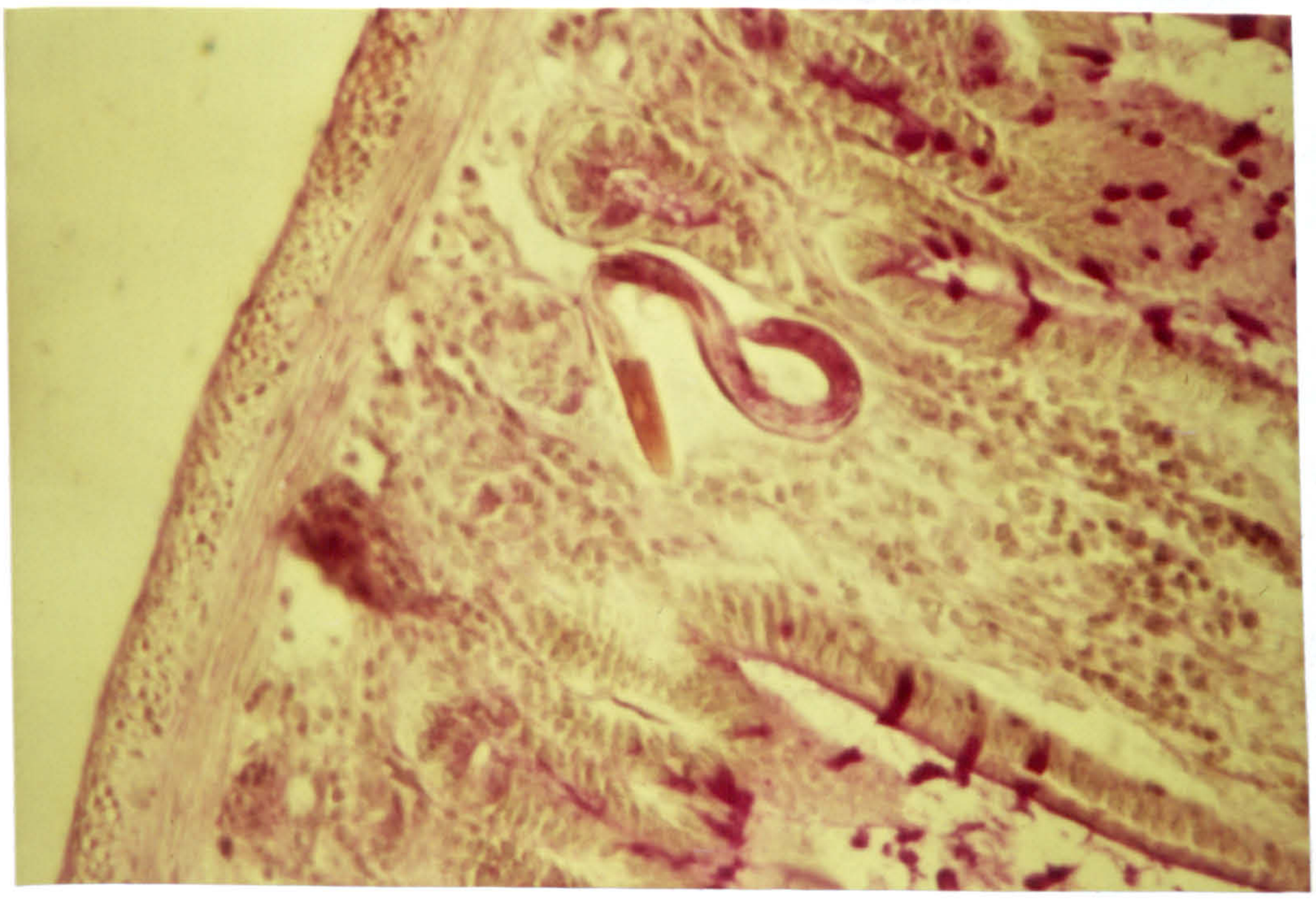


PLATE 11

Section of mouse small intestine infected with 10,000 infective A. suum eggs and sacrificed 10 hr. post-infection; showing entry of the infective larva into the muscle layer; stained with PAS, counter stained with Orange G. Mag. 480. Note the oblique nature of the larval entry into the muscle layer.

PLATE 11

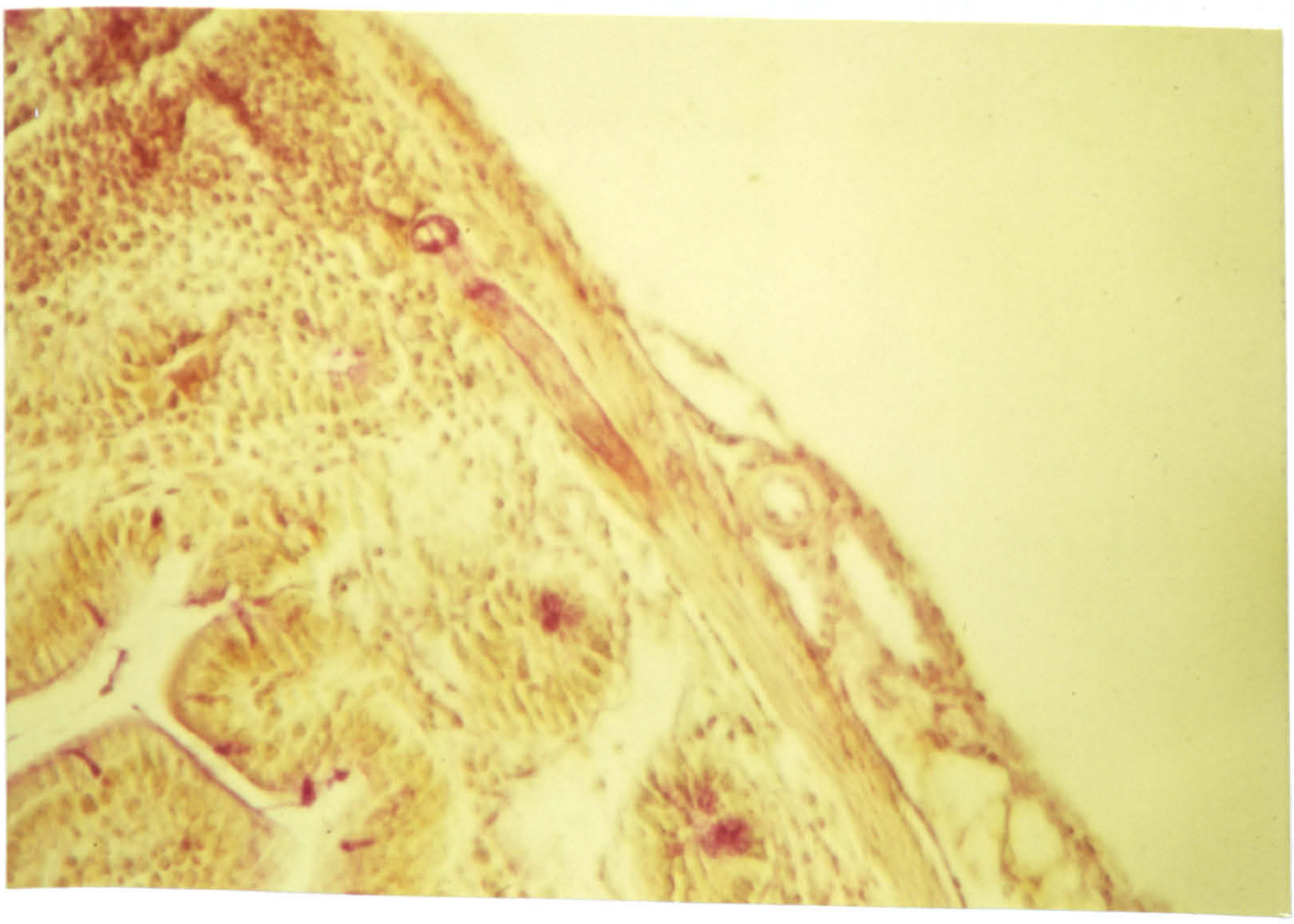


PLATE 12

Electron micrograph of oesophagus of infective A. suum larva. Note the absence of any extensive oesophageal glands as have been described in some hookworm infective larvae.

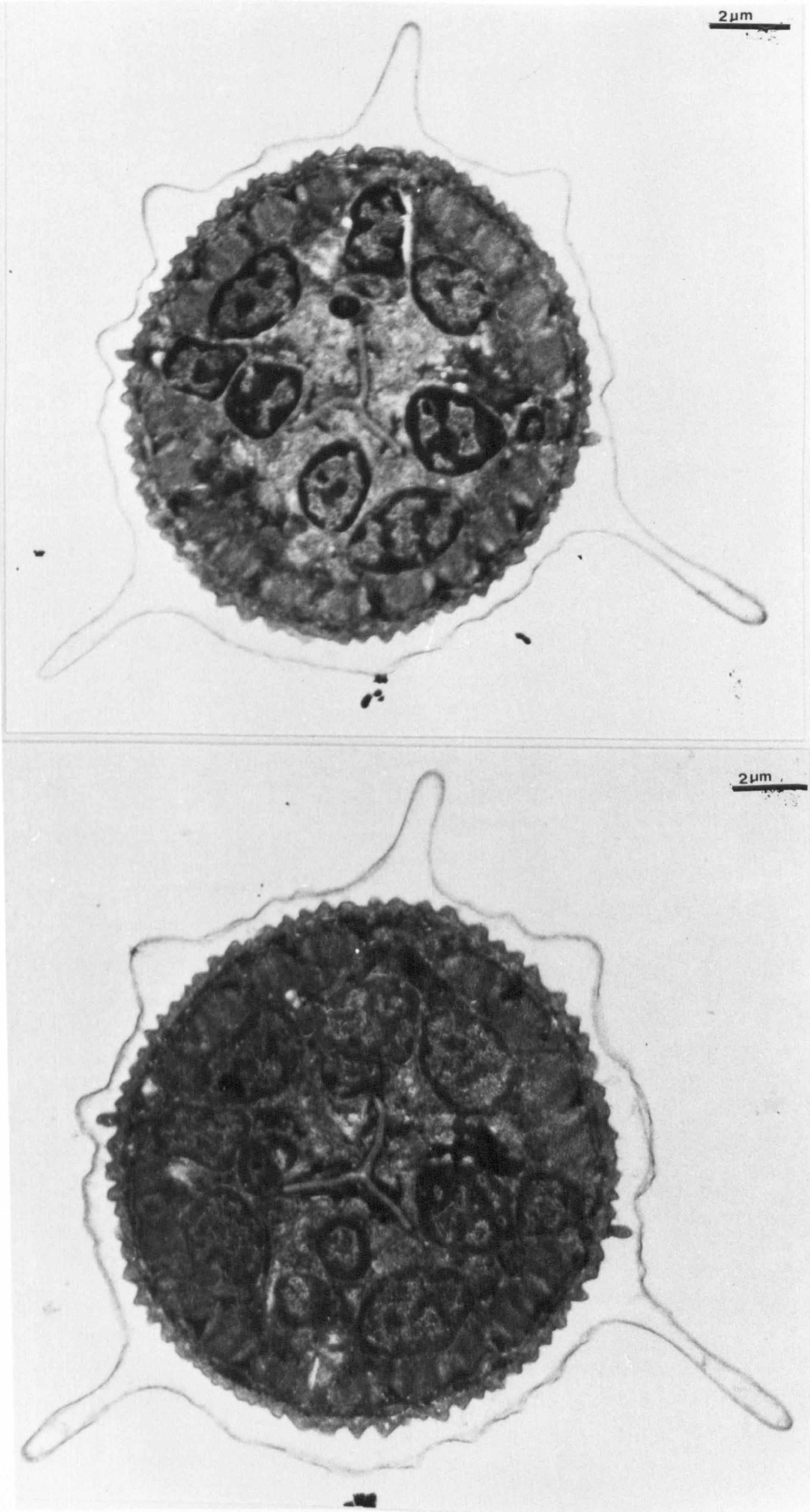
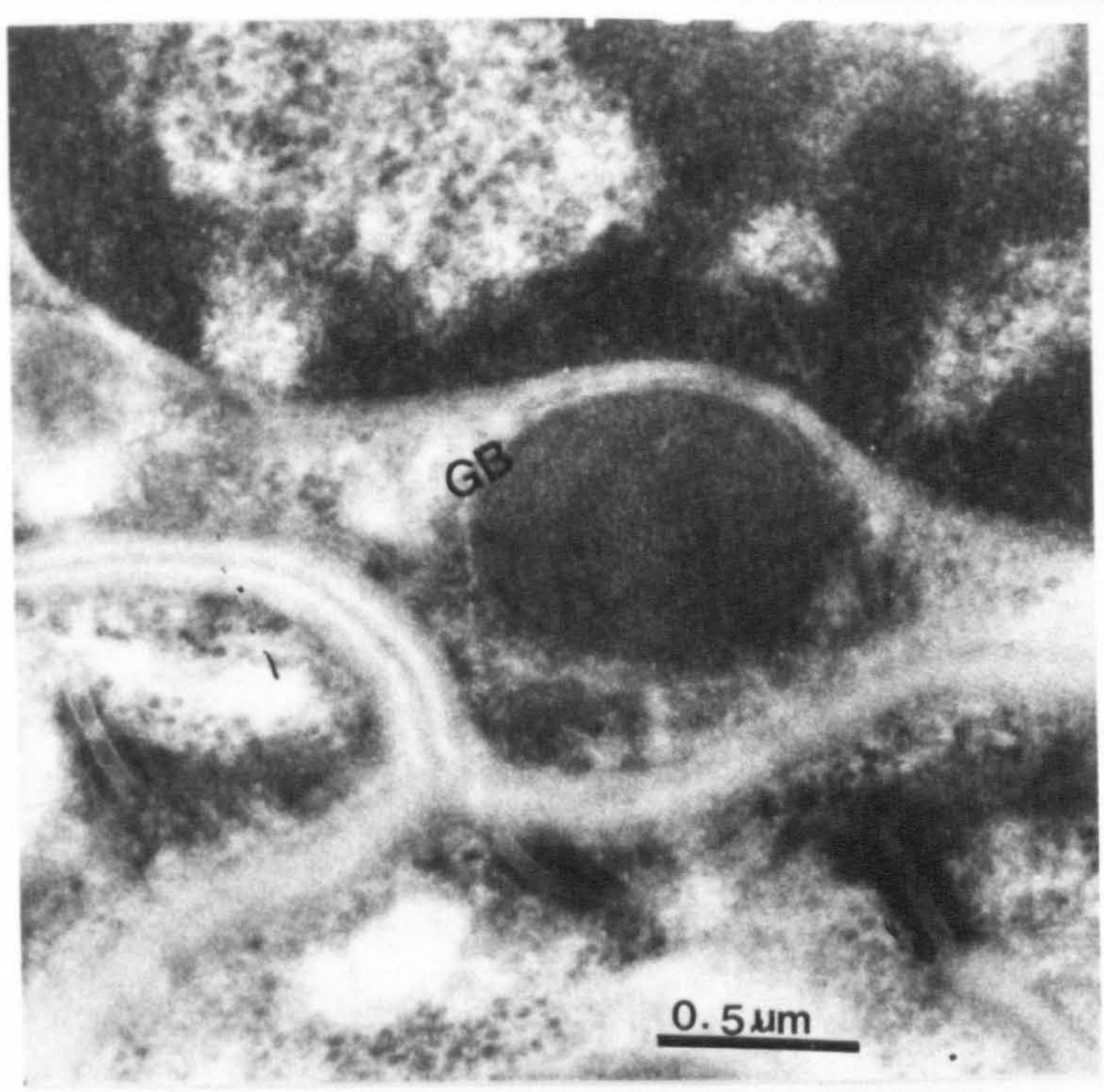

2µm
2µm

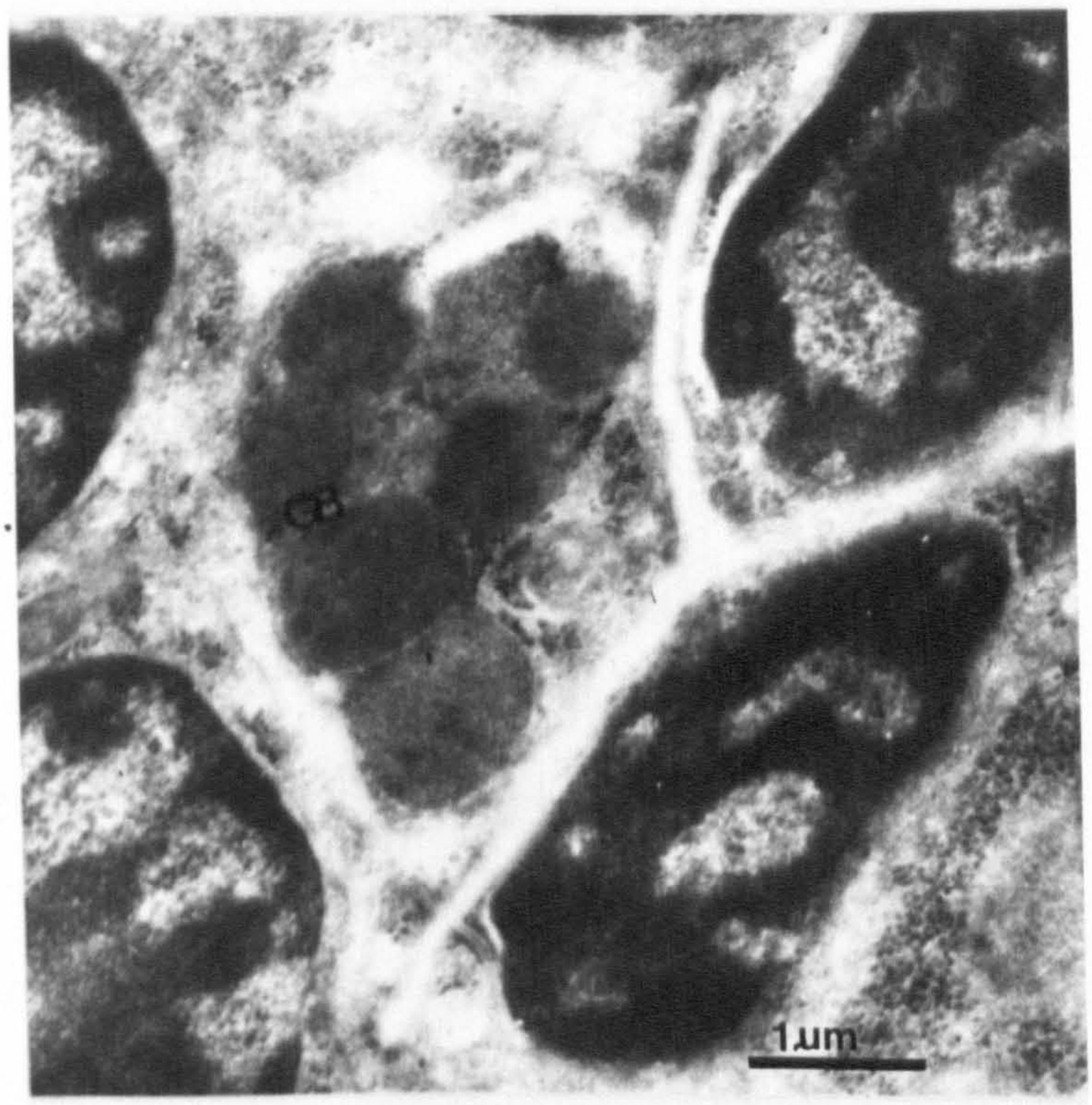
PLATE 13

A. suum infective larva: Electron micrograph of a transverse section through the posterior region of the oesophagus of infective larva (a) physically removed from the egg-shell, (b) hatched physiologically, (c) recovered from the liver of mice infected with 20,000 infective A. suum eggs. Note the absence of the granular body (GB) in (c).

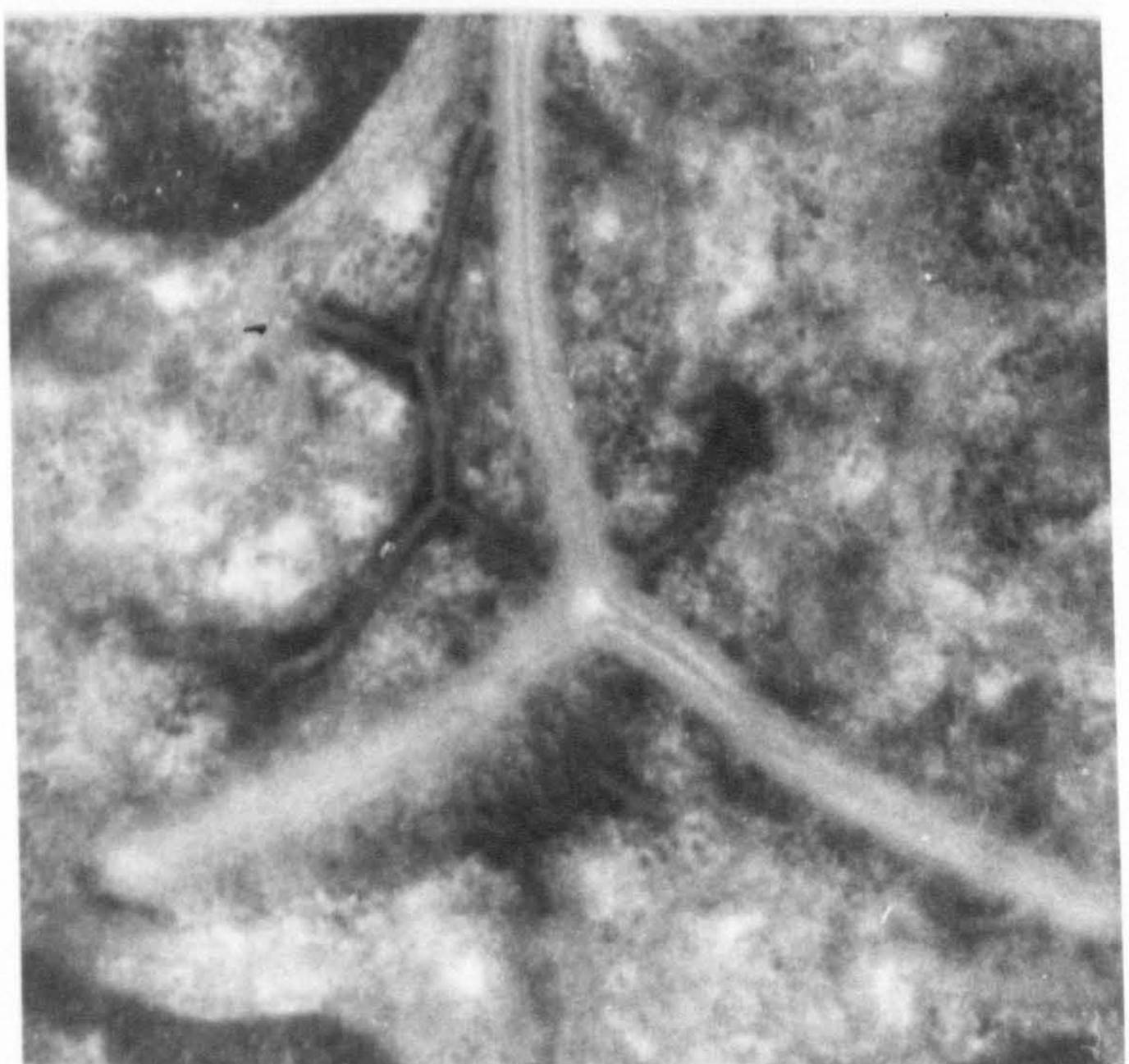
a



b



c



Larvae were first found in the external muscle layers 4 hours after infection. The larvae generally appeared to wander about in the connective tissues of the submucosa before entry into the muscle layer. Invasion of the muscle layer was oblique (Plate 11). In none of the numerous tissue sections examined were larvae found attempting to traverse the muscle layer perpendicularly. Similarly, passage through the muscle layer as indeed the other layers of the intestinal tissue, appeared to be accomplished by larval separation of the host cells and individual fibres (Plate 11).

The number of larvae entering the muscle layers within 5 hours of infection was small compared to the numbers which had reached the liver within the same time (Table VI).

Although larval passage through the intestinal tissue was relatively rapid, some host cellular response occurred (Plate 11). This consisted mainly of mononuclear cells and eosinophils and occurred as early as about 8 hours after infection. None of the mice used in these experiments had any previous record of exposure to A. suum.

Dye release from Azocoll

The results of incubating the various test solutions with Azocoll are plotted in Figure 23. There was some increase in the absorbance of the supernatant from samples containing living larvae or larval secretions over the control samples but the activity was low compared with purified pepsin. There was no difference between any of the negative controls and a single representative curve has been plotted for clarity.

Gelatinolytic activity

When tested against thin gelatin films, the test samples (containing the living larvae or the larval secretions) did not show any gelatinolytic activity as evidenced by the presence of lighter areas on the stained slides (Plate 14). Only in areas where pepsin (10 mg/ml), acting as positive control, and the larval homogenate were placed was there any digestion of the gelatin.

Histolytic Activity

Similarly, neither living larvae nor larval secretions had any apparent effect on frozen sections of host intestinal tissue, whereas pepsin resulted in marked histolysis (Plate 15).

FIGURE 23

Azocoll dye release from living larvae and larval extracts of infective larvae of A. suum over 6 hours at 37°C.

- ● 10 mg/ml pepsin
- ▼ Larval Extract (collected after 4 hours incubation)
- ▽ Living larvae
- ■ Larval extract (collected after 2 hours incubation)
- ○ (boiled) heat killed larvae.

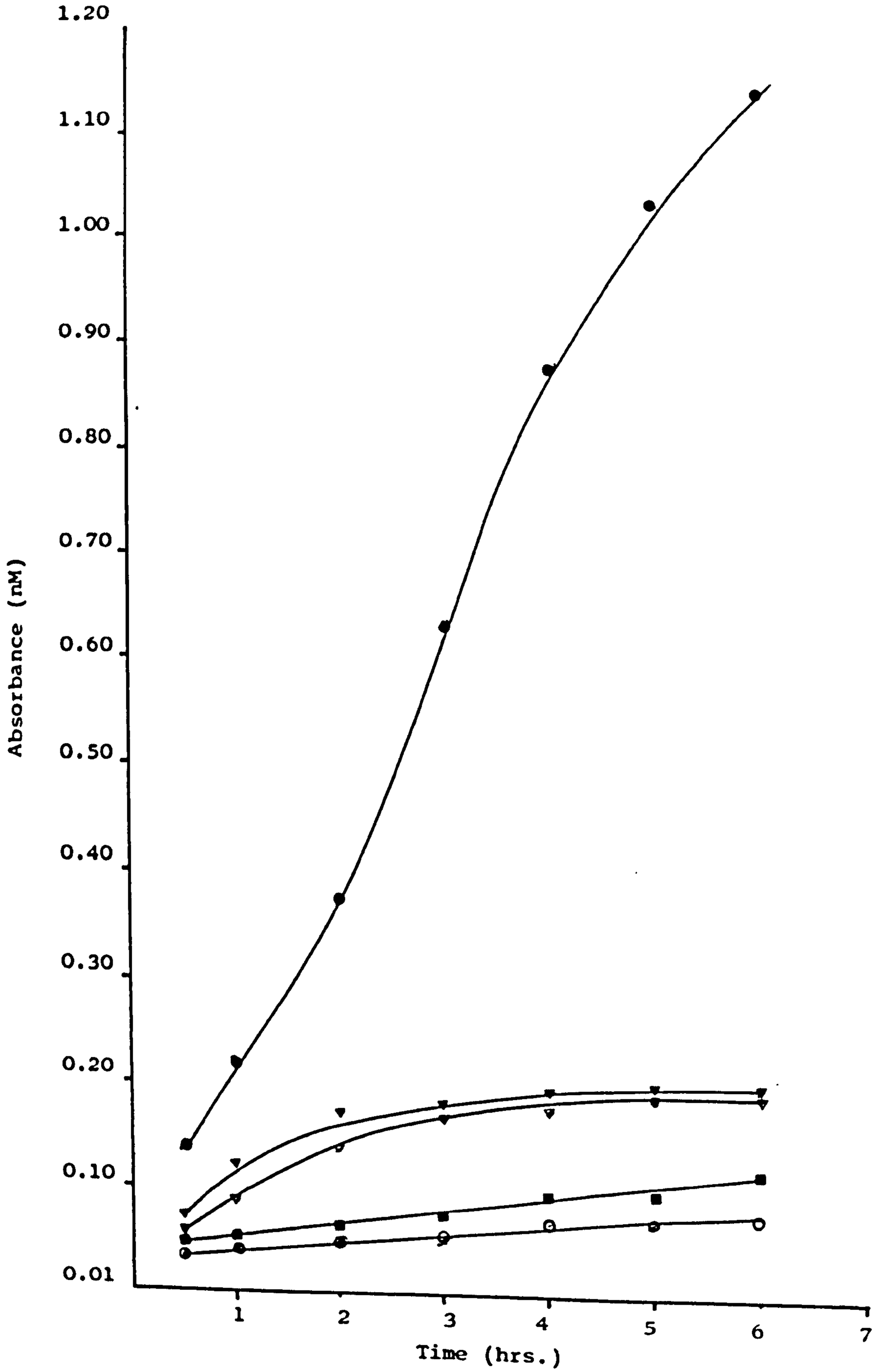

1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.01
Absorbance (nM)
1
2
3
4
5
6
7
Time (hrs.)

PLATE 14

Digestion of thin gelatin film by living larvae and larval extracts of A. suum after 5h of incubation at 37°C; 95% RH.

A_1 Living larvae (without antibiotics)
A_2 Heat killed larvae (without antibiotics)
A_3 10 mg/ml Pepsin (without antibiotics)
A_4 Larval extract (without antibiotics)
A_5 Incubation medium (without antibiotics)
A_6 10 mg/ml Pepsin (without antibiotics)

B_1 Living larvae (with antibiotics)
B_2 Heat killed larvae (with antibiotics)
B_3 10 mg/ml Pepsin (with antibiotics)
B_4 Larval extract (with antibiotics)
B_5 Incubation medium (with antibiotics)
B_6 10 mg/ml Pepsin (with antibiotics)

C_1 Larval homogenate (with antibiotics)
C_2 Incubation medium (with antibiotics)
C_3 10 mg/ml Pepsin (with antibiotics)

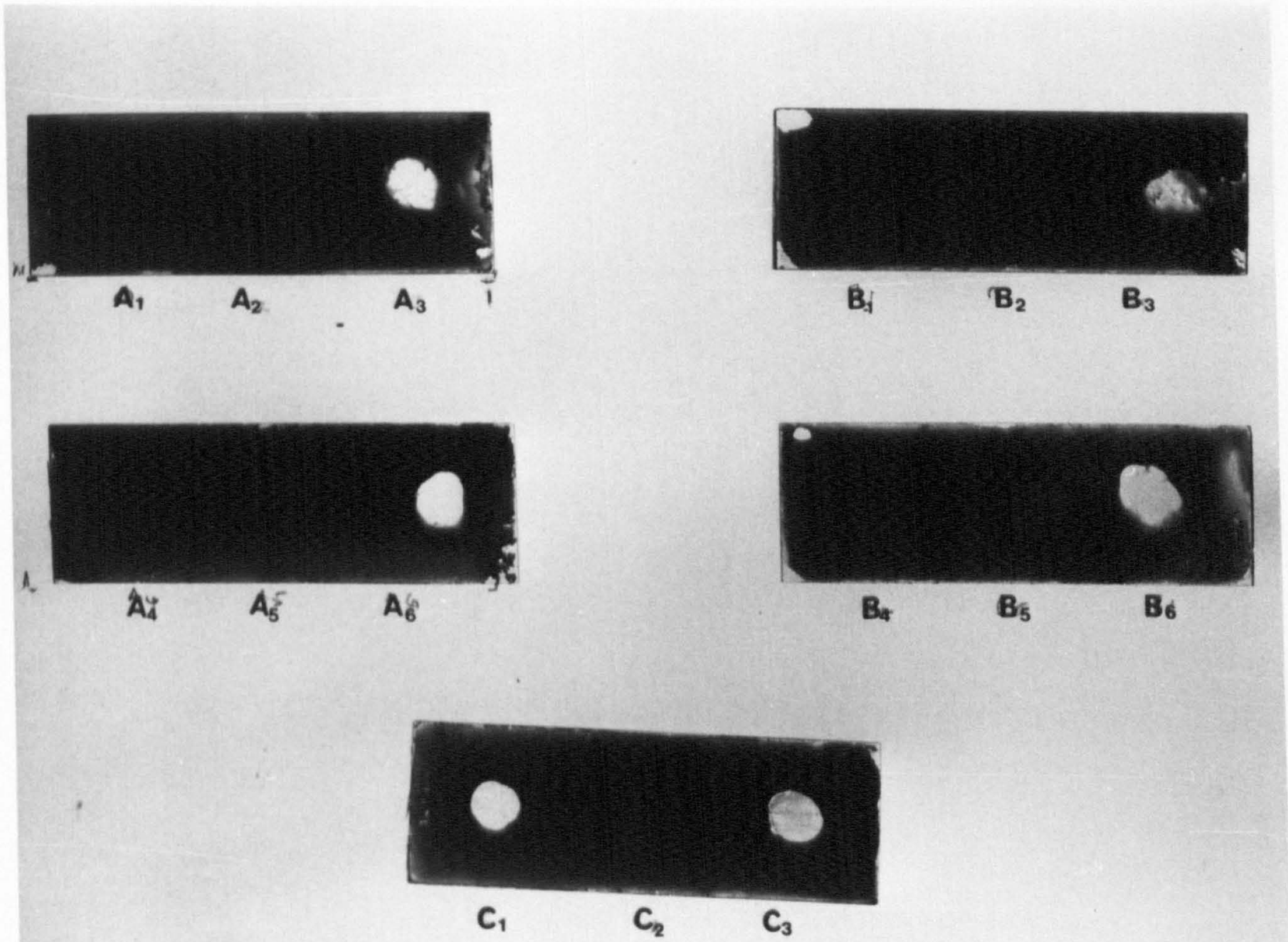

A1
A2
A3
B1
B2
B3
A4
A5
A6
B4
B5
B6
C1
C2
C3

PLATE 15

Photomicrographs of frozen sections of mouse intestine showing the effect of incubation of the tissue with living larvae and larval extracts: stained with PAS, counterstained with Orange G. Mag. X168. The sections were incubated at 37°C for 5 hours.

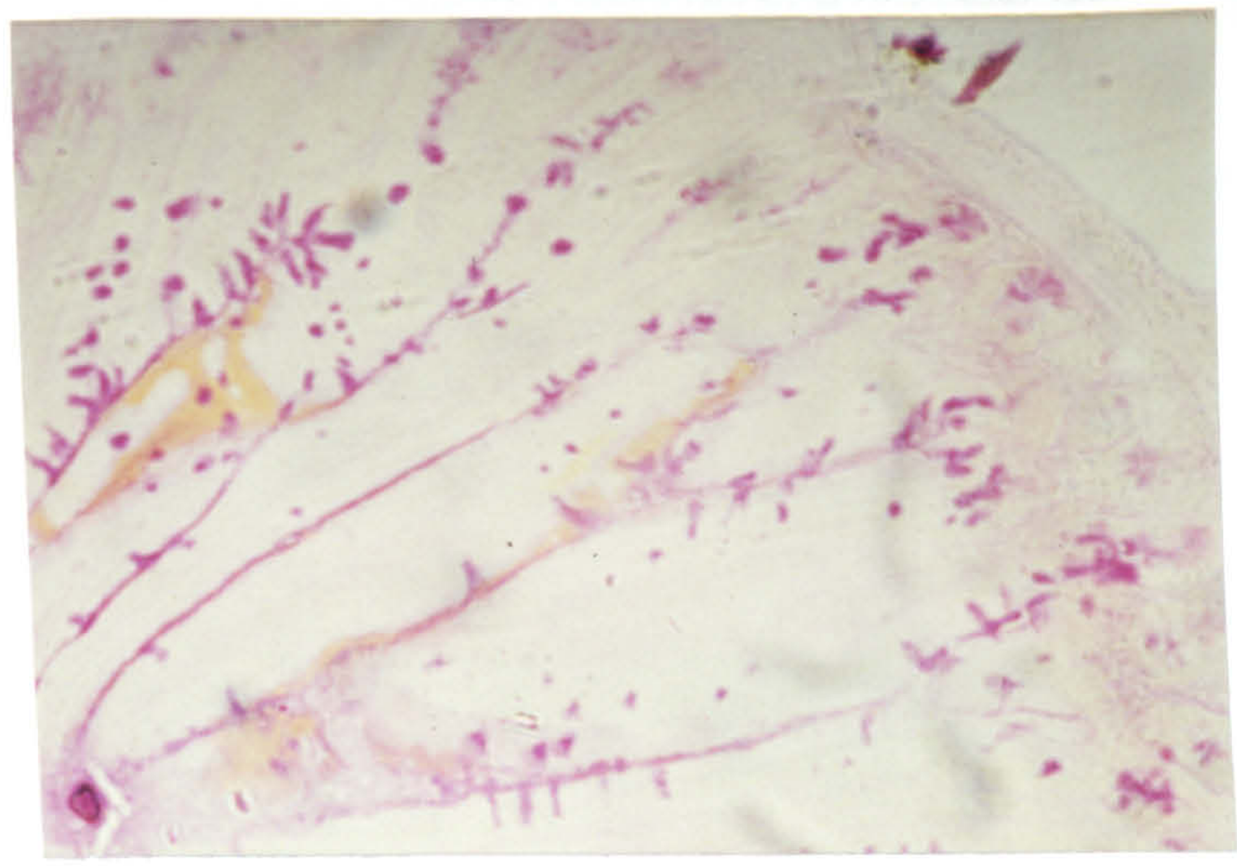
a. Section incubated with living larvae.

b. Section incubated with larval extract.

c. Section incubated with phosphate buffered saline (pH 7.2) - as negative control.

d. Section incubated with 10 mg/ml pepsin - as positive control.

a



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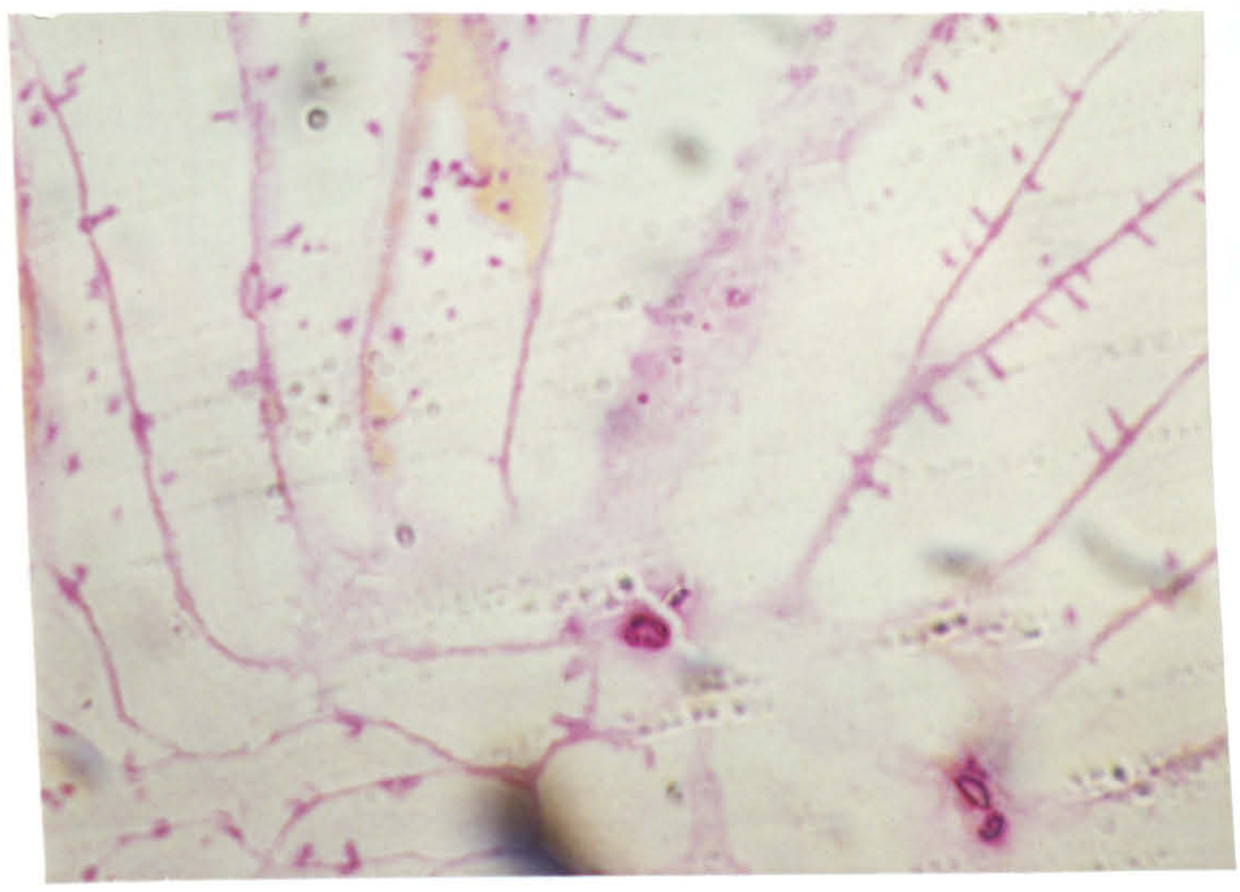

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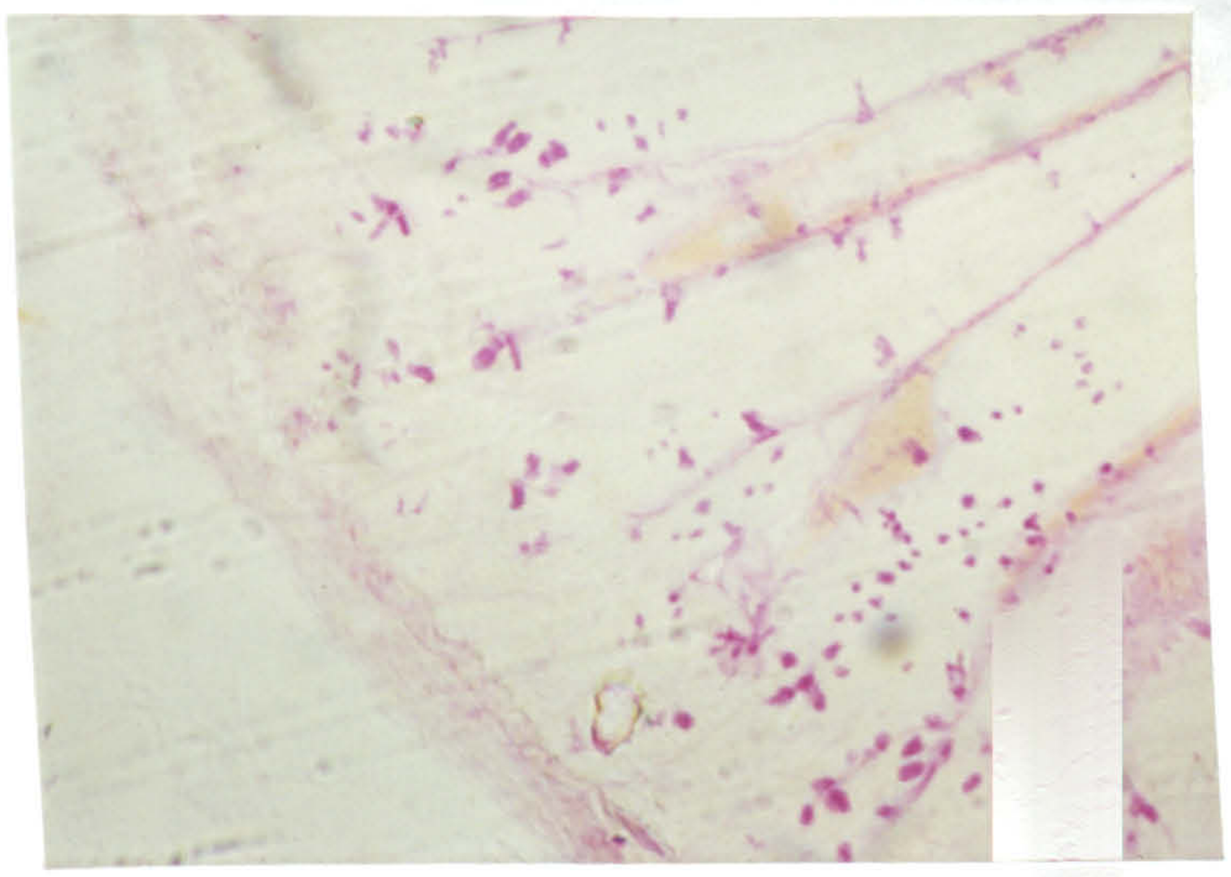
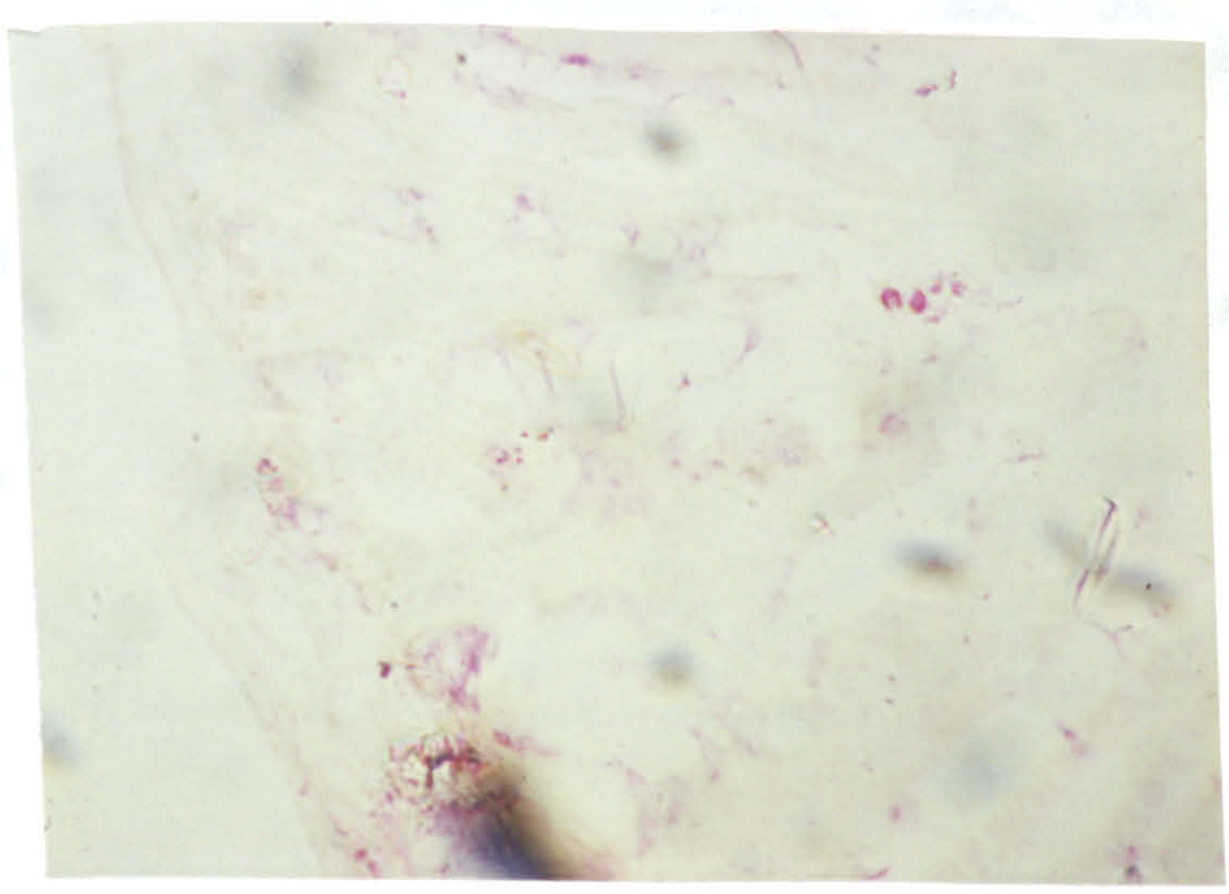


d



DISCUSSION

During their passage through the gut en route to the liver and the lungs, the infective larvae have to traverse the single layered epithelial cell-lining of the gut mucosa and the extracellular barriers presented by the basement membrane and ground substances of the submucosal connective tissue. In a number of tissue migrating parasites which lack obvious mechanical devices for effecting entry into their host tissue, as is the case with A. suum (Alicata, 1935; Douvres, Tromba and Malakatis, 1969), penetration into and subsequent migration through the tissue is attributed largely to the action of the larval secretions (enzymes). Detailed cytological studies of the changes associated with penetration, particularly in the case of S. mansoni and F. hepatica (Vogel, 1932; Pinto and Almeida, 1945; Gordon and Griffiths, 1951; Griffiths, 1953; Lewert and Lee, 1954; Stirewalt, 1959; Stirewalt and Kruidenier, 1961; Stirewalt and Fregeau, 1966; Wilson, Pullin, Denison, 1971), have strongly supported this hypothesis.

Following histological observations, Jenkins (1968) in his studies on early migration of A. suum

in white mice, reported excavations in the host intestinal tissue which he suggested could be attributed to the activity of larval enzyme.

In view of the fact that the standard histological procedures are inadequate in ascertaining the cause and precise nature of host tissue changes following infection, it was decided that the adoption of a histochemical technique which enables changes in the ground substance of the host tissue along the route of larval passage to be easily visualised would be a more fruitful approach in an effort to elucidate the nature of host tissue alteration caused by the migrating larvae. The technique is based on a freeze-dry technique first described by Gersh (1948) and since utilised by a number of workers for various studies including studies on host tissue changes caused by penetration of various helminth larvae by Lewert and Lee (1954).

The results of the present investigations confirm findings on the early migration of infective A. suum larva by previous workers (Ransom and Foster, 1920; Ransom and Cram, 1921; Roberts, 1934; Sprent, 1952; Nichols, 1956a; Olsen and Kelly, 1960). They especially consolidate

Jenkins (1968) observation on the pattern of early distribution of the infective eggs in the host. Jenkins (1968) observed that larvae penetrated the host intestinal tissue mostly via the caecum and colon and migrated to the liver via the blood stream.

Hatch of the infective eggs occurred in the anterior of the small intestine (Figure 19) and larvae invade more posteriorly. While it is possible that the hatched larvae may have penetrated the intestinal tissue at one point, re-emerged and re-entered another part, the occurrence of the vast majority of penetrating larvae in the posterior $^{1}/_{3}$ of the small intestine, the caecum and the colon (Figure 20) seems to suggest that these regions may present a more favourable environment for invasion. How far penetration of the host tissue via these regions is due to active selection by the larvae is not known. It is probable that this observation has more to do with the length of the host gut in relation to the rate of hatching of the infective eggs and passage, of food material than active site selection by the parasite. Jenkins (1968) observed that the same pattern of early distribution

of infective larvae was maintained in rats and rabbits with longer guts than mice.* The other possible explanation for this pattern of larval distribution is, of course, the physical and/or physio-chemical differences between the various gut regions and their contents, which may be affecting the larvae. The effects of such variations on helminth penetration have not been investigated. The infective larvae of A. suum leave their protected environment in the egg-shell under high Co_2 atmosphere (Rogers, 1958; Fairbairn, 1961). Penetration of the intestinal mucosa appears to start soon after hatching (Figure 20). An active cytochrome system has been shown to exist in the infective A. suum larvae through to the third stage. Accordingly, these stages of the life cycle are aerobic (Faure-Fremiet, 1912; Brown, 1928; Jaskoski, 1952; Passey and Fairbairn, 1955). An oxygen requirement may be one of the factors that leads the infective larvae into the host tissue soon after hatching. Cleeland and Lawrence (1962) showed that during the first 30 to 50 days after hatching in vitro the infective larvae require an atmosphere in which oxygen is available. When the larvae were

*Keittivuti (1979) also recently reported similar distribution of hatched larvae in the intestines of mice and pigs following oral inoculation with embryonated A. suum eggs.

cultured in an oxygen free atmosphere, no growth was reported and survival was greatly impaired (Cleeland, 1963).

Larval migration from the intestine to the liver is generally considered to be via the hepatic portal system (Ransom and Tram, 1921; Roberts, 1934; Jenkins, 1968). However, the persistent occurrence of larvae in the lymphatic vessels (Jenkins, 1968, see Appendix) and the muscle layers indicated the possibility of at least some limited migration via these routes. The occurrence in the lungs of larvae significantly smaller in size than those found in the liver is interesting and quite contrary to what one would normally expect if all the larvae had taken the same route of migration. There was general increase in size as larvae progressed along their migratory route. The presence of such small larvae has been reported by previous workers who have recorded measurements of the infective larvae during the early phases of migration. Roberts (1934) recovered two-day old larvae from the liver of pigs and guinea pigs. These ranged in size from 0.27 to 0.31 mm long, while in the lungs the larvae measured 0.27 to 0.365 mm. Kelley

et al (1957) recorded larvae from the liver of two days after infection which averaged 0.33 mm long, while larvae from lungs of pigs averaged only 0.30 mm in length.

The results suggest that there may be two routes by which larvae reach the lungs. The majority of larvae after penetrating the gut wall enter the vessels of the portal system and are then transported to the liver. Some, however, enter the lymphatic system and may reach the heart via the thoracic duct and thence the lungs having by-passed the liver. In other words, the organ first reached by the migrating larvae as they leave the small intestine depends on whether they left the small intestine *via* the hepatic portal or the lymphatic system. The liver would seem to be providing a more favourable growth environment for the migrating larvae judging from the difference in the size of those larvae which migrated to the liver first.

It is not known how far the infective larvae obtain their nutrient from the host tissue. Soon after hatching the intestine of the infective larva is without a lumen consisting only of a solid column of cells and is non-functional (Jenkins and Erasmus, 1971). Development of

the intestinal lumen in the infective larva is considered to start about 3 days after infection and becomes fully developed 8 days post-infection (Jenkins, 1971). However, through a series of *in vitro* culture experiments (Pitts and Ball, 1953, 1955; Pitts, 1960, 1962, 1963; Cleeland and Lawrence, 1962; Cleeland, 1963; Levine and Silverman, 1969), it has been shown that little or no development of the infective larva takes place *in vitro* except in highly nutritious complex media. This suggests that nutrient uptake must be occurring apparently in the absence of a fully functional gut. Transcuticular absorption has been reported in a number of nematodes (Riding, 1970; Chen and Howells, 1979a and b). During hatching the infective larvae of *A. suum* are known to liberate a hatching fluid containing a lipase, a chitinase and a proteinase (Rogers, 1958, 1960; Fairbairn, 1961; Hincks and Ivey, 1977) which it has been suggested may also be acting to assist larval entry into the host tissue. However, neither the site of production of this fluid nor its definite involvement in larval invasion of the host tissue has been established. Site of

production, nature and role of larval secretions in host tissue penetration and subsequent passage have received some attention amongst other helminths (Lutz, 1919; Faust and Maleey, 1924; Faraust, Jones and Hoffman, 1934; Kuhlow, 1953; Silverman and Maneely, 1955; Welch, 1959; Erasmus, 1959; Stirewalt and Kruidenier, 1961; Vik, 1964; Bird, 1967; Lee, 1969; Colley, 1970). Hexacanth larvae of a number of cyclophyllidian tapeworm possess three pairs of hooks and a pair of glands which open between the laterial pairs of hooks (Reid, 1946, 1948; Smyth, 1966). In Raillietina cesticillus it has been shown that these glands contain PAS-positive materials prior to penetration. When onchospheres were recovered from the haemoceal after penetration, Sawada (1961) was unable to demonstrate further staining. Lethbridge (1971) in a study of hatching and penetration of Hymenolepis diminuta in Tenebrio molitor found that both size of the penetration glands and the non-glycogen, PAS-positive material of their contents decreased as penetration proceeded, with the paths of larval passage marked by local destruction of the epithelial cells. Amongst nematodes, secretions of the oesophageal glands have been implicated as sources of larval "invasive" enzymes. Welch (1959)

working on Howardula acronymphium, a nematode parasite of larval drosophilid flies, noted the collapsed dorsal oesophageal gland of the adult female which was much distended prior to penetration. Smith (1976) following his studies on ultrastructure of the oesophageal region of larval hookworms has shown that both the third stage larvae of Necator americanus and A. tubaeforme possess three oesophageal glands which, however, is rather less well developed in the case of A. tubaeforme - one dorsal and two subventral. These glands (particularly the subventral gland) became considerably reduced in size after penetration of rabbit skin by the infective larvae of N. americanus. Similar reduction in the size of these glands following penetration through the tissue was not reported in the case of A. tubaeforme whose entry into host tissue is purely mechanical (Matthews, 1975). The present study showed that infective larva of A. suum lack similar extensive oesophageal glands. However a few granular structures, which have been described as secretory (Jenkins, 1971), were present in the "pre-penetrated" infective larvae. These materials were lost in "penetrated"

larvae. This suggests that they may have been utilised during larval passage. However, the precise role(s) of these larval products in the invasion process remains uncertain.

The result of the histochemical investigations demonstrated that movement of the infective larvae through the host tissue imposes alteration to the basement membrane and ground substance of the host tissue. Jenkins (1968) in his studies on the early migration of larvae of A. suum in white mice observed that the penetrating larvae produced a demonstrable spreading factor reaction with Evans' blue in the mucosa of the gut. Based on this observation, he suggested that the ability of the larvae to burrow through the host tissue may be due in part to the secretion of lytic enzymes. The present finding suggest that this is only partly right. Entry into the host tissue by the infective larva is aided by the activity of larval secretion. However, there was no evidence that these secretions played a histolytic role in the invasion process. Jenkins (1968) reported excavations in the lumina propria of the small intestine which he suggested could be attributed to the activity of the migrating larvae. Except for a transient change in the

host tissue along the path of larval passage, as indicated by the change in the staining affinity no area of permanent excavations or digestion of the host tissue was observed.

Similar tissue reactions following the passage of a number of parasitic helminths have been reported (Lewert and Lee, 1954, 1956). In the case of S. mansoni, Lewert and Lee (1954) showed that penetration of mammalian skin by the cercariae produces profound alteration of the extracellular glycoprotein containing materials. They observed that the basement membrane swelled in advance of the penetrating cercariae changed staining characteristics and finally disappeared at the site of penetration, water-soluble glycoproteins appeared in the ground substance of the tissue. They attributed these reactions to the effect of larval enzymes.

The present study failed to show any extensive digestion of the host tissue. It is therefore probable as suggested by Lewert and Lee (1954) in the case of S. mansoni, S. douthilli, S. simiae, S. ratti and A. caninum that the activity of the larval secretion is directed against the ground substances of the host tissue

causing them to loosen or "soften". Thus altering the resistance of the acellular material to larval passage without the host tissue being necessarily digested. It is quite possible that the larval secretion may be acting by cleaving the bonds of the glycoproteins (the polysaccharide-containing proteins) of the ground substance of the host tissue thereby loosening the cells and fibres and making it easier for the migrating larvae to "unzip" or pull them apart.

The sloughing of the softened larval pathway as a result of the histological method employed in his study may perhaps account for the apparent excavations reported by Jenkins (1968). The freeze-dry technique of tissue preparation used in the present study is really indispensible in this type of work. This method of histochemical investigation was developed by investigators studying the state of, and changes in, the extracellular elements of connective tissue with particular emphasis on the basement membrane and ground substance. The Hotchkiss technique demonstrates polysaccharide-containing proteins. The intensity of staining is believed to be related to the number of reactive groups

or to the amount of material present and also to the degree of association or dissociation of the material. Up to a point, more intense staining indicates more polysaccharide and thus, the presence of more glycoprotein. However, it has been shown that highly polymerized glycoprotein may give less intense staining reactions. In the latter case, the reaction of the highly polymerized glycoprotein may be increased following the exposure to enzymes which presumably depolymerize it and allow more reactive groups to be exposed, thus giving a highly coloured product with the reagent. Lewert and Lee (1954) suggested that enzymes may cause even greater alteration of the glycoprotein material. This they showed may result in the production of soluble products with loss of reactive groups and decrease in the intensity of the staining when the material is removed.

Direct penetration into the submucosa *via* the Crypts of lieberkuhm appears to be the common route of larval entry. Although it is quite possible that some larval penetration may occur *via* the villi, evidence of larval penetration was never observed. Contrary to the present observation, entry *via* the villi has been reported in a number of gut penetrating helminths

(De Wᵭele, 1933; Silverman and Maneely, 1955; Bilqees, 1968; Banerjee and Singh, 1969; Baᵭker, 1970).

Passage between the epithelial cells at the base of the Crypts and between the paneth cells appears to demand physical effort on the part of the larvae as exemplified by the contraction and contortion of the body during passage (Plate 5). In S. mansoni physical activity has been associated with squeezing out of larval secretions which assist the breakdown of the acellular materials of the host tissue during passage (Gordon and Griffiths, 1951). The physical effort required for the initial displacement of the epithelial cells of the mucosa may be the influence behind the tendency for penetrating larvae to use a common entry point. Such opportunistic behaviour has been reported amongst some other tissue penetrating helminths (Stirewalt, 1966). The situation may have been accentuated by the large number of the infective eggs used. Aggregation behaviour has not been widely reported amongst parasitic nematodes.

Passage of larvae through the host intestinal

tissue was fairly constant up to 10 hours post-infection. However, the number of larvae entering the muscle layer was low compared to the number which had reached the liver within 5 hours (Table VI). This suggests that passage across the muscle layer may not represent a major route of early larval migration to the liver and lungs. Although larval passage through the host intestinal tissue is rapid, the prompt induction of host cellular response in mice without previous records of infection with A. suum is puzzling. Whether the response is directed against the migrating larvae or as a protective measure against subsequent infection or both is not certain. Perhaps the quick host response may have something to do with antigenic nature of the larval secretion.

In the tissue sections, the surface of some of the larvae as well as the host tissue in their migratory pathways stained brilliantly indicating possible coating by larval secretions. Kruidenier (1953,1954) suggested that apart from acting to lessen the resistance of the host tissue to larval passage, such secretions produced by migrating helminths may also play the role of both a lubricant and anti-enzyme.

There have been few studies on secretions released by the infective larvae of animal parasitic nematodes (Lewert and Lee, 1954, 1956; Devi, Murthy and Reddy, 1969; Matthews, 1975). Devi, Murthy and Reddy (1969) observed that larvae of Dracunculus medinensis release secretions that are active against a number of protein substrates. Matthews (1975) also showed that N. americanus and S. fulleborni infective larvae released an azocoll positive secretion when incubated at 37°C. Similar azocoll-positive results have been demonstrated in the present study in the case of A. suum, confirming an earlier observation by Hincks and Ivey (1977). These workers found that the infective larvae of A. suum after hatching continued to produce secretions capable of releasing dye from azocoll. However, the inability of these larval secretions to digest either the thin gelatin film or frozen sections of host tissue suggest that the secretions are neither gelatinolytic nor histolytic. It would therefore seem that these secretions are not likely to have any pronounced histolytic role in the host tissue penetration process. This observation confirms the results of the histochemical observations which showed the apparent inability of the infective larvae to digest the host tissue along their route.

PART THREE

SOME OBSERVATIONS (AT THE S.E.M. LEVEL) ON THE EXTERNAL MORPHOLOGY OF EARLY STAGES OF INFECTIVE LARVAE OF A. SUUM

INTRODUCTION

Parasite invasion has been studied in relatively few species (Matthews, 1977). In many of the examples that have been investigated, especially among the cestodes and trematodes and also some filarial nematodes, mechanical disruption of the tissue is achieved by the use of sclerotised cuticular structures which may take various forms (Ishii and Miyazaki, 1968; Robson and Erasmus, 1970; Loverde, 1975; Bennett, 1975; Kóie and Frandsen, 1976; Sakamoto and Ishii, 1976). Although a case has been made for at least one species of nematode, Ancylostoma tubaeforme to invade purely mechanically without the presence of such structures (Matthews, 1972, 1975) this does not appear to be the general rule. The presence in Ascaris suum larvae of a "boring tooth" in the form of a small sclerotised knob-like structure has been mentioned by Chandler, Alicata and Chitwood (1974). Rather surprisingly such a structure has not been recorded elsewhere despite the fact that there have been a number of life cycle studies which have included

detailed morphological descriptions of the larval stages (Alicata, 1935; Roberts, 1934; Douvres, Tromba and Malakatis, 1969; Douvres and Tromba, 1971). All these have been at the light microscope level and no scanning electron microscope study of Ascaris larvae has been published although the adult worms have been subjected to examination at this level on a number of occasions (Madden, Tromba and Vetterling, 1970; Weise, 1973; Ansel and Thilbaut,1973).

There is little doubt that larval activity is the most important factor in the actual process of entry and movement within the tissues, although the present study has suggested that larval secretions may be released during invasion and that these may ease the passage of the larvae through the tissues.

At a time when increasing use is being made of the scanning electron microscope in elucidation of the morphology of various parasites, re-examination of the morphology of infective larvae using SEM was considered worthwhile. The objective was to ascertain if the larvae possessed any morphological structures that might be associated with larval invasion.

PREPARATION OF LARVAE FOR S.E.M.

Larvae hatched *in vitro* as described earlier, as well as those recovered from the intestine, liver and lungs of mice dosed with large quantities of infective eggs 4 hours, 24 hours, 48 hours and 96 hours previously, were isolated from egg-shells and debris using a Baermann funnel. The isolated larvae were then rinsed in phosphate buffered saline (PBS) pH 7.2 and fixed in 3% gluteraldehyde in 0.1m cqcodylate buffer and treated for 1 hour in cqcodylate buffered 1% osmium tetroxide (pH 7.2). The larvae were then dehydrated through a graded series of alcohols into acetone and finally placed in a miniature stainless wire basket and transferred to a critical point drier. Care was taken to ensure that the larvae were kept moist during the transfer.

Critical point drying was accomplished with liquid Co_2. The dried specimens were then mounted on standard SEM specimen stubs, coated with a layer of Gold-palladium (150-200Å) and examined using an SEM 151 M-7 scanning microscope operating at 30 kv.

OBSERVATIONS AND DISCUSSION

Plate 16a illustrates the general appearance of critical point dried infective second stage larvae of A. suum. The larvae measure 250 ± 6.80 µm long by 13.00 ± 1.64 µm, at the broadest region of the body - just anterior to the pharyngo-intestinal junction.

The body wall is marked by complete transverse striations. There are two lateral longitudinal alae, which extend to within about 8 µm of each end. As the larvae progress from second to third stage, the cuticular markings become broader and the lateral alae, which initially appear fin-like become shorter and increase in thickness. It is interesting to note that following his light microscope study of the morphology of the infective second stage larva of A. lumbricoides, Nichols (1968) reported that the body of the larva is without annulation. The annulated nature of the body wall (Plate 16b) is perhaps in keeping with the great body flexibility displayed by the larvae during movement.

Dourves, Tromba and Malakatis (1969) in their light microscope study of the morphogenesis and migration of A. suum larvae developing to the fourth stage in swine, rabbits and mice,

referred to the larvae in all phases of development as having a head comprised of three cuticular lips, one dorsal and two subventral. This description of the infective larvae immediately conjures up the picture of a miniature adult Ascaris (Madden, et al, 1970; Weise, 1973). There was no evidence that larvae in the early stages of development (up to 6 days after infection in mice) possessed 3 lips (Plates 16c, d, 18b, 19b). In larvae hatched in vitro as well as those obtained from infected mice 3 to 10 hours and 1 to 2 days after infection, the heads were generally observed to be smoothly tapered, ending in a circular cuticular lip (Plate 16c). The lip was generally not constricted from the rest of the body. In a few specimens as also noted by Dourves et al (1969) the body appeared to "shoulder" externolateral to the lip (Plate 16d). On a few such specimens, cuticular knobs which varied in prominance were occasionally encountered just beneath the protruding lip. Such specimens were not typical of the larvae observed. In the vast majority of larvae hatched in vitro and recovered from the livers of infected mice, the heads were

PLATE 16

Scanning electron micrograph of infective 2nd stage larvae of A. suum. The larvae were hatched in vitro and fixed less than one hour after hatching, critical point dried and prepared for scanning.

(a) Shows the general appearance of the larvae.

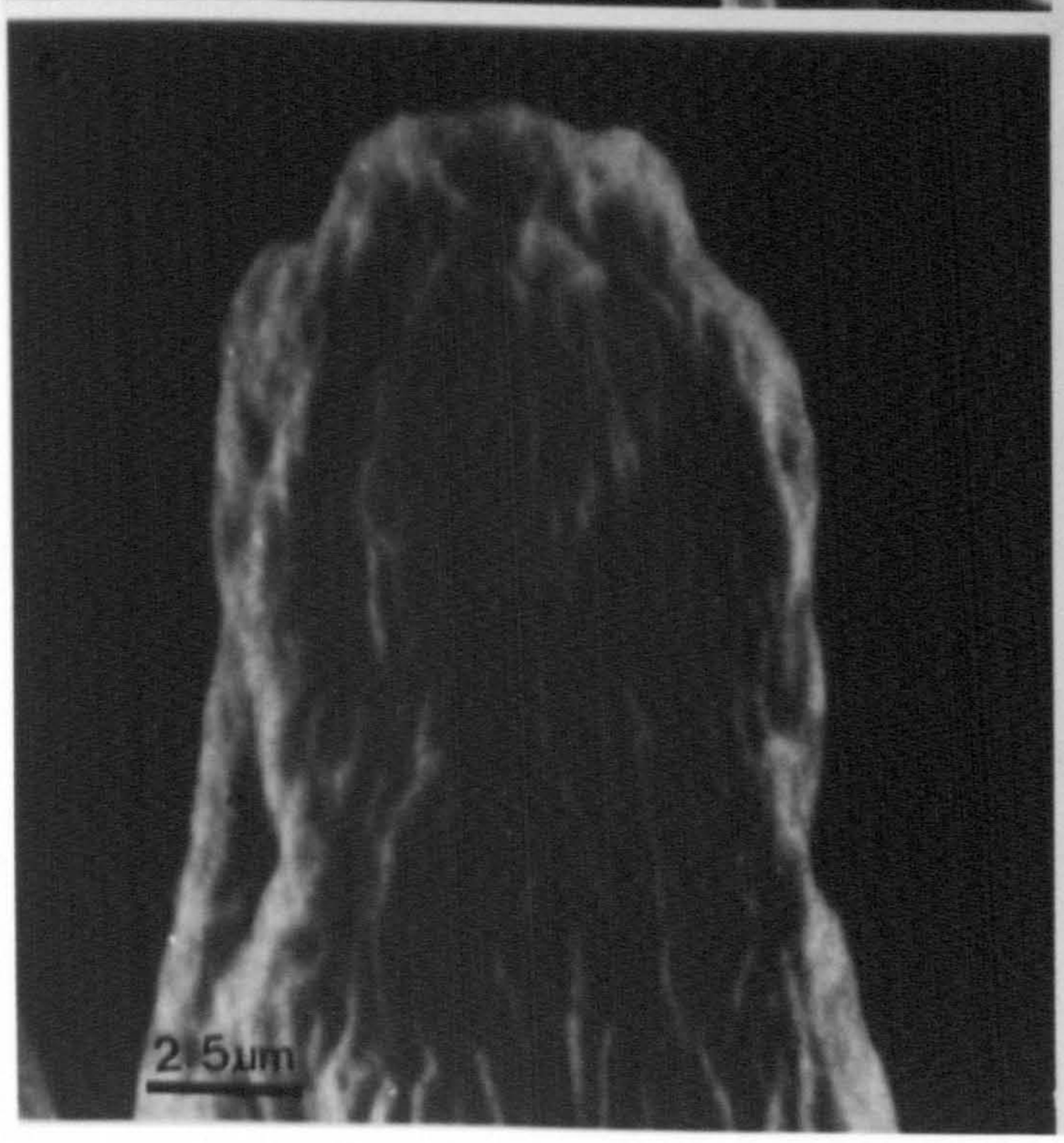
(b) Details of the body wall: note the transverse striation of the body wall.

(c) Anterior end of the infective larva: note the smooth tapering and blunt termination of the head into a circular cuticular lip.

(d) Anterior end of one of few specimens in which the lip appeared to protrude from the rest of the body.


a
10 µm


5 µm


2·5µm


5µm

generally blunt, and the protrusion of the lip less marked. Thus, the predominance of the infective larvae with such blunt heads in the liver soon after infection suggests that the cuticular knobs observed on those few specimens may be of little significance in larval penetration and are probably artifactual. Abnormalities in organogenesis of nematodes developing in abnormal hosts or in vitro are well known (Clunies Ross,and Kauzal, 1932; Weinstein, 1966).

Following their comparative study of the development of A. suum in rabbits, guinea pig, mice and swine up to 11 days after infection, Dourves and Trombo (1971) suggested that A. suum larvae recovered from these animals can be readily classified as follows - late second stage L2; early (E3), Middle (M3) and late third (L3); Third moult (3M) and early fourth stage (E4). However, they reported seeing no larvae in the second moult (2M) in any of the animals.

The second stage of A. suum is generally regarded as the infective larval stage (Stewart, 1917, 1918a and b, 1919a, 1921; Ransom and Foster, 1917, 1920; Ransom and Cram, 1921; Alicata, 1936; Olsen and Kelley, 1960). However,

a number of workers have suggested that the larvae may undergo two moults inside the egg (Henner, 1959; Thust, 1966; Araujo, 1972 and Maung, 1978). In which case, the infective larva is the 3rd larval stage. Maung (1978) reported that the first moult starts about 15 days after incubation of the eggs at 28°C and that by the 16th day of incubation, the sheath is completely lost. So that when pressure is applied on the egg, a naked second stage larva is released. He also reported that although most of the larvae lost their first-stage sheaths, a few do retain them so that at about the 19th day after incubation, when the second moult begins, the larvae possess two sheaths instead of one.

Larvae were pressed out of eggs at various stages of development and examined. In none of these larvae was more than one sheath observed (Plate 17). Most of the infective larvae obtained in this manner were ensheathed in the cast skin of the first stage larvae. When hatched <u>in vitro</u> many of the hatched larvae were naked. So presumably the sheath was lost during hatching.

PLATE 17

Photomicrograph showing the anterior end of 2nd stage larva of A. suum "pressed" out of the egg, 20 days after embryonation. Note that the larva is still enclosed within the sheath of the first-stage larva.

2.5µm

Larval migration to the liver of infected mice is very rapid. As reported in the previous Section, a large number of the larvae reach the liver within 3 to 4 hours of infection. In pigs it may be delayed until about 6 hours after infection (Dourves and Tromba, 1971). On reaching the liver, rapid development of the larvae occurs. By the second day after infection, some of the larvae begin to moult to give rise to the 3rd larval stage (Dourves et al, 1969) and by the 4th day after infection, all the larvae in the liver have become either early 3rd or middle 3rd stage larva (Dourves and Tromba, 1971).

The morphology of the larvae at these stages of development is quite distinct and remarkably different from that of second stage larvae. Plates 18a, b, c and 19a, b, c show features of the early and middle third larval stages. Apart from the suggestion that the larvae at all stages of development possessed 3 lips, the distinct features of these larval stages at the light microscope level were more or less accurately described by Dourves et al (1969). Both larval stages can further be separated at the ultrastructural level on the morphology of their anterior ends. The cuticular circular lip surrounding the oral opening in the early third

larval stage is generally thicker (Plate 18b) than that of the middle third larval stage (Plate 19b). Although distinction between the two larval stages based on size can be misleading, due to much overlapping of the body measurements. By the 3rd day after infection the early 3rd stage larvae measure 309 ± 4.5 /um long by 17.50 ± 1.36 um, and the middle 3rd stage larvae 341.36 ± 8.60 /um long by 18.00 ± 2.45 /um. By the 4th day after infection, the early 3rd larval stage measures 498.50 /um ± 6.10 /um long by 25.10 /um ± 1.26, and the middle 3rd stage 576.00 /um ± 6.12 /um long by 26.43 ± 3.48 /um. This growth trend appeared abnormal as one would have expected a normal transformation from the early 3rd to middle 3rd stage larvae with increase in time post-infection. Perhaps this anomaly may not be unconnected with mice being a "foreign" host.

Although Roberts (1934) described larvae at all stages of development as having amphids, these do not become distinct until the middle 3rd larval stage is reached. In both the early and middle third larval stages, the triradiate muscular pharynx is easily seen (Plate 18c) and

PLATE 18

Scanning electron micrograph of early 3rd stage larvae of A. suum. The specimens were collected from the liver of infected mice 3 days after infection; critical point dried and prepared for scanning.

(a) Gross external appearance of the larva.

(b) Anterior end: note the much thickened cuticular lip.

(c) Detail of the oral opening of the early third larval stage. Note the triradiate muscular wall of the oesophagus.

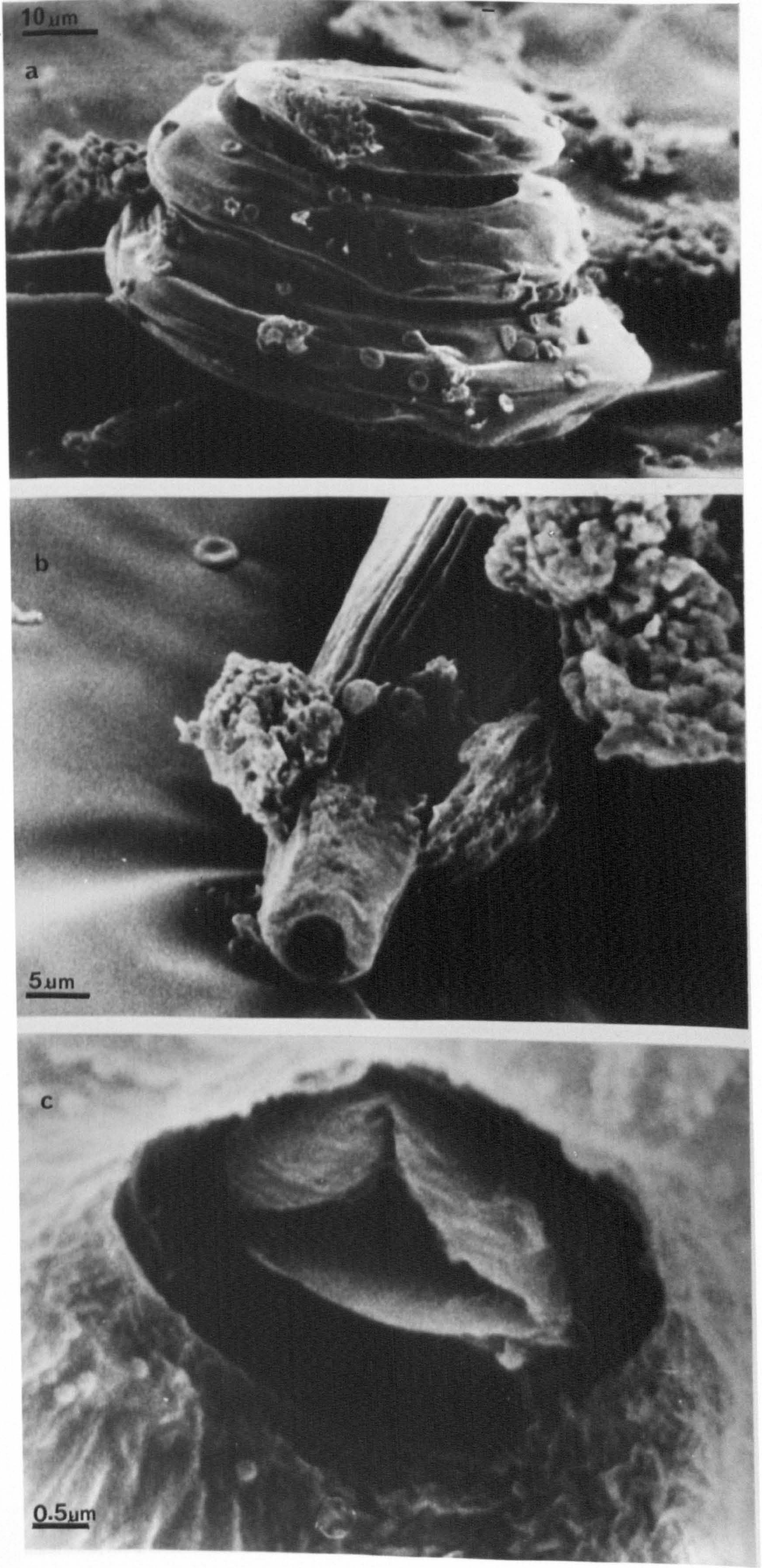

10 µm
a
b
5µm
c
0.5µm

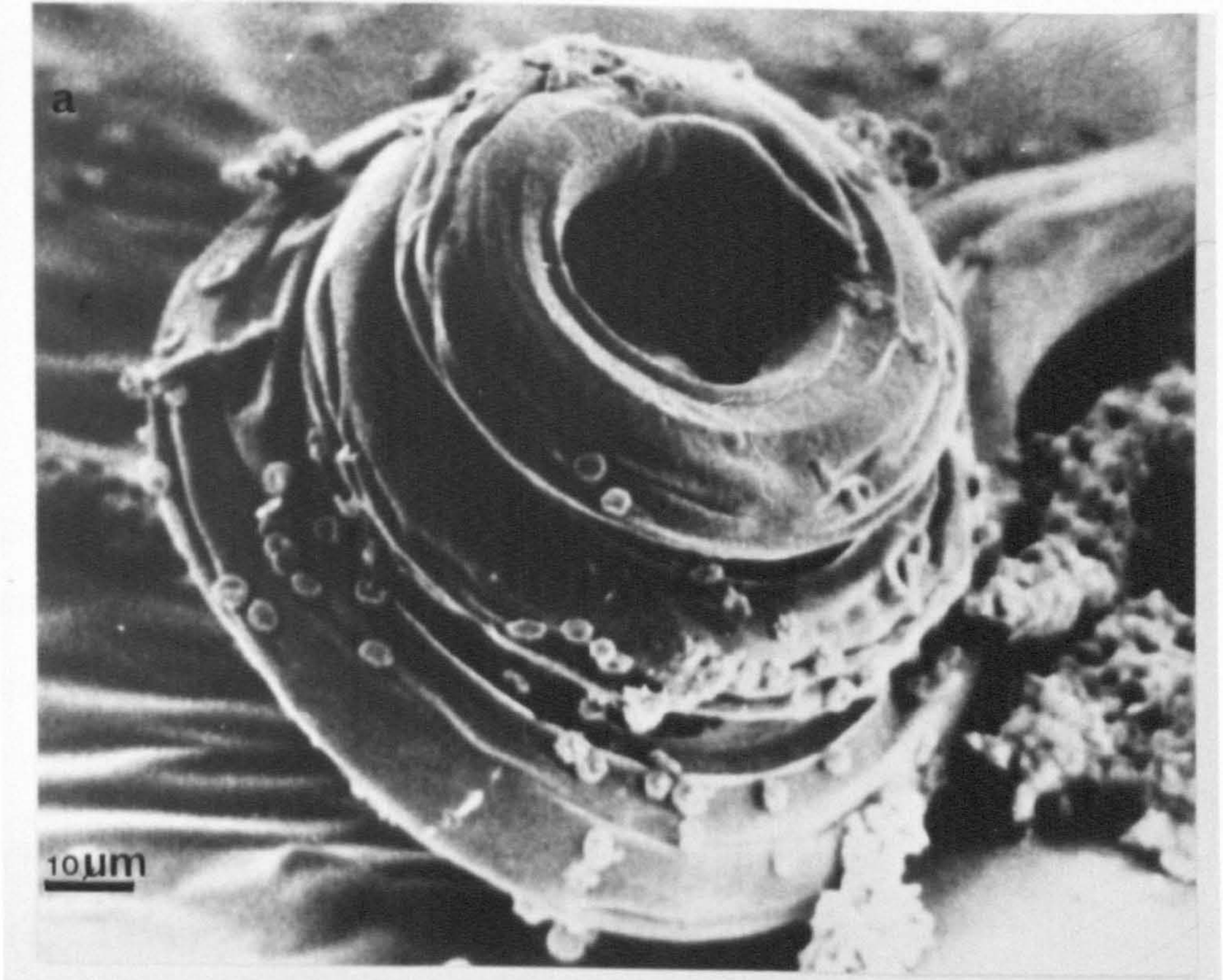
PLATE 19

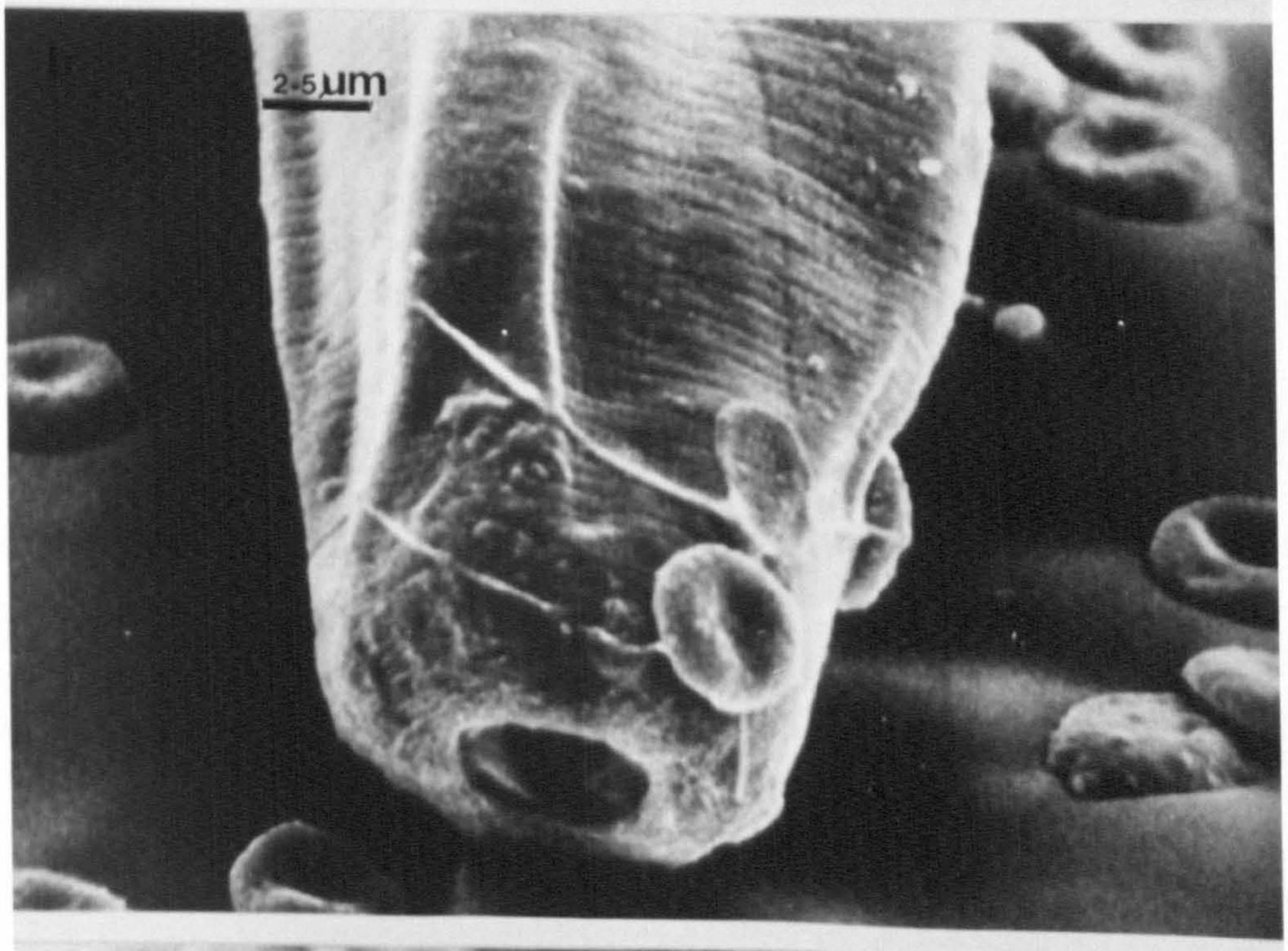
Scanning electron microscope of middle 3rd stage larvae of A. suum. The specimen were recovered from the liver of infected mice 3 days after infection, critical point dried and prepared for scanning.

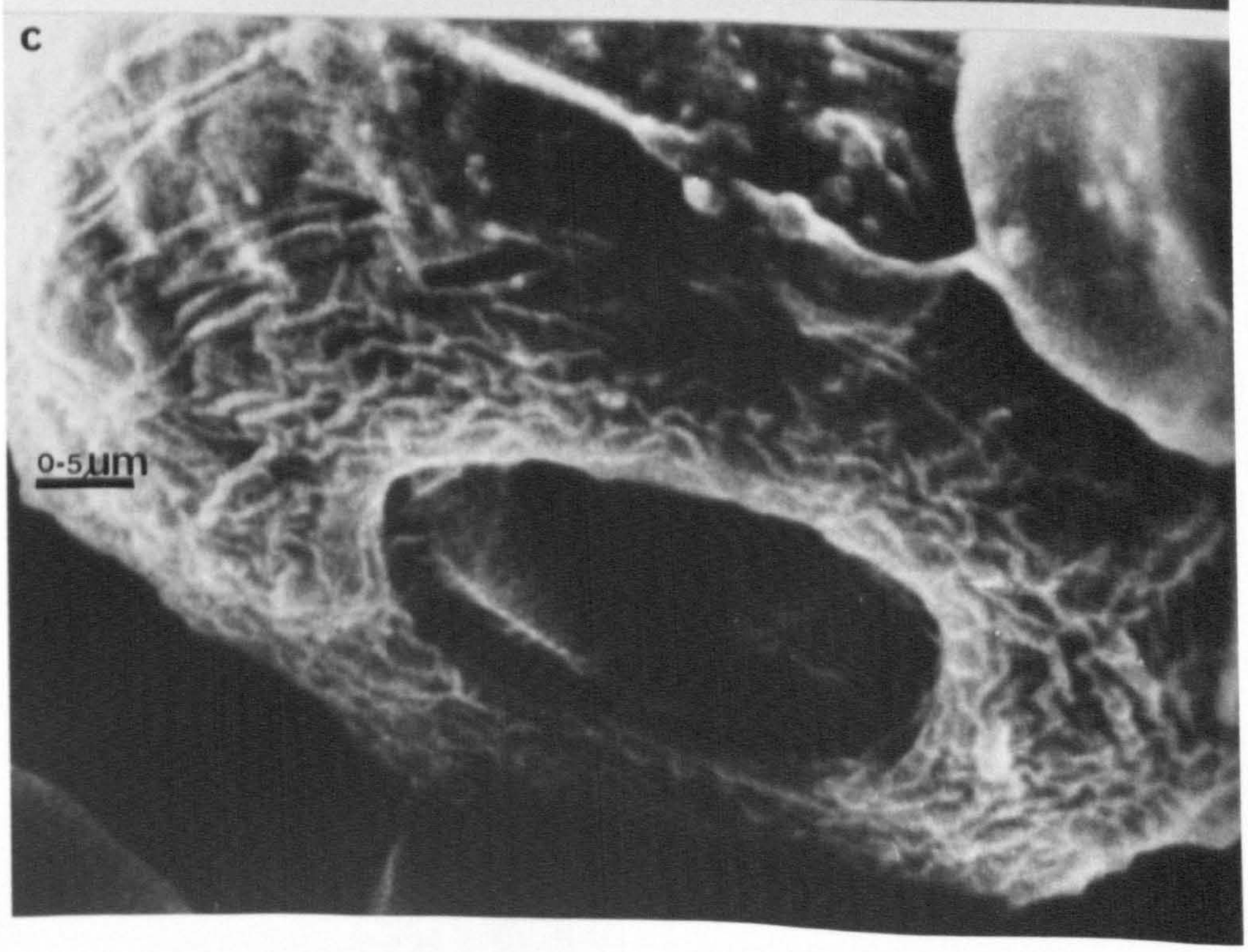
(a) Gross external appearance of the larva.

(b) Anterior end of the larva.

(c) Higher magnification of (b). Note the amphidial opening.


a
10μm


2·5μm


c
0·5μm

judging from the structure of the oral opening it may easily be associated with development of suction pressure.

There is no evidence that any of the larval stages possess a "boring tooth" as suggested by Chandler et al (1974).

Although Roberts (1934) reported two moults of the infective larva of A. suum in the lungs (of pig) the second and the third, no difference was observed in the morphology of the larvae obtained from the liver and lungs in the present study. In any case, larval development in mice and guinea pig is reported to be terminated at the beginning of the late third stage (Douvres and Tromba, 1971).

GENERAL DISCUSSION

There are three major ways by which parasites gain entry into their hosts. Firstly direct innoculation by agents like the arthropod vectors; secondly active percutaneous invasion and finally by oral ingestion. Invasion of host tissue via the oral route demands that the infective stage be resistant to the hosts digestive enzymes and immediately infective. Most parasites undergo a period of development outside their hosts before becoming infective. The length of time required varies between species and has also been shown to depend on a number of external environmental factors of which temperature is probably the most significant. The present study has shown that increase in temperature increases the rate of development of A. suum eggs. However, eggs embryonated at lower temperatures with consequent lower rates of development gave rise to infective larvae with greater viability than those embryonated at higher temperatures. This suggests that the eggs need to develop fairly slowly.

The natural host of A. suum is the pig, a coprophilic animal. Female Ascaris produce a tremendous number of eggs. If the eggs were immediately infective on production, there is

the possibility that through ingesting its own faeces, the host may become hyperinfested. The pathogenetic nature of Ascaris infection would lead to disease and possibly lead to the death of the host. This is against the principle of successful parasitism. Delayed development of the infective eggs may therefore be a natural device designed to prevent this happening and allowing sufficient time for the eggs to be dispersed. The extreme resistance of Ascaris eggs to environmental factors which is attributed to the egg-shell is well known. A. suum eggs often pass through the gut of experimentally infected hosts and out in the faeces without being digested. It thus seems possible in the light of the recent work by Perry and Clarke ($^{pers.}_{comm.}$) which showed that A. suum eggs are permeable to water, that this high resistance of the egg-shell may be as important in resisting digestion by the host as it is in resisting dessication.

Hatching of the infective eggs occurs mainly in the small intestine and the hatched larvae invade the host tissues soon afterwards. Although the number of species so far studied is limited, Matthews (1977) in his review on the

passage of larval helminths through host tissue barriers has suggested that parasites may be grouped into three broad categories (which are not necessarily mutually exclusive) based on their host tissue invasion mechanisms. Firstly, there are those parasites like the infective larvae of A. tubaeforme which have no apparent adaptations for penetration; normal body movement and feeding result in passage through the barrier. Secondly, there are the parasites with specialised cuticular structures, stylets and/or hooks that are used to mechanically disrupt tissues and finally, those with powerful enzymatic secretions which chemically dissolve constituents of the membranes. From the results of the present studies it seems that A. suum may belong more to the third category than either of the first two. The present investigations revealed that the infective larvae have neither specialised cuticular structures that might aid their entry into the host tissue, nor do they browse their way into the host intestinal tissue. Instead, it has been shown that after hatching from the egg-shell, A. suum larvae continue to produce proteolytic

secretions which cause changes in the glyco-proteins that form the basement membrane and the ground substance of the host tissue altering them in such a way that suggests that they are depolymerised (Perl and Catchpole, 1950; Gersh, 1952; Lewert and Lee, 1954).

The basement membrane and ground substance of host tissues present major barriers to penetrating helminths. Regardless of the mechanism involved few helminths confront their host tissue directly. Most exploit natural lines of weakness in the tissue. The relative success of invading helminths has been shown to be influenced greatly by the integrity and nature of the host tissue (Stirewalt, 1956). The present study revealed that entry of infective A. suum larvae into the host intestinal tissues occurs mainly via the crypts of liberkuhn. These represent a hyaline transitional area subjacent to the base of the villi. They are areas of the intestine where rapid mitotic division occurs producing new cells to replace the cells of the villi and the mucosa. How far the immature nature of the acellular cement, subepitheial basement membrane and ground substance of the tissue in these regions is instrumental in this area of the host tissue being used as entry sites

is not certain. The basement membrane and the ground substance of the host tissue present a barrier that fluctuates in its effectiveness with the age and physical status of the host. In young animals they are diffused and non-resistant allowing penetrating helminths to traverse them with ease and rapidity. In older animals they are thicker and dense and more resistant to enzymatic alteration. Consequently not only is penetration slower in older animals but a relatively high proportion of the infective larvae are unable to traverse the tissue (Lewert and Lee, 1954). Hypophysectomized young rats have been shown to develop highly polymerised basement membranes in the skin, and resemble aged animals in their resistance to infection by helminths (Lewert and Lee, 1954). It has also been reported by Sadun (1953, 1954) and by Ackert and Dewhurst (1956) that moderate doses of testosterone, alphaestradol and diethylstibestrol induce early maturation of the connective tissue barriers such as the basement membranes and increase resistance to infection with Ascaridia in immature male and female chickens. The incidence of Ascaris infections is generally much

higher in young rather than adult pigs (Gitter, Kidd and Davies, 1965; Davidson and Taffs, 1965). It therefore seems reasonable to suggest that such "age immunity" operative in Ascaris infection may be associated with the state of organisation of these acellular materials in the host tissues and the ability of the larval secretions to alter or depolymerise them. That is to say that increased resistance to infection with A. suum in adult pigs may have non-humoral aspects and may develop in the absence of natural antibody or antibody response engendered by previous infection.

Infective larvae of A. suum have been reported to migrate through the tissues of various hosts including rat, rabbit, cattle and sheep (Roberts, 1934; McDonald and Chevis, 1965). Following the above line of thought, it would seem that this fairly wide host range and relative susceptibility of the hosts to infection may also be associated with a chemical similarity in their acellular ground substance, and its susceptibility to the larval secretions.

Other parasites affecting the acellular glycoproteins of the host tissue in the same

manner include S. mansoni, Schistosomatium douthitti and Strongyloides ratti (Lewert and Lee, 1954). A further example of this type of activity is found in the hexacanth embryo of Taenia saginata (Silverman and Maneely, 1955). As it penetrates the intestinal mucosa, larval secretions alter the intercellular cement substances. These secretions have also been shown to have some cytolytic effects. There is no evidence in the present study, however, that the A. suum larval secretion is histolytic. From reports of other investigators (Stirewalt, 1966), it appears that cytolysis is not always a prominent feature accompanying penetration of host tissues by helminths. The usual picture (as it is in the case of the present study) is one in which one or two cells of the epithelium in the immediate vicinity of penetrating larvae may be displaced or dislodged. It seems therefore that penetrating larvae of various helminths may vary in their enzymatic endowments. No precise identification of the biochemical nature of the "invasive" enzymes possessed by larval helminths is yet possible, although the presence of lipase, amylase and hyaluronidase in the larval extracts of various species has been suggested (Levine,

Garzoli, Kuntz and Killough, 1948; Kuntz, 1953). An earlier report by Jenkins (1968) as well as the results of the present investigations showed that entry of infective A. suum larvae into host tissue induces an Evans blue reaction in the gut mucosa. Although Evans blue reaction is often associated with hyaluronidase, it is important to remember that the reaction may be caused by a wide range of substances not containing hyaluronidase (Lewert, 1956). It is more probable that larval helminth secretions, as in the case of bacterial toxins, represent a complex mixture of various substances rather than a simple chemical substance. Future work aimed at precise characterisation of these secretions and development of "anti-enzymes" to inhibit their action may be helpful in control of the parasites.

A final point which may be considered in the light of the present study is the phenomenon of visceral larva migrans. This is one of the disease syndromes often associated with larval ascarid invasion. The condition is generally associated with cat and dog ascarids in abnormal (human) hosts; migration of A. suum in man has not been implicated. It is remarkable that in none of the in vivo experiments were larvae found anywhere but in the gut, liver and lungs. The

physical contraints of the tissues involved and the limited penetrative ability of the infective larvae may help to explain this rather confined migratory pattern.

It is envisaged that on penetrating the host intestinal tissue, infective A. suum larvae enter the vessels of the submucosa, the speed with which they reach the liver precludes direct migration through the peritoneal cavity. The first capillary bed the larvae encounter would be in the liver. The average diameter of the sinusoids in the lobules, about 2.8 /u (in pig) is smaller than that of the larvae and any motion is likely to rupture the vessel walls and release the larvae into the tissue. By this time, the larvae are feeding and growing. The central vein of the lobules is larger than the sinusoides (about 84 /um in diameter in pig) and it is assumed that the larvae enter these and are carried through the hepatic portal vein to the heart and thence to the lungs.

The lungs act as a sort of non-return valve. Larvae can easily break out through the thin walled vessels of the alveoli but by this time they are much larger than the fine capillaries

and are not able to re-enter the circulatory system. They are thus trapped within the lungs. The normal motion of ciliary activity in the lungs and associated air-ways is towards the pharynx and the larvae are carried upwards and thence back to the intestinal tract.

Toxocara canis, T. cati and Toxascaris leonina, the dog and cat ascarids, have been shown to migrate to many organs following oral infection in mice (Al Zubarde pers. comm.). This suggests an alternative invasion pattern, possibly with the use of more powerful penetrative enzymes. These might permit direct entry into the peritoneal cavity and distribution to the organs or enzymatic damage to the lungs allowing re-entry into the circulatory system and thus retention within the somatic organs for longer periods. In human cases this would result in visceral larva migran$.

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APPENDIX TO FIGURE 2

Percentage of 14-18 week old pigs carrying natural A. suum infection

Time of Inspection	No. of Pigs Examined	% number of Pigs Infected
1. 11. 77	30	26.66
15. 11. 77	30	40.00
6. 12. 77	30	36.66
13. 12. 77	30	33.33
10. 1. 78	30	43.33
24. 1. 78	30	26.66
7. 2. 78	30	36.66
21. 2. 78	30	23.33
7. 3. 78	30	40.00
21. 3. 78	30	33.33
4. 4. 78	30	26.66
18. 4. 78	30	43.33
2. 5. 78	30	30.00
16. 5. 78	30	23.33
1. 6. 78	30	36.66
27. 6. 78	30	30.00
11. 7. 78	30	16.66
18. 7. 78	30	43.33
7. 8. 78	30	30.00
5. 9. 78	30	33.33
19. 9. 78	30	53.33
17. 10. 78	30	36.66
31. 10. 78	30	26.66

APPENDIX TO FIGURE 3

Time of Inspection	% Infection	Mean (S.E.) Worm Burden/Pig	Proportion of male worm to total worm burden
November 1977	33.333	16.15±9.1473	0.2941
December 1977	35.000	14.190±4.0491	0.3520
January 1978	35.000	7.143±3.6617	0.30667
February 1978	30.000	11.333±6.4526	0.2866
March 1978	36.667	11.636±4.1721	0.2393
April 1978	35.00	8.714±4.3191	0.2677
May 1978	26.667	19.438±6.9178	0.2786
June 1978	33.333	16.85±2.2569	0.3115
July 1978	30.000	14.778±8.4911	0.2376
August 1978	30.000	8.333±3.0615	0.2400
September 1978	38.333	13.522±5.6403	0.2386
October 1978	31.667	14.579±5.9602	0.2563

APPENDIX TO FIGURE 7

Mean (S.E.) of 15 determinants of percentage of *A. suum* eggs embryonated at 30° ± 1°C in 0.1N H_2SO_4 for 26 days, in which the enclosed larvae were observed to be motile at various time intervals after being stimulated to hatch *in vitro* at 37°C.

Time (min.)	Mean percentage number of eggs containing motile larvae
0	14.13 ± 1.27
15	85.12 ± 2.73
30	97.68 ± 1.95
45	90.00 ± 2.15
60	65.13 ± 2.23
75	47.49 ± 1.31
90	41.56 ± 1.55
105	38.74 ± 1.23
120	36.49 ± 1.87
135	35.00 ± 1.05
150	34.68 ± 1.26
165	33.15 ± 1.19
180	32.00 + 1.16

APPENDIX TO FIGURE 8

Mean (S.E.) of 15 determinants of percentage hatch of A. suum infective eggs embryonated at 30° ± 1°C in 0.1N H_2SO_4 for 26 days and subjected to hatching stimulus *in vitro* at 37°C.

Time (min.)	Percentage Hatch (S.E.)
15	0
30	0
45	12.16 ± 2.13
60	35.18 ± 2.96
75	62.73 ± 1.46
90	67.05 ± 2.63
105	69.48 ± 2.15
120	73.59 ± 1.82
135	79.45 ± 2.05
150	80.00 ± 1.98
165	79.83 ± 1.44
180	81.15 ± 1.12

APPENDIX TO FIGURE 10

Mean (S.E.) percentage hatch of 25 replicate samples of *Ascaris suum* eggs embryonated for 60 days in 0.1N H_2SO_4 at 22° ± 1°C and hatched at 37°C.

Time (min.)	Mean (S.E.) percentage hatch
15	0
30	0
45	9.038 ± 2.119
60	42.354 ± 4.554
75	58.210 ± 3.322
90	63.592 ± 1.539
105	65.888 ± 1.394
120	81.198 ± 2.704
135	83.466 ± 1.667
150	83.464 ± 1.573
165	83.686 ± 1.678
180	83.996 ± 1.385
195	83.614 ± 1.475

APPENDIX TO FIGURE 11

Log of total index of development of eggs incubated at various temperatures

Day of Observation	Temperature of incubation				
	$16^{\circ} \pm 1^{\circ}C$	$18^{\circ} \pm 1^{\circ}C$	$20^{\circ} \pm 1^{\circ}C$	$22^{\circ} \pm 1^{\circ}C$	$24^{\circ} \pm 1^{\circ}C$
2	Ø	Ø	Ø	Ø	Ø
4	Ø	Ø	Ø	Ø	Ø
6	Ø	Ø	Ø	1.49	1.38
8	Ø	Ø	Ø	1.84	1.82
10	Ø	Ø	1.43	2.12	2.23
12	Ø	Ø	1.77	2.30	2.47
14	Ø	1.45	2.05	2.39	2.59
16	1.42	1.86	2.23	2.50	2.68
18	1.82	2.20	2.43	2.62	2.70
20	2.05	2.42	2.53	2.66	2.70
22	2.10	2.57	2.64	2.70	2.70
24	2.46	2.65	2.68	2.70	2.70
26	2.62	2.69	2.70	2.70	2.70
28	2.67	2.69	2.70	2.70	2.70
30	2.68	2.70	2.70	2.70	2.70
32	2.69	2.70	2.70	2.70	2.70
34	2.70	2.70	2.70	2.70	2.70
36	2.70	2.70	2.70	2.70	2.70
38	2.70	2.70	2.70	2.70	2.70
40	2.70	2.70	2.70	2.70	2.70

APPENDIX TO FIGURE 11 continued

Log of total index of development of eggs incubated at various temperatures

Date of observation	26° ± 1°C	28° ± 1°C	30° ± 1°C	32° ± 1°C	34° ± 1°C	36° ± 1°C	38° ± 1°C
2	0	1.26	1.34	1.49	1.38	Ø	Ø
4	1.08	1.67	1.81	2.10	2.03	Ø	Ø
6	1.68	2.02	2.15	2.36	2.11	Ø	Ø
8	1.98	2.31	2.40	2.55	2.37	1.43	Ø
10	2.34	2.53	2.43	2.68	2.57	1.66	Ø
12	2.37	2.67	2.67	2.70	2.60	1.77	0.08
14	2.66	2.70	2.69	2.70	2.63	1.95	0.20
16	2.70	2.70	2.70	2.70	2.67	2.35	Ø
18	2.70	2.70	2.70	2.70	2.70	2.31	Ø
20	2.70	2.70	2.70	2.70	2.70	2.08	Ø
22	2.70	2.70	2.70	2.70	2.70	1.96	Ø
24	2.70	2.70	2.70	2.70	2.70	Ø	Ø
26	2.70	2.70	2.70	2.70	2.70	Ø	Ø
28	2.70	2.70	2.70	2.70	2.70	Ø	Ø
30	2.70	2.70	2.70	2.70	2.70	Ø	Ø
32	2.70	2.70	2.70	2.70	2.70	Ø	Ø
34	2.70	2.70	2.70	2.70	2.70	Ø	Ø
36	2.70	2.70	2.70	2.70	2.70	Ø	Ø
38	2.70	2.70	2.70	2.70	2.70	Ø	Ø
40	2.70	2.70	2.70	2.70	2.70	Ø	Ø

APPENDIX TO FIGURE 12

Mean (S.E.) time to achieve 60% larval development in eggs incubated at various temperatures

Incubation Temperature	Time (hours)
16° ± 1°C	722 ± 6.60
18° ± 1°C	620 ± 6.46
20° ± 1°C	562.2 ± 12.31
22° ± 1°C	464.4 ± 9.60
24° ± 1°C	427.6 ± 4.79
26° ± 1°C	367.2 ± 8.97
28° ± 1°C	317.2 ± 8.06
30° ± 1°C	262.8 ± 10.11
32° + 1°C	222.0 + 8.49
34° ± 1°C	314.4 ± 7.96

APPENDIX TO FIGURE 13

Mean (S.E.) percentage hatch of A. suum eggs embryonated at various incubation temperatures for 60 days in 0.1N H_2SO_4 and hatched in vitro at 37°C for 2 hrs. (mean of 25 determinatives)

Embryonation Temperature	Percentage Hatch
16° ± 1°C	77.10 ± 5.66
20° ± 1°C	77.20 ± 7.58
22° ± 1°C	84.73 ± 5.13
24° ± 1°C	82.81 ± 6.03
26° ± 1°C	80.72 ± 5.10
28° ± 1°C	81.31 ± 5.93
30° ± 1°C	79.45 ± 5.18
32° ± 1°C	67.13 ± 9.50
34° ± 1°C	35.41 ± 12.64

APPENDIX TO FIGURE 14

Effect of embryonation temperature on *in vitro* survival of hatched larvae in phosphate buffered saline (pH 7.2) at 37°C. The eggs were embryonated for 60 days.

Temperature of embryonation	Time (hrs.) to achieve 90% mortality (mean ± S.E. of 5 determinators)
16° ± 1°C	126.83 ± 6.50
18° ± 1°C	144.2 ± 8.149
20° ± 1°C	140.31 ± 3.21
22° ± 1°C	138.56 ± 3.95
24° ± 1°C	84.2 ± 2.26
26° ± 1°C	36.06 ± 1.63
28° ± 1°C	32.02 ± 1.64
30° ± 1°C	28.04 ± 3.05
32° ± 1°C	6.00 ± 1.49
34° ± 1°C	2.01 ± 0.82

APPENDIX TO FIGURE 15

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 16° ± 1°C and aged for times indicated in phosphate buffered saline pH 7.2 at 37°C.

Age of larvae	No. of larve in the sample	No. of penetrants (mean of 5 replicates, S.E.)
0 h.	30	17.30 ± 1.10
1 h.	30	17.23 ± 1.10
4 h.	30	17.30 ± 0.69
8 h.	30	17.00 ± 0.97
12 h.	30	16.15 ± 0.94
16 h.	30	14.20 ± 0.83
20 h.	30	11.33 ± 0.94
24 h.	30	8.46 ± 1.03
28 h.	30	6.82 ± 0.70
32 h.	30	5.31 ± 0.93
36 h.	30	4.11 ± 0.60
40 h.	30	3.00 ± 0.53
44 h.	30	2.10 ± 0.45
48 h.	30	1.60 ± 0.45
52 h.	30	0.89 ± 0.06
56 h.	30	0.20 ± 0.01
60 h.	30	0
64 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 22° + 1°C and aged for times indicated in phosphate buffered saline (pH 7.2) at 37°C.

Age of larvae	Number of Larvae in the sample	Number of Penetrants (mean of 5 replicates S.E.)
0 h.	30	22.59 ± 0.48
1 h.	30	22.51 ± 0.55
4 h.	30	22.43 ± 0.64
8 h.	30	22.10 ± 0.61
12 h.	30	20.90 ± 0.56
16 h.	30	17.81 ± 0.53
20 h.	30	14.00 ± 0.21
24 h.	30	10.62 ± 0.32
28 h.	30	8.56 ± 0.10
32 h.	30	7.00 ± 0.59
36 h.	30	5.95 ± 0.43
40 h.	30	5.10 ± 0.50
44 h.	30	4.22 ± 0.13
48 h.	30	3.45 ± 0.30
52 h.	30	2.55 + 0.09
56 h.	30	1.70 ± 0.04
60 h.	30	0.82 ± 0.01
64 h.	30	0
68 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 18° + 1°C and aged for times indicated in phosphate buffered saline (pH 7.2) at 37°C.

Age of Larvae	Number of larvae in the sample	Number of penetrants (mean of 5 replicates S.E.)
0	30	17.86 ± 0.95
1	30	17.83 ± 0.56
4	30	17.70 ± 0.59
8	30	17.41 ± 0.43
12	30	16.00 ± 0.94
16	30	13.51 ± 0.63
20	30	10.36 ± 1.06
24	30	8.13 ± 0.48
28	30	6.37 ± 0.50
32	30	5.00 ± 1.05
36	30	4.02 ± 0.89
40	30	3.15 ± 0.51
44	30	2.35 ± 0.40
48	30	1.60 ± 0.10
52	30	1.00 ± 0.10
56	30	0.35 ± 0.02
60	30	0
64	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 20° + 1°C and aged for times indicated, in phosphate buffered saline at 37°C.

Age of Larvae	No. of larve in the sample	No. of penetrants (mean of 5 replicates) S.E.
0 h.	30	18.35 ± 1.01
1 h.	30	18.34 ± 0.97
4 h.	30	18.40 ± 1.10
8 h.	30	18.00 ± 1.00
12 h.	30	17.15 ± 1.00
16 h.	30	15.35 ± 0.65
20 h.	30	12.73 ± 0.91
24 h.	30	9.51 ± 0.53
28 h.	30	7.81 ± 0.88
32 h.	30	6.64 ± 0.31
36 h.	30	5.48 ± 0.50
40 h.	30	4.22 ± 0.20
44 h.	30	3.41 ± 0.16
48 h.	30	2.35 ± 0.02
52 h.	30	1.70 + 0.19
56 h.	30	1.00 ± 0.16
60 h.	30	0.50 ± 0.01
64 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 24° ± 1°C and aged for times indicated in phosphate buffered saline at 37°C.

Age of Larvae	No. of larvae in the sample	No. of penetrants (mean of 5 replicates, S.E.)
0 h.	30	18.26 ± 0.81
1 h.	30	18.21 ± 0.90
4 h.	30	17.89 ± 0.48
8 h.	30	17.65 ± 0.97
12 h.	30	16.45 ± 1.24
16 h.	30	15.10 ± 0.86
20 h.	30	12.20 ± 0.36
24 h.	30	9.00 ± 0.55
28 h.	30	7.26 ± 0.31
32 h.	30	5.74 ± 0.99
36 h.	30	4.68 ± 0.85
40 h.	30	3.71 ± 0.21
44 h.	30	2.93 ± 0.55
48 h.	30	1.45 ± 0.26
52 h.	30	0.71 ± 0.06
56 h.	30	0.10 ± 0.04
60 h.	30	0
64 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 26° + 1°C and aged for times indicated in phosphate buffered saline at 37°C.

Age of Larvae	No. of larvae in the sample	No. of penetrants (mean of 5 replicates, S.E.)
0 h.	30	12.10 ± 0.59
1 h.	30	12.10 ± 1.09
4 h.	30	12.00 ± 0.87
8 h.	30	11.71 ± 0.73
12 h.	30	11.25 ± 0.55
16 h.	30	9.10 ± 0.56
20 h.	30	6.25 ± 0.43
24 h.	30	4.50 ± 1.09
28 h.	30	3.51 ± 0.89
32 h.	30	2.64 ± 0.42
36 h.	30	1.80 ± 0.45
40 h.	30	1.20 ± 0.42
44 h.	30	0.45 ± 0.05
48 h.	30	0.10 ± 0.10
52 h.	30	0
56 h.	30	0
60 h.	30	0
64 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 28° + 1°C and aged for times indicated, in phosphate buffered saline at 37°C.

Age of Larvae	No. of Larvae in the sample	No. of penetrants (mean of 5 replicates, S.E.)
0 h.	30	10.93 ± 1.03
1 h.	30	10.90 ± 0.63
4 h.	30	10.96 ± 1.10
8 h.	30	10.10 ± 1.05
12 h.	30	7.83 ± 0.91
16 h.	30	5.95 ± 0.53
20 h.	30	4.21 ± 0.41
24 h.	30	3.15 ± 0.36
28 h.	30	2.35 ± 0.48
32 h.	30	1.45 ± 0.31
36 h.	30	0.61 ± 0.50
40 h.	30	0
44 h.	30	0
48 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 30° + 1°C and aged for times indicated, in phosphate buffered saline at 37°C.

Age of larvae	No. of larvae in the sample	No. of penetrants (mean of 5 replicates, S.E.)
0	30	7.76 ± 0.96
1	30	7.60 ± 0.64
4	30	7.45 ± 0.98
8	30	6.81 ± 0.47
12	30	6.00 ± 0.53
16	30	4.65 ± 0.18
20	30	3.40 ± 0.21
24	30	2.30 ± 0.93
28	30	1.38 ± 0.20
32	30	0.26 ± 0.10
36	30	0
40	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 32° + 1°C and aged for times indicated, in phosphate buffered saline at 37°C.

Age of Larvae	No. of larvae in the sample	Mean no. of penetrants (mean of 5 replicates, S.E.)
0 h.	30	2.73 ± 0.56
1 h.	30	2.74 ± 0.43
4 h.	30	2.72 ± 0.45
8 h.	30	2.45 ± 0.58
12 h.	30	2.00 ± 0.43
16 h.	30	1.45 ± 0.41
20 h.	30	6.95 ± 0.08
24 h.	30	0.49 ± 0.10
28 h.	30	0
32 h.	30	0

Passage through filter paper No. 42 after 90 mins. exposure by larvae obtained from eggs embryonated at 34° + 1°C and aged for times indicated, in phosphate buffered saline at 37°C.

Age of Larvae	No. of larvae in the sample	No. of penetrants (mean of 5 replicates, S.E.)
0	30	2.81 ± 0.66
1	30	2.68 ± 0.40
4	30	2.50 ± 0.13
8	30	2.23 ± 0.48
12	30	1.98 ± 0.33
16	30	1.35 ± 0.20
20	30	0.80 ± 0.10
24	30	0.10 ± 0.10
28	30	0
32	30	0

APPENDIX TO FIGURE 17

Measurement of lipid levels of larvae embryonated at 22° ± 1°C, hatched at 37°C (for 2 hours) and held in phosphate buffered saline at various temperatures as indicated, for 24 hours.

Temperature	Lipid level of 520nM
37°C	758.9 ± 49.6
35°C	567.4 ± 40.9
28°C	1673.8 ± 71.4
25°C	1751.7 ± 62.2
20°C	1730.1 ± 57.1
15°C	1666.7 ± 62.1
12°C	1705.7 ±363.8

APPENDIX TO FIGURE 18

Measurement of lipid levels of larvae embryonated at 22° ± 1°C, hatched at 37°C (for 2 hours) and held in phosphate buffered saline for the given lengths of time at 37°C.

Time/Age	Lipid level at 520nM
1	1694.56 ± 50.35
2	1899.28 ± 61.51
4	1671.61 ± 44.06
6	833.48 ± 54.63
8	1207.33 ± 50.47
10	755.52 ± 39.22
12	708.50 ± 49.66
14	329.83 ± 36.08
16	381.24 ± 42.78
18	667.82 ± 42.42
20	256.6 ± 42.98
22	252.8 ± 26.64
24	192.00 ± 34.71
26	147.50 ± 22.07

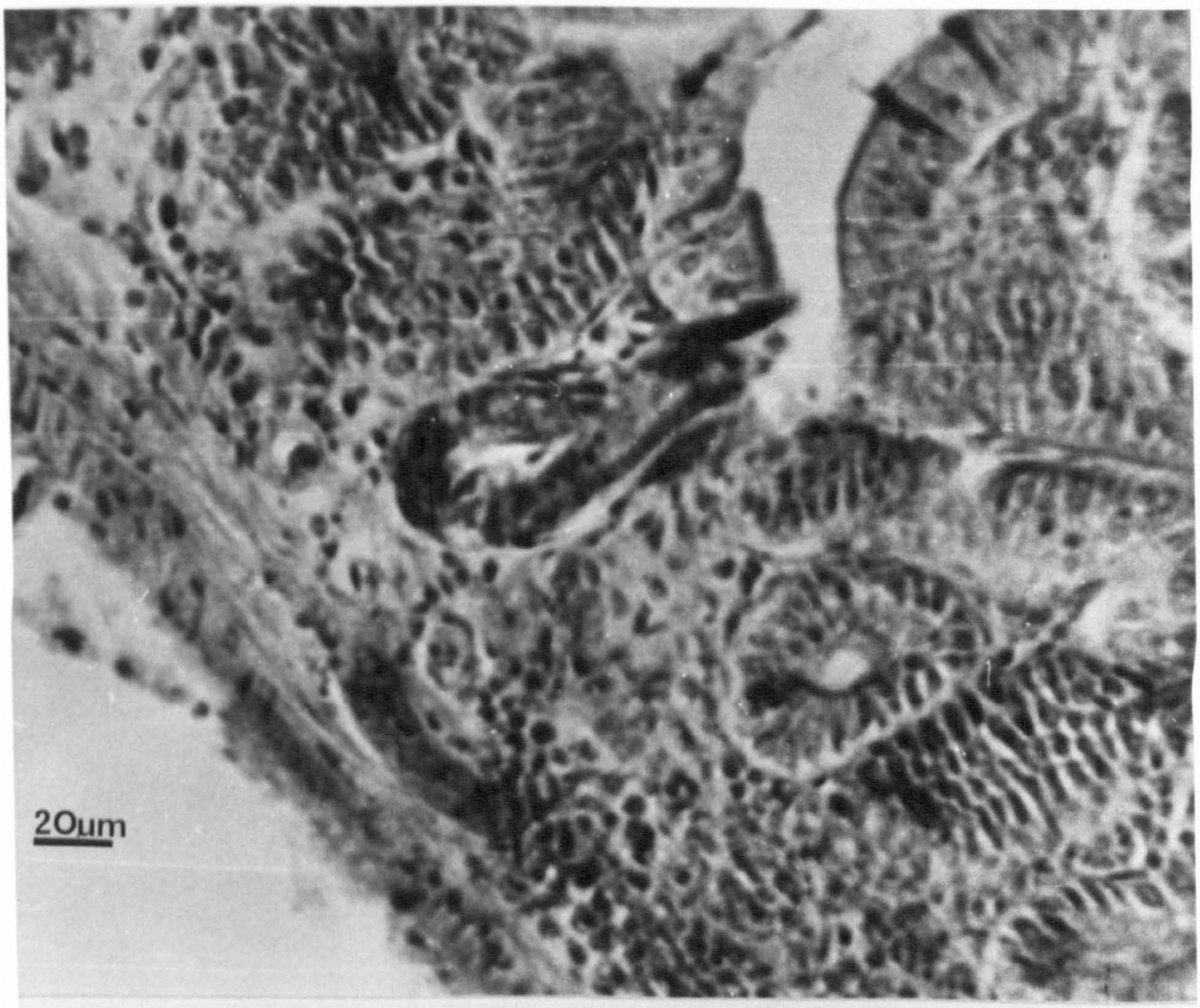
PLATE APPENDIX 1a

Section of small intestine of mouse infected with 10,000 infective A. suum eggs, showing larvae entering the submucosa and the muscle layer 6 hours after infection. Section was stained with PAS and counter stained with Orange G.

PLATE APPENDIX 1b

Section of small intestine of mouse infected with 10,000 infective A. suum eggs, showing larva in the lymphatic vessel 4 hours after infection. Section was stained with H and E.

a



b

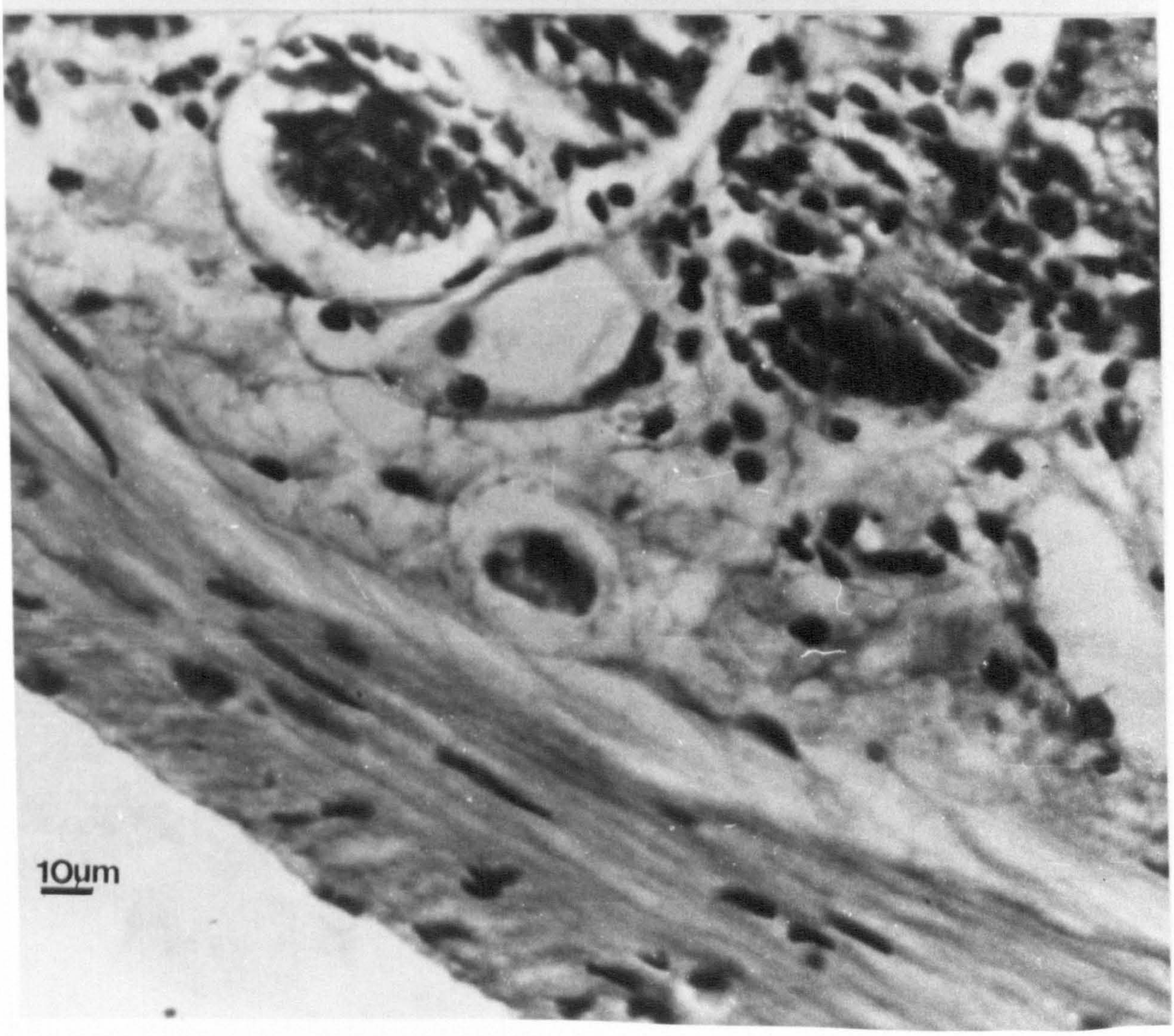


PLATE APPENDIX 1c

Section of mouse liver showing infective *A. suum* larva in the organ 4 hours after infecting the animal with 10,000 infective *A. suum* eggs. Section was stained with H. and E.

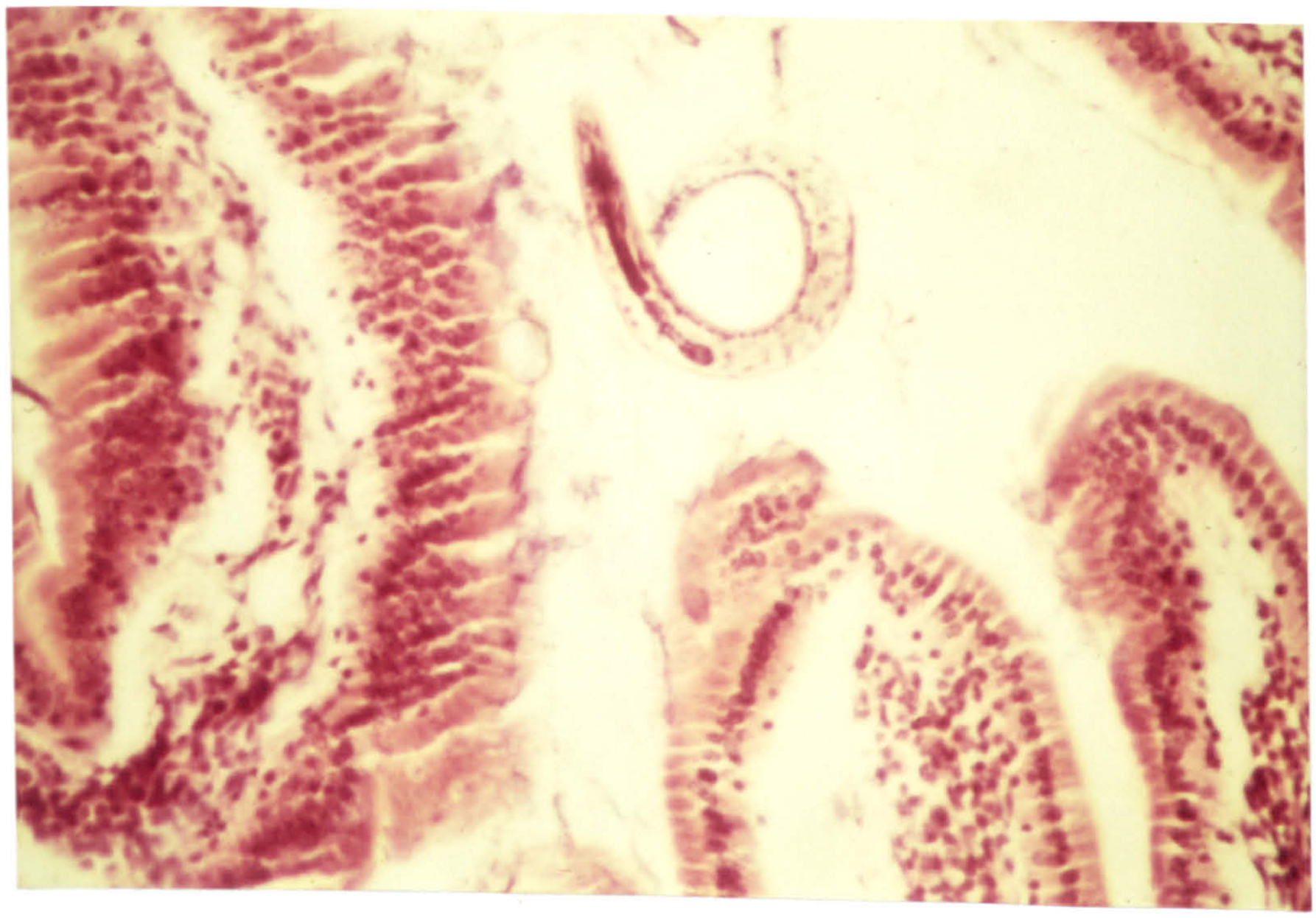
c

PLATE APPENDIX 2a

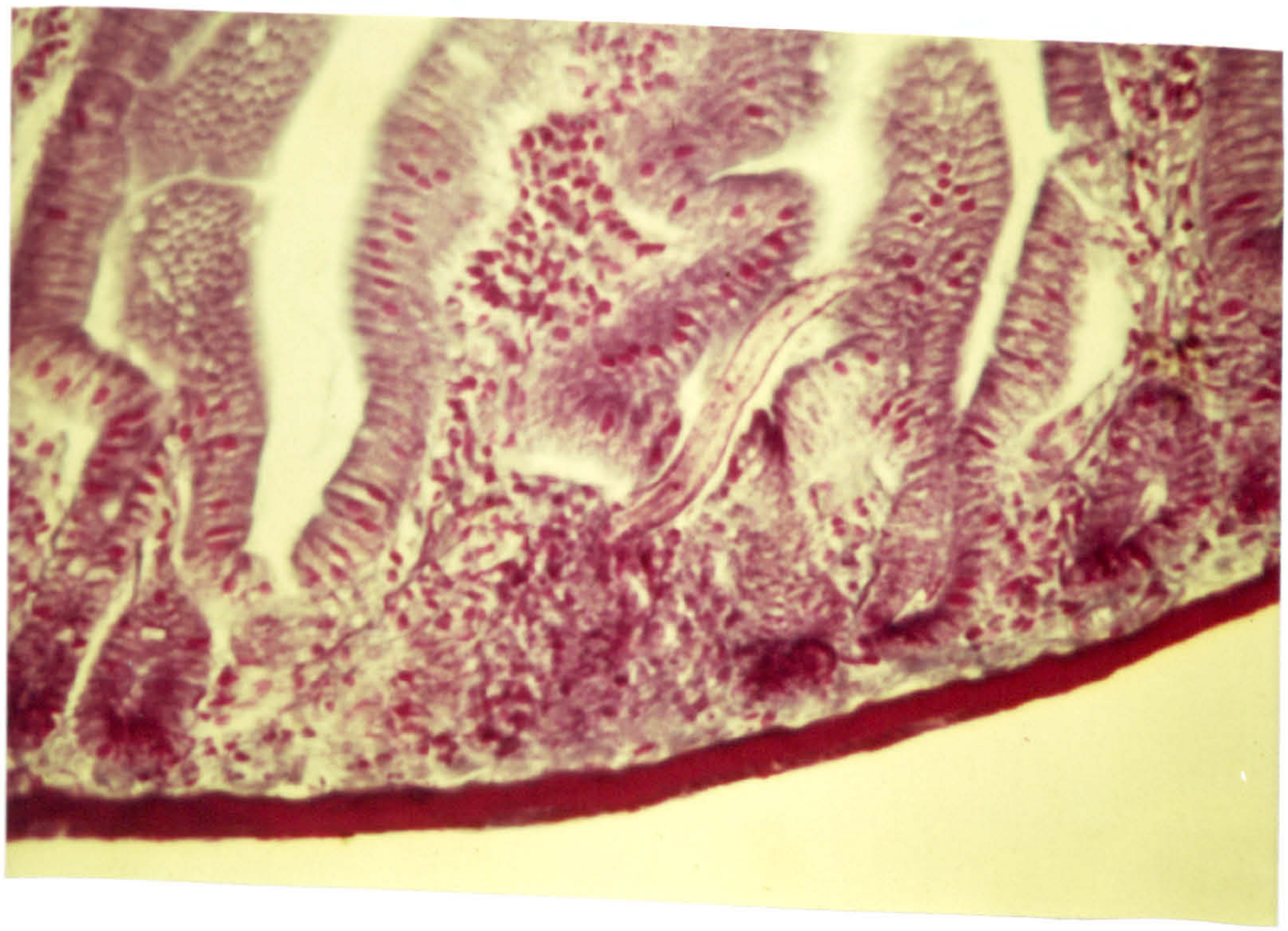
Section of the small intestine of mouse infected with 10,000 infective A. suum eggs, showing infective larva in the lumen. The mouse was sacrificed 60 mins. after infection; stained with Mallory Triple stain Mag X480. Note the intense staining of the Oesophagus.

PLATE APPENDIX 2b

Section of the small intestine of mouse infected with 20,000 infective A. suum eggs and sacrificed 2 hours after infection; showing entry of the infective larva into the submucosa; stained with Alcian Blue. Chlorantine Fast Red Mag X480.



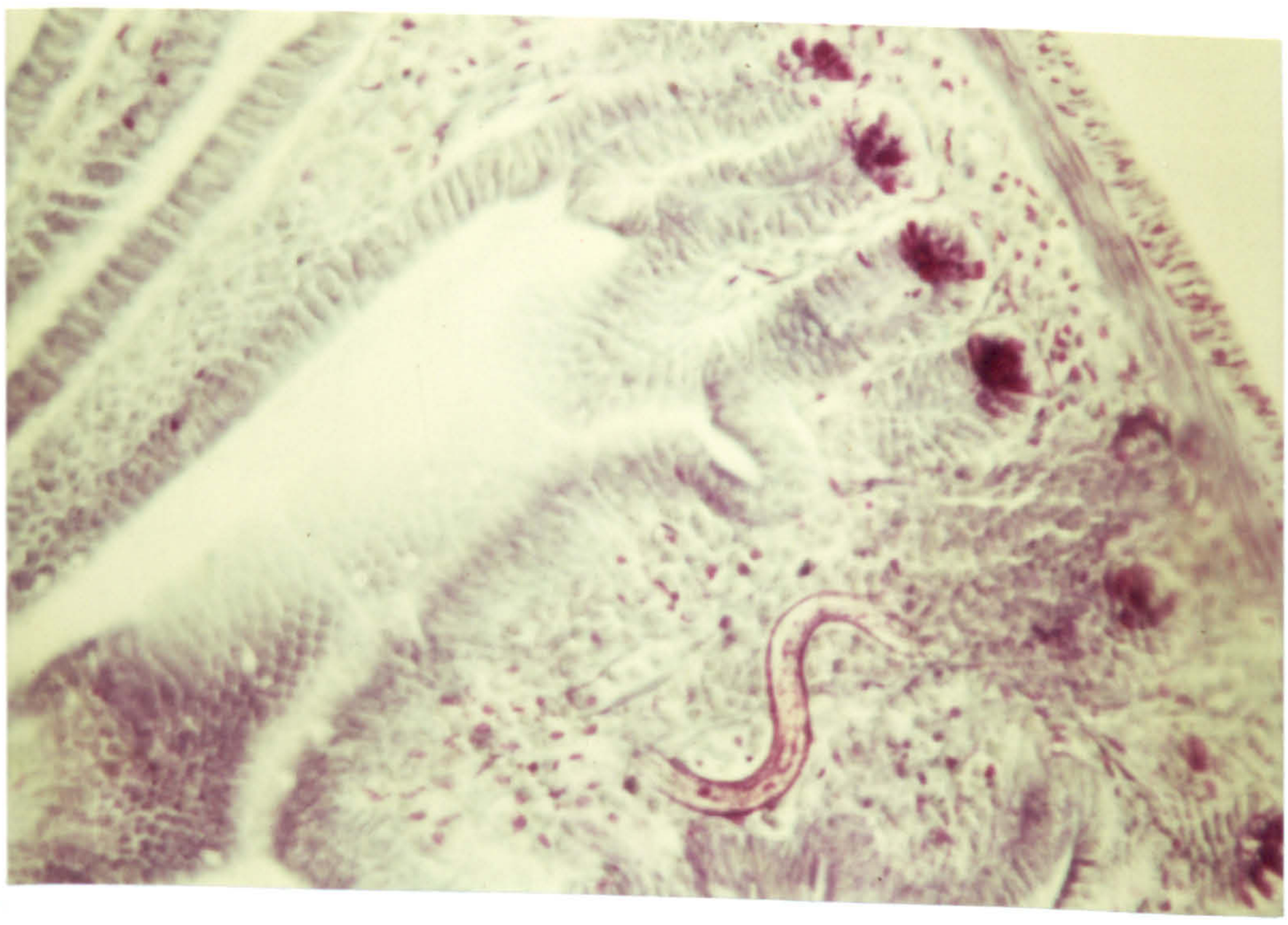
2a



2b

PLATE APPENDIX 2c

Section of the small intestine of mouse infected with 20,000 infective A. suum eggs and sacrificed 8 hours after infection; showing infective larva in the lamina propria; stained with Alcian Blue-Chlorantin Fast Red Mag X480. Note the absence of any clear digestion of the host tissue by the infective larva.



2c

Appendix To Figure 23

Mean (± SE) of 4 separate spectrophotometric readings at 520 nM of Azocoll dye release from living larvae and larval extracts of infective A. suum larvae over 6 hours at 37°C.

Pepsin 10 mg/ml in phosphate buffer + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
0.130	0.228± 0.005	0.373± 0.005	0.635± 0.001	0.875± 0.29	1.03± 0.022	1.13± 0.024

Pepsin 10 mg/ml in phosphate buffer + antibiotics + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
0.094± 0.008	0.223± 0.096	0.42± 0.016	0.64± 0.016	0.855± 0.031	1.01± 0.045	1.15± 0.016

Phosphate buffer + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
0.035	0.040	0.045	0.049± 0.002	0.061± 0.001	0.066± 0.002	0.071± 0.001

Phosphate buffer + Antibiotics + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
0.035	0.040	0.045	0.051± 0.001	0.060± 0.001	0.065± 0.001	0.071± 0.001

Living larvae + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
0.052± 0.002	0.080± 0.004	0.131± 0.006	0.162± 0.011	0.174± 0.005	0.187± 0.002	0.192± 0.002

Living larvae + antibiotics + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
$0.055^{+}_{-0.001}$	$0.081^{+}_{-0.002}$	$0.132^{+}_{-0.003}$	$0.154^{+}_{-0.006}$	$0.175^{+}_{-0.007}$	$0.188^{+}_{-0.003}$	$0.195^{+}_{-0.001}$

Larval Secretion (collected after 4 hours of Incubation) + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
$0.070^{+}_{-0.007}$	$0.129^{+}_{-0.009}$	$0.178^{+}_{-0.006}$	$0.189^{+}_{-0.006}$	$0.195^{+}_{-0.003}$	$0.201^{+}_{-0.007}$	$0.208^{+}_{-0.006}$

Larval Secretion (collected after 4 hours of incubation) + 25 mg Azocoll + Antibiotics

30 min.	1h	2h	3h	4h	5h	6h
$0.069^{+}_{-0.006}$	$0.134^{+}_{-0.004}$	$0.179^{+}_{-0.005}$	$0.186^{+}_{-0.006}$	$0.194^{+}_{-0.002}$	$0.211^{+}_{-0.009}$	$0.219^{+}_{-0.005}$

Larval secretion (collected after 2 hours of incubation) + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
$0.040^{+}_{-0.004}$	$0.056^{+}_{-0.008}$	$0.065^{+}_{-0.001}$	$0.074^{+}_{-0.002}$	$0.090^{+}_{-0.004}$	$0.094^{+}_{-0.002}$	$0.119^{+}_{-0.023}$

Larval secretion (collected after 2 hours of incubation) + 25 mg Azocoll + antibiotics

30 min.	1h	2h	3h	4h	5h	6h
$0.041^{+}_{-0.003}$	$0.055^{+}_{-0.004}$	$0.064^{+}_{-0.003}$	$0.071^{+}_{-0.003}$	$0.085^{+}_{-0.004}$	$0.094^{+}_{-0.003}$	$0.105^{+}_{-0.004}$

Boiled larvae (at 100°C for 15 min.) + 25 mg Azocoll

30 min.	1h	2h	3h	4h	5h	6h
0.035	0.040	$0.045^{+}_{0.001^{-}}$	$0.052^{+}_{0.002^{-}}$	$0.062^{+}_{0.002^{-}}$	0.065	$0.071^{+}_{0.003^{-}}$